Hepatic copper accumulation in Labrador retriever and Wilson's disease

Where human and canine diseases come together

Yara Roelen
Honours Program 2012-2013
Department of companion animals
Faculty of veterinary medicine, University of Utrecht

Hepatic copper accumulation in human and dog

Hepatic copper accumulation in Labrador retriever & Wilson's disease in human patients

This research project is embedded in the Tissue Repair research program of the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, the Netherlands.

Yara Roelen

Principal investigator:	Yara Roelen Cambridgelaan 283 3584 DZ Utrecht y.s.roelen@students.uu.nl
Daily supervisor:	Drs. Hille Fieten
Supervisors:	Dr. Bart Spee Dr. P. Leegwater Prof. Dr. Jan Rothuizen

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General introduction

Chapter 1: General introduction

Copper and copper transport

Copper plays an essential role in a variety of biological processes including mitochondrial respiration, antioxidant defense, neurotransmitter synthesis, connective tissue formation, pigmentation, and iron metabolism (1).

Copper is a transition metal able to cycle between two redox states; oxidized Cu^{+} (cupric ion, stable) and reduced Cu2+(cuprous ion, unstable). Therefore copper can function as an electron donor and acceptor for different enzymes, including cytochrome -c-oxidase, superoxide dismutase 1(SOD1) and ceruloplasmin (2).

Copper is an essential element but toxic when overloaded. Therefore copper metabolism has to be tightly regulated to prevent accumulation of free copper.

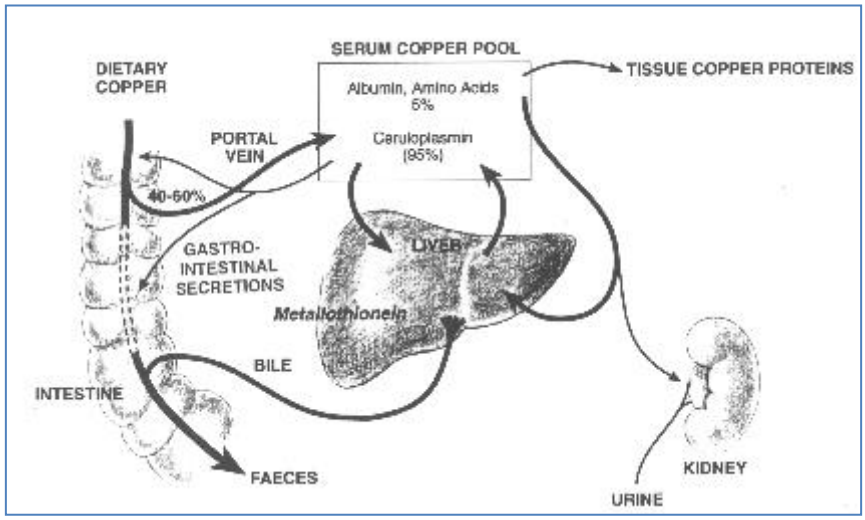

Figure 1: Copper transport in the body.

After oral intake copper is absorbed by enterocytes and is transported via the portal circulation to the liver and kidney. In the portal circulation copper is bound to albumin, transcuprein and other plasma proteins for transport to the liver (Gollan JC et al, 1998)

Body copper homeostasis

Copper distribution in the body is a biphasic process. In the first phase dietary copper is absorbed by enterocytes and transported via the portal circulation to predominantly the liver whereas a small proportion enters the kidney. Copper loosely bound to amino acids is filtered in the kidney and reabsorbed in the tubulus (3). In the second phase hepatic copper is distributed to other organs (4) (Figure 1).

First phase of copper transport

Copper uptake from the diet occurs in the small intestines. Copper enters the enterocyte in the intestine. In this first step copper homeostasis is already being monitored since the intestine is able to modulate the rate of copper absorption (5).

After absorption in the enterocytes, copper enters the portal circulation and is bound with high affinity to albumin and transcuprein (macroglobulin) for transport to the liver. Albumin and transcuprein have

accessible binding sites for Cu++(II) and can rapidly exchange copper with each other (6). Albumin is an amino acid store and carrier for free fatty acids, bilirubin and contains 15% of the total amount of plasma copper. Transcuprein is responsible for trapping and inactivating proteases, zinc and holds 10% of the total amount of plasma copper (7).

Copper enters the hepatocytes via copper transporter 1(CTR1) and may be stored within hepatocytes, secreted into plasma, or excreted in bile.

Second phase of copper transport

To facilitate the excretion of copper from the liver copper is incorporated into ceruloplasmin which is facilitated by ATP7B(16) . Copper bounded ceruloplasmin is then secreted into the plasma (8). Ceruloplasmin comprises 70% of the total plasma copper pool and delivers copper to other tissues such as the heart and the brain. Other functions of ceruloplasmin are: neutralization of radicals, catalysation of oxidation reactions involving molecular oxygen and various natural and synthetic amines ferroxidase activity(7). Ceruloplasmin is synthesized in hepatocytes and secreted into the plasma following the incorporation of six atoms of copper in the secretory pathway. Incorporation of copper leads to a conformational change of apoceruloplasmin (without copper) to copper bound holoceruloplasmin (9) which is enzymatically active. Once copper is incorporated into ceruloplasmin, this structure is not dialyzable at neutral pH and only extractable with disruptive procedures(10). Certain organs preferably accept copper from ceruloplasmin compared to albumin or transcuprein(6).

Cellular copper transport

In the cell, different copper binding proteins transport copper and help to maintain copper homeostasis. The copper held by hepatocytes is mostly bound to copper chaperones (1) and metallothionein, or incorporated into cuproenzymes (11)(Figure 2).

Copper enters the enterocyte via copper transporter 1 (CTR1)(12), probably copper transporter 2(CTR2)(13) and divalent cation transporter(DMT1)(14) after reduction from ++ to + state. Copper is transported from the enterocytes into the portal circulation by ATP7A, that is located at the basal membrane of the enterocyte under high copper conditions(15).

In the hepatocyte Copper transport protein ATOX1 shuttles copper to ATP7B and is required for proper biliary excretion of excess copper delivering copper to transgolgi network. In tissues other than liver ATOX1 also interacts with ATP7A.

ATP7A and ATP7B are located in the final compartment of the Golgi complex, known as the trans golgi network. ATP7A is expressed in many tissues, including muscle, kidney, heart, intestine and plays an important role in neurons and astrocytes (17). However, ATP7A has a low expression in the liver (18). ATP7B is predominantly expressed in the liver

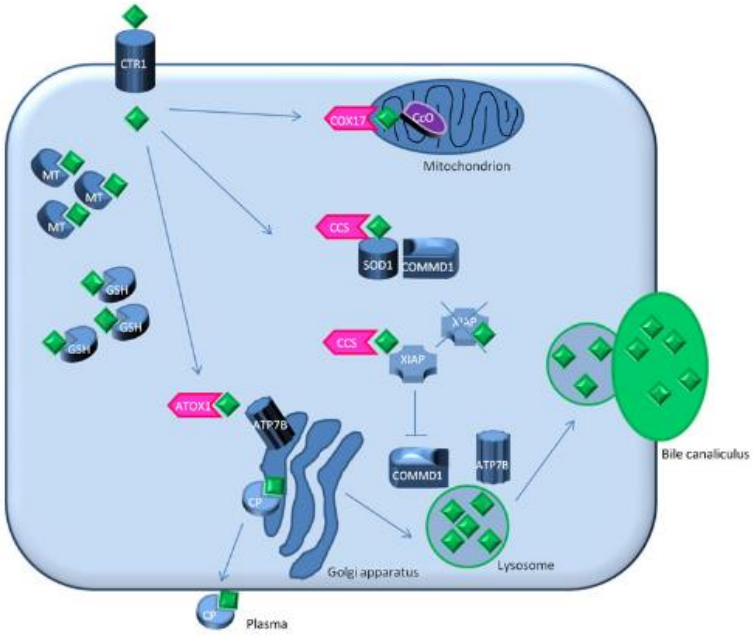

Figure 2: Copper metabolism in the hepatocyte. Copper enters the hepatocyte via CTR1. In the hepatocyte copper is shuttled by copper chaperones to destination molecules. Copper can be stored in the hepatocyte, secreted into the plasma or excreted into the bile. Figure derived from Fieten et al(2012)(1)

and kidney and has minor expression in brain, lung and placenta (3). Both ATP7A and B have a copper dependent localization. During elevated copper levels, ATP7A is relocated from the trans golgi network to the basal membrane. ATP7B is not recruited to the plasma membrane, but will transfer to cytoplasmic vesicles and apical vacuoles of the bile canaliculi. This trafficking of ATP7A and B is a reversible process if the copper concentration will normalize to basal levels(19). ATP7A is also required for the transfer of copper to other cuproenzymes such as peptidylglycine amidating monoxygenase (PAM) (8).

Copper chaperone for superoxide dismutase (CCS) shuttles copper to superoxide dismutase 1(SOD1) after entering the cell. Superoxide dismutase 1(SOD1) is a 154 amino acid protein and is present in both cytosol and mitochrondrial intermembrane space(IMS)(20). SOD1 is one of the first lines of defense against oxidative damage catalyzing the dismutation of superoxide to hydrogen peroxide and oxygen(21). Free copper ions are responsible for toxic superoxide anion radicals, which are bound by SOD1. SOD1 binds one atom of copper by four histidine residues in the SOD1 molecule. For the activity of SOD1, SOD1 requires three posttranslational modifications; copper and zinc acquisition, intramolecular disulfide bond formation and dimerization. SOD1 receives copper molecules from its copper chaperone, CCS in the cytosol and mitochondria.

CCS is a homodimer of 33 kDA subunits and is mainly localized in the cytoplasm(22). CCS has striking homology to SOD1 in its amino acid sequence (23). Human CCS is a 274-amino acid protein that contains three domains. CCS is responsible for copper insertion and disulfide bond formation of SOD1. Whether SOD1 is fully dependent on CCS for activity is not clear yet. Mammalian SOD1 can be dependent on CCS for activity (24), but recent studies found that SOD1 can be activated in a CCS and oxygen dependent manner(25).

Proteins COX17 and COX11 are copper chaperones for Cytochrome C Oxidase (CCO). CCO is the key enzyme of the respiratory chain of eukaryotic mitochondria that catalyzes reduction of molecular oxygen to water. CCO requires several cofactors, including three copper ions. COX17 and 11 deliver and insert copper into CCO(26).

In the hepatocyte COMMD1 may interact with ATP7B**.** COMMD1(previously called Murr1) plays a critical role in copper excretion as it is able to bind to ATP7B and ATP7A which is probably regulated by X-linked inhibitor of apoptosis XIAP(27). Data suggest that COMMD1 and ATP7B cooperate in the excretion of copper from hepatocyte(28).

Copper toxicosis and oxidative stress

As described before, copper can exist in a cupric and cuprous state. Transitions from Cu (II) to Cu(I) can in certain circumstances also results in the generation of reactive oxygen species (ROS). Both cupric and cuprous copper ions can participate in oxidation and reduction reactions. This radical forming is done via the Haber-Weiss reaction(29)(Figure 3).

Figure 3: Haber-Weiss reaction

This reaction results in several products and one of them is the toxic hydroxyl radical(•HO). The hydroxyl radical is the most powerful oxidizing radical in biological systems and is capable of reacting with practically every biological molecule (30). Hydroxyl radical can extract the hydrogen from an amino-bearing carbon to form protein radical as well as from an unsaturated fatty acid to form a lipid radical(31).

Because free copper results in toxic radicals, copper metabolism is tightly regulated preventing cellular free copper accumulation. Copper homeostasis is maintained by an increased intestinal copper absorption or urinary excretion and endogenous excretion via primarily the biliary route(2). This protects the body against respectively copper toxicity or deficiency. But when copper reaches an extremely high concentration, this mechanisms protecting the body from copper overload will fail. Copper toxicity primarily affects the liver because this is the first site of copper depositions after it enters the blood(32).

Copper related diseases

Human copper related diseases

Menke's disease

Menke's disease (MD) is an X-linked multisystemic lethal disorder of copper metabolism. There are several forms of MD. Patients can die in early childhood, but variable forms exist. Occipital horn syndrome (OHS) is the mildest form. The classical form can be recognized by progressive neurodegeneration and connective tissue dysfunction, and death occurs before the third year (43). OHS is characterized by radiographic observation of occipital horns, where exostoses can be found (43)

MD is caused by mutations in *ATP7A*(44). In patients with MD copper is trapped in enterocytes due to a dysfunction of *ATP7A*, leading to systemic copper deficiency(43). The incidence of this disease is 1 in 298.000 (100). Symptoms can be related to the lack of activity of copper-dependent enzymes (Table 1).

Mutations in ATP7A protein lead to a less functional ATP7A. Copper will accumulate to abnormal levels in organs except for brain and liver tissue. In the liver the low copper content is due to requirement of the metal in other tissues, rather than disturbed copper metabolism. This is because ATP7B is the main copper transporter in the liver (44).

Enzyme	*Biological activity*	*Symptom*
Cytochrome *c* oxidase	Cellular respiration	CNS degeneration
		Ataxia
		Muscle weakness
		Respiratory failure
Superoxide dismutase	Free radical scavenging	CNS degeneration
Ceruloplasmin	Iron and copper transport	Anemia
Hephaestin	Iron transport	Anemia
Tyrosinase	Pigment formation	Hypopigmentation
Dopamine	Catecholamine	Ataxia
β-hydroxylase	production	Hypothermia
		Hypotension
		Diarrhea
Peptidyl α-amidating enzyme	Activation of peptide hormones	Wide spread effects
Lysyl oxidase	Collagen an elastin cross-linking	Premature rapture of fet membranes
		Cephalohematoma
		Abnormal facies
		High-arched palate
		Emphysema
		Hernias
		Bladder diverticula
		Arterial aneurysms
		Loose skin and joints
		Osteoporosis
		Petechial hemorrhage
		Poor wound healing
		CNS degeneration
Sulfhydryl oxidase	Cross-linking of keratin	Abnormal hair
		Dry skin

Table 1: Consequences of loss of ATP7A function for enzymes, leading to different symptoms, figure of Tümer et al(2002)(44). CNS = central nerve system

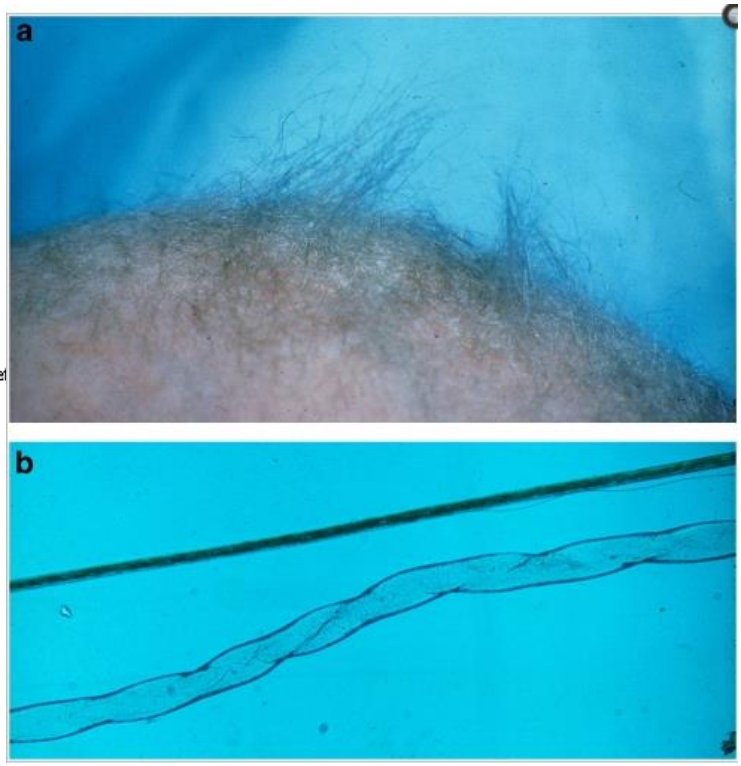

Figure 4: Kinky hair disease, picture from Tümer et al, 2010(44). a. clinical view of Menke's disease. b. typical hair changes seen with a microscope.

Genetics

In total 370 mutations in *ATP7A* are found in relation to Menke´s disease (44). Out of 370 mutations identified in Menke´s disease patients, the most frequently encountered mutation is a missense mutation Gly727Arg. Mutation analysis revealed 281 mutations that affect the mRNA transcription resulting in a nonfunctional truncated protein or nonsense-mediated decay mechanism.

Phenotype and genotype

There is no correlation identified between the mutations and clinical course of Menke's disease. A inter and intrafamilial phenotype variability is seen in MD/OHS patients carrying the same *ATP7A* mutation (44). In general, one patient with OHS have more mutations in *ATP7A*, but leads to a partially functional protein or reduced amount of normal protein.

Diagnostics and treatment, prognosis

Menke's disease can be diagnosed by typical hair changes and poor muscular development. Typical hair changes can also be seen at 1-2 months of age with a microscope, where a twisted hair shaft is seen(44) (Figure 4). At this age only subtle changes in the skin and muscle are present, which is very often overseen. Around 2-3 months patients develop seizures and additional symptoms of failure thrive including poor eating, vomiting and diarrhea. Muscular tone is often decreased in early life, but is later worsened by spasticity and weakness of extremities (45).

A reduced level of copper and ceruloplasmin in plasma is seen, however these findings are not specific for Menke's disease since copper and ceruloplasmin are low in newborns anyway (46). Identification of mutations in *ATP7A* is the ultimate diagnostic method.

Treatment of Menke's disease is mainly symptomatic and is based on providing copper to tissues and copper-dependent enzymes. Copper should be supplemented parenterally oral because when copper is supplemented orally it is trapped in the intestines (46). Early treatment of Menke's disease may have a beneficial effect on neurodevelopment, but does not remove all symptoms. There is no cure for Menke's disease. Response to copper treatment is also dependent on the type of underlying *ATP7A* mutations. Patients with mutations resulting in partial activity of the ATP7A protein are likely to have a better prognosis with treatment (46).

Wilson's disease

Wilson's disease is an autosomal recessive disorder of copper metabolism caused by mutations in the *ATP7B* gene. Wilson's disease occurs on average in 30 individuals per million (1 per 30,000) population (3).

Symptoms seen in Wilson's disease result from copper accumulation in tissues and can be divided in neurologic and hepatic symptoms although a mixture of these symptoms is often seen. Clinical signs of liver disease can vary between asymptomatic forms with elevated liver enzymes or liver enlargement, chronic hepatitis, and chronic liver failure. Also hemolytic anemia, coagulopathy and acute liver failure are often seen (47). Neurological symptoms are characterized by motoric dysfunction and can be unspecific, mild and therefore may be unrecognized(48). Symptoms can vary from changes in behavior, tremor, dystonia and spasticity(47).

Also psychiatric symptoms can be present in Wilson's disease patients, where depression is the most common symptom. Also neurotic or aggressive behavior may occur (3).

Age at the onset of symptoms is usually from 6 years old to 45 years old (3), but Wilson's disease with hepatic involvement is found in patients in their 70s (49). Wilson's disease is manifested more commonly as liver disease in children, but should be considered as the cause of any acute or chronic liver disease in adults (3). Signs and symptoms of Wilson's disease are rarely observed before age of 3 years, reflecting capacity of the liver to store excess copper.

In children hepatic symptoms of Wilson's disease are seen at an average age of 10–13 years, a decade younger than patients with neurological disease (51). Approximately 45% of all affected individuals present with liver disease, 35% with neurological signs and symptoms and 10% with psychiatric symptoms.

Diagnosis

Diagnosing Wilson's disease is difficult because there is no gold standard. The diagnosis is usually based on clinical findings and laboratory measurements.

Diagnosing neurologic Wilson's disease is straight forward if two of the following symptoms are present: Kayser-Fleischer rings, typical neurologic symptoms, and low serum ceruloplasmin levels.

Granular copper deposition in the cornea causes the Kayser-Fleisher rings seen with a slit lamp examination. Almost all neurologic Wilson's disease have Kayser-Fleisher rings, however, only 50% of the Wilson's disease patients have this clinical feature. Also patients with biliary cirrhosis can show Kayser-Fleisher rings (50).

Ceruloplasmin is an important copper transporter, which comprise 70% of all copper in plasma. In Wilson's disease patients, loss of ATP7B impairs holoceruloplasmin synthesis and biliary copper excretion. Because copper is trapped into the cell, copper cannot be incorporated into ceruloplasmin which results in no holoceruloplasmin and a degradation of the non-active ceruloplasmin (apoceruloplasmin). Therefore, low ceruloplasmin concentration can be a useful indicator of copper status (51). However, ceruloplasmin is an acute phase protein and therefore patients with chronic active hepatitis can have a normal ceruloplasmin activity(51).

Also hepatic and urinary copper could help diagnosing Wilson's disease. Unfortunately, they both have their limitations. Urine copper excretion will be increased in patients with Wilson's disease, but

copper excretion is also increased in other hepatic disorders and urine is often incorrectly collected (50).

A liver biopsy is needed for hepatic copper measurement. Hepatic copper is increased in 82% of patients with WD and usually exceeds 250 µg/g dry weight. In some patients liver biopsies have a high risk because of coagulopathy or ascites. Also in later stage of hepatic Wilson's disease, measurement of hepatic copper is less reliable because of unequally distribution of copper due to liver cirrhosis(3).

Because of the genetic background of this disease, the use of mutation analysis of *ATP7B* in patients could be useful. However, this approach is time-consuming and very costly. But, with allele-specific probes or haplotype analysis mutation diagnosis would be much easier and may be clinically relevant (50).

Gitlin et al. (2003) developed a scoring system for Wilson's disease and combines clinical symptoms and results of diagnostic tests (Table 2) (51).

Typical clinical symptoms and signs		Other tests	
Kayser-Fleischer rings		Liver copper (in absence of cholestasis)	
Present	2	>5 × ULN (>250 µg/g)	2
Absent	0	50–250 µg/g	1
Neurologic symptoms		Normal (<50 µg/g)	−1
Severe	2	Rhodamine positive granules[a]	1
Mild	1	Urinary copper (in absence of acute hepatitis)	
Absent	0		
Serum ceruloplasmin		Normal	0
Normal (>0.2 g/L)	0	1–2x ULN	1
0.1–0.2 g/L	1	>2x ULN	2
<0.1 g/L	2	Normal, but >5× ULN after D-penicillamine	2
Coombs' negative hemolytic anemia		Mutation analysis	
Present	1	Mutations on both chromosomes	4
Absent	0	Mutation only on one chromosome	1
		No mutations detected	0
Total score	Evaluation		
4 or more	Diagnosis established		
3	Diagnosis possible, more tests needed		
2 or less	Diagnosis very unlikely		

ULN, upper limit of normal.
[a]If no quantitative liver copper available.

Table 2: Scoring system for diagnosing Wilson Disease from Gitlin et al, 2003(51)

Treatment

Treatment of Wilson's disease consist of chelation therapy using d-penicillamin, trientine, or zinc acetate.

Both d-penicillamine and trientine are chelators that bind copper resulting in an increase of urinary excretion of copper (69). Trientine also enhances fecal excretion of copper. D-penicillamine causes a greater negative copper balance than trientine and is also more toxic (70). Together with chelation therapy zinc supplementation is recommended (70) because zinc interferes with the absorption of copper in the gastro-intestinal tract (71). Side effects seen during d-penicillamine treatment are hypersensitivity reactions, bone marrow suppression and development of autoimmune disease (51). Zinc acetate is used to inhibit copper absorption in the gastrointestinal tract and thus maintain neutral or negative copper balance in patients (72). Zinc treatment alone is recommended in patients after the return of normal copper balance with chelation therapy(73). Liver transplantation may be necessary in cases with severe cirrhosis. Future therapies for Wilson's disease are gene therapy and cell transplantation. These therapies are fully described elsewhere(73).

Prognosis

Patients with Wilson's disease have a good prognosis if the disease is diagnosed promptly and treated consistently. The best time to treat Wilson's disease has not been establish yet. Patients with early hepatic disease have a generally favorable diagnosis. Severe neurologic disease may not resolve entirely with treatment (3). Prognostic factors for Wilson's disease are the presence of jaundice and ascites, both associated with a higher mortality(74). Future therapies may help treat patients suffering from severe hepatic or neurologic clinical signs.

Genetics

Mutations in the ATP7B gene

The Wilson's Disease (WD) gene (OMIM 277900) was identified in 1993 and codes for a copper transporting P-type ATPase, ATP7B. ATP7B is a large gene of 1411 amino acids with a molecular mass of 165 kDa (3). It contains several functional domains: six copper binding domains, a transduction domain involved in the transduction of the energy of ATP hydrolysis to cation transport, a cation channel and phosphorylation domain, an ATP-binding domain and eight hydrophobic transmembrane sequences (50). Each domain has his own function and contribute to copper transport. ATP7B links copper to ceruloplasmin and removes excess copper via the bile. Both functions of ATP7B are impaired in Wilson´s disease, leading to copper accumulating in liver tissue and brain (Figure 5). This figure also shows copper disorder Menke's disease.

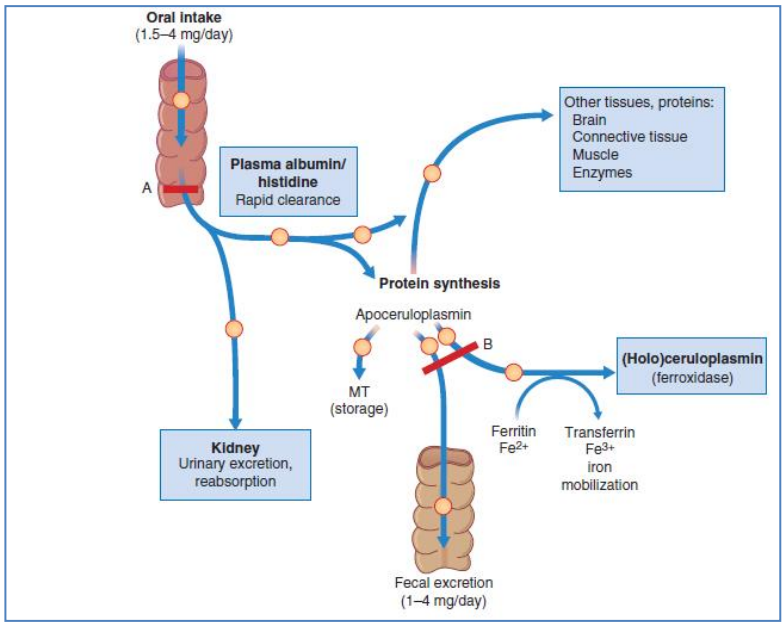

Figure 5: Simplified overview of the pathways for copper ion transport, with excretion predominantly via bile. MT, metallothionein (copper storage), represents copper. Two sites of transport disorders are shown: A, Menke's disease (*ATP7A* gene); B, Wilson disease. Figure from Roberts et al (3)

Molecular genetic analysis of patients with WD has revealed more than 518 variants (379 probable disease-causing and the remainder possible normal variants) from populations worldwide (Available at: www.medicalgenetics.med.ualberta.ca/wilson /index.php; Last accessed: 20 June 2007) (52).The mutations include deletions, insertions, and missense and nonsense mutations, which make up half of all mutations. Which mutation occurs most differs among population areas (Table 3).

Area	Most common mutation(exon)
Central Eastern, Northwestern Europe	H1069Q(14)
Sardinia	-441/-421 del(5'UTR)
Canary Islands	L708P(8)
Spain	M645R(6)
Turkey	R969Q, A1003T(13)
Brazil	3400delC(15)
Saudi Arabia	Q1399R(21)
Far East	R778L(8)

Table 3: Common mutations of Wilson's Disease Gene in different populations. Figure derived from Ferenci et al(2012)(50)

Depending on the domain where a mutation is located, each mutation leads to a different dysfunction of *ATP7B*. For example: *ATP7B* with mutation E1064A l loses to the ability to bind ATP entirely, whereas H1069Q mutant showed minimal binding (53). In contrast, the R1151H mutant exhibits only a 1.3 fold reduction in affinity for ATP. The C1104F mutation alters protein folding, whereas C1104A mutation does not affect the structure or function of the N-domain at all.

Relation between ATP7B mutations and phenotype of WD patients

Many articles have focused on explaining the phenotype of a patient with the ATP7B mutation they have.

Homozygosity for H1069Q is known to be associated with neurologic disease or late onset (54-56)(50). Contrary on this finding, no association was found between H1069Q and onset or occurrence of neurologic disease (57). R969Q appeared to show a late onset of disease with milder severity(54). In contrast, patients with 1711-IG→C showed an earlier onset of liver disease(56). Mutations in exon 8 commonly are also associated to liver disease (50).

Nonsense and frameshift mutations are associated with a severe phenotype, early disease onset and lower ceruloplasmin values (54,58,59).

In most cases no association between genotype and phenotype is found in Wilson's disease patients(60,61). Results of studies indicate that factors other than *ATP7B* mutations play a significant role in the pathogenesis(60). The age of onset is also hard to determine, as the diagnosis can be overlooked in asymptomatic patients. Also the absence of neurologic disease in patients with liver disease in childhood is often explained by the limited exposure time of the central nervous system, it is surprising that late-onset neurologic Wilson's disease can occur without any evidence of liver involvement.

This wide range of this disease cannot be explained just by different mutations in ATP7B. The number of different mutations makes it hard to find a clear association, because it is hard to determine which mutation or mutations cause the phenotype. Also many Wilson's disease patients carry two different mutations (so-called compound heterozygous).

Environmental, epigenetic and other genetic factors are thought to play a role in pathogenesis of Wilson's disease (62). Also gender is thought to play a role: the hepatic form of Wilson's disease occurs more frequently in women and women develop the neuropsychiatric form of this disease also

on average two years later compared to men. This may be due to the protective effect of estrogens and iron metabolism differences (63).

Modifier genes in Wilson's disease

A modifier gene affects the gene expression of other genes. Over the last years many articles have been published about modifier genes for certain disease. A new disease therapy has been proposed which is based on mimicking and perhaps to enhance the effects of naturally occurring genetic modifiers which can be not functional in a certain disease. This could improve the prediction, treatments and prevention of human diseases (64). For example; if ATP7A is less functional in Wilson's disease patients, therapy can be focused on restoring the function.

Nonsynonymous single nucleotide polymorphism(nsSNP) affect the amino acid sequence of a gene product and this can change protein function and contribute to diseases like Wilson disease. Different studies focused on COMMD1(Murr1) as modifier genes for Wilson's disease, but all concluded that this gene is not implicated in the pathogenesis (65-67). Also BIRC4/XIAP and ATOX1(67) have been tested as modifier genes. In the BIRC4/XIAP gene only significant differences in exon 6 concerning white blood cells count and lymphocytes were found (68). However, this difference could only be linked to XIAP function and not to phenotype and pathogenesis of Wilson's disease.

Based on literature it can be concluded that there´s probably a modifier gene for Wilson´s disease, however, the question remains; which gene modifies the phenotype of Wilson's disease patients? Therefore research has to be focused on finding this modifier genes which will unravel the genetic background of Wilson´s disease.

Canine copper related diseases

Canine copper toxicosis

In inherited primary copper storage disease copper accumulation is always centrolobularly localized (33). In secondary copper storage disease, due to cholestasis, copper is localized in the periportal parenchyma(33). Also hepatic copper values differ among primary and secondary copper storage disease; whereas secondary hepatic storage never accumulate much copper, hepatic copper values in primary copper disease diseases can be extremely high (34).

In 1968 normal hepatic copper concentrations in the dog were already reported to be around 82 µg/g (same as ppm) wet weight (35), which is extremely high in comparison with human and rat liver (12 and 19 times less). Hoffman et al found even higher hepatic copper concentrations in different breeds(Table 1) (36). In Dobermanns a wide range of hepatic copper concentrations is found, 100-700 ppm dw in healthy dogs. This is because copper in Doberman pinschers copper is often increased before the development of clinical hepatitis. Hepatic copper concentration in affected dogs with primary storage disease vary from 600 to above 2,200 ppm(33).

Typically, primary defects results in higher hepatic copper concentrations compared to secondary defects due to decreased hepatic function. However, the amount of hepatic copper accumulation does not reflect the severity of the hepatic disease in all cases. The proliferation of scar tissue during the development of cirrhosis dilutes the hepatocyte copper and this results in a relative lower hepatic copper concentration. Besides this, scar and cirrhotic tissue cannot take up copper(37).

The copper content found in the canine liver is from the same order of magnitude that is found in some patients with Wilson's disease (35). It is described that due to a high hepatic copper concentration, dogs are more sensitive to copper accumulating and are more sensitive to develop disease when dietary copper intake is high (35). This can be the result of a lack(38) or less(39) specific binding of copper to albumin which is specific for the dog, but more recent studies contradict this (40).

Canine copper associated liver diseases

Dogs with hepatic copper accumulation can appear normal over years before developing clinical signs, although copper may begin to accumulate by five to six months of age. In hereditary copper related diseases the severity of copper accumulation depends on severity of dysfunction (36). Also per breed hepatic copper value can differ (table 4).

Range ppm dw	Reference range	Dogs	Breed	Method
120-304	<400	6	Labrador retriever	NAA
100-700	197±113	13	Doberman pinschers	NAA
91-358	206±56	22	Bedlington terriers	SP
94-270	190±56	15	Mix breed dogs	SP

Table 4: Normal hepatic copper range per breed, listed by Hoffman et al, 2009(36). NAA: neutron activation analysis, SP: spectroscopy, ppm: parts per million(µg/g, mg/kg)

In Bedlington terrier copper toxicosis is caused by deletions in exon 2 of the COMMD1 gene. However, the presence of one or two normal copies of *COMMD1* exon 2 does not exclude BT from

copper toxicosis (1).These dogs accumulate hepatic copper, with values rising as high as 10,000 ppm dry weight (37).

Also in West Highland White Terrier (WHWT) a hereditary copper accumulation is found. However, in this breed the highest hepatic copper value recorded was 3,500 ppm. Also a difference is found in symptoms where some affected WHWT accumulate moderate excess and never develop liver damage, whereas affected Bedlington terriers always develop hepatitis (75).

In the Dalmatian a primary metabolic defect is described causing hepatic copper accumulation with hepatic copper above 3,000 ppm. The mechanism and genetic basis of this defect is unknown(76). Copper accumulation is also described for Sky terrier (77), Anatolian shepherd (78), and Dobermann (79).

Copper accumulation in the Labrador retriever

Symptoms of copper accumulation in Labrador retrievers are very non-specific, resulting from liver dysfunction. Symptoms start with mild decrease of activity or appetite with periods of normal behavior. After months till years, symptoms can vary from intermittent vomiting and nausea, polyuria and polydipsia, icterus, diarrhea, and ascites (36). There is a strong female predisposition and also nutrition plays an important role in the development of this disease (80). Labradors are often presented to the veterinarian in end-stage liver disease, when presence of liver cirrhosis precludes successful treatment (81).

Copper accumulation over time in Labradors

Affected Labradors start to accumulate copper around 5-6 months of age, and these dogs will accumulate copper over time and this occurs in three stages(36). First, excess copper accumulate in hepatocytes of zone 3, but will not result in inflammation. As the copper concentration increases further, the zone of hepatocyte with histochemically demonstrable copper granules expanding to zone 2. At the highest copper concentration, even the zone 1 hepatocytes contain hepatocytes with copper accumulation(37). After a period of several years, the second stage begins when copper accumulation results in inflammation, but no clinical signs are seen yet(41). This copper accumulation will eventually causes hepatitis. In this third stage, inflammation of the liver causes clinical signs. In this stage non-specific clinical signs such as anorexia, vomiting and weight loss can be seen(42). If no treatment is given in this stage, irreversible liver damage will be develop due to liver cirrhosis, which can be fatal.

Genetics

Copper accumulation in Labrador retriever is a disease with a complex genetic background. In 2008 Hoffman et al found (82) a complex inheritance pattern with a calculated heritability for copper accumulation traits of up to 0.85. In 2009 it was suggest to reveal the genetic defect causing hepatic copper accumulation in Labrador retriever. In 2013 Fieten et al (99) did this and found mutations in copper transporters *ATP7A* and *ATP7B* associated with respectively lower and higher hepatic copper levels. In both *ATP7A* and *ATP7B* a missense mutation was found, respectively T327I and R1452Q. These mutations were also evaluated functionally (99).

Diagnosis

Laboratory findings reveal an increase of ALT and ALP, which suggests liver disease and is not specific for hepatic copper accumulation (36). Currently, the only way to diagnose this disease in breeds other than Bedlington terrier, and to monitor treatment efficacy, is by quantitative evaluation of copper in hepatic biopsies (81). Histologically the disease is characterized by centrolobular copper accumulation together with inflammatory cell infiltrate (99). However, as said before, it is possible to find a copper lower than 2,000 ppm dw if significant cirrhosis is present. In this situation, primarily centrolobular inflammation is present(37).

Therapy

Therapy of copper accumulation is mainly focused on two goals; decrease copper intake and increase copper excretion. To decrease copper intake, copper-restricted diets are available for dogs. These diets are also enriched with zinc since zinc suppresses copper uptake (42). An increased copper excretion can be achieved by chelation therapy with d-penicillamine, a highly soluble degradation product of penicillin. d-penicillamine binds copper and promotes urinary copper excretion (69). Monitoring is needed during treatment since there is an inter-individual variation in treatment response (83), this may be due to variation in mutations in *ATP7A* and *ATP7B*. Copper and zinc deficiency are possible

side effects of therapy with d-penicillamine, therefore monitoring during treatment is necessary(83). I is recommended to use a combined therapy with both diet and d-penicillamine. Other side effects seen in human are not described in the dog except from nausea and vomiting.

Prognosis

When cirrhosis is present, the prognosis is very uncertain; however, preliminary data suggests that chelation therapy is even at this late time point beneficial (37). When the liver is not cirrhotic yet, the prognosis is good with treatment of d-penicillamin.

Biomarkers of copper status for in the dog

Evaluation of hepatic copper status

A biomarker for copper status in the dog would help to diagnose this disease at subclinical stage, or when the dog is presented at a clinical stage. A biomarker could also be used for monitoring during treatment of d-penicillamin.

Serum or plasma copper and ceruloplasmin are widely used to evaluate hepatic copper status in human. However, serum plasma does not correlate to hepatic copper status and ceruloplasmin only reacts on severe copper deficiency (84) or in patients with dysfunction of ATP7B . Also both measurements are influenced by age, sex and non-copper related processes like inflammatory diseases(85).

A recent study shows there is no correlation between hepatic and urine copper values (80), but there is a correlation between urine Cu/Zn ratio and hepatic copper status. Platelet cytochrome-c activity (CCO)(86) and diamine oxidase(DAO)(87,88) are described to reflect hepatic copper status. However, these enzymes have their limitations; CCO is a labile enzyme and DAO activity is also an indicator of tissue injury(89).Therefore these enzymes cannot function as biomarkers for copper status.

Some of these enzymes are already tested in the dog, but most of them not in relation to copper status. One study focused on blood copper enzymes during sustained strenuous exercise in sled dogs (90). Copper decreased during exercise, this might be due to increased urinary copper excretion. Also diamine oxidase, ceruloplasmin and SOD1 activity where decreased. No study has been performed which measures these enzymes during copper overload.

CCS and SOD1

Erythrocyte protein levels of CCS and SOD1 are known to reflect copper deficiency in rats, mice (91,92), and cattle (93). Whereas CCS levels tend to increase during copper deficiency, SOD1protein levels decrease during copper deficiency. However others describe that protein levels of CCS tend to be more sensitive compared to SOD1 protein levels(92), or even that SOD1 protein levels do not reflect copper deficiency (94).

Limited data is available about the effect of copper overload on erythrocyte CCS and SOD1 protein/mRNA levels. In rats CCS protein levels are influenced by an increased body copper load, leading to a decrease in CCS protein levels(95). In humans SOD1 protein levels decrease during copper deficiency(96).

On mRNA levels not all studies find a consistent result; It is reported that copper deficiency decrease SOD1 expression in organs of rats(97), but other studies report no change in mRNA levels (23,95,98). Since erythrocytes do not possess a nuclei, mRNA levels must be effected at posttranscriptional levels. It is described that copper loaded CCS is degraded faster than apoCCS and therefore CCS protein levels decrease with high copper levels (22).

However, as biomarker for copper accumulation for Labrador retriever CCS and SOD1 protein levels have not been tested yet in relation to hepatic copper levels. Therefore the question remains; are CCS and SOD1 biomarkers for hepatic copper status in the Labrador retriever?

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Chapter 2

Objectives

Chapter 2: Objectives

Scientific field

The research groups at faculty of Veterinary Medicine are divided over five thematic and interdisciplinary programs. This research program is embedded in the "Tissue Repair" group, which focusses on the pathophysiology of tissue dysfunction and potential repair mechanisms that adult stem cells could provide.

The Tissue Repair group contains three different research lines. The first research line focus on muscoskeletal system, the second on internal organs, especially the liver. The third research line focus on tumourigenesis.

This project is part of the second research line, the liver research group. Because of the great similarities between certain inherited and spontaneous diseases in animals and human patients, animals can be used as a model for comparative studies of pathogenesis and therapeutic intervention. This type of research is called translational medicine.

This project uses the similarities between animal and human patients for inherited liver diseases, where canine inherited copper storage disease is used as a model for human Wilson's disease and also the other way around.

Objectives

The objectives of the current honours program project were two-fold:

In the first part of the project, biomarkers for hepatic copper status in human, erythrocyte CCS and SOD1 protein levels, were tested in the Labrador retriever. Also in other species including mice, rats, cattle erythrocyte CCS and SOD1 protein levels are known to reflect both copper deficiency and overload. Therefore the aim of this project is to investigate whether erythrocyte CCS and SOD1 protein levels reflect hepatic copper status in Labrador retriever. Erythrocytes and liver biopsies of Labrador retrievers are used for quantifying CCS and SOD1 protein levels by Western blot. Also erythrocyte follow-up samples will be tested for erythrocyte CCS, SOD1 and CCS/SOD1 protein levels. First correlation is tested between liver CCS, SOD1 protein levels and hepatic copper. After this correlation is tested between erythrocyte and liver CCS (and SOD1) protein levels. Last correlation will be tested between erythrocyte CCS, SOD1 protein levels, CCS/SOD1 ratio and hepatic copper status, also in follow-up samples.

A hypothesis that was generated in the dog was tested in human patients. A genome wide association study was performed on Labrador retrievers and revealed missense mutations in *ATP7A* and *ATP7B*, associated with respectively lower and higher hepatic copper levels. The hypothesis that ATP7A could be a modifier gene in copper accumulation disorders was generated for Wilson´s disease patients. WD patients with mutations in *ATP7A*, that partly impair the function of the gene, may have a later age of onset than patients with no mutations in *ATP7A* . Therefore the aim of this project was to investigate whether copper transporter *ATP7A* could be a modifier gene with a gatekeeper function for copper absorption and this way influence age of diagnosis in Wilson's disease patients. Sanger sequencing of the exons and intron-exon boundaries of *ATP7A* will be performed on Wilson's disease patients. Association between mutation and age of diagnosis will be tested.

Chapter 3

Testing specificity of CCS and SOD1 antibodies for biomarker assays in the dog

Testing specificity of CCS and SOD1 antibodies for biomarker assays in the dog

Roelen Y[1] and Dirksen K[1], Fieten H[1], Spee B[1], Rothuizen J[1]

1. Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, 3584 CM Utrecht, The Netherlands

Abstract

Keywords: CCS, SOD1, transfection

Background: Copper toxicosis is a hereditary disease in the Labrador retriever. A non-invasive biomarker for hepatic copper status is needed to detect increased hepatic copper levels before clinical signs become apparent. Two promising biomarkers are copper chaperone for superoxide dismutase (CCS) and superoxide dismutase 1 (SOD1) which can be quantified in erythrocytes. CCS and SOD1 levels are known to reflect copper status in mice and rat. However, CCS and SOD1 antibodies are not validated for use in protein quantification of canine erythrocytes.
Objectives: To prove specificity of antibodies directed against human CCS and SOD1 and test whether they are applicable for use in protein extracts from canine erythrocytes.
Material and Methods: The specificity of antibodies for CCS and SOD1 was tested with siRNA mediated knock-down in canine bile-duct epithelial (BDE) cells and human hepatocellular carcinoma (HepG2) cells. Knockdown of CCS and SOD1 was tested on mRNA and protein level using qPCR and Western blot.
Results: qPCR confirmed a knockdown on mRNA level for CCS and SOD1 of respectively 93 and 73 percent in BDE cells compared to non-target siRNA controls 48 hours after transfection. In HepG2 cells, knockdown for CCS and SOD1 mRNA was respectively 97 and 94 percent. Western blot confirmed the knock-down in both BDEs and HepG2s given by the disappearance of the immunoreactive band for CCS and SOD1 for three consecutive days after transfection.
Conclusion: The loss of the immunoreactive band of CCS and SOD1siRNA treated cells confirmed the specificity of the antibodies. Therefore these antibodies can be used for CCS and SOD1 protein quantification in canine erythrocytes to investigate their use as biomarkers for copper toxicosis.

Introduction

Copper toxicosis is a hereditary disease in the Labrador retriever (1). Due to a disturbed copper metabolism copper accumulates in the liver leading to liver damage. Currently, the only way to establish the diagnosis is by means of a liver biopsy. Therefore, a non-invasive biomarker is needed. Two promising biomarkers in erythrocytes are CCS and SOD1, which are known to reflect copper status in mice and rats (2-4). Antibodies for human CCS (FL-274, Santa Cruz Biotechnology, Santa Cruz, CA) and SOD1 (FL-154, Santa Cruz Biotechnology, Santa Cruz, CA) were available for Western blotting. However, CCS and SOD1 antibodies were not validated for use in canine samples. Both antibodies are used for detection of CCS and SOD1 of mouse (5) and human (6,7) origin. This study proved specificity of antibodies for CCS and SOD1 by knockdown of both proteins in canine bile-duct epithelial (BDE) cells and human hepatocellular carcinoma (HepG2) cells. qPCR confirmed knock-down in HepG2 and BDE cells after siRNA mediated transfection and diminished protein levels were confirmed by Western blot analysis.

Material and methods

Cell-lines

In this study canine bile duct epithelial BDE cells, developed by the AMC experimental liver group, were used. These cells display characteristics of hepatocytes such as production of albumin and ceruloplasmin (8). As a control, human HepG2 cells (ATCC, HB 8065) were used. HepG2 and BDE cells were grown in DMEM (Life Technologies Inc., Invitrogen, Breda, The Netherlands) supplemented with 1% Penicillin streptomycin and 10% fetal calf serum (FBS; Harlan Sera-lab, Loughborough, United Kingdom).

Establishment of CCS and SOD1 knockdown

Canine and human specific siRNAs against CCS and SOD1 were transfected into BDE and HepG2 cells. For subsequent experiments, HepG2 cells were seeded in 6-well and 96-well plates at a concentration of respectively 200,000 and 7,500 cells per well on the day of transfection (reversed transfection). BDE cells were seeded in 6-well and 96-well plates at a concentration of respectively 30,000 and 1,000 cells per well one day prior to both transfection techniques (forward transfection).

For silencing experiments, specific Stealth RNAi™ siRNA duplex oligoribonucleotides were obtained from Invitrogen (Invitrogen Life Technologies) using the BLOCK-iT™ RNAi Designer. Specific sequences for silencing were selected for canine and human CCS and SOD1 and were designed based on their corresponding Genbank accession numbers (Table 1). Canine siRNA for CCS and SOD1 were used to establish knockdown in BDE cells and human siRNA for CCS and SOD1 were used to establish knockdown in HepG2 cells. A predesigned nonsense Stealth RNAi™ siRNA, which is not homologous to the vertebrate transcriptome, was used as a negative control (NT). Besides CCS, SOD1and NT siRNA transfected cells, a fourth group consisted of not treated transfected cells as these were only transfected with the transfection reagent and not with siRNA. Transfection was performed in triplicate on 96-well plates for mRNA analysis and in duplicate on the 6-well plates for protein isolation.

Species	Target	Sequence(5'→ 3')
Canine	CCS	CAGGCAUCCAGAGUGUUAAAGUGCA
Canine	SOD1	UGUACUAGUGCAGGUCCUCACUUUA
Human	CCS	GCAACAGCUGUGGGAAUCACUUUAA
Human	SOD1	AGGGCAUCAUCAAUUUCGAGCAGAA

Table 1: Sequences of canine and human siRNAs for CCS and SOD1.

HepG2 cells were transfected with a reverse transfection technique according to the manufacturer's instructions. For HepG2 cells, complexes of 10 nM siRNA and 3 µl/ml lipofectamine™ RNAiMAX were prepared inside the wells. After an incubation period of 20 minutes at room temperature, cells and medium were added. After 24 hours of incubation growth media including antibiotics replaced the transfection media.

For BDE cells, two transfection techniques were used. First BDE cells were transfected a forward transfection, using complexes of 10 nM siRNA and 3 µl/ml lipofectamine™ RNAiMAX were prepared outside the wells. After an incubation period of 20 minutes at room temperature, complexes of 10 nM siRNA and lipofectamine were added to the cells. After 24 hours of incubation growth media including antibiotics replaced the transfection media.

The second transfection technique that was used for BDE cells, a forward transfection with Lipofectamine 2000™ (Invitrogen) was performed in combination with a Magnet Assisted Transfection (MATra) technique (IBA BioTAGnology/Westburg b.v., Leusden, The Netherlands). Lipofectamine 2000™ (1.5 µl/ml), Lipofectamine MATRa enhancer reagent (1.5 µl/ml), and siRNA molecules (50nM) were added to the cells and incubated for 20 minutes on the plate magnet under cell-culture conditions. After 24 hours of incubation growth media including antibiotics replaced the transfection media.

RNA isolation and reverse-transcription polymerase chain reaction

For both BDE and HepG2 cell-lines total RNA was isolated for each group (CCS siRNA, SOD1 siRNA, nonsense siRNA, non-treated control) at day 2, 3, 4, and 7 after transfection. Hereto cells were washed twice with sterile filtered PBS, incubated with 20 µl/well Sample Preparation Reagent (SPR; Bio-Rad, Veenendaal, the Netherlands) for 1 minute before transfer to RNAse free strips. From all RNA samples cDNA was synthesized with the the iScript™cDNA Synthesis Kit (Bio-Rad).

Quantitative measurements of mRNA levels after transfection

Quantitative real-time polymerase chain reaction (qPCR) was performed on CCS, SOD1 and three endogenous reference genes; ribosomal protein S19 (RPS19), beta-2 microglobulin (B2M) and ribosomal protein S5(RPS5) as described previously (9). Perlprimer v1.1.14 was used for primer design on Genbank annotated transcripts and the amplicon was tested for secondary structures using MFold (The RNA Institute, University at Albany, http://mfold.rna.albany.edu/). Primer specificity was validated by DNA sequencing and melt-curves of the amplicon. Primers are listed in table 2. The qPCR reaction was performed in triplicate and was based on the high affinity double stranded DNAbinding dye SYBR green I (iQ SYBR green Supermix, BioRad, Veenendaal, The Netherlands) using a 2-step qPCR protocol. Standard curves, automatically constructed by plotting the relative starting amount *versus* threshold cycles, were generated using serial 4-fold dilutions of pooled cDNA. The endogenous reference genes were used to normalize the expression of the genes of interest, CCS and SOD1. Normalized expression of the target genes was calculated using the $2^{-\Delta Ct}$ method, corrected if efficiencies were not 100%. The average gene expression was normalized to the corresponding non target transfected to account for effects associated by the experimental transfection procedure.

Gene	Primers	Sequence(5'-3')	Tm(°C)	Product size	Accession no
Canine SOD1	Forward	tggtggtccacgagaaacgagatg	64	99	NM_001003035.1
	Reverse	tcagcaaaccgaacaccacagtaac			
Canine CCS	Forward	tgtggcatcatcgcacgctctg	64	96	NM_001194970.1
	Reverse	ctcctcgctccggccggg			
Human SOD1	Forward	cgagcagaaggaaagtaatgg	60	146	NM_000454.4
	Reverse	caggagtgaaattaggagataggt			
Human CCS	Foward	tcactttaaccctgatggagc	62,7	141	NM_005125.1
	Reverse	gcacttccacaccctaca			

Table 2: Sequences of primers for CCS and SOD1 used for qPCR

Western blot analysis

At day 2,3,4, and 7 after transfection BDE and HepG2 cells were washed twice with HBSS, centrifuged and resuspended in 350 µl RIPA buffer containing 1 % Igepal, 1 mM, PhenylMethylSulfonyl Fluoride (PMSF), 1 µg/ml aprotinine, and 1 mM sodium orthovanadate (Sigma

chemical Co., Zwijndrecht, The Netherlands) for 30 minutes on ice. Protein concentrations were obtained using a Lowry-based assay (DC Protein Assay, Bio-Rad) and subsequently proteins were denatured for 2 min at 95°C. If protein concentrations were too low, samples were concentrated using Amicon Ultra-0.5 Centrifugal Devices. As a control also canine erythrocyte were taken for Western blot analysis (Chapter 4). For the detection of SOD1 4 µg and for CCS 60 µg of protein from either BDE or HepG2 cells were separated over 15% Tris-HCl polyacrylamide gels (Bio-Rad) and transferred onto Hybond-C Extra Nitrocellulose membranes (Amersham Biosciences Europe, Roosendaal, The Netherlands). The membranes were blocked in TBS-Tween (0.1%) supplemented with 4% w/v nonfat dry milk (Bio-Rad) for 1 hour at room temperature. Membranes were incubated with a polyclonal antibody against CCS (FL-274, Santa Cruz Biotechnology, Santa Cruz, CA) and SOD1 (FL-154, Santa Cruz Biotechnology, Santa Cruz, CA) in a dilution of 1:1,000 in TBST with 4% BSA overnight at 4°C. After washing with TBS-Tween (0.1%), membranes were incubated with a mouse anti-rabbit horse radish peroxidase-conjugated secondary antibody (RD systems, Netherlands) at a 1:5,000 dilution in TBS with 4% BSA for 1 hour at room temperature. As a loading control a Beta-Actin antibody (Pan Actin AB-5, Neomarkers, Fremont, USA) in a 1:2,000 dilution was used. After washing with TBS-Tween (0.1%), antibody bound proteins were detected using the ECL Western blot analysis system, performed according to the manufacturer's instructions (Amersham Biosciences Europe). Images were captured with ChemiDoc XRS Chemi Luminescent Image Capture (Biorad). Density of immunoreactive bands were measured using Quantity one (Version 4.6.9, Biorad). Density of immunoreactive bands were corrected for background and normalized to actin.

Statistics

Knockdown of CCS and SOD1 was compared between target siRNA and non-target siRNA, where non target was treated as 100% and on this basis knockdown of siRNA treated cells was calculated in percentages (Table 1, column 6). qPCR results were analyzed with the statistical environment R (R 2.7.2. package for Windows, http://www.Rproject.org). A generalized linear model with relative expression as outcome variable was used with day and target protein (siRNA CCS or siRNA SOD1, and non-treated cells) as factor covariates. The best fitted model was chosen based on lowest AIC values. If there was a day*protein interaction, estimate knockdown with 95% confidence intervals was described per day including estimate knockdown in protein expression. If the best fitted model was only with target protein as covariate, estimate knockdown with 95% confidence intervals was described comparing non treated cells and CCS or SOD1 siRNA treated cells compared to day 2.

Results

Establishment of CCS and SOD1 knockdown

Knockdown of CCS and SOD1 in HepG2 cells was performed using SiRNA mediated knockdown using lipofectamine™ RNAiMAX. HepG2 cells were 90 % confluence at time of transfection

The first knockdown of CCS and SOD1 in BDE cells was performed using SiRNA mediated knockdown using lipofectamine™ RNAiMAX. BDE cells were 70 % confluence at time of transfection.

The second knockdown of CCS and SOD1 in BDE cells was performed using Lipofectamine 2000™ (Invitrogen) in combination with a Magnet Assisted Transfection (MATra) technique. BDE cells were 30 % confluence at time of transfection.

Quantitative measurements of mRNA levels after transfection

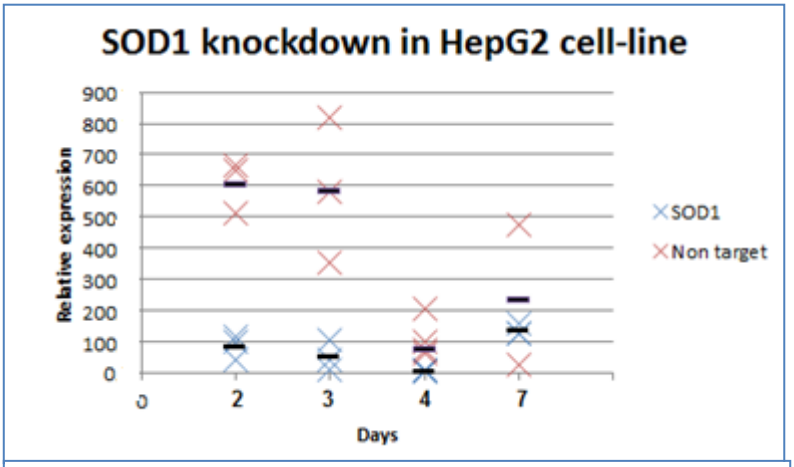
A linear model was used with relative expression of CCS or SOD1 as outcome variable and day, target protein(either non target or CCS/SOD1 siRNA treated cells) and the interaction between day and target protein as covariates. First the best fitted model was chosen based on AIC values.

For CCS and SOD1 expression in BDE cells SiRNA mediated knockdown using lipofectamine™ RNAiMAX was below 60%(data not shown). Therefore the second transfection technique using was performed.

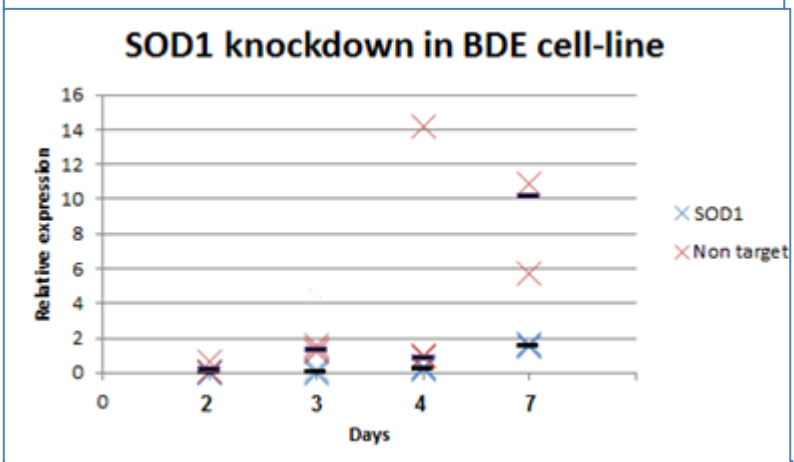
For CCS and SOD1 expression in BDE cells the best fitted model was with interaction between day and target protein as covariates. For CCS expression in HepG2 cells the best fitted model was with target protein as covariate. For SOD1 expression in HepG2 cells the best fitted model included day and target protein interaction.

For all models significant knockdown of CCS and SOD1 was established, for CCS in BDE cells at day two, for SOD1 in BDE cells at day 4 and 7. For HepG2 cells significant knockdown of CCS was established at day 3,4,7 and for SOD1 at day 4 and 7(Figure 1).

A



B



C

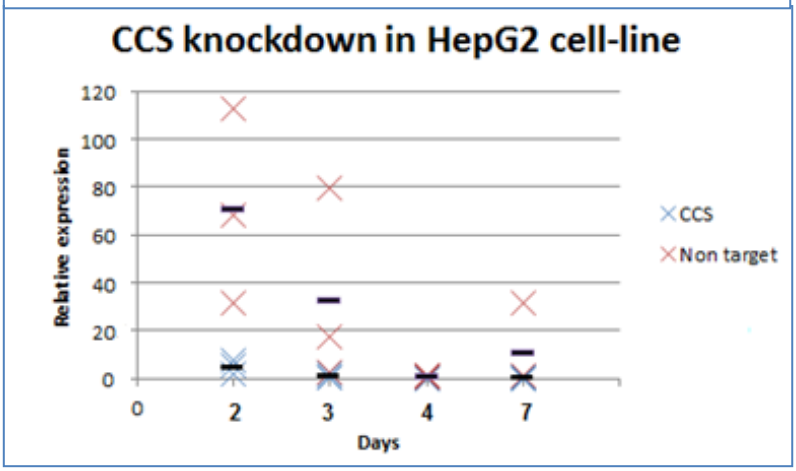
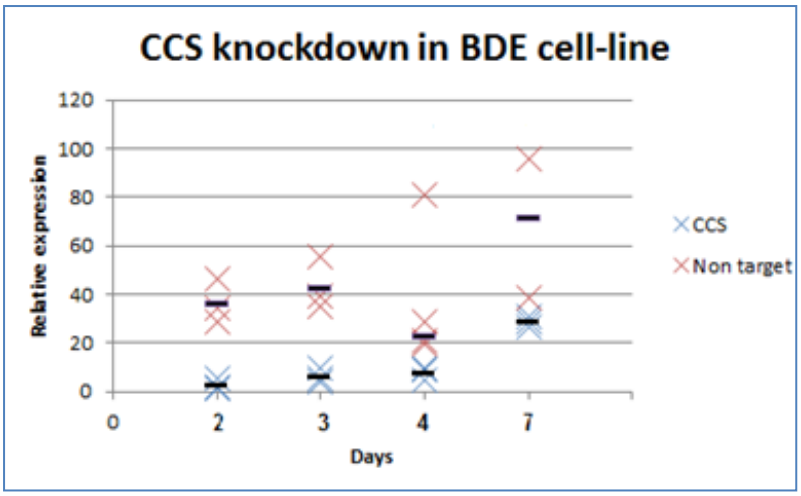


Figure 1: qPCR results of knockdown of CCS and SOD1 in HepG2 and BDE cells.

Figure A shows SOD1 knockdown in HepG2 cells, figure B shows SOD1 knockdown in BDE cell, figure C shows CCS knockdown in HepG2 cells and figure D shows CCS knockdown in BDE cells. X-axes shows time in days(2,3,4 and 7 days), y-axes shows relative expression normalized to references genes RPS19, RPS6 and B2M. Horizontal stripes indicate the median of CCS, SOD1 and non target protein expression. SiRNA mediated knockdown is performed per target in triplicate and also qPCR is performed in triplicate. In this graphs average of these qPCR triplicates is shown.

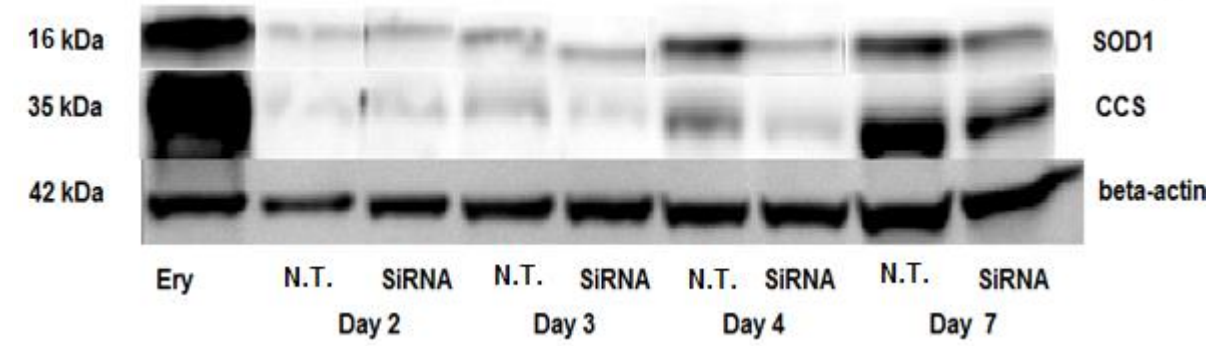
D



Western blot analysis

qPCR results of BDEs mRNA showed a low relative mRNA expression of CCS and SOD1(data not shown). Therefore protein samples of BDE cells were concentrated using Amicon Ultra – 0.5 Centrifugal Filter devices. Western blotting yielded a 35 kDA and 16 kDA immunoreactive band of respectively CCS and SOD1 in non-target siRNA transfected samples in BDE and HepG2 cells (Figure 2A.1 and 2A.2). Specificity was proven in controls without primary antibody, which were deemed negative (data not shown). The first lane shows canine erythrocytes as a control. It is notable that canine SOD1 is smaller compared to human SOD1(Figure 2.A.2). Densitometric analysis indicated a strong reduction of both CCS and SOD1protein levels in siRNA treated samples in both BDE and HepG2 cell line. Knockdown was calculated comparing non-target and CCS or SOD1 siRNA treated cells, where non-target was treated as 100%. CCS knockdown at protein levels was highest at day 4 for BDE cells with 75 percent (Figure 2B.2.1) and for HepG2 cell line at day 7 with 74 percent compared to control (Figure 2B.1.1). SOD1 knockdown at protein level was highest at day 4 for BDE cells with 77 percent (Figure 2B.2.2) and for HepG2 cells highest at day 4 with 88 percent (Figure 2B.1.2).

2.A.1



2.A.2

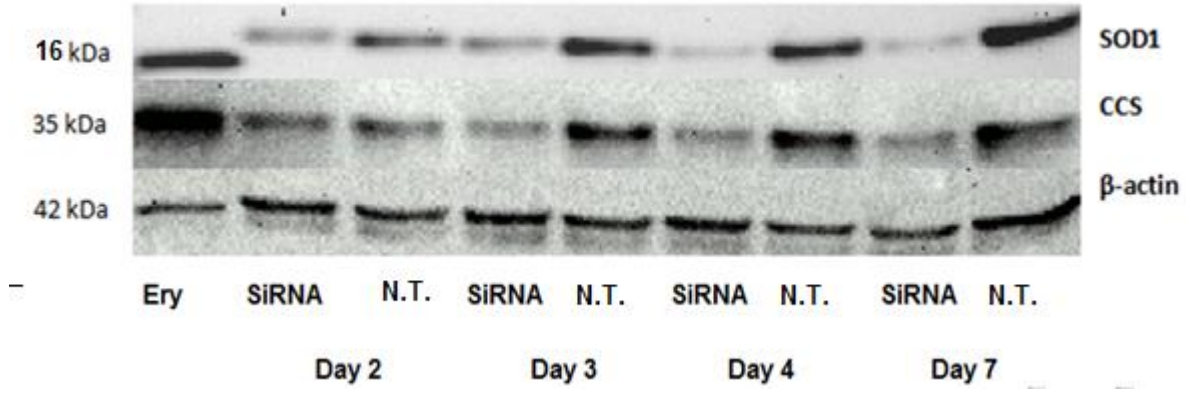


Figure 2: Western blot of knockdown of CCS and SOD1

A.1: Western blot of knockdown of CCS and SOD1 in BDE cells at day 2,3,4, and 7, normalized to actin. First lane shows canine erythrocytes. N.T. is an abbreviation for non treated.

A.2: Western blot of knockdown of CCS and SOD1 in HepG2 cells at day 2,3,4,7. N.T. is an abbreviation for non treated.

B.1.1

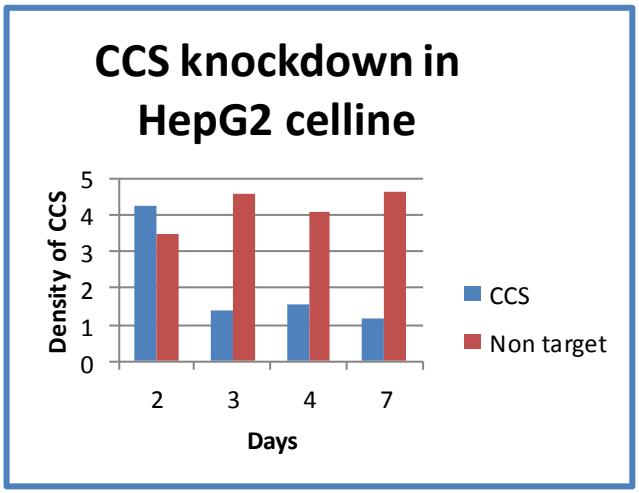


Figure 2B1.1: Quantification of Western blot: Knockdown of CCS in HepG2 cell line. Density of immune reactive bands was corrected with background staining and normalized to actin. Highest knockdown on protein level is established at day 7 with 74% knockdown

B.1.2

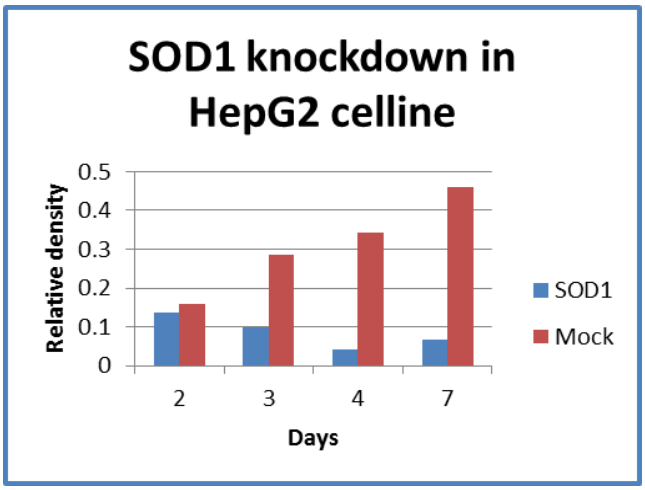


Figure 2B1.2: Quantification of Western blot: Knockdown of SOD1 in HepG2 cell line. Density of immune reactive bands was corrected with background staining and normalized to actin. Highest knockdown on protein level is established at day 7 with 88% knockdown.

B.2.1

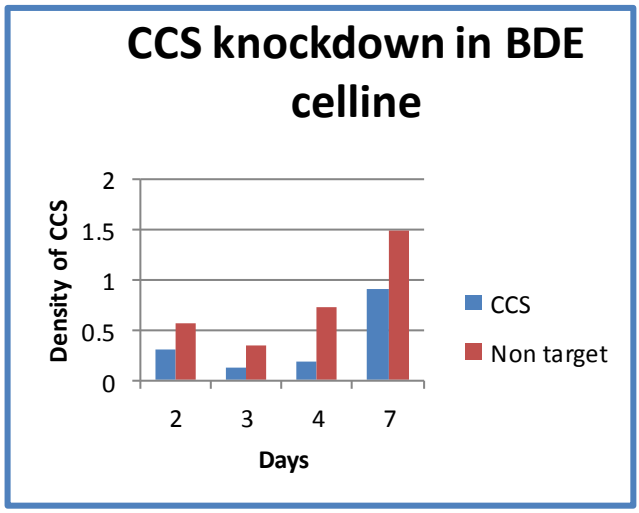


Figure 2B2.1: Quantification of Western blot: Knockdown of CCS in BDE cell line. Density of immune reactive bands was corrected with background staining and normalized to actin. Highest knockdown on protein level is established at day 4 with 75% knockdown.

B.2.2

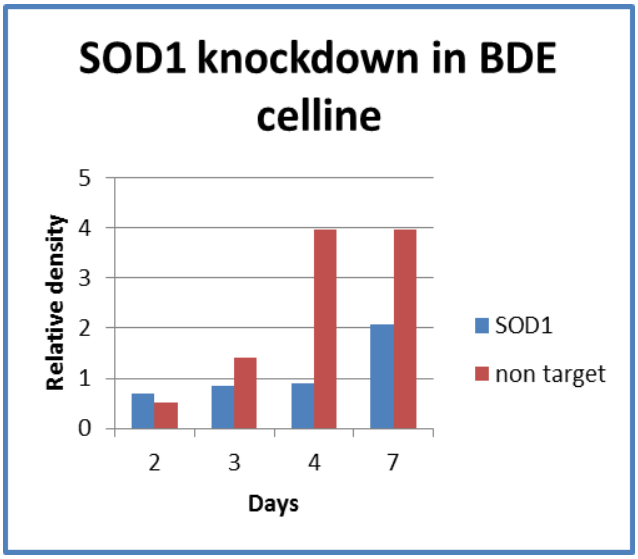


Figure 2B2.2: Quantification of Western blot: Knockdown of SOD1 in BDE cell line. Density of immune reactive bands was corrected with background staining and normalized to actin. Highest knockdown on protein level is established at day 4 with 77% knockdown.

Conclusion

Disappearence of the immunoreactive band with CCS and SOD1 siRNA treated cells confirmed the specificity of the antibodies used for CCS and SOD1. Therefore these antibodies can be used for biomarker assays in canine samples.

Discussion

Knockdown of CCS and SOD1 resulted in disappearence of the immunoreactive band of CCS and SOD1. This proves the specificity of the antibodies for CCS and SOD1.

The first transfection technique with lipofectamine™ RNAiMAX used on BDEs was not successful. Knockdown of CCS and SOD1 were less than 60% percent. Together with the wrong transfection technique for this cell line, this low knockdown can be due to over-confluence of cells at day 0. It is known that over-confluence at day 0 negatively influences siRNA transfection efficiency. HepG2 cells grow less fast compared to BDEs so this problem did not appear with HepG2 cells. In general it is recommended to start a transfection of BDEs with a 30 – 60% confluence. The Matra siRNA transfection technique used in this study was already proven to be successful on BDE cells, therefore this result is not surprising (10).

Western blot analysis showed a difference in the molecular weight between human and canine SOD1 protein. Human SOD1 protein showed an immunoreactive band on 23 kDa, where canine SOD1 shows an immunoreactive band on 16 kDa. The difference in molecular weight of canine SOD1 has already been reported before (11) but not shown on Western blot. The human SOD1 antibody is applicable for canine erythrocytes due similarity in target sequence between both species. Despite of this weight difference, BLAST analysis showed 80% similarity between human and canine SOD1 at the protein level. The difference in molecular weight might therefore be explained by difference on protein level. Human SOD1protein contains 154 amino acids, whereas canine SOD1 protein contains 150 amino acids. Ensemble shows a molecular weight of 15.5 kDA for canine SOD1, and 15.9 for human SOD1. Why this difference seems to be greater than 0.4 kDa, has to be further investigated.

QPCR analysis of CCS and SOD1 in BDE cells, transfected with MATra technique, showed a low relative mRNA expression, even in non-treated and non-target transfected cells. Also Western blot analysis of CCS and SOD1 in BDE cells showed a low protein level. This result is not shown in other studies, but magnetic beats may increase cytotoxicity. In this transfection technique siRNA and magnetic beats are drawn onto cells using a strong magnetic field. Magnetic beats may have a cytotoxic side effect. When cells have cellular stress, SOD1 mRNA levels will increase, because SOD1 prevents cellular damage (12). Our data indeed show an increase of SOD1 on mRNA level over time. Due to low mRNA levels of CCS and SOD1, CCS and SOD1 protein levels were also low. How this MaTra transfection technique leads to low mRNA levels of CCS and SOD1, has to be further investigated. The cytotoxic side effects could be investigated with a MTT assay which will prove the effect of the MATRA transfection technique on the BDE cells.

Antibodies for CCS and SOD1 are shown to be applicable for canine erythrocytes. As said before, differences between canine and human SOD1 makes this result surprising. This is also the case in CCS, where canine CCS has two transcript variants and human only one (Ensemble,database version 72). The antibodies are probably designed on parts of the canine and human antigen that are similar and do not have a mismatch.

Erythrocyte CCS and SOD1 levels will be tested as a potential biomarker of copper toxicosis in the Labrador retriever. If they show a correlation with hepatic copper, they will be used to identify dogs at risk so illness can be prevented. Moreover, this biomarker could be useful in monitoring copper status during and after treatment.

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Chapter 4

CCS protein levels and CCS/SOD1 ratio in erythrocytes reflect hepatic copper status in Labrador retriever

CCS protein levels and CCS/SOD1 ratio in erythrocytes reflect hepatic copper status in Labrador retrievers

Roelen Y[1] and Dirksen K[1], Fieten H[1], Spee B[1], Rothuizen J[1]

1. Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, 3584 CM Utrecht, The Netherlands

Keywords: Copper; hepatitis; Labrador; Biomarker; CCS; SOD1

Abstract

Background: Copper associated hepatitis is a hereditary disease in the Labrador retriever. Until now, diagnosis can only be made by means of a liver biopsy. A biomarker that can be obtained non-invasively and that correlates with hepatic copper concentration is necessary to identify dogs at risk in the subclinical phase, in which treatment is most effective. Promising biomarkers are erythrocyte CCS and SOD1 protein levels. Both are described to reflect both copper deficiency and copper accumulation in mice and rats.
Objectives: To determine whether CCS and SOD1 protein levels in erythrocytes reflect hepatic copper status in the Labrador retriever.
Material and methods: Erythrocytes of 25 Labrador retriever with hepatic copper ranging from 28 till 1,445 mg/kg dry weight liver were used for quantifying CCS and SOD1 protein levels by Western blot. Linear regression was performed and the model of best fit (with hepatic copper as a continuous variable or as a factor variable), including the covariates: sex (M,F) and previous treatment (nothing, D-penicillamin, diet) was determined. Follow up samples of two Labrador retrievers were used to test effects of CCS and SOD1 protein levels in association with hepatic copper levels over time.
Conclusions: Both erythrocyte CCS protein levels and CCS/SOD1 ratio showed a non-linear relationship with hepatic copper (ppm) in which hepatic copper was entered as a factor variable. Hepatic copper (ppm) was divided in three groups; hepatic copper of <400, 400-800, and >800 ppm. Erythrocyte CCS/SOD1 protein levels distinguish between hepatic copper <400 and >400 ppm whereas erythrocyte CCS protein levels distinguish between hepatic copper of 400-800 ppm and >800 ppm. Erythrocyte SOD1 protein levels were not significantly associated with hepatic copper concentration. Follow-up samples in Labrador retriever did not always show the same trend as individual animals. Further research has to focus on testing erythrocyte CCS protein levels and erythrocyte CCS/SOD1 ratio in more follow-up samples of Labrador retrievers and replicating these results in a bigger cohort of Labradors retrievers.
Clinical relevance:
Erythrocyte CCS protein levels and CCS/SOD1 ratio are significantly associated to hepatic copper levels and can serve as biomarkers for hepatic copper status. These parameters can be very useful for early diagnosis of hepatic copper toxicosis and can aid in non-invasive monitoring of chelation therapy.

Introduction

Copper accumulation in the Labrador retriever is a hereditary disease with a complex genetic background with a strong female predisposition (1,2). In affected Labrador retrievers copper accumulation may start at 5-6 months of age, and these dogs will accumulate copper over time. This copper accumulation will eventually cause hepatitis. When hepatitis is present, initially non-specific clinical signs appear, such as anorexia, vomiting and weight loss and when the disease progressed, clinical signs typical for parenchymal liver disease such as icterus, ascites and hepatic encephalopathy may appear (3). Diagnostic measurement reveals an elevation of alanine aminotransferase (ALT) activity. Histologically centrolobular localization of copper accumulation is found. Without chelation therapy affected Labradors will eventually develop chronic hepatitis and cirrhosis leading to liver

failure (4). At this stage signs of liver failure will appear, but in this phase treatment will not restore the normal liver function in most cases. Copper accumulation in Labrador retriever is diagnosed by means of a liver biopsy. A biomarker that can be obtained non-invasively and that correlates with hepatic copper concentration will be of great benefit to identify dogs at risk in the subclinical phase and thus to prevent clinical illness. Also, a biomarker can help monitoring during treatment to prevent d-penicillamin overtreatment, leading to copper deficiency (5). However, a biomarker for hepatic copper status does not exist yet.

Recent studies revealed promising biomarkers for hepatic copper status. Copper chaperone CCS is shown to reflect both copper deficiency and overload in erythrocytes in mice and rats (6-8). In cattle, erythrocyte CCS protein levels are described as biomarker for copper deficiency (9), where during copper deficiency erythrocyte CCS protein levels significantly increase. In humans, copper supplementation leads to a decrease in protein levels of CCS (10). Also superoxide dismutase 1 (SOD1) in erythrocytes ,which receives copper molecules from CCS, shows reflection on copper status(11), where during copper deficiency erythrocyte SOD1 protein levels significantly increase. Different reviews suggest erythrocyte CCS as most promising potential marker for deficiency as well as excess of copper (12,13). However, both CCS and SOD1 protein levels are not tested yet as biomarker for copper status in dogs.

The aim of this study was to determine whether erythrocyte CCS and SOD1 protein levels and CCS/SOD1 ratio reflect hepatic copper status and therefore can be applied as biomarkers. First the correlation between liver and erythrocyte CCS and SOD1 protein levels were tested to see if erythrocytes protein levels reflect liver protein levels. Furthermore CCS, SOD1 and CCS/SOD1 protein levels were tested in erythrocytes, and as a proxy for hepatic copper status. Results of this study can be used to develop a non-invasive diagnostic test for copper accumulation in Labrador retriever and may aid in early diagnosis and prevention of clinical illness.

Materials and Methods

Animals

All Labrador retrievers were client owned and presented to the Department of Clinical Sciences of Companion Animals, Utrecht University, the Netherlands in 2011 and 2012. Diagnosis of copper associated hepatitis was established through histological assessment of liver biopsy specimens and quantitative copper determination in liver tissue. Blood and liver samples were obtained after informed consent of the owners. Procedures were approved by the University's Ethical Committee on Animal Experiments as required under Dutch legislation.

Set up of the study

This study contains four parts. In the first part correlation between erythrocyte and liver CCS and SOD1was tested. In the second part correlation between liver CCS and hepatic copper was tested. Third, erythrocyte CCS and SOD1 protein levels and CCS/SOD1 ratio were tested for association with hepatic copper. In the fourth part follow-up samples in the same Labrador retriever were tested for erythrocyte CCS, SOD1 and CCS/SOD1 ratio.

Liver biopsies

Liver tissue was collected using a Tru cut device (14G needle) under ultrasound guidance. Two biopsies were used for histopathological examination and copper staining after fixation in 4% neutral buffered formalin and paraffin embedding. Two other biopsies were collected in cryo vials, fixed in RNAlater (Ambion, Austin, TX, USA) for a maximum of 24 hours or snap-frozen in liquid nitrogen

and stored at −70°C. A separate biopsy specimen of minimally 5mg was freeze dried prior to quantitative copper determination by instrumental neutron activation analysis. Dogs were considered to have normal hepatic copper concentrations if concentrations were below 400 mg/kg dry weight liver (dwl)(14).

Erythrocytes

EDTA blood samples were washed 3 times with PBS in a dilution of 1:10 and then centrifuged at 2,500 rpm for 5 minutes. Supernatant was pipetted off the erythrocyte pellet and the pellet was stored at -70 degrees until protein isolation.

Protein denaturation

The snap-frozen liver tissue was suspended in 200 µl of RIPA buffer containing 1 mM PhenylMethylSulfonyl Fluoride (PMSF, Sigma Aldrich, Steinheim, Zwitserland, 1 µg/ml aprotinine (Sigma Aldrich, Steinheim, Zwitserland), and 1 mM sodium orthovanadate (Sigma Aldrich, Steinheim, Zwitserland). Erythrocytes were suspended 1:1 RIPA buffer. Protein concentrations were obtained using a Lowry-based assay (DC Protein Assay, BioRad, Veenendaal, The Netherlands). Protein denaturation was performed by heating the samples for 2 min at 95°C prior to Western blot.

Western blot

For the detection of SOD1 10 µg erythrocyte protein and 4 µg liver protein was used and for the detection of CCS loading of protein respectively 60 µg and 17 µg of protein. Electrophoresis and blotting was performed as described in chapter 3. Membranes were incubated with a polyclonal antibody against anti-rabbit CCS (FL-274, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-rabbit SOD1 (FL-154, Santa Cruz Biotechnology, Santa Cruz, CA) in a dilution of 1:1,000 in TBST with 4% BSA overnight at 4°C. Specificity of the antibodies was tested by siRNA mediated knockdown of CCS and SOD1 (Chapter 3). As a loading control a polyclonal anti-mouse GAPDH antibody (Sigma-Aldrich, Missouri, USA) in erythrocytes and a monoclonal anti-mouse Actin (Pan Actin Ab-5 Neomarkers, Fremont, California) in liver tissue was used, both in a dilution of 1:1,000. Membranes were probed with GAPDH overnight in 4°C. After washing with TBS-Tween (0.1%), antibody bound proteins were detected using the ECL Select Western blot Detection Reagent, performed according to the manufacturer's instructions (GE Healthcare Life sciences, Italy). Images were captured with ChemiDoc XRS Chemi Luminescent Image Capture(Biorad). Density of immunoreactive bands was measured using Bio-Rad Version 4.6.9. Density of immunoreactive bands were corrected with background staining and normalized to the density of immunoreactive bands of GAPDH.

Statistics

Results were analyzed with the statistical environment R (R 2.7.2. package for Windows, http://www.Rproject.org). First a linear regression model with liver CCS, SOD1, and CCS/SOD1 protein expression as outcome variable was used to test the association between the protein levels and hepatic copper concentration. Second a linear regression model with erythrocyte CCS protein levels as outcome variable was used to the test the association between the erythrocyte and liver CCS protein levels. The same model was used for SOD1 protein levels. Second a linear regression model with erythrocyte CCS, SOD1 and CCS/SOD1 protein expression as outcome variable was used to test the association between the protein levels and hepatic copper concentration. For all models age of the dog at the time of biopsy (years), gender (male, female) and treatment in the period preceding the collection of biopsy (no treatment, dietary treatment with Royal Canin hepatic dry diet, D-penicillamin treatment in a dose of 20 mg/kg/day) were added in the model as covariates. The model was compared to a model without hepatic copper and a model in which hepatic copper was modeled as a factor by creating three groups (low copper (<400 mg/kg dwl), medium copper (400-800 mg/kg dwl), and high

copper (800-1000 mg/kg dwl). The model of best fit was determined by using Akaike's information criterium.

Results

Animals

Labrador retrievers used for this study had an average hepatic copper of 559±360.6 ppm and an average age of 8.6±2.4 years (Table 1). As said before, this study contains four parts, which are named 1 till 4 (Table 1). The treatment six months before liver biopsy is also listed in Table 1. Labrador retrievers could either receive d-penicillamin treatment or diet, or no treatment. Part one and two are only performed at 15 Labrador retrievers, because only for these samples both liver tissue and erythrocytes were available. For this fourth part of this study, follow-up samples of two Labrador retriever were available.

Number	Gender	Years	Hepatic copper value	Follow-up	Diet/Treatment	Study
168_9	F	6.5	28		Pen	1,2,3
189_6	F	7.2	177		Diet	3
236_7	F	9.5	224		Diet	1,2,3
255_4	F	7.2	229		Diet	1,2,3
371_1	M	11.5	250		Nothing	1,2,3
377_1	M	7.9	318		Nothing	3
369_1	F	3.6	356		Nothing	3
141_5	M	9.3	393		Diet	3
215_6	M	6.5	409		Diet	1,2
365_1	F	9.1	444		Nothing	1,2,3
141_6	M	9.9	446		Diet	1,2
186_8	F	7.3	482		Diet	1,2,3
283_3	M	10.8	509		Diet	1,2,3
208_7	M	8.4	526		Pen	3
277_4	F	10.9	570		Pen	3
252_6	F	11.9	601		Diet	3
212_7	M	5.8	643		Diet	3
237_5	F	6.1	664		Diet	3
208_6	M	7.9	677		Diet	1,2,3
301_3	F	5.3	720		Diet	1,2,3
376_1	M	12.3	795		Nothing	1,2,3
362_1	M	6.2	1146		Nothing	1,2,3
375_1	M	10.1	1310		Nothing	3
275_6	F	7.0	741		Diet	3
275_4	F	6.0	55	1	Pen	1,2,3,4
275_5	F	6.5	462	2	Diet	3,4
361_1	F	10.8	1270	1	Nothing	1,2,3,4
361_2	F	11.3	121	2	Pen	3,4
361_3	F	11.4	756	3	Nothing	3,4

Table 1: Table of used Labrador erythrocytes with corresponding hepatic copper value. Gender(Female or male) and age when liver biopsy was taken(years) are listed. Follow-up samples of two Labrador retrievers were available and are named 1,2 and 3. Labrador retrievers received no treatment(nothing), d-penicillamine(Pen) or diet(Diet). Samples were used for different parts of this study. Samples which were used for the first part of the study(1) were used to test association between erythrocyte and liver CCS/SOD1 protein level. Samples for the second part(2) of this study were used to test association between liver CCS/SOD1 protein levels and hepatic copper status Samples used for the third part of this study(3) were used to test association between erythrocyte CCS,SOD1 protein levels and CCS/SOD1 ratio and hepatic copper status. Samples used for the fourth part of this study(4) are follow-up samples of Labrador retrievers and were used for measurements CCS, SOD1 protein levels and CCS/SOD1 ratio over time.

Western blot analysis

Relation between liver and erythrocyte CCS protein levels

Liver and erythrocyte CCS protein levels were positively correlated (r^2=0.37, *p=0.02*) (Figure 1).

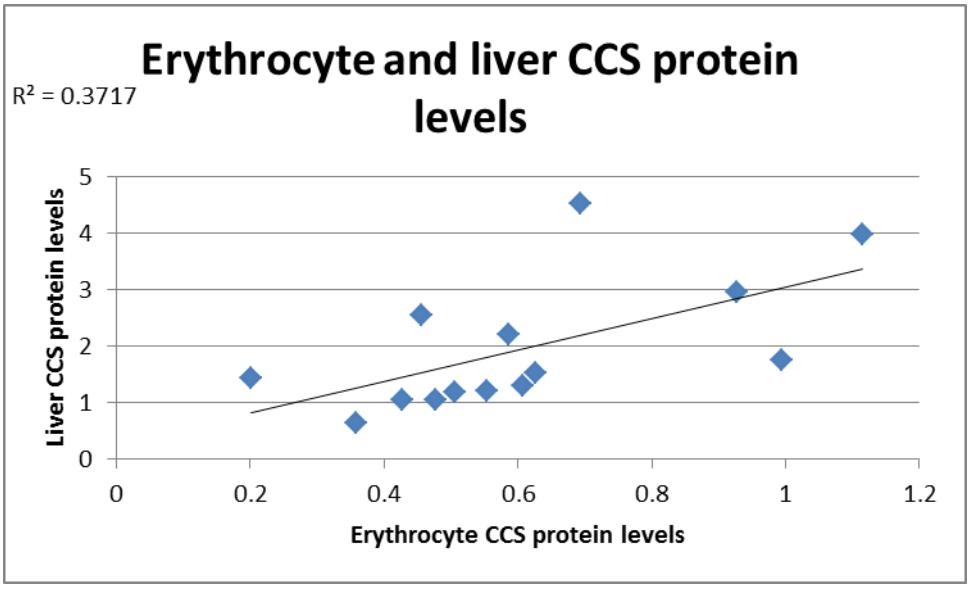

Figure 1: Scatterplot of correlation between erythrocyte and liver CCS protein levels

Erythrocyte and liver CCS protein levels were positively correlated(R^2=0.37, p=0.02)

Relation between liver and erythrocyte SOD1 protein levels

Erythrocyte and liver SOD1 protein levels were positively correlated (R^2=0.32, *p=0.03*) (Figure 2).

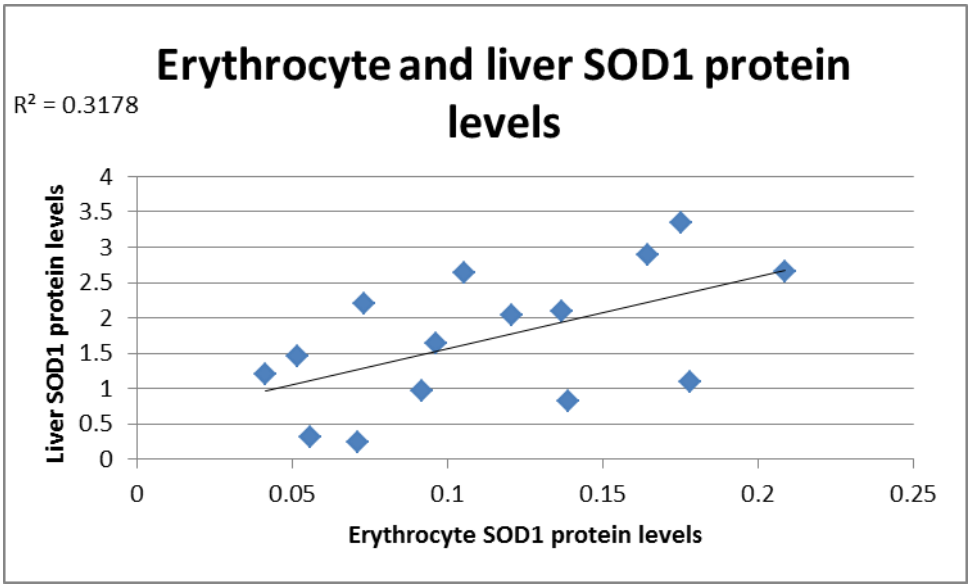

Figure 2: Scatterplot of correlation between erythrocyte and liver SOD1 protein levels

Erythrocyte and liver SOD1 protein levels were positively correlated(R^2=0.32, p=0.03)

Relation between hepatic copper values and liver CCS and SOD1 protein levels

Liver CCS protein levels were negatively correlated with hepatic copper values ranged from 28 to 1,270 ppm (r^2=0.37, p=0.02) (Figure 3 and 4). Liver SOD1 protein levels showed a positive correlation with hepatic copper levels, however this correlation was not significant (*p>0.05* (Figure 3 and 5). Beta-actin was used as a loading control for both CCS and SOD1.

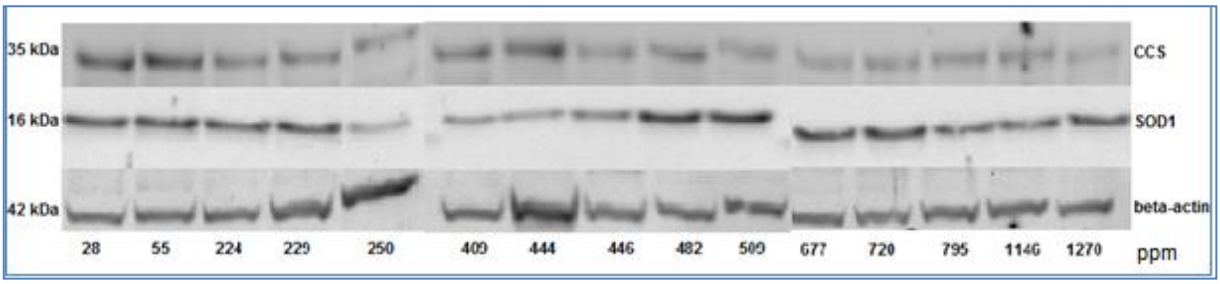

Figure 3: Western blot of CCS, SOD1 and beta-actin in liver tissue with hepatic copper(ppm) of 28 to 1270 mg/kg. Beta-actin was used as loading control. X-as shows hepatic copper(ppm), y-as shows on the left the weight(kDa) and on the right CCS, SOD1 and beta-actin.

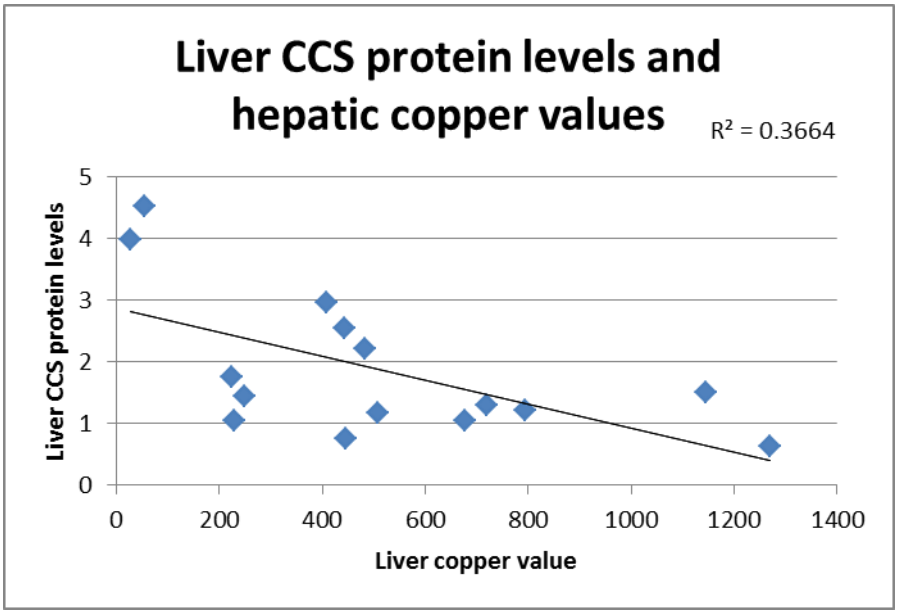

Figure 4:Scatterplot correlation between liver CCS protein levels and hepatic copper values(ppm).

Liver CCS protein levels were negatively correlated with hepatic copper values(r^2=0.37, *p=0.02*).

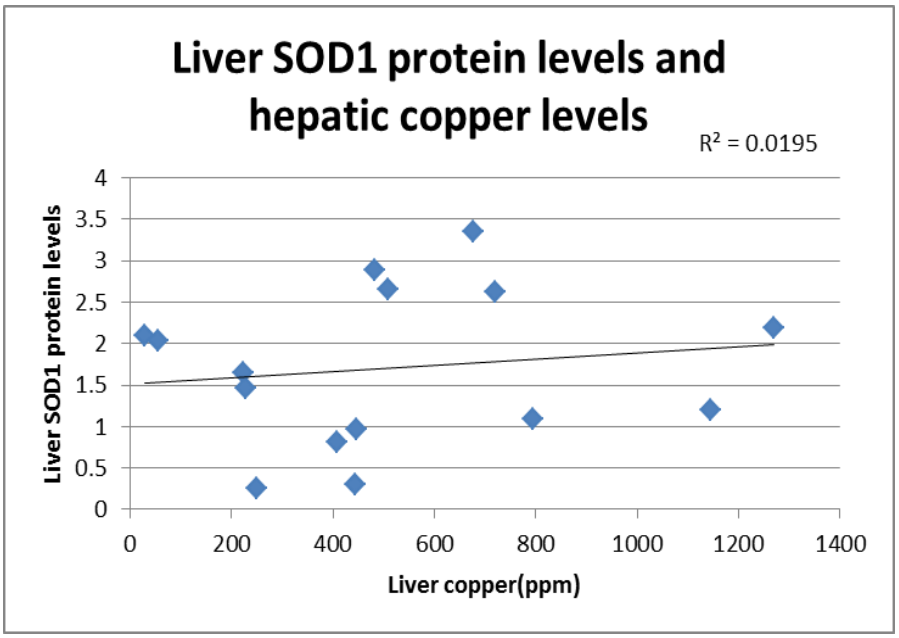

Figure 5:Scatterplot of liver SOD1 protein levels and hepatic copper values(ppm).

Liver SOD1 protein levels were not significant correlated with hepatic copper values(r^2=0.02, *p>0.05*).

Relation between hepatic copper concentration and erythrocyte CCS protein levels

Gender, treatment and age did not have a significant effect on CCS protein levels. CCS protein levels and hepatic copper concentration showed a non-linear relationship and the model in which hepatic copper was entered as a factor variable showed the lowest AIC.

Three groups of erythrocyte CCS protein levels were detected (Figure 6 and 7). One group of hepatic copper levels below 400, which had high CCS protein levels, one group of hepatic copper levels between 400 and 800 ppm, which had lower CCS protein levels and one group of hepatic copper levels above 800 ppm, which had higher CCS protein levels than the medium hepatic copper levels group. Therefore we used a regression model with erythrocyte CCS protein levels as outcome and these three groups as factors. This model showed a significant difference between these groups. Between the group with hepatic copper <400 and hepatic copper between 400-800 the estimate decrease of 1.41 CCS protein levels was seen (95% confidence interval[CI], 1.72 - 1.10). Between <400 hepatic copper and hepatic copper >400 an estimate decrease of 0.79 was seen(95% CI, 1.18 – 0.40).

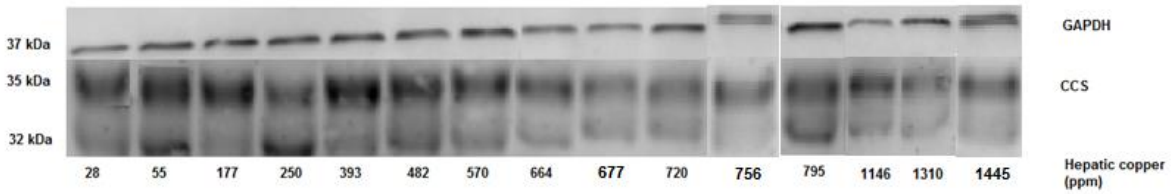

Figure 6: : Western blot of CCS protein levels of different hepatic copper levels ranged from 28 till 1445 ppm. GAPDH was used as loading control.

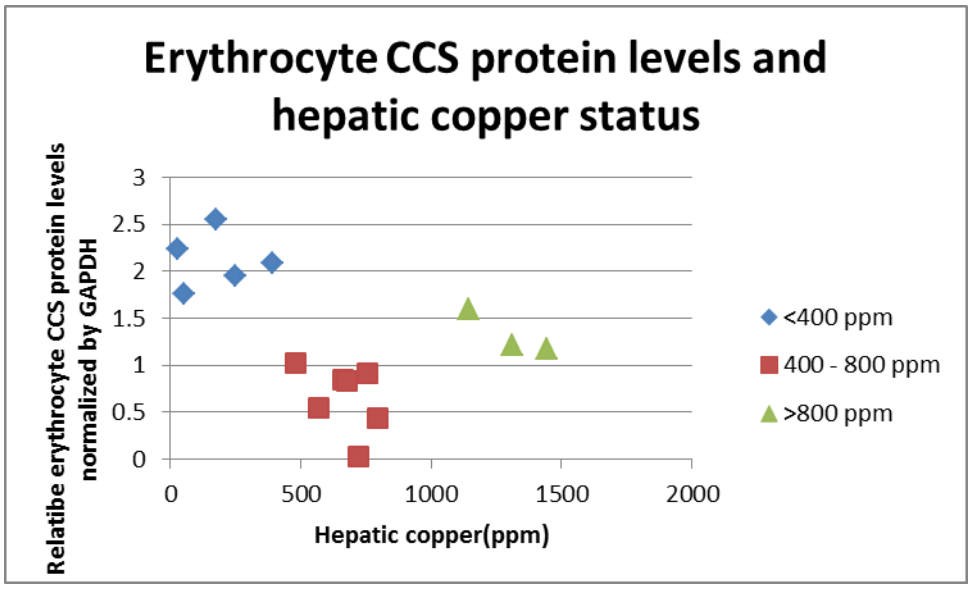

Figure 7:Scatterplot of erythrocyte CCS protein levels and hepatic copper status. Three groups were detected in this data; hepatic copper levels below 400 ppm, hepatic copper levels between 400-800 ppm and hepatic copper levels above 800 ppm. These data were analyzed with a regression model with hepatic copper as factor variable.

Relation between hepatic copper concentration and erythrocyte SOD1 protein level

Erythrocyte SOD1 protein levels were not significantly correlated with hepatic copper values, ranged from 28 till 1,445 ppm (r^2=0.23, *p>0.05*) (Figure 8 and 9). Gender, treatment and age did not have a significant effect on SOD1 protein levels. GAPDH was used as loading control.

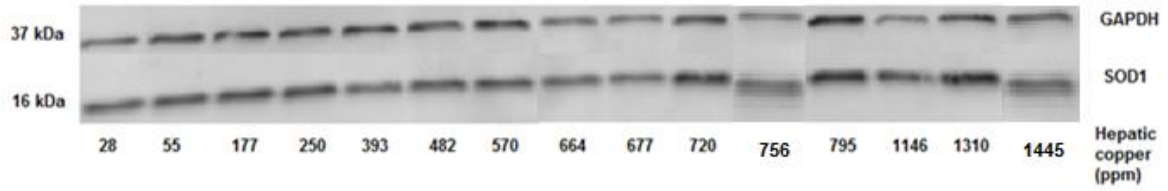

Figure 8: : Western blot of CCS protein levels of different hepatic copper levels ranged from 28 till 795 ppm. GAPDH was used as loading control.

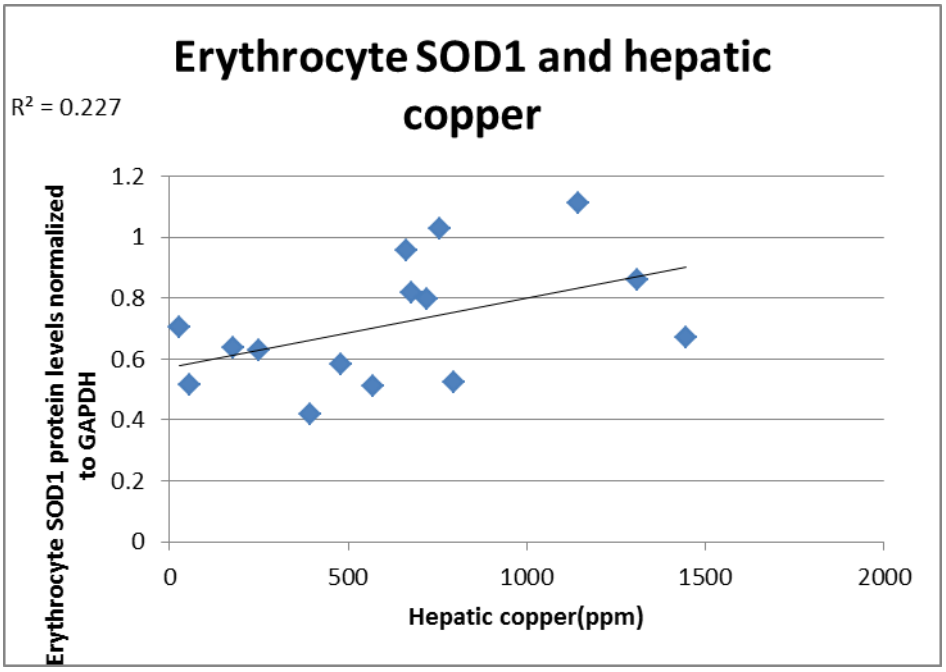

Figure 9:Scatterplot of erythrocyte SOD1 protein levels and hepatic copper values(ppm).

Erythrocyte SOD1 protein levels not significantly correlated with hepatic copper values(r^2=0.23, p>0.05).

Relation between hepatic copper concentration and erythrocyte CCS/SOD1 protein level

Gender, treatment and age did not have a significant effect on CCS/SOD1 protein levels. CCS/SOD1 protein levels and hepatic copper concentration showed a non-linear relationship and the model in which hepatic copper was entered as a factor variable showed the lowest AIC. This model showed a significant difference between these three groups(Figure 10). Between the group with hepatic copper <400 and hepatic copper between 400-800 the estimate decrease of 2.75 CCS/SOD1 ratio was seen(95% confidence interval[CI], 3.37 – 2.13). Between <400 hepatic copper and hepatic copper >400 an estimate decrease of 2.27 was seen(95% CI, 3.04 – 1.49).

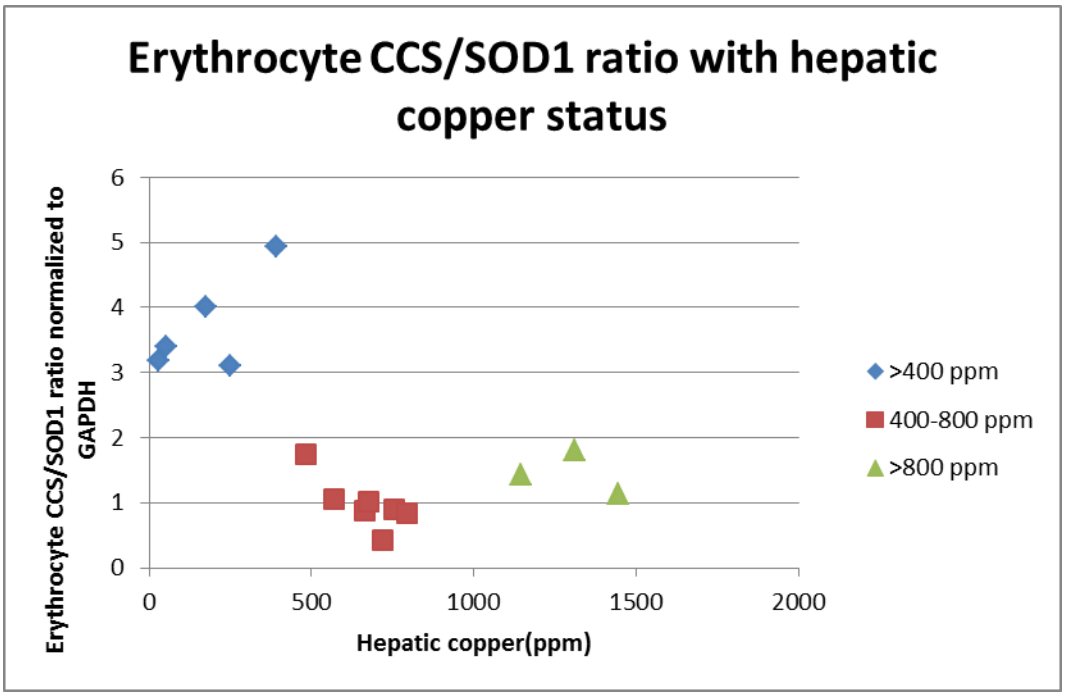

Figure 10: Graph of erythrocyte CCS/SOD1 protein levels in hepatic copper from 28 till 1445 ppm.

Relation between erythrocyte CCS protein levels and hepatic copper values in follow-up samples.
In two Labrador retrievers erythrocytes were collected corresponding with two or three liver biopsies taken at different time points.

The first Labrador (number 275) received d-penicillamin, a control liver biopsy showed a hepatic copper of 54 ppm. After this the treatment changed to low copper diet. After a half year a control liver biopsy was taken and hepatic copper was increased to 462 ppm. After this the diet was continued, and after half a year a liver biopsy was taken. Hepatic copper was increased from 462 to 741 ppm.

For follow-up samples 1 and 2 the same trend was seen as in individual Labrador retrievers. Between time point 1 and 2 hepatic copper increased, which leads to an decrease on erythrocyte CCS protein levels, increase of SOD1 protein levels and decrease of CCS/SOD1 protein levels. For follow-up sample 3 a different trend is seen. Between time point 2 and 3 hepatic copper increases, which leads in these sample to an increase of CCS protein levels, a decrease of SOD1 protein levels and an increase of CCS/SOD1 protein levels (Figures 11,12, and 13).

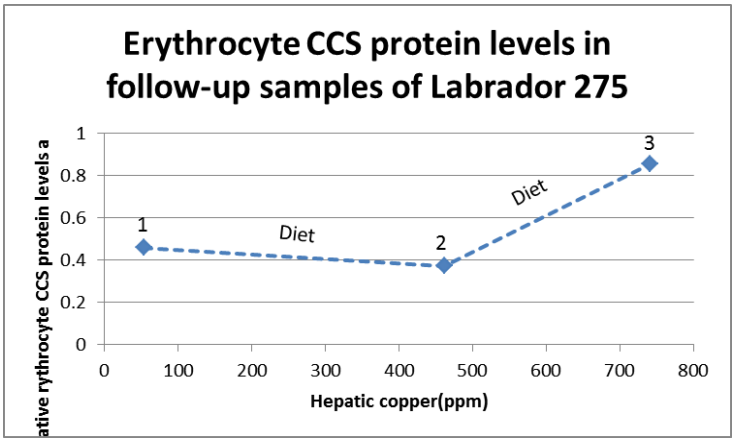

Figure 11: Erythrocyte CCS protein levels in follow-up samples of Labrador retriever. After d-penicillamin treatment, a liver biopsy was taken, hepatic copper was 54 ppm(Time point 1). After this diet was given to this Labrador retriever and after half a year a liver biopsy was taken and hepatic copper was increased to 462(Time point 2). This diet were continued, and after half a year hepatic copper was increased from 462 to 741 ppm(time point 3).

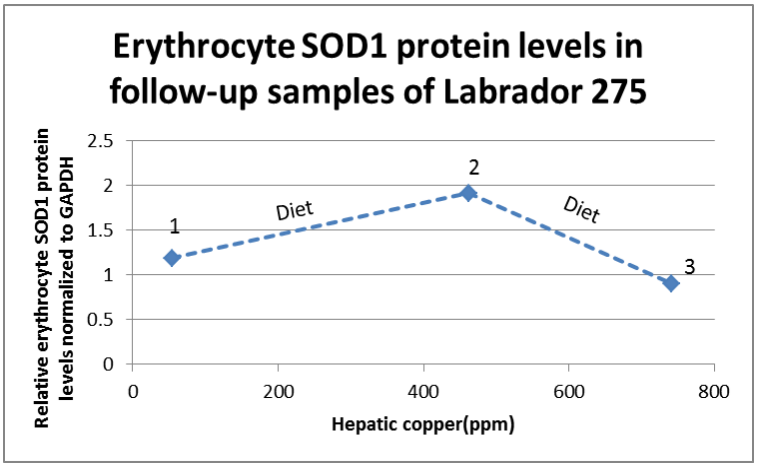

Figure 12: Erythrocyte SOD1 protein levels in follow-up samples of Labrador retriever. After d-penicillamin treatment, a liver biopsy was taken, hepatic copper was 54 ppm(Time point 1). After this diet was given to this Labrador retriever and after half a year a liver biopsy was taken and hepatic copper was increased to 462(Time point 2). This diet were continued, and after half a year hepatic copper was increased from 462 to 741 ppm(Time point 3).

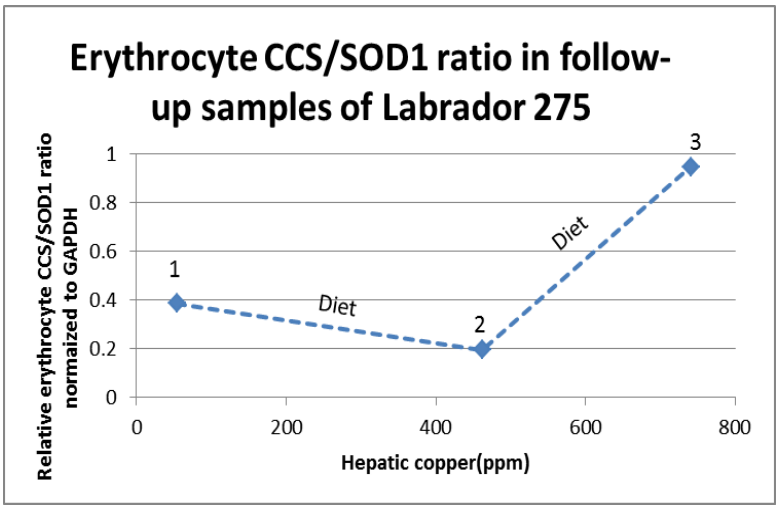

Figure 13: Erythrocyte CCS/SOD1 ratio in follow-up samples of Labrador retriever. After d-penicillamin treatment, a liver biopsy was taken, hepatic copper was 54 ppm(Time point 1). After this diet was given to this Labrador retriever and after half a year a liver biopsy was taken and hepatic copper was increased to 462(Time point 2). This diet were continued, and after half a year hepatic copper was increased from 462 to 741 ppm(Time point 3).

The second Labrador retriever(361) had a hepatic copper of 1270 ppm when the first biopsy was taken. After this, treatment of metalcaptase(d-penicillamin) was given. After a half year control biopsy was taken, showing a decrease of hepatic copper from 1270 to 121 ppm. In this Labrador the same trend was seen as in individual animals in CCS protein levels, where CCS and CCS/SOD1 ratio were both negatively correlated with hepatic copper values(Figure 14 and 15). SOD1 protein levels in these samples did not show the same trend as in individual animals(Figure 16).

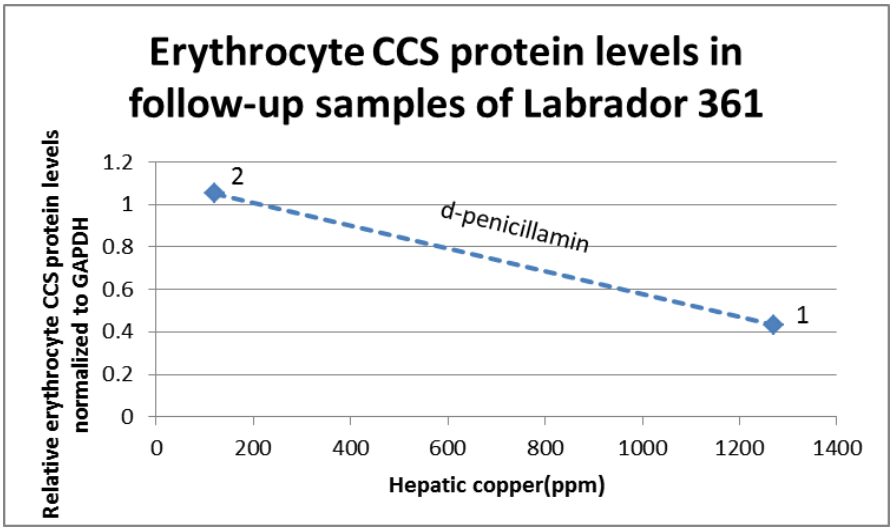

Figure 14: Erythrocyte CCS protein levels in follow-up samples of Labrador retriever. After d-penicillamin treatment hepatic copper decreased from 1270 ppm(time point 1) to 121 ppm(time point 2).

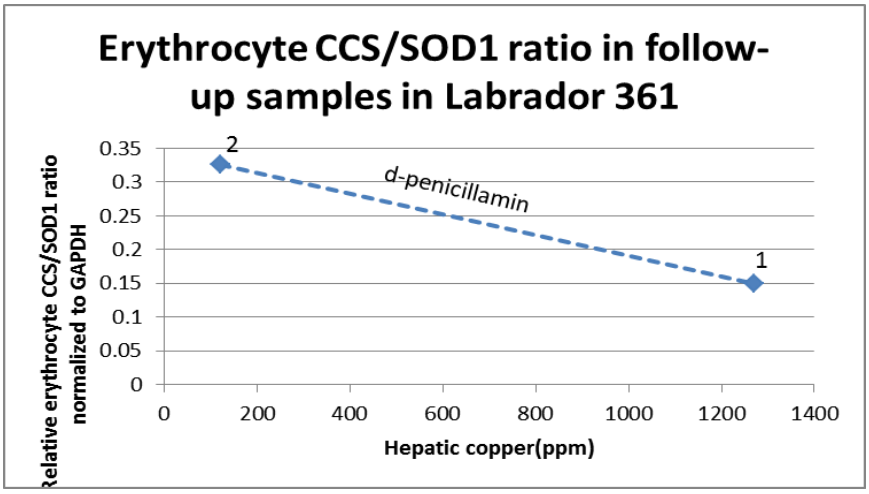

Figure 15: Erythrocyte CCS/SOD1 ratio in follow-up samples of Labrador retriever. After d-penicilamin treatment hepatic copper decreased from 1270 ppm(time point 1) to 121 ppm(time point 2).

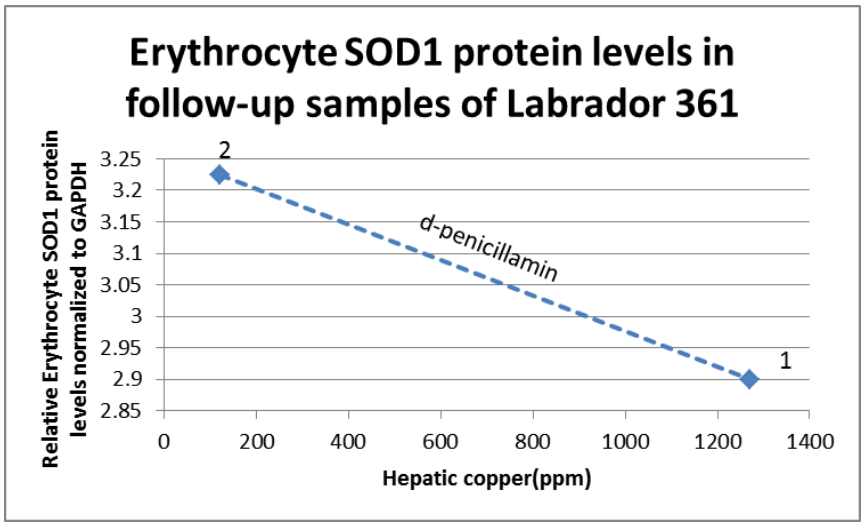

Figure 16: Erythrocyte SOD1 protein levels in follow-up samples of Labrador retriever. After d-penicillamin treatment hepatic copper decreased from 1270 ppm(time point 1) to 121 ppm(time point 2).

Discussion

Currently the only way to diagnose hepatic copper accumulation in the Labrador retriever is by means of a liver biopsy. Therefore a non-invasive biomarker is needed that correlates with hepatic copper status.

Many proteins and enzymes in blood plasma or serum are used to evaluate hepatic copper status, but most of them are non-specific or not sensitive. Serum or plasma copper and ceruloplasmin are widely used to evaluate hepatic copper status. Ceruloplasmin comprise 70% of the total plasma copper. It is function is to deliver copper to tissues, to neutralize radicals, to catalyze oxidation reactions involving molecular oxygen en various natural and synthetic amines. Furthermore it is an important ferroxidase (15). However, serum copper plasma does not correlate to hepatic copper status(16) and ceruloplasmin only reacts by decreasing in activity on severe copper deficiency (17). Also ceruloplasmin is influenced by ATP7B deficiency. Both serum copper and ceruloplasmin are influenced by age, sex and non-copper related processes like inflammatory diseases(17). A recent study showed there is no correlation between hepatic and urine copper values(18), however there is a correlation between hepatic copper and urine copper/zinc ratio. Platelet cytochrome-c activity (CCO) and diamine oxidase(DAO) are described to reflect hepatic copper status(18,19), because of their interaction with copper atoms. Cytochrome c-oxidase binds two copper and diamine oxidase is responsible from oxidative deamination of diamines. However, these enzymes have their limitations; CCO is a labile enzyme and DAO activity is also an indicator of tissue injury (20). Therefore these enzymes cannot function as biomarker for copper status.

On western blot CCS antibody detected two immunoreactive bands at 35 kDa and 32 kDa. Only the 35 kDa band is correlated with hepatic copper status. The second immunoreactive band of 32 kDa is not described on the antibody datasheet, therefore this immunoreactive band was not expected. Only one study shows this second band of CCS on a Western blot figure and conclude with peptide blocking experiments that this band is immunospecific and present in white and red blood cells and platelets(4). All other studies did not notice this second immunoreactive band. This second immunoreactive band can be explained by the number of transcript of the canine CCS gene, which has two transcripts, both protein coding with respectively 350 and 274 amino acids(Ensemble). Transcript 1 and 2 have a molecular weight of respectively 36 and 29 kDa. Why only transcript 1 has a correlation with copper cannot be explained, because both of transcripts have 3 Cu,Zn,SOD1 binding sites. The difference between the two transcript has to be further functional investigated.

Based on results of this study erythrocyte CCS protein level reflects hepatic copper status in the Labrador retriever. However, the reflection is different than expected. CCS protein levels and hepatic copper concentration showed a non-linear relationship. Erythrocyte CCS protein levels significantly differ between hepatic copper levels <400, 400-800 and >800 ppm. However, the biggest difference is seen between hepatic copper levels<400 and >400 ppm, which shows little overlap. Hepatic copper levels >800 ppm result in higher erythrocyte CCS protein levels than hepatic copper levels between 400-800, which was not expected. Other studies already showed that CCS reflects both copper overload in rats (7), where erythrocyte CCS protein levels significantly decrease by copper overload. Copper deficiency in mice (11), rats (8), and cattle (9) result in a significantly increase of erythrocyte CCS protein levels. This is because copper bounded CCS is faster degraded by 26S proteasome in erythrocytes, leading to a decrease of protein levels of erythrocyte CCS (8). High hepatic copper levels resulting in high erythrocyte CCS protein levels is not described before. Bertinato et al fed 36 rats three different copper diets: 5 mg Cu/kg(normal), 1,000 mg/kg(high) and 2,000 mg/kg (very high) copper a day for 13 weeks. Erythrocyte CCS protein levels significantly differ between the normal and

very high copper fed groups. Hepatic copper levels above 1,000 ppm were reached, however an average of all erythrocyte CCS protein levels were taken to calculate the difference between the groups. Therefore it is hard to determine whether in this study high hepatic copper levels results in high erythrocyte CCS protein levels. Therefore further research has to focus on the relation of hepatic copper levels above 1,000 and erythrocyte CCS protein levels.

Erythrocyte CCS protein levels were five times higher (80%) in hepatic copper of 28 ppm compared to hepatic copper of 795 ppm. Bertinato found a 30% reduction of CCS when rats where fed with high hepatic copper diet instead of a normal copper diet (7).Hepburn found even a 65% increase between cattle fed with normal and copper deficient diets (9). A reduction of 80% between hepatic copper of 28 and 795 ppm is therefore not surprising, representing a very low(considered copper deficient) and high (considered copper overload) hepatic copper.

This study shows that erythrocyte CCS, SOD1 protein levels were not influenced by age, treatment or gender, only by hepatic copper levels, which was expected. Limited data is available about this subject, so further research with more follow-up samples of Labrador retriever will reveal more about this. For example, low copper diet contains high zinc values, which has an antioxidant function(30). This may lead to less SOD1 protein levels and therefore more CCS protein levels, because CCS has an overcapacity compared to SOD1. D-penicillamin is described to may reduce the rate of liver fibrogenesis in patients with Wilson's disease(31).

Measuring erythrocyte CCS protein levels in follow-up samples in Labradors did not show the same trend as in individuals animals, which was not expected. Protein levels of copper chaperone CCS are dependent on hepatic copper levels, therefore it is surprising if treatment has an influence on erythrocyte CCS protein levels. The time between liver biopsies vary per Labrador retriever, this is important because we suspect erythrocyte CCS protein levels only change in the bone marrow. Erythrocytes have a turnover of 120 days, changes in CCS protein levels probably after four months or more, as each erythrocyte has a different age. More follow-up samples have to be taken from different Labrador retrievers with different treatments, which makes the results more reliable.

Erythrocyte SOD1 protein levels were not significantly correlated to hepatic copper status. It is described that erythrocyte SOD1 protein levels could reflect copper deficiency (24,33). Also previous studies that describes that erythrocyte CCS levels are more sensitive to copper status compared to SOD1 protein levels (22). In the liver this is explained by higher SOD1 protein levels compared to CCS protein levels (28). Therefore a small fraction of total SOD1 stabilizing CCS in a complex would be sufficient to produce large fold increases in CCS (28). If this is the same mechanism as for erythrocytes, has to be further investigated.

Erythrocyte SOD1 protein levels in follow-up samples did not show the same trend as in individual animals. This Labrador had a low copper diet before this biopsy was taken. We expected that in these follow-up samples erythrocyte SOD1 protein levels were positively correlated with hepatic copper values. The results indicate that d-penicillamin has a negative effect on SOD1 protein levels, or diet has a positive effect on SOD1 protein levels. SOD1, Cu,Zn Superoxide dismutase, needs both copper and zinc for its activity. May be a high zinc diet leads to more Cu,Zn superoxide dismutase. The effect of high zinc, low copper diet on erythrocyte SOD1 protein levels has to be further investigated.

Based on results of this study erythrocyte CCS/SOD1 ratio and hepatic copper concentration showed a non-linear relationship. Erythrocyte CCS/SOD1 protein levels significantly differ between hepatic copper<400, 400-800 and >800 ppm. We also found that hepatic copper levels between <400 and >400 ppm are better reflected by erythrocyte CCS/SOD1 protein levels than erythrocyte CCS protein

levels. It is described that erythrocyte CCS/SOD1 protein levels reflect hepatic copper status (11,24) and one study proposed that CCS/SOD1 ratio is a better biomarker for copper deficiency then CCS and SOD1 considered separately.

The mechanism of changes in erythrocyte SOD1 and CCS protein levels as a reflection of copper status is not yet understood. Latest studies reveal that for both proteins mRNA levels remains unaffected during copper deficiency in several organs including liver tissue (25). Bertinato described that copper bounded CCS is faster degraded than unbound CCS, leading to a decrease of erythrocyte CCS protein levels during copper overload. However, West(11) revealed that proteolysis in erythrocytes is very low, which means a decrease of CCS cannot be the result of a fast degradation of CCS. Changes in erythrocyte SOD1 levels can be due to post transcriptional changes in SOD1. SOD1 requires three posttranslational modifications; copper and zinc acquisition, intramolecular disulfide bond formation and dimerization(26). During hepatic copper accumulation more copper is present in the hepatocyte, which leads to activation of more SOD1. This may lead to more SOD1 activity and this may be detected on protein level in the hepatocyte and maybe also in erythrocytes. Another hypothesis can be that unbound SOD1 is degraded by a proteasome, but copper bounded SOD1 is active and therefore is not degraded. Also changes in protein levels of SOD1 can be the result of changes in protein expression in the bone marrow when erythrocyte are made. This would explain that CCS only reflects chronic copper deficiency and overload(7). Further research has to be performed on the exact mechanism of changes in CCS and SOD1 protein expression in erythrocyte as reflection of copper status.

As expected, liver CCS protein levels are negatively correlated with hepatic copper values. This finding is also seen in rats (7). Probably the same mechanism as in erythrocyte is responsible for this finding. Copper bounded CCS is faster degraded than unbound CCS by 26S proteasome. As hepatocytes also contain this 26S proteasome (27), this mechanism is also present in the liver. However, as said before this mechanism is not fully understood yet, so further research is necessary.

Liver SOD1 protein levels are positively correlated with hepatic copper values, however this correlation is not significant. Also in rats a not significant change in liver SOD1 protein levels is described (6). As said before, in the liver SOD1 protein levels are much higher than CCS protein levels (28). Also apoSOD1(SOD1 without activity) can accumulate, which makes correlation between hepatic copper status and SOD1 protein levels less reliable (29). However, in this study Western blot is used and with this method there is no distinction between apo and holo SOD1. Future research can focus on measuring SOD1 activity assay in relation to hepatic copper status.

Liver and erythrocyte CCS were positively correlated with each other. As described before, both erythrocyte and liver CCS decrease when hepatic copper increase, so it is expected that both erythrocyte and liver CCS correlate. In cattle erythrocyte and liver protein levels are also described (9). In this study both erythrocyte and liver CCS significantly differ between copper sufficient and copper deficient feed cattle, but no correlation was calculated between these two.

Liver and erythrocyte SOD1 were also positively correlated. This is surprising, because both liver and erythrocyte SOD1 protein levels did not correlate with hepatic copper. Limited data is available, so further research has to explain this correlation.

Liver and erythrocyte CCS and SOD1 protein levels were measured using Western blot analysis. However, this method has limitations. First, only samples on the same western blot can be compared, due to limitations in standardization of Western blotting. Therefore only 18 samples could be measured each time. Second, western blot is a semi-quantitative method and immunoreactive bands

can be diffuse and therefore hard to quantify. Future steps will focus on developing a quantitative diagnostic test for CCS and SOD1 protein levels.

Erythrocyte CCS/SOD1 protein levels better reflect hepatic copper levels between <400 ppm and >400-800 ppm than erythrocyte CCS protein levels. Therefore erythrocyte CCS/SOD1 could maybe used as biomarker for copper deficiency during treatment of d-penicillamin preventing overtreatment. However, erythrocyte CCS, CCS/SOD1 protein levels in follow-up samples of Labrador retrievers often do not show the same trend as in individual animals. Future research will be focused on testing erythrocyte CCS and CCS/SOD1 ratio in more follow-up samples of Labrador retrievers and replicating these results in a bigger group of Labrador retrievers. After this a diagnostic test can be developed, which can be tested for sensitivity and specificity.

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Chapter 5

Copper transporter ATP7A as modifier gene in Wilson's disease patients

Copper transporter *ATP7A* as modifier gene in Wilson's disease patients

Roelen Y[1], Fieten H[1], Houwen R H J[2], Rothuizen J[1]

1. Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, 3584 CM Utrecht, The Netherlands
2. Department of Pediatric Gastroenterology, Wilhelmina Children's Hospital, Postbox 19008, 3501 CA Utrecht, The Netherlands

Abstract

Keywords: *ATP7A*, Wilson's disease, modifier gene, copper associated hepatitis
Background: Wilson´s disease is a autosomal recessive disorder of copper metabolism caused by mutations in the *ATP7B* gene, which leads to copper accumulation in liver and brain. Over 500 different mutations have been identified in the *ATP7B* gene, of which the H1069Q mutation is most commonly encountered in the Caucasian population. Age of onset and symptoms are highly variable in Wilson's disease, but both cannot be completely explained by the variety in mutations in *ATP7B*. Patients with identical *ATP7B* mutations can differ in age of onset and phenotype. Recently, a genome wide association study in the Labrador retriever with Wilson's disease like copper associated hepatitis revealed the candidate modifier gene *ATP7A*, which may perform a gate keeper function for copper uptake.
Objectives: To investigate whether copper transporter *ATP7A* can be a modifier gene with a gatekeeper function for copper absorption and this way influence age of onset in Wilson's disease patients.
Material and methods: Sanger sequencing of the exons and intron-exon boundaries of *ATP7A* was performed on DNA of 60 Wilson's disease patients, which were homozygous (n=48) or heterozygous (n=12) for the *ATP7B* H1069Q mutation. In all patients age of diagnosis was determined. Patients with an age of onset earlier than 20 years where named 'early onset', and patients with an age of diagnosis later than 19 years were named 'late onset'. In our dataset 26 patients had an early onset, 64 had a late onset of Wilson's disease.
The mutation was evaluated for the association with age of diagnosis., A linear regression model was used with age of diagnosis as continuous outcome variable and gender, *ATP7B* mutation (modeled as H1069Q homozygous or heterozygous), and mutations found in *ATP7A* gene (modeled as homozygous mutant(2), heterozygous mutant(1) and wild-type(0)) and origin of patients as covariates. Also a logistic regression was tested with age of diagnosis (binomial) as outcome and also variables were gender, *ATP7B* mutations and mutations found in *ATP7A* gene.
Results: Sanger sequencing revealed a non-synonymous SNP (snSNP) in exon 10 (V767L). No association was found between V767L snSNP in the *ATP7A* gene and the age of diagnosis modeled either as a continuous or as a binomial outcome variable. Conclusion: In the current data set of Wilson's disease patients, there was no association between mutations in *ATP7A* and age of onset of the disease. However, we cannot exclude whether mutations in introns or promotor regions of *ATP7A* influence *ATP7A* expression and therefore have an effect on the function of *ATP7A*.

Introduction

Wilson's disease is an autosomal recessive disorder of copper metabolism caused mutations in ATP7B gene. Mutation analysis of different Wilson´s disease patients revealed more than 500 mutations in *ATP7B* gene. Most Wilson´s disease patients are compound heterozygous, which means they carry more than one mutation, clinical symptoms can vary between hepatic, neurologic and psychological symptoms. Age at the onset of symptoms is usually from 6 years to 45 years old (1), but Wilson's disease with hepatic involvement may be detected for the first time in patients in their 70's (2).

Clinical symptoms and age of onset are highly variable between patients. However, this diversity in clinical symptoms and age of onset cannot be explained by variation in mutations in the *ATP7B* gene only. Patients carrying identical *ATP7B* mutations can have different clinical signs and age of onset (3). Environmental, epigenetic and other genetic factors are thought to play a role in the variety of the phenotype (4).

Gender and modifier genes are thought to play a role in age of onset in Wilson´s disease patients. Women have a later age of onset than man, probably due to hormonal influences of estrogens(5). Also other genes than *ATP7B* are tested as modifier gene for Wilson´s disease and therefore affect the phenotype, especially age of onset, of WD patients. Several genes were tested for association with phenotype of Wilson's disease, but no association is found(6)(7).

A recent study revealed a promising candidate gene as potential modifier of Wilson's disease. Hepatic copper accumulation in the Labrador retriever is a hereditary disease with a complex genetic background (8). Recently a genome wide association study was performed in Labradors with Wilson's disease like copper associated hepatitis and revealed significant mutations in both *ATP7B* and *ATP7A* gene(9), known as disease causing gene in the copper deficiency disorder Menke´s disease (10). In this study a mutation in *ATP7A* was associated with lower hepatic copper levels and mutations in *ATP7B* were associated with higher hepatic copper levels.

Copper transporters *ATP7A* and *ATP7B* have an important function in respectively the enterocyte and hepatocyte. In the enterocyte ATP7A ensures copper transport to the portal circulation(11) and to other cuproenzymes. Because ATP7A facilitates excretion of copper into the portal circulation, ATP7A may perform a gate keeper function in protecting the body copper homeostasis. ATP7B links copper to ceruloplasmin(12) and facilitates excretion of copper into the bile(3). In Wilson's disease, both functions of ATP7B are impaired in Wilson´s disease, leading to copper accumulating in liver tissue and brain.

Based on the results in the Labrador retriever, we hypothesize that the *ATP7A* gene may perform a gatekeeper function in enterocytic copper uptake and that this phenomenon may modify the age of onset in people with decreased hepatic copper excretion caused by mutations in *ATP7B*. WD patients with mutations in *ATP7A*, that partly impair the function of the gene, may have a later age of onset than patients with no mutations in *ATP7A*, this would explain diversity in phenotype in Wilson´s disease patients. However, *ATP7A* has not been tested yet as modifier gene in WD.

This study will investigate whether *ATP7A* can be a modifier gene with a gatekeeper function for copper absorption and in this way influence the age of onset in Wilson's disease patients. Sanger sequencing of 90 WD patients has established a clear view on the possibility of *ATP7A* as modifier gene.

Material and methods

Patients

Data from patients with WD were collected from four different institutes in France, Poland, Germany and the Netherlands. In appendix 2 *ATP7B* mutations, age of onset and age of diagnosis were listed of all Wilson's disease patients. Of all patients is known if they were heterozygous or homozygous for the most common *ATP7B* mutation, H1069Q. Diagnosis of WD was reviewed according to the criteria of the 8th International conference on Wilson and Menkes Disease(13).

Definition of age of onset

The age of onset is the age when the first clinical symptoms attributable to Wilson's disease occurred. Age of onset was divided in early and late age of onset. Patients with an age of onset earlier than 20

years where named ‘early onset’, and patients with an age of onset later than 19 years were named ‘late onset’.

DNA analysis

Twenty-three exons and intron-exon boundaries of *ATP7A* were investigated by Sanger sequencing (Table 1) of DNA of 60 Wilson’s disease patients. Primers were developed in Perl primer and tested for their specificity with NCBI Blast. Polymerase chain reactions (PCR) for amplification of gDNA was performed using 1,25 ng/µl gDNA and Taq polymerase (Invitrogen, Carlsbad, USA) following the manufacturer’s instructions. PCR product was purified with ExoI using 2U/µl. 1 µl of purified PCR product was incubated with 1 µl of a forward or reverse primer, 2 µl BigDye(Applied Biosystems, Foster City, CA, USA) mix, 2 µl of 5 x Sequence buffer (Applied biosystems, Foster City, CA, USA) and 4 µl of MilliQ in a volume of 10µl. This reaction was performed for 25 cycles of 96 ºC for 30 seconds, 50 ºC for 15 seconds and 60 ºC for 2 minutes(Appendix 1). The product was purified using sephadex columns using a multiscreen 96-filtration plate and were analyzed on an ABI Prism 3130xl Genetic analyzer(Applied Biosystems, Foster City, CA, USA). The sequences were analyzed using SeqMan Pro(DNA star, Version 10.1.1). Sequences were compared with the reference genome on NCBI Ensemble(Build 3.1). Polymorphisms were named according to nomenclature recommendations listed on the Human Genome Variation Society website (http://www.hgvs.org/mutnomen/). If polymorphisms were found, mutation analysis of this polymorphisms were also performed on thirty other Wilson’s disease patients.

Statistical analysis

Statistical analysis were performed with R 2.7.2 (package for Windows, http://www.Rproject.org). The mutation was evaluated for the association with age of diagnosis, where age of diagnosis was binomial and continuous described for late(1) and early(0) diagnosis. A linear regression model was used with age of diagnosis as continuous outcome variable and gender, *ATP7B* mutation(modeled as H1069Q homozygous or heterozygous), and mutations found in *ATP7A* gene(modeled as homozygous mutant(2), heterozygous mutant(1) and wild-type(0)) and origin of patients as covariates. Also a logistic regression was tested with age of diagnosis(binomial) as outcome and also variables were gender, ATP7B mutations and mutations found in *ATP7A* gene. The best model was chosen based on Akaike’s information criterium. Validity of the model was checked by inspecting the QQ-plots and inspecting the residual plots for normality and constance of variance

Results

Patients

Mutation analysis of all 23 exons were performed on 60 WD patients. Of these patients, 30 Wilson’s disease patients were male, 30 were female. Age of diagnosis ranged from 4 till 41 years(23±10.6). Mutation found in *ATP7A* was also analyzed in 30 Polish Wilson’s disease patients. Of these patients, 6 Wilson’s disease patients were male, 6 were female. Age of diagnosis ranged from 31 till 63 years(41 ±7.2). Mutations found in *ATP7A*, age, gender, clinical symptoms and age of diagnosis of these patients are listed in Appendix 2.

Exon	Primer	Sequence(5’→ 3’)	Location(Build 2.1)	Amplicon size	Tm
1	NA				55
	NA				
2	Yara8_Fw	GGAAAGATTATTTGCATGGCTG	77,227,068-77,227,286	219	55
	Yara8_Rv	TGACTTCAGTTTCCAAAGAG			
3	Yara23_Fw	ACTCTTCTTGAATGTGGTGTG	77,243,648-77,244,240	593	55
	Yara23_Rv	CCAGACACATTACCTTTAATTCGC			
4	Yara19_Fw	TTTCTGAAGTAGCCCAGGA	77,244,674-77,245,198	525	55
	Yara19_Rv	GCTGGAGGGAGAGTTTGAG			
4	Yara20_Fw	TTATCTGCACTCCAATATGTAAGC	77,245,013-77,245,554	524	55

	Yara20_Rv	TACAGTGCCTATGAATCATTTCTC			
5	Yara15_Fw	GAATCTTTCCCTTTCTACCAG	77,253,954-77,254,189	236	55
	Yara15_Rv	TGTCTCACCTTCTTCCCG			
6	Yara22_Fw	TTTCTTATCAATGCTCTTAGGAAT	77,258,550-77,258,814	265	55
	Yara22_Rv	ATTATCTTCACAACTCTTCTCTG			
7	Yara17_Fw	GCTTTGTCTTTAACAGGTGAGGG	77,264,583-77,264,847	265	55
	Yara17_Rv	GCCTCTATCTGATGCAAACCA			
8	Yara12_Fw	ACTGTTCTTAATGACAATACCA	77,266,581-77,266,756	176	55
	Yara12_Rv	TACTTACTGTCTTATTTCTCGTT			
9	NA				55
	NA				
10	Yara21_Fw	AAAGCCAAAGAAAGTGACTAT	77,268,276-77,268,658	383	55
	Yara21_Rv	TCATTTATCCACCCAACATT			
11	Yara4_Fw	TTTGGGTAAATGCTAAAGATTGTA	77,270,066-77,270,281	216	55
	Yara4_Rv	AAAGAGAAGAGGGAAGAGT			
12	Yara1_Fw	GGTAGGAAGCATATTATCATGG	77,271,203-77,271,390	188	55
	Yara1_Rv	GAAAGAACATACCTGTGATGAG			
13	Yara14_Fw	TCTATCTTTACTCTCCATACAGGG	77275719-77275962	244	55
	Yara14_Rv	GGTGAAGATTTATACTGAGAAGGC			
14	Yara10_Fw	TTAATTCTAGGCTCCTATCCAGCA	77,276,432-77,276,588	157	55
	Yara10_Rv	CAAGTCACTTACAGGAAAGTAGGT			
15	Yara24_Fw	GGGCTACAATAGAAGTATCTCC	77,284,746-77,284,947	202	55
	Yara24_Rv	TCTTACCTTATGAGCCATCTCC			
16	Yara18_Fw	TCTGGAAAGGTGATGTGG	77,286,843-77,287,170	328	55
	Yara18_Rv	GCAAATCACAGTCAAACAC			
17	NA				55
	NA				
18	Yara3_Fw	ATTTCTGTGCCTACTTTCAG	77,294,314-77,294,489	176	55
	Yara3_Rv	AAACCTTACCATCAACTGCT			
19	Yara6_Fw	CCCTATATTAGATGAGCTGTG	77,296,078-77,296,243	116	55
	Yara6_Rv	CCTATCAATTACCTGAGAAGC			
20	Yara13_Fw	TTGCTCAGTTATGTTTCACG	77,298,018-77,298,325	308	55
	Yara13_Rv	CAGTCTCATACTCCTCAAAGG			
21	Yara7_Fw	GAATGATCTTCTGGATGTAGTG	77,298,814-77,298,948	135	55
	Yara7_Rv	GTTAAGAGTCACATACCAGCA			
22	Yara5_Fw	CCATACTTGTTTCTTAGGAG	77,300,950-77,301,087	138	55
	Yara5_Rv	GCAAGCTATCATACTTACAG			
23	Yara2_Fw	TTGCATATGTCCAGTTACAGG	77,301,777-77,302,107	311	55
	Yara2_Rv	GTGCATGACAAGTTAAACTG			

Table 3: Sequences of primers used to sequence *ATP7A* gene. Primers which are not optimized yet are listed as NA. Exon 4 is divided into two overlapping fragments. Tm is the optimal temperature used for PCR.

Mutation analysis

Sanger sequencing of 23 exons and intron-exon boundaries revealed a non-synonymous single nucleotide polymorphisms (snSNP) in exon 10 (Table 3 and figure 1). This polymorphism is located in

one of the eight the transmembrane segment of ATP7A protein (14). V767L snSNP found was statistical analyzed. An allele frequency of 0.28 was found for the non reference C-allele.

	SNP Exon 10
SNP on mRNA level	2299 G→C
Amino acid change	767 V→L
Frequency of observed genotypes (n=90)	GG: 24(26.7%) GY: 39(43.3%) GC: 20(22.2%) CY: 6 (6.7%) CC: 1(1.1%)
Allele frequency	G: 0.72 C: 0.28

Table 3: Frequency of single nucleotide polymorphisms(snSNP) found in exon 10 within the ATP7A gene.

```
CDS: Putative 1       40    I  L  L  V  A  M  Y  E  R  A  K  V  N  P  I  T  F  F  D  T
Query                 121   TTCTTCTAGTTGCAATGTATGAGAGAGCCAAAGTGAACCCTATTACTTTCTTTGACACAC  180
                            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct                 2451  TTCTTCTAGTTGCAATGTATGAGAGAGCCAAAGTGAACCCTATTACTTTCTTTGACACAC  2510
CDS:copper-transport  764   I  L  L  V  A  M  Y  E  R  A  K  V  N  P  I  T  F  F  D  T
```

Figure 4: SNP of guanine in cytosine results in an amino acid change of Valine in Leucine.

snSNP V767L and age of diagnosis

Age of diagnosing showed no correlation with snSNP V767L(Figure 2). Also analyzing age of diagnosis as a binomial trait in early and late (0 or 1), didn't correlate with snSNP V767L, data not shown). In statistical analysis gender and origin of the sample were taken as covariate, however no significant difference was found between age of onset between gender and origin of the sample (data not shown).

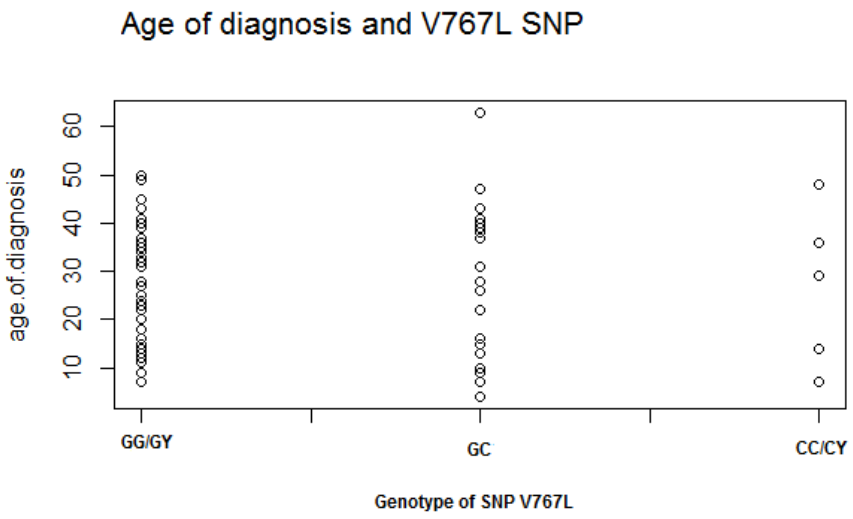

Figure 2: Dotplot of age of diagnosis in relation to snSNP V767L. x-as shows age of diagnosis in years, y-as shows genotype; GG/GY, GC and CC/CY. No association was found between the age of diagnosis and snSNP v767L(p>0.05).

Discussion

Wilson's disease is a difficult disease to diagnose for different reasons. As said before, there is no golden standard for diagnosing Wilson's disease. The age of onset is also hard to determine, as the diagnosis can be overlooked in asymptomatic and symptomatic patients. Overlooked WD patients often do not received treatment, which they certainly need.

The diversity of clinical symptoms and age of onset cannot be explained yet. Obviously it could be that different *ATP7B* mutations lead to different phenotypes and therefore also different age of onset. Many researches has focused on explaining WD phenotype with most common mutation H1069Q. However, no consensus about association between *ATP7B* mutations and phenotypical diversity in Wilson's disease patients is found in literature. Correlation between *ATP7B* mutations and clinical symptoms has been described for this disease. Homozygosity for H1069Q is described to be associated with neurologic disease or late disease (15-17), or both(18), however this mutation is also described to have no association with any phenotype(19).

Also many patients that carry identical *ATP7B* mutations appear to have different clinical symptoms (3). Clinical signs even differ between identical twins(20), which suggest environmental, epigenetic and other genetic factors are involved in the onset of this disease(4).

Many researchers have focused on modifiers genes for Wilson's disease, especially genes which are known to contribute to regulation of copper metabolism. Copper metabolism is tightly regulated and many copper transporting and binding proteins are involved in this process. Variants in genes of these copper binding proteins could explain the difference in WD phenotype in patients with identical *ATP7B* mutations. *BIRC4/XIAP*(21) and *Murr1(COMMD1)*(6) are already tested as modifier gene. *COMMD1* physically interact with *ATP7B* and *XIAP* is suggested as a regulator of copper-induced cell injury. Mutations were found in these genes, but no clear genotype phenotype correlation was identified, and mutations were also seen in non-WD humans. In general no modifier gene for WD is found yet, which may be due the rarity WD and the high occurrence of compound heterozygous Wilson's disease patients impede genotype-phenotype studies.

In this study we found no correlation between age of onset and mutations in *ATP7A* gene. We hypothesized that *ATP7A* could function as gatekeeper and in that way influence age of onset in Wilson's disease patients. Fieten et al(2013) showed that hepatic copper accumulation in Labrador retriever is caused by mutations in *ATP7A* and *ATP7B*, where mutations in *ATP7A* leads to lower hepatic copper levels (9). *ATP7A* transports copper to the portal circulation. When *ATP7A* is less functional, this leads to less copper in the circulation and therefore less hepatic copper accumulation. We hypothesized that in Wilson's disease patients this could result in an later age of onset. Maybe other copper binding proteins that aren't tested yet, function as modifier gene for Wilson's disease patients. However, three exons still have to be sequenced, so we can't exclude *ATP7A* gene as modifier gene for Wilson's disease yet. Further sequencing has to be performed on Wilson's disease patients to investigate whether *ATP7A* is a modifier gene for WD patients.

In this studies only exons and intron-exons boundaries were sequenced. However, introns can influence and enhance gene expression. Intron-containing and intronless versions of otherwise identical genes can exhibit dramatically different expression profiles(22). Therefore mutations in *ATP7A* could also be located in introns and are missed in this way of sequencing. If no SNP is found during sequencing of the other exons of *ATP7A*, sequencing of the introns of *ATP7A* may be a good approach for identification of mutations.

One problem in research after modifier genes in Wilson's disease patients is the age of diagnosis is hard to determine. Most studies don't explain what their definition is of age of onset, or use age of diagnosis for age of onset. Also the difference between late and early onset is hard to generalize. We set the age of onset boundary to 20 years, however this isn't a golden standard. Ferenci describes age of onset as age above 40 year(4)s, but most studies don't describe their boundaries(16). Also WD is often overlooked, so this makes the age of diagnosis less valuable as a reflection of the age of onset. This could explain why no association is found between snSNP V767L in *ATP7A* and age of diagnosis. However, the mutant allele(CY and CC) was found in early and late age of diagnosis, from 7 till 48 years old. Sequencing more WD patients for this snSNP would probably reveal the same diversity of age of diagnosis for the mutant allele.. In future research early and late age of onset has to be described in the report and maybe a standard boundary has to be set generalized.

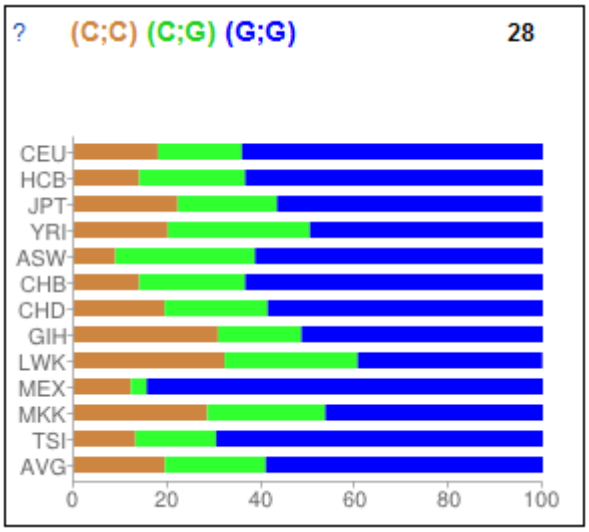

Figure 3: Frequency of GG/GY, GC, CC/CY differs between countries. CEU=Europe, CHB: China, JPT: Japan, YRI: Afrika, ASW: USA, CHD: Colorado, GIH: Houston, LWK: Kenya, MEX: California, MKK: Kenya, TSI: Italia, AVG: average of samples of above groups. Derived from www.snpedia.com(28)

snSNP V767L, is already known and has an overall frequency of 0.26 in the general population. However, this differs between countries(Figure 3). In accordance to this data, we found in our dataset an allele frequency of 0.28. In Europe this SNP has a frequency of 0.25, which corresponds with the allele frequency of our dataset.

This snSNP is also described in Menke's disease patients (24-26) but no association is found between V767L and pathogenesis of Menke's disease. Menke's disease is caused by mutations in *ATP7A* gene. Mutation analysis revealed 370 mutations in *ATP7A* in MD patients(27). In patients with MD copper is trapped in enterocytes due to a dysfunction of ATP7A protein, leading to systemic copper deficiency(10). However, identical to Wilson's disease, no association is found between phenotype of MD and mutations in *ATP7A*. It is described that phenotype of MD is not influenced by V767L. However, it is not tested if V767L functionally influence ATP7A expression in enterocyte or other cells. V767L as is located one of the eight transmembrane segment of ATP7A(14). In other transmembrane segments are missense mutations associated with Menke's disease. Therefore it's possible that V767L influences the function of *ATP7A*. Also, as modifier gene for Wilson's disease we don't expect a SNP that leads to no copper absorption in the enterocyte, but less copper absorption. In Menke's disease mutations in *ATP7A* gene has to lead to severely decreased copper absorption by enterocytes. Further research has to focus whether V767L functional influences *ATP7A* expression.

In conclusion, these data suggest that *ATP7A* gene is unlikely to influence the age of onset by playing a gatekeeper function in Wilson's disease patients. However, it is still likely that modifier genes other than *ATP7A* maybe influence phenotype and especially age of onset/diagnosis in Wilson's disease patients. If this is the case, finally genotype-phenotype correlation for Wilson's disease is established. Mutation analysis of modifier genes could predict the phenotype of WD patients. Treatment of d-penicillamine could be started before symptoms arise, which leads to less liver damage and a better prognosis.

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Chapter 6

General discussion

General discussion

Canine models are widely used to diagnose and cure human liver diseases(1,2). Favier et al concluded that man and dog share the same hepatopathies and have identical clinical, pathological and pathogenetic reaction patterns during the development of liver disease. Therefore the dog seems to be a good model to test new human therapeutic strategies(1). Also knowledge obtained in human research can be used in research of canine diseases. Treatment of Wilson's disease with d-penicillamine is used as example for treatment of canine copper toxicosis diseases. This treatment has shown to be effective for the treatment of copper toxicosis in Labrador retriever(3). Also biomarkers for copper status found in human can be an example for biomarker studies in the dog. Erythrocyte CCS and SOD1 protein levels reflect hepatic copper status in human(4), therefore these proteins are tested as biomarker during this honours program.

This honours program combines both canine and human knowledge obtained in research, to fill gaps in the pathogenesis and genetic background of copper associated liver diseases.

To test erythrocyte CCS and SOD1 protein levels as biomarker for hepatic copper status, antibodies for these protein have to be validated for biomarker assay in the dog. In this honours program specificity of CCS and SOD1 antibodies were tested using SiRNA mediated knockdown and was confirmed qPCR and Western blot(Chapter 3).

The first aim in this honours program was to determine whether CCS and SOD1 protein levels in erythrocytes reflect hepatic copper status in the Labrador retriever. Both erythrocyte CCS protein levels and CCS/SOD1 ratio were shown to reflect copper status. Erythrocyte CCS/SOD1 protein levels distinguishes between hepatic copper <400 and >400 ppm whereas erythrocyte CCS protein levels distinguishes between hepatic copper of 400-800 ppm and >800 ppm. Erythrocyte SOD1 protein levels were not significantly associated with hepatic copper concentration. Erythrocyte CCS/SOD1 could possibly be used as biomarker for copper deficiency during treatment of d-penicillamin preventing overtreatment. Erythrocyte CCS protein levels could possibly be used to identify dogs at risk. In this study one Labrador receiving chelation therapy, turned out to have a hepatic copper value of 28 ppm, which is extremely low. Monitoring erythrocyte CCS protein levels could possibly prevent this overtreatment. Hepatic copper accumulation in Labrador retriever is known to have a very long subclinical stage, so early detection of hepatic copper accumulation could prevent illness.

In addition it was established that follow-up samples in Labrador retriever often do not show the same trend as individual Labrador retrievers. This is worrying, because this biomarker was meant for monitoring during treatment. If this is not possible, the role of erythrocyte CCS protein levels or CCS/SOD1 ratio as biomarker for copper status becomes debatable.

Further research has to focus on testing erythrocyte CCS protein levels and erythrocyte CCS/SOD1 ratio in more follow-up samples of Labrador retrievers. This will increase the knowledge on the effect of treatment on CCS and SOD1 levels as well as the connection between CCS, CCS/SOD1 and hepatic copper status.

Another important goal of further research will be replicating the results in a bigger cohort of individual Labradors. However, the method we use for protein measurement has its limitations. In this method only 18 samples can be measured at the same time and various Western blots cannot be compared with each other, because it is impossible to accomplish this method each time under the

same conditions. We tried to use one or two standard samples at each western blot and normalize the dataset to these standard samples, but this was not successful. The only way to test more samples for these proteins is to use another method. Preferably this method would be ELISA, which is very sensitive and the same antibodies used for Western blot could be used for this method. However, this method has to be optimized for the measurement of these proteins. Future research will be focused on quantification of CCS and SOD1 protein levels in combination with hepatic copper status. Standard lines for these proteins have to be developed for ELISA, by which we could test more samples.

If erythrocyte CCS protein levels can be used as biomarker for hepatic copper status in Labrador retriever, monitoring during disease with erythrocyte CCS protein levels or CCS/SOD1 ratio in Wilson's disease patients could also replace liver biopsies.

Furthermore results of canine studies generated a hypothesis for the genetic background of Wilson's disease. A genome wide association study was performed on Labrador retrievers and revealed missense mutations in *ATP7A* and *ATP7B*, associated with respectively lower and higher hepatic copper levels. The hypothesis that ATP7A could be a modifier gene in Wilson's disease in human patients.Sanger sequencing revealed a non-synonymous SNP(snSNP) in exon 10 (V767L). In the current dataset no association was found between V767L snSNP in the *ATP7A* gene and the age of onset at diagnosis. However, we cannot exclude whether mutations in introns or promotor regions of *ATP7A* influence *ATP7A* expression and therefore have an effect on the function of *ATP7A*. Also three more exons have to be sequenced, so there is still a possibility we'll find in this dataset a SNP that is associated with age at diagnosis. It is described that this SNP is not associated with Menke's disease, this may be because this SNP does not influence the ATP7A expression. However, the phenotype we expect is not as extreme as seen in Menke's disease patients. Also the SNP is located in a transmembrane segment, where as well Menke's causing SNP's are located(5). Therefore it cannot be concluded whether this snSNP is associated with age at diagnosis in Wilson's disease patients and whether ATP7A is a modifier gene for Wilson's disease. Further research has to focus on sequencing three more exons, sequence introns and promotor regions that could influence gene function.

Although many similarities are seen between human and canine copper associated liver diseases, many differences are also seen in phenotype of these diseases. Both Wilson's disease and hepatic copper accumulation in Labrador retriever are caused by mutations in ATP7B,however clinical symptoms differ between these diseases. Symptoms seen in Wilson's disease patients are neurological, hepatic or psychological , whereas affected Labrador's develop hepatic symptoms after a very long subclinical phase .. In Wilson's disease patients hemolytic anemia and coagulopathy are also seen(6), contrary to Labrador retrievers, in which these symptoms are never present. Both diseases are treated with chelation therapy, however more symptoms are present in human than in the dog. In human hypersensitivity reactions, bone marrow suppression and development of autoimmune diseases are seen as side effects of chelation therapy(7). In the dog only nausea and vomiting are described as side effects(3).

In summary, this honours program research project is a beautiful example of using similarities and differences between species to fill gaps in pathogenesis and diagnosis of different copper associated liver diseases. It shows that both liver diseases could be used as an example for each other, where human and canine diseases come together.

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Appendices

Appendix 1: PCR protocol Chapter 4

Appendix 2: Samples from Chapter 4

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Appendix 1

Protocol sequencing *ATP7A*

PCR: 10 µl reaction

	µl	Final concentration
10 x PCR buffer	1	1
MgCl2(50 mM)	0.5	2.5 mM
dNTP's(10 mM)	0.2	200 µM
Forward primer(10µM)	0.5	0.5 µM
Reverse primer(10µM)	0.5	0.5 µM
Taq polymerase	0,05	
MiliQ	2.25	
gDNA(1,25 ng/µl)	5	6.25
Total	10	

95.0 °C	**5:00**	
95.0 °C	0:30	25 times
55.0 °C	0:30	
72.0 °C	0:30	
72.0 °C	10:00	
20.0 °C	∞	

Purify reaction: Exo I

	µl
PCR product	10,0
Exo I(2U/µl)	1,0

37.0 °C	**60:00**
75.0 °C	20:00
12.0 °C	∞

Tercycle reaction

	µl
Terminator Ready reaction mix	2
PCR product	1
Primer(Forward or Reverse)	1
5 x sequence buffer	2
MiliQ	4
Total	**10**

96.0 °C	**0:30**	
50.0 °C	0:15	25 times
60.0 °C	2:00	
4.0 °C	∞	

Appendix 2 – Wilson's disease patients

	Country	Gender	age of diagnosis	age of diagnosis binomial	H1069Q mutation in ATP7B(homozygous=0, not homozygous=1)	V767L	V767L, 0=G, GC=1, CC=2
Parijs_1	France	F	13	0	0	GC	1
Parijs_10	France	F	22	1	0	GC	1
Parijs_11	France	M	23	1	0	GY	0
Parijs_12	France	M	24	1	0	GY	0
Parijs_13	France	M	28	1	0	GY	0
Parijs_14	France	F	28	1	0	GC	1
Parijs_15	France	M	29	1	0	CY	2
Parijs_16	France	M	31	1	0	GY	0
Parijs_17	France	M	31	1	0	GY	0
Parijs_18	France	F	31	1	0	GG	0
Parijs_19	France	M	32	1	0	GY	0
Parijs_2	France	F	16	0	0	GG	0
Parijs_20	France	M	35	1	0	GY	0
Parijs_21	France	M	40	1	0	GY	0
Parijs_22	France	F	40	1	0	GC	1
Parijs_3	France	M	16	0	0	GY	0
Parijs_4	France	F	18	0	0	GG	0
Parijs_5	France	F	18	0	0	GG	0
Parijs_6	France	F	18	0	0	GG	0
Parijs_7	France	M	20	1	0	GY	0
Parijs_9	France	F	22	1	0	GG	0
19	Netherlands	F	11	0	0	GG	0
26	Netherlands	F	9	0	1	GC	1
32	Netherlands	M	37	1	0	GY	0
34	Netherlands	M	37	1	1	GY	0
43	Netherlands	M	13	0	0	GY	0
58	Netherlands	M	12	0	0	GY	0
74	Netherlands	F	9	0	1	GG	0
75	Netherlands	F	38	1	1	GC	1
87	Netherlands	F	7	0	0	GC	1
96	Netherlands	M	7	0	0	CY	2
116	Netherlands	M	7	0	0	GY	0
137	Netherlands	F	27	1	0	GG	0
139	Netherlands	M	36	1	1	CY	2
144	Netherlands	F	27	1	0	GG	0
149	Netherlands	F	4	0	1	GC	1
163	Netherlands	F	26	1	0	GC	1
179	Netherlands	M	33	1	1	GY	0
191	Netherlands	F	27	1	0	GG	0
214	Netherlands	F	9	0	1	GG	0

228	Netherlands	F	40	1	1	GG	0
237	Netherlands	M	31	1	0	GY	0
239	Netherlands	F	10	0	1	GC	1
256	Netherlands	M	9	0	1	GY	0
260	Netherlands	M	33	1	1	GY	0
264	Netherlands	M	32	1	0	GY	0
2007D05040 D01	Germany	M	41	1	0	GY	0
2008D02551 D01	Germany	M	14	0	0	GY	0
2009D10013 D01	Germany	F	31	1	0	GC	1
D20709	Germany	F	15	0	0	GC	1
D209713	Germany	M	14	0	0	CY	2
D209714	Germany	F	25	1	0	GG	0
D940271	Germany	F	16	0	0	GG	0
D940298	Germany	M	37	1	0	GY	0
D940920	Germany	M	15	0	0	GY	0
D940975	Germany	F	16	0	0	GC	1
D992358	Germany	M	36	1	0	GY	0
8	Germany	F	15	0	0	GC	0
266	Germany	M	14	0	0	CY	2
1240	Germany	F	25	1	0	GG	0
508	Poland	M	43	1	0	GY	0
626	Poland	F	35	1	0	GG	0
704	Poland	F	50	1	0	GG	0
726	Poland	F	49	1	0	GG	0
758	Poland	F	32	1	0	GG	0
771	Poland	M	31	1	0	GY	0
777	Poland	M	31	1	0	GY	0
885	Poland	F	37	1	0	GC	1
897	Poland	M	40	1	0	GY	0
898	Poland	M	37	1	0	GY	0
903	Poland	M	43	1	0	GY	0
1047	Poland	M	34	1	0	GY	0
1325	Poland	F	63	1	0	GC	1
1369	Poland	M	43	1	0	GY	0
1441	Poland	M	50	1	0	GY	0
1508	Poland	M	48	1	0	CY	2
1564	Poland	F	43	1	0	GC	1
1570	Poland	M	40	1	0	GY	0
1573	Poland	F	41	1	0	GC	1
1587	Poland	F	47	1	0	GC	1
1621	Poland	F	33	1	0	GG	0
1662	Poland	F	45	1	0	GG	0

1686	Poland	F	49	1	0	GG	0
1710	Poland	M	39	1	0	GY	0
1742	Poland	M	31	1	0	GY	0
1746	Poland	M	39	1	0	GY	0
1759	Poland	F	36	1	0	CC	2
1770	Poland	F	40	1	0	GG	0
1785	Poland	F	39	1	0	GC	1
1809	Poland	M	40	1	0	GY	0