Copper-associated hepatitis in the Labrador retriever

diagnosis, treatment and genetics

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diagnose, therapie en genetica

(met een samenvatting in het Nederlands)

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Opgedragen aan mijn vader

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Chapter 1

General introduction: Canine models of copper toxicosis for understanding mammalian copper metabolism

Hille Fieten, Peter A.J. Leegwater, Adrian L. Watson, Jan Rothuizen

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Abstract

Hereditary forms of copper toxicosis exist in man and dogs. In man, Wilson disease is the best studied disorder of copper overload, resulting from mutations in the gene coding for the copper transporter ATP7B. Forms of copper toxicosis for which no causal gene is known yet, are recognized as well, often in young children. Although advances have been made in unraveling the genetic background of disorders of copper metabolism in man, many questions regarding disease mechanisms and copper homeostasis remain unanswered.
Genetic studies in the Bedlington terrier, a dog breed affected with copper toxicosis, identified *COMMD1*, a gene that was previously unknown to be involved in copper metabolism. Besides the Bedlington terrier, a number of other dog breeds suffer from hereditary copper toxicosis and show similar phenotypes to humans with copper storage disorders. Unlike the heterogeneity of most human populations, the genetic structure within a purebred dog population is homogeneous, which is advantageous for unraveling the molecular genetics of complex diseases. This article reviews research performed in the Bedlington terrier, summarizes what was learned from studies into COMMD1 function, describes hereditary copper toxicosis phenotypes in other dog breeds and discusses the opportunities for genome wide association studies on copper toxicosis in the dog to contribute to the understanding of mammalian copper metabolism and copper metabolism disorders in man.

Introduction

The trace element 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. However, it is extremely toxic when present in excessive amounts. Therefore, copper concentrations in the body are tightly regulated[1]. The importance of proper functioning of its homeostatic regulation is illustrated by the genetic disorders Menkes disease (OMIM #309400) and Wilson disease (OMIM #277900), that result from mutations in genes coding for the homologous copper-transporting P-type ATPases ATP7A and ATP7B, respectively.

Dietary copper uptake takes place in the small intestine[2], where CTR1[3] and possibly CTR2[4] and DMT1[5] can facilitate copper uptake into enterocytes. 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[6]. In the blood, copper is bound to small molecules such as histidine, and to serum proteins like α2-macroglobulin and albumin[7] for transport to the liver, the primary site of copper storage[8-10].

Copper enters the hepatocytes via CTR1[11] and is sequestered by small molecules like metallothionein[12] and glutathione[13] in the cytosol. Specialized copper chaperones shuttle copper to their destination molecules. CCS shuttles copper to SOD1, which participates in oxidative stress defense[14]. COX17 is the copper chaperone for the cytochrome C oxidase, which resides in the mitochondrial inner membrane and plays a critical role in the electron transport chain for cellular respiration[15].

The copper chaperone ATOX1[16] delivers copper to ATP7B that is located in the trans Golgi compartment[17-19]. Here, copper is necessary for the formation of holo-ceruloplasmin, which is subsequently secreted into the blood[20]. In addition, ATP7B facilitates the excretion of excess copper into the bile[21] (Figure 1). Mutations in ATP7B can result in Wilson disease (WD), an autosomal recessive disorder[22;23] characterized by copper accumulation in the liver, brain and cornea[24]. Clinical signs manifest in the form of hepatic, neurologic or psychiatric impairment and often become evident in people in the 2nd or 3rd decades of

life[25] (Table 1). The disease incidence is estimated to be 1 in 30,000[26]. A wide variety of mutations in ATP7B have been described (http://www.wilsondisease.med.ualberta.ca) and most patients are compound heterozygotes[27]. There is a lack of correlation between the genotype and the phenotype and individuals carrying the same mutation can show distinct clinical signs, which poses major difficulties for diagnosing the disease[28;29]. Other genes or environmental influences are thought to modify clinical expression of the disease, but have not yet been identified[30].

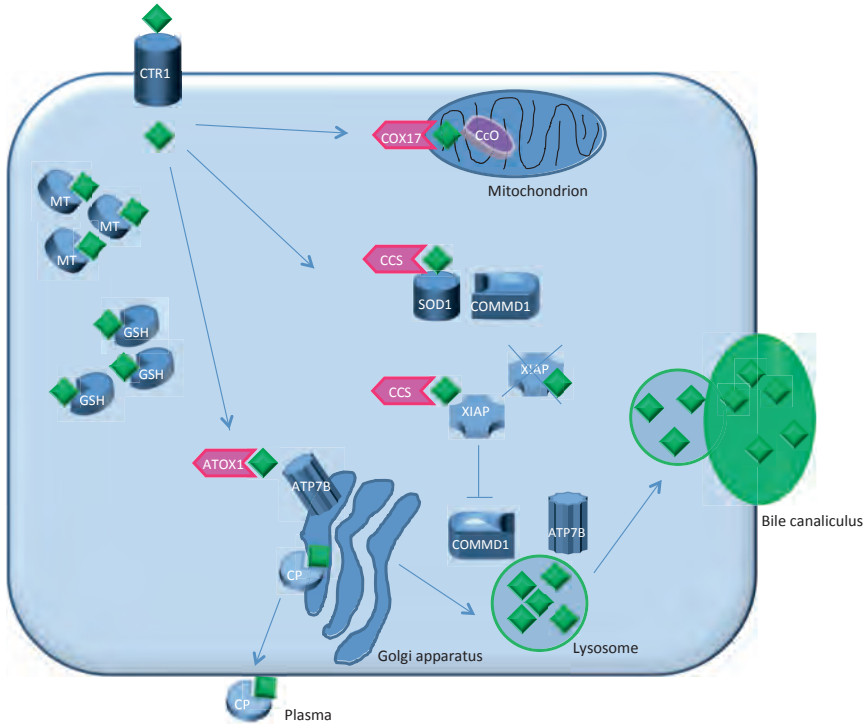


Figure 1 Model of hepatocyte copper metabolism. Copper (green diamond) enters the cell via copper transporter 1 (CTR1) and is sequestered in the cytoplasm by the small molecules metallothionein (MT) and glutathione (GSH). Shuttling of copper to the destination molecules takes place via copper chaperones (pink). COX17 shuttles copper to the cytochrome C oxidase (CcO) in the mitochondria. CCS is the chaperone for superoxide dismutase (SOD1). Recently COMMD1 was shown to interact with SOD1 and this interaction requires CCS-mediated copper incorporation in SOD1. ATOX1 transports copper to ATP7B in the trans Golgi network, where incorporation of copper in apo-ceruloplasmin (CP) takes place. Holo-ceruloplasmin is subsequently excreted in the plasma. The precise mechanism for export of excess copper in the bile is not completely resolved, but it is hypothesized that ATP7B and COMMD1 mediate fusion of copper-loaded vesicular compartments to the apical membrane. Furthermore, COMMD1 may play a role in the maintenance of ATP7B. XIAP can inhibit COMMD1 by promoting its degradation, resulting in cellular copper accumulation. XIAP itself can receive copper from CCS, and copper binding of XIAP results in its degradation and decrease in caspase inhibition, which may result in enhanced apoptosis.

Non-Wilsonian forms of hepatic copper toxicosis in man that often occur in early childhood include Indian childhood cirrhosis (ICC)[31], endemic Tyrolean infantile cirrhosis (ETIC)[32] and idiopathic copper toxicosis (ICT)[33] (Table 1). Although an increased incidence can occur in certain populations, overall these diseases are rare. Genetic defects in these forms of copper toxicosis have not been identified yet, but consanguinity and high dietary copper intake are reported to be involved in the disease pathogenesis, pointing towards a genetic cause, modified by environmental factors.

Besides in man, copper storage disorders have also been identified in other mammals including dogs. Hereditary canine copper toxicosis is identified with a high incidence in a number of purebred dog populations including the Bedlington terrier[34], Skye terrier[35], West Highland white terrier[36], Dalmatian[37], Dobermann[38] and Labrador retriever[39]. Although the disease is characterized by copper accumulation in the liver leading to inflammation and eventually liver cirrhosis in all breeds, phenotypic differences in the magnitude of copper accumulation, sex predisposition and severity of the disease exist between breeds (Table 1). One of the best studies copper storage disorders in dogs is Bedlington terrier copper toxicosis (BTCT). The identification of the causal mutation in *COMMD1* in this breed[40] was a breakthrough in the understanding of mammalian copper homeostasis and the use of purebred dogs to identify disease causing genes. No mutations are currently known in the other affected breeds, suggesting that there are more, currently unidentified, genes involved in canine copper homeostasis.

Recently the purebred dog has emerged as a powerful model to study genetic diseases, due to the unique population structure[41]. With the availability of the complete DNA sequence of the canine genome and new techniques for high-throughput genotyping and DNA sequencing, opportunities are now open to perform genome wide association studies in dogs. The high incidence of copper storage disorders in certain dog breeds, the resemblance with the human phenotypes, the apparent complex etiology, and the possibility to study dietary copper intake, makes copper toxicosis a promising phenotype for genetic studies in the dog.

Table 1 Comparison of phenotypes between different forms of copper toxicosis in man and dogs

	WD	ICC	ETIC	ICT	Bedlington	Labrador	Dobermann	WHW terrier	Dalmatian
Gene	*ATP7B*	UK	UK	UK	*COMMD1*	UK	UK	UK	UK
Mode of inheritance	Autosomal recessive	UK	UK	UK	Autosomal recessive	Complex	UK	UK	UK
Sex predisposition	No	Male	No	No	No	Female	Female	No	No
Age of onset	Adolescence	Early childhood	Early childhood	Early childhood	Adolescence-middle age	Adolescence-middle age	Adolescence-middle age	Middle age-older dogs	Adolescence-middle age
Liver pathology	Cirrhosis	Cirrhosis	Cirrhosis	Cirrhosis	Cirrhosis	Cirrhosis	Cirrhosis	Cirrhosis	Cirrhosis
Reported liver copper increase compared to reference value	10 X	100 x	UK	50x	50x	10x	10x	20x	20x
Neurological impairment	Yes	No	No	No	No	No	No	No	No
Ceruloplasmin	Decreased	Normal or raised	UK	Normal or raised	Normal or raised	UK	UK	UK	
Dietary influence	Minor	Major	Major	Major	Minor	Major	UK	UK	UK

Normal hepatic copper concentration in human liver is <50 µg/g dwl and in dog liver <400 µg/g dwl; UK, unknown

Here we will review the unraveling of the genetics of Bedlington terrier copper toxicosis and how it contributed to the knowledge gain of the functional aspects of COMMD1. Further, we will describe copper toxicosis phenotypes in several dog breeds and discuss the opportunities and possible pitfalls of genome wide association studies in canine copper storage disorders for the detections of new genes underlying copper homeostasis disorders.

Discovery of the copper toxicosis gene in the Bedlington terrier

The appearance of a progressive form of chronic hepatitis accompanied by high hepatic copper concentrations in the Bedlington terrier was first described in the United States[34]. Subsequently, Bedlington terrier copper toxicosis (BTCT) was recognized in Australia[42] and Europe[43-47]. The prevalence was very high, ranging from 25% to 46% in different Bedlington terrier populations[48; 49]. Familial predisposition and an increased incidence of a disease in a closed population, such as the Bedlington terrier breed, indicate a hereditary etiology. Test matings confirmed this assumption and showed an autosomal recessive inheritance pattern[50; 51]. Selective breeding by excluding affected dogs which were diagnosed based on a liver biopsy, led to a decrease in incidence of BTCT. In the well-documented Dutch Bedlington terrier population the incidence dropped dramatically from 46% to 11% [49]. However, because carrier dogs could not be identified by a liver biopsy, a DNA test was needed for complete eradication of the disease.

A search for the causal gene was initiated by several research groups resulting in the exclusion of ATP7B[52;53], metal transporter ATP6H[54], copper transporters CTR1 and CTR2[53] and the copper chaperone ATOX1[52;55] as candidates for BTCT based on mapping criteria or resequencing efforts. The application of the first whole genome linkage study with microsatellite markers in dogs, led to the detection of linkage between the microsatellite marker C04107 and BTCT[56]. Two alleles were present in the Bedlington terrier population, and allele 2 co-segregated with BTCT. The frequency of the disease associated allele was very high in the European, American and Australian populations, and varied from 0.31 to 0.5 [56-59]. Whereas implementation of the microsatellite marker test in the

Bedlington terrier

Although there are some reports of atypical copper toxicosis in Bedlington terriers, a homozygous *COMMD1* exon 2 deletion is causative for BTCT in the majority of dogs[40]. Impaired biliary copper excretion leads to a massive accumulation of copper in the liver, which is the highest that is recognized in any dog breed. Copper concentrations as high as 2,000 µg/g dwl are already recognized in 1 year old dogs, however, often no histological signs of hepatitis are present then[84;122]. Hepatitis develops around 2-5 years of age and the dogs become clinically ill. Successful treatment is possible with D-penicillamine. Without treatment the hepatic copper concentration tends to increase over time and usually reach values of 5,000 µg/g dwl, but in some cases extremely high liver copper levels of 15,000 µg/g dwl have been reported. A tendency of decrease in hepatic copper concentration is present in old animals or in advanced stages of liver cirrhosis[122].

West Highland white terrier

Hepatitis associated with hepatic copper accumulation was first reported in this breed in the Unites States[36]. Later, the same authors reported a larger group of 71 dogs, of which many were related[123]. The disease had a clear familial distribution and when two affected dogs were mated, all dogs in the offspring showed increased hepatic copper concentrations, indicating a hereditary background. Of the 71 cases investigated by Thornburg et al., 44 had a highly increased copper concentration with an equal distribution over both sexes. Copper concentrations do not reach extremely high values, as in the Bedlington terriers, the highest value reported was 6,800 µg/g dwl, however the majority of affected West Highland White terriers has copper concentrations around 2,000 µg/g dwl.

Dobermann

Dobermanns have been reported to have a very severe form of hepatitis and cirrhosis, which is almost exclusively seen in females and has a fatal course within weeks or a few months after diagnosis. Reports from the USA[124], Finland[125], and the Netherlands[38;126-128] describe increased copper concentrations and a predominant monocellular infiltrate in the liver of affected Dobermanns. In the Dutch population a random sample of 15% of a cohort of three year old Dobermanns was followed over time. In 6% of these dogs, copper-associated

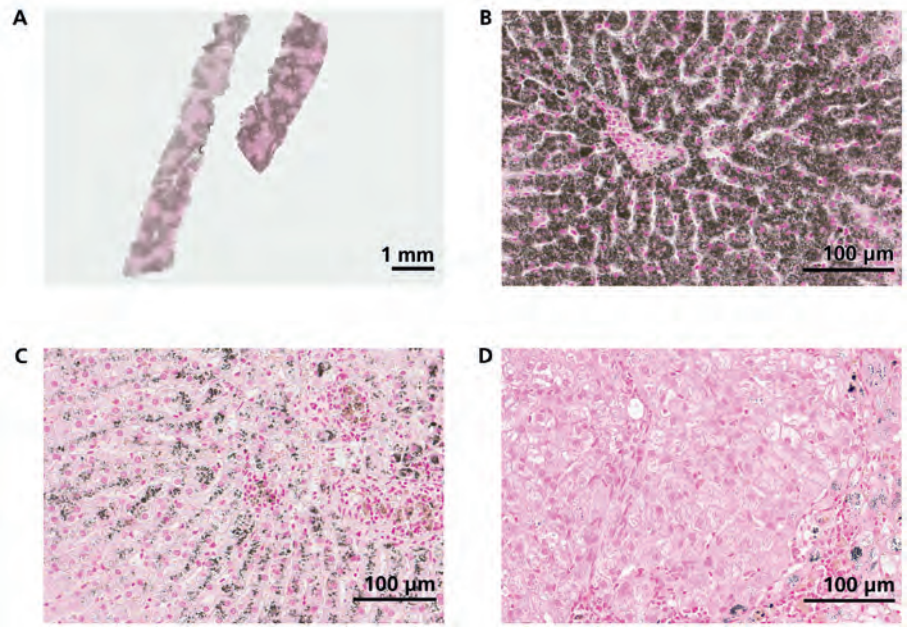


Figure 2 Histological appearance of copper-associated hepatitis in different dog breeds. Liver biopsies are stained with rubeanic acid and haematoxylin counterstain. **A**) Liver biopsy of a Bedlington terrier clearly showing centrolobular distribution of copper. **B)** Liver biopsy of a Bedlington terrier (female, 3 years old, hepatic copper concentration 11,500 µg/g dwl). A large number of copper granules are visible mainly in hepatocytes but also in Kupffer cells. The central vein is located in the middle of the picture. **C)** Liver biopsy of a Labrador retriever (female, 5 years old, hepatic copper concentration 2,360 µg/g dwl). Copper granules are present in hepatocytes and macrophages in the centrolobular area. The centrolobular region at the right area of the picture is characterized by loss of hepatocytes, mild fibrosis and moderate numbers of lymphocytes and plasma cells. **D)** Liver biopsy of a Dobermann (female, 6 years old, hepatic copper concentration of 1,700 µg/g dwl). The centrolobular area is characterized by mild fibrosis with multifocal accumulation of macrophages containing lipofuscin pigment and copper granules. This area shows moderate infiltration with lymphocytes. Hepatocytes in the centrolobular region contain moderate amounts of copper granules.

subclinical hepatitis was present and hepatic copper concentrations increased to 1,000 µg/g dwl over time. The etiological role of copper was demonstrated by the dramatic improvement of the prognosis upon treatment with D-penicillamine associated with a normalization of copper concentrations[115]. Mandigers et al. also demonstrated that the biliary excretion of intravenously injected ^{64}Cu tends to be decreased in affected Dobermanns[128]. MHC class II antigen expression was detected in hepatocytes in cases with Dobermann hepatitis, but not in control tissue[129], therefore Dobermann hepatitis was suggested to be an auto-immune disease. Induction of MHC class II antigen

76. Coronado V. A., B. O'Neill, M. Nanji *et al*. 2008. Polymorphisms in canine ATP7B: candidate modifier of copper toxicosis in the Bedlington terrier. Vet. J. 177: 293-296.

77. Burstein E., L. Ganesh, R.D. Dick *et al*. 2004. A novel role for XIAP in copper homeostasis through regulation of MURR1. EMBO J. 23: 244-254.

78. Spee B., B. Arends, A.M. van Wees *et al*. 2007. Functional consequences of RNA interference targeting COMMD1 in a canine hepatic cell line in relation to copper toxicosis. Anim. Genet. 38: 168-170.

79. Miyayama T., D. Hiraoka, F. Kawaji *et al*. 2010. Roles of COMM-domain-containing 1 in stability and recruitment of the copper-transporting ATPase in a mouse hepatoma cell line. Biochem. J. 429: 53-61.

80. Su L.C., C.A. Owen Jr., P. E. Zollman *et al*. 1982. A defect of biliary excretion of copper in copper-laden Bedlington terriers. Am. J. Physiol. 243: G231-G236.

81. de Bie P., B. van de Sluis, L. Klomp *et al*. 2005. The many faces of the copper metabolism protein MURR1/COMMD1. J. Hered. 96: 803-811.

82. Lutsenko S. & M.J. Petris. 2003. Function and regulation of the mammalian copper-transporting ATPases: insights from biochemical and cell biological approaches. J. Membr. Biol. 191: 1-12.

83. Voskoboinik I., J. Camakaris & J.F. Mercer. 2002. Understanding the mechanism and function of copper P-type ATPases. Adv. Protein Chem. 60: 123-150.

84. Su L. C., S. Ravanshad, C.A. Owen Jr *et al*. 1982. A comparison of copper-loading disease in Bedlington terriers and Wilson's disease in humans. Am. J. Physiol. 243: G226-30.

85. Weiss K.H., J.C. Lozoya, S. Tuma *et al*. 2008. Copper-induced translocation of the Wilson disease protein ATP7B independent of Murr1/COMMD1 and Rab7. Am. J. Pathol. 173: 1783-1794.

86. Kaler S.G. 2011. ATP7A-related copper transport diseases-emerging concepts and future trends. Nat. Rev. Neurol. 7: 15-29.

87. Vonk W.I., P. de Bie, C.G. Wichers *et al*. 2011. The copper-transporting capacity of ATP7A mutants associated with Menkes disease is ameliorated by COMMD1 as a result of improved protein expression. Cell Mol. Life Sci.

88. Vonk W.I., C. Wijmenga, R. Berger *et al*. 2010. Cu,Zn superoxide dismutase maturation and activity are regulated by COMMD1. J. Biol. Chem. 285: 28991-29000.

89. van den Berghe P.V. & L.W. Klomp. 2010. Posttranslational regulation of copper transporters. J. Biol. Inorg. Chem. 15: 37-46.

90. Muller P., H. van Bakel, B. van de Sluis *et al*. 2007. Gene expression profiling of liver cells after copper overload in vivo and in vitro reveals new copper-regulated genes. J. Biol. Inorg. Chem. 12: 495-507.

91. Maine G.N., X. Mao, P.A. Muller *et al*. 2009. COMMD1 expression is controlled by critical residues that determine XIAP binding. Biochem. J. 417: 601-609.

92. Lian M. & X. Zheng. 2009. HSCARG regulates NF-kappaB activation by promoting the ubiquitination of RelA or COMMD1. J. Biol. Chem. 284: 17998-18006.

93. Zoubeidi A., S. Ettinger, E. Beraldi *et al*. 2010. Clusterin facilitates COMMD1 and I-kappaB degradation to enhance NF-kappaB activity in prostate cancer cells. Mol. Cancer. Res. 8: 119-130.

94. Huang Y., M. Wu & H.Y. Li. 2008. Tumor suppressor ARF promotes non-classic proteasome-independent polyubiquitination of COMMD1. J. Biol. Chem. 283: 11453-11460.

95. Brady G.F., S. Galban, X. Liu *et al*. 2010. Regulation of the Copper Chaperone Ccs by Xiap-Mediated Ubiquitination. Mol. Cell. Biol.

96. Mufti A.R., E. Burstein, R.A. Csomos *et al*. 2006. XIAP Is a copper binding protein deregulated in Wilson's disease and other copper toxicosis disorders. Mol. Cell. 21: 775-785.

97. Biasio W., T. Chang, C.J. McIntosh *et al*. 2004. Identification of Murr1 as a regulator of the human delta epithelial sodium channel. J. Biol. Chem. 279: 5429-5434.

98. Ke Y., A.G. Butt, M. Swart *et al*. 2010. COMMD1 downregulates the epithelial sodium channel through Nedd4-2. Am. J. Physiol. Renal Physiol. 298: F1445-56.

99. Maine G.N. & E. Burstein. 2007. COMMD proteins and the control of the NF kappa B pathway. Cell. Cycle. 6: 672-676.

100. van de Sluis B., P. Muller, K. Duran *et al*. 2007. Increased activity of hypoxia-inducible factor 1 is associated with early embryonic lethality in Commd1 null mice. Mol. Cell. Biol. 27: 4142-4156.

101. van de Sluis B., X. Mao, Y. Zhai *et al*. 2010. COMMD1 disrupts HIF-1alpha/beta dimerization and inhibits human tumor cell invasion. J. Clin. Invest. 120: 2119-2130.

102. Muller T., B. van de Sluis, A. Zhernakova *et al*. 2003. The canine copper toxicosis gene MURR1 does not cause non-Wilsonian hepatic copper toxicosis. J. Hepatol. 38: 164-168.

103. Coronado V.A., J.A. Bonneville, H. Nazer *et al*. 2005. COMMD1 (MURR1) as a candidate in patients with copper storage disease of undefined etiology. Clin. Genet. 68: 548-551.

104. Thomas G.R., J.R. Forbes, E.A. Roberts *et al*. 1995. The Wilson disease gene: spectrum of mutations and their consequences. Nat. Genet. 9: 210-217.

105. Schaefer M., R.G. Hopkins, M.L. Failla *et al*. 1999. Hepatocyte-specific localization and copper-dependent trafficking of the Wilson's disease protein in the liver. Am. J. Physiol. 276: G639-46.

106. Stuehler B., J. Reichert, W. Stremmel *et al*. 2004. Analysis of the human homologue of the canine copper toxicosis gene MURR1 in Wilson disease patients. J. Mol. Med. 82: 629-634.

107. Lovicu M., V. Dessi, M. B. Lepori *et al*. 2006. The canine copper toxicosis gene MURR1 is not implicated in the pathogenesis of Wilson disease. J. Gastroenterol. 41: 582-587.

108. Weiss K.H., U. Merle, M. Schaefer *et al*. 2006. Copper toxicosis gene MURR1 is not changed in Wilson disease patients with normal blood ceruloplasmin levels. World J. Gastroenterol. 12: 2239-2242.

109. Gupta A., I. Chattopadhyay, S. Mukherjee *et al*. 2010. A novel COMMD1 mutation Thr174Met associated with elevated urinary copper and signs of enhanced apoptotic cell death in a Wilson Disease patient. Behav. Brain Funct. 6: 33.

110. Wu Z.Y., G.X. Zhao, W.J. Chen *et al*. 2006. Mutation analysis of 218 Chinese patients with Wilson disease revealed no correlation between the canine copper toxicosis gene MURR1 and Wilson disease. J. Mol. Med. 84: 438-442.

111. Hayashi H., S. Wakusawa, M. Yano *et al*. 2007. Genetic background of Japanese patients with adult-onset storage diseases in the liver. Hepatol. Res. 37: 777-783.

112. Bosje J.T., T.S. van den Ingh, A. Fennema *et al*. 2003. Copper-induced hepatitis in an Anatolian shepherd dog. Vet. Rec. 152: 84-85.

113. Thornburg L.P., G. Rottinghaus, M. McGowan *et al*. 1990. Hepatic copper concentrations in purebred and mixed-breed dogs. Vet. Pathol. 27: 81-88.

114. Poldervaart J.H., R.P. Favier, L.C. Penning *et al*. 2009. Primary hepatitis in dogs: a retrospective review (2002-2006). J. Vet. Intern. Med. 23: 72-80.

115. Mandigers P.J., T.S. van den Ingh, P. Bode *et al*. 2005. Improvement in liver pathology after 4 months of D-penicillamine in 5 doberman pinschers with subclinical hepatitis. J. Vet. Intern. Med. 19: 40-43.

116. Hoffmann G., P.G. Jones & S.J. Mesu. 2006. Randomized, doubleblind, placebo-controlled treatment with D-penicillamine against hepatic copper accumulation in Labrador Retrievers. *In* Research Abstract Program of the 24th Annual ACVIM Forum Louisville, KY, may 31–June 3, 2006.

117. Twedt D.C., H.A. Hunsaker & K.G. Allen. 1988. Use of 2,3,2-tetramine as a hepatic copper chelating agent for treatment of copper hepatotoxicosis in Bedlington terriers. J. Am. Vet. Med. Assoc. 192: 52-56.

118. Allen K. G., D.C. Twedt & H.A. Hunsaker. 1987. Tetramine cupruretic agents: a comparison in dogs. Am. J. Vet. Res. 48: 28-30.

119. Hoffmann G. 2009. Copper-associated liver diseases. Vet. Clin. North Am. Small Anim. Pract. 39: 489-511.

120. Brewer G.J., R.D. Dick, W. Schall *et al*. 1992. Use of zinc acetate to treat copper toxicosis in dogs. J. Am. Vet. Med. Assoc. 201: 564-568.

121. Hoogenraad T.U. & J. Rothuizen. 1986. Compliance in Wilson's disease and in copper toxicosis of Bedlington Terriers. Lancet. II: 170.

122. Twedt D.C., I. Sternlieb & S.R. Gilbertson. 1979. Clinical, morphologic, and chemical studies on copper toxicosis of Bedlington Terriers. J. Am. Vet. Med. Assoc. 175: 269-275.

123. Thornburg L.P., G. Rottinghaus, G. Dennis *et al*. 1996. The relationship between hepatic copper content and morphologic changes in the liver of West Highland White Terriers. Vet. Pathol. 33: 656-661.

124. Thornburg L.P. 1998. Histomorphological and immunohistochemical studies of chronic active hepatitis in Doberman Pinschers. Vet. Pathol. 35: 380-385.

125. Speeti M., J. Eriksson, S. Saari *et al*. 1998. Lesions of subclinical doberman hepatitis. Vet. Pathol. 35: 361-369.

126. van den Ingh T.S., J. Rothuizen & R. Cupery. 1988. Chronic active hepatitis with cirrhosis in the Doberman pinscher. Vet. Q. 10: 84-89.

127. Spee B., P.J. Mandigers, B. Arends *et al*. 2005. Differential expression of copper-associated and oxidative stress related proteins in a new variant of copper toxicosis in Doberman pinschers. Comp. Hepatol. 4: 3.

128. Mandigers P.J.J., P. Bode, A.M.T.C. van Wees *et al*. 2007. Hepatic ^{64}Cu excretion in Dobermanns with subclinical hepatitis. Res. Vet. Sci. 83: 204-209.

129. Speeti M., A. Stahls, S. Meri *et al*. 2003. Upregulation of major histocompatibility complex class II antigens in hepatocytes in Doberman hepatitis. Vet. Immunol. Immunopathol. 96: 1-12.

130. Dyggve H., L.J. Kennedy, S. Meri *et al*. 2011. Association of Doberman hepatitis to canine major histocompatibility complex II. Tissue Antigens. 77: 30-35.

131. Lequarre A.S., L. Andersson, C. Andre *et al*. 2011. LUPA: A European initiative taking advantage of the canine genome architecture for unravelling complex disorders in both human and dogs. Vet. J. 189: 155-159.

132. Cooper V.L., M.P. Carlson, J. Jacobson *et al*. 1997. Hepatitis and increased copper levels in a dalmatian. J. Vet. Diagn. Invest. 9: 201-203.

133. Noaker L.J., R.J. Washabau, C.J. Detrisac *et al*. 1999. Copper-associated acute hepatic failure in a dog. J. Am. Vet. Med. Assoc. 214: 1502-6, 1495.

134. Andersson M. & E. Sevelius. 1991. Breed, sex and age distribution in dogs with chronic liver disease: a demographic study. J. Small Anim. Pract. 32: 1-5.

135. Shih J.L., J.H. Keating, L.M. Freeman *et al*. 2007. Chronic hepatitis in Labrador Retrievers: Clinical presentation and prognostic factors. J. Vet. Intern. Med. 21: 33-39.

136. Smedley R., T. Mullaney & W. Rumbeiha. 2009. Copper-associated hepatitis in Labrador Retrievers. Vet. Pathol. 46: 484-490.

137. Hoffmann G., H.C. Heuven, P.A. Leegwater *et al*. 2008. Heritabilities of copper-accumulating traits in Labrador retrievers. Anim. Genet. 39: 454.

138. Hoffmann G., P.G. Jones, V. Biourge *et al*. 2009. Dietary management of hepatic copper accumulation in Labrador retrievers. J. Vet. Intern. Med. 23: 957-963.

139. Karlsson E.K. & K. Lindblad-Toh. 2008. Leader of the pack: gene mapping in dogs and other model organisms. Nat. Rev. Genet. 9: 713-725.

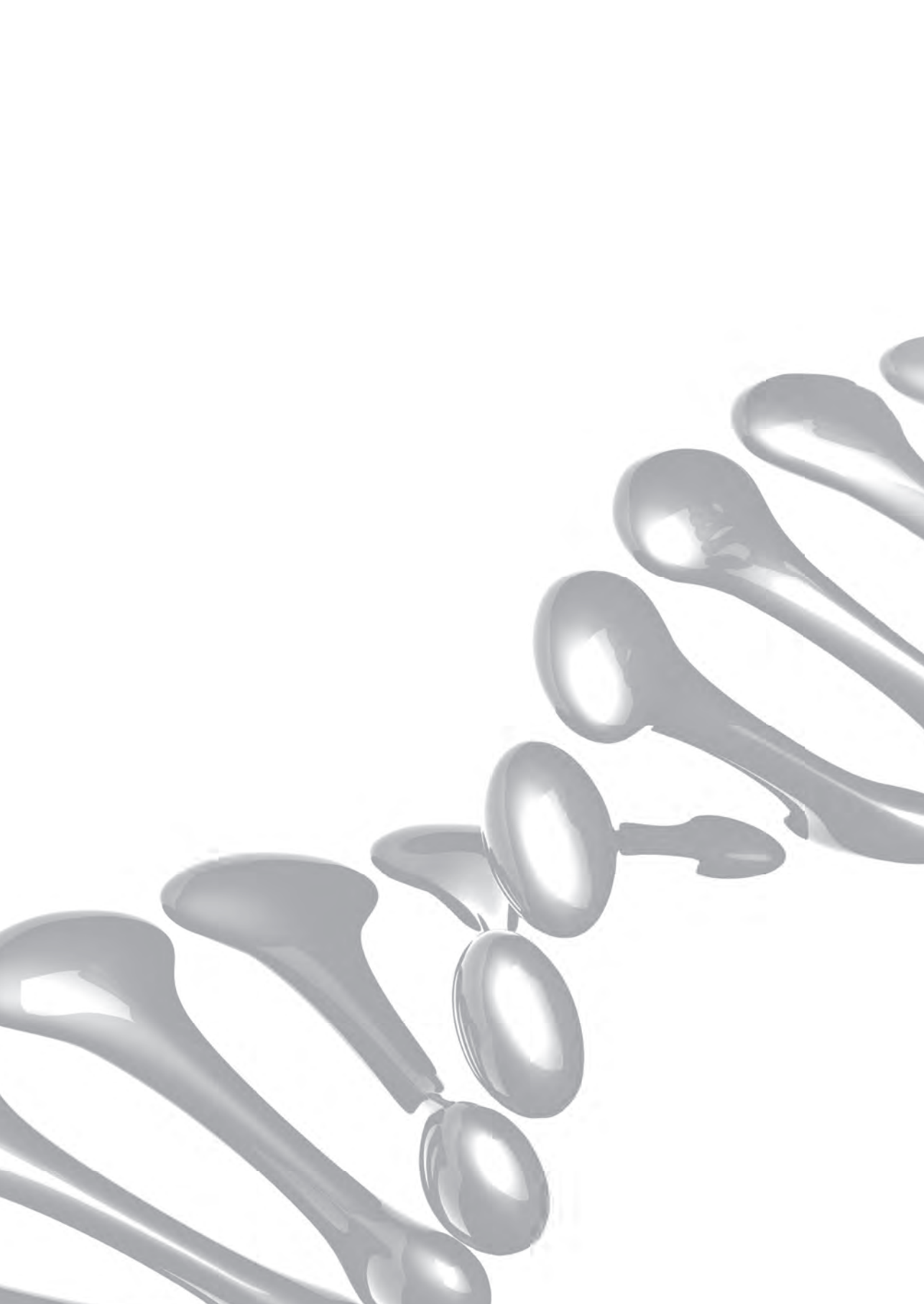
140. Wilbe M., P. Jokinen, K. Truve *et al*. 2010. Genome-wide association mapping identifies multiple loci for a canine SLE-related disease complex. Nat. Genet. 42: 250-254.

141. Lindblad-Toh K., C.M. Wade, T.S. Mikkelsen *et al*. 2005. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature. 438: 803-819.

142. Sutter N.B., M.A. Eberle, H.G. Parker *et al*. 2004. Extensive and breed-specific linkage disequilibrium in Canis familiaris. Genome Res. 14: 2388-2396.

143. Karlsson E.K., I. Baranowska, C.M. Wade *et al*. 2007. Efficient mapping of mendelian traits in dogs through genome-wide association. Nat. Genet. 39: 1321-1328.

144. Aulchenko Y.S., S. Ripke, A. Isaacs *et al*. 2007. GenABEL: an R library for genome-wide association analysis. Bioinformatics. 23: 1294-1296.



Chapter 2

Aims and Scope

Copper is an important trace element and is indispensable for a large number of biological processes in the body. Therefore, copper metabolism is tightly regulated. The liver is the main organ involved in regulation of copper metabolism, but is also the first to be affected in copper overload. In both dogs and humans aberrant hepatic copper accumulation eventually results in liver cirrhosis.

Copper-associated hepatitis accounts for approximately 1/3 of all primary hepatitis cases in dogs and is recognized with increasing frequency. Whilst the disease is described in a large number of dog breeds, the focus of this thesis will be on the Labrador retriever. This breed is the most popular dog breed worldwide and is an important example of a dog breed with a complex form of copper-associated hepatitis, where multiple gene mutations and environmental influences contribute to the disease phenotype.

The recognition of an increased incidence of chronic hepatitis in certain families of Labrador retrievers, which was refractory to traditional prednisolone therapy, prompted the researchers at the Faculty of Veterinary Medicine, Utrecht University to start an investigation into the etiological background. The application of specific copper staining to histological slides of liver biopsies of affected Labrador retrievers, identified centrolobular copper accumulation. Chelation therapy with D-penicillamine led to a markedly improved outcome, indicating an etiological role of copper in the disease pathogenesis. This was the starting point of a large study in collaboration with the Dutch Labrador retriever breed club, and funded by Waltham Centre of Pet nutrition. Labrador retriever cases of copper-associated hepatitis were identified from patient files from the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine and dogs related to affected individuals were recruited and admitted for a liver biopsy and DNA collection for genetic studies. A tremendously valuable database of Labrador retrievers in various (subclinical) stages of the disease was built in this way and was a useful starting point for the clinical studies described in this thesis involving optimization of diagnosis and therapeutic protocols.

The central aims for the studies presented in this thesis were:

1. To optimize diagnosis and medical treatment of copper-associated hepatitis in the Labrador retriever

2. To evaluate the role of diet in the disease pathogenesis and long-term management strategies

3. To discover causal gene mutations involved in copper-associated hepatitis in the Labrador retriever and to provide functional evidence for their involvement in the disease

The first part of this thesis (**Part I Diagnosis and D-penicillamine therapy**) focused on optimization of diagnosis (**Chapter 3**) and D-penicillamine treatment (**Chapter 4**). In humans with Wilson disease, urinary excretion of copper and zinc is used for diagnostic purposes and for monitoring efficacy of chelation therapy. The aim of the studies described in **Chapter 3** was to investigate the possibility of urinary excretion of copper, zinc and iron in diagnosis and follow-up of therapy in dogs with copper-associated hepatitis. Therefore, we explored urinary excretion patterns of copper, iron and zinc with and without administration of D-penicillamine in Labrador retrievers, COMMD1-deficient dogs and Beagles. Furthermore, we studied the association between urinary copper in a single urine sample with hepatic copper concentrations in these 3 groups of dogs. Finally we studied the urinary copper/zinc ratios in relation to hepatic copper concentration in Labrador retrievers at first biopsy as well as in follow-up samples during therapy.

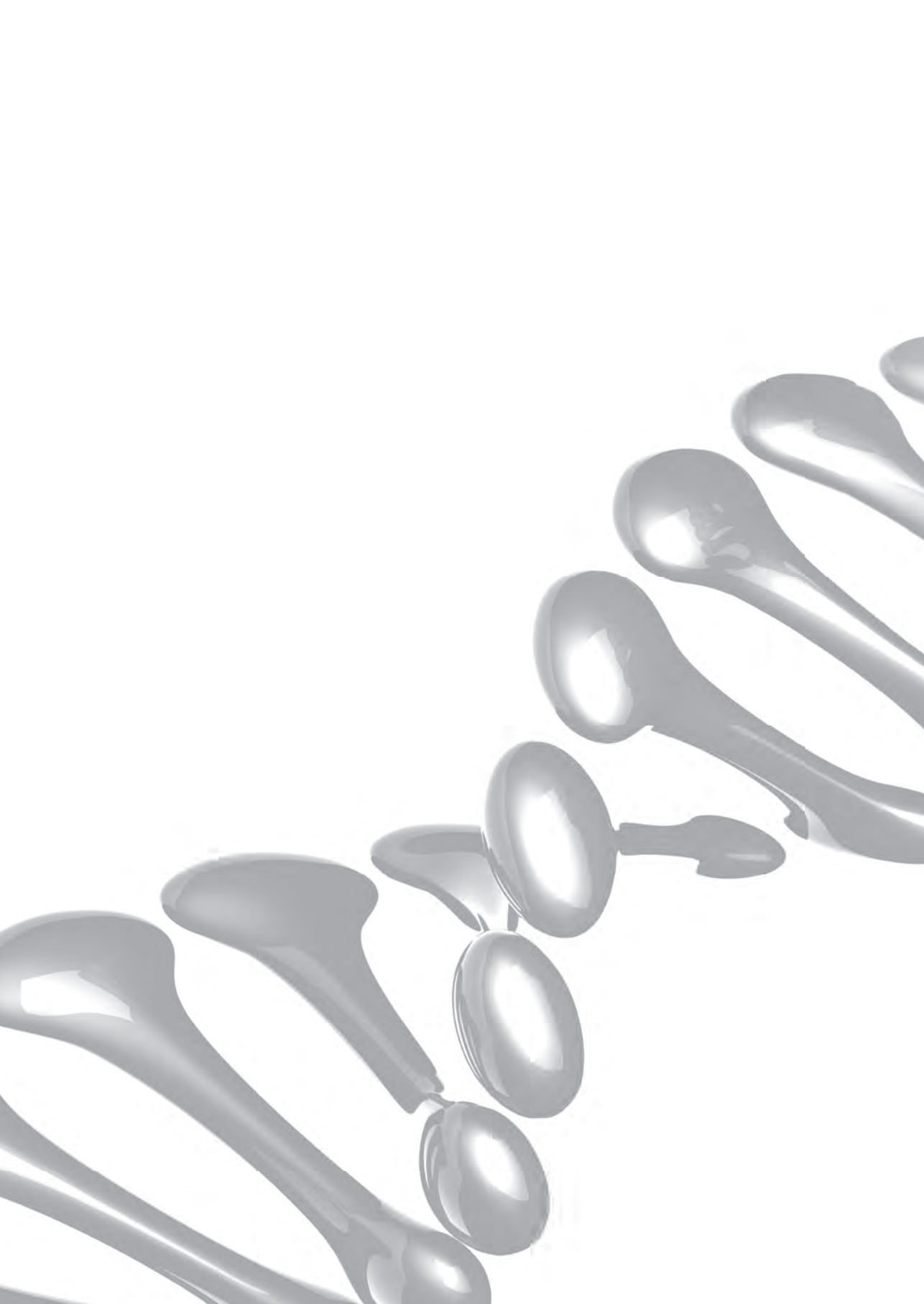
As illustrated by the results obtained in **Chapter 3**, D-penicillamine is a highly effective copper chelator, promoting urinary copper excretion. Traditionally, COMMD1-deficient dogs are usually treated continuously during their entire lifetime. However, dogs with a complex form of copper-associated hepatitis, do not show extreme high levels of copper in their liver and optimal treatment durations for these dogs were not established. In **Chapter 4** we aimed to contribute to the development of an optimal treatment protocol for dogs with complex copper-associated hepatitis. Therefore, we determined three main goals: (1) establishing a model to predict the duration of D-penicillamine

treatment required to reach a normal hepatic copper concentration in Labrador retrievers, (2) evaluating the effect of D-penicillamine treatment on the activity and stage of copper-associated hepatitis, and (3) determining the effect of D-penicillamine treatment on hepatic iron and zinc concentrations for identification of possible metal deficiencies upon chelation therapy.

The second part of this thesis (**Part II Diet**) focused on the effect of intake of dietary copper and zinc in copper-associated hepatitis in Labrador retrievers. Within the community of specialists in Veterinary Internal Medicine there has been an ongoing debate, as to whether copper-associated hepatitis is an expression of an increase in copper levels in pet food over the last decade rather than a genetic disease. In **Part II** we aimed to provide insights into a possible etiological role of diet and explored the potential of diet for long-term management of affected dogs. No reliable data was available regarding a possible effect of dietary copper and zinc levels in generally fed commercial dog food on hepatic copper and zinc levels. Therefore, the aim of the study presented in **Chapter 5** was to investigate this effect in Labrador retrievers. The study confirmed that hepatic copper concentrations were positively correlated to dietary copper- and negatively correlated to dietary zinc levels. In addition, we observed that lifelong continuous D-penicillamine therapy may bear the risk of copper and zinc deficiency in Labrador retrievers with complex copper-associated hepatitis and may therefore not be an ideal long-term management strategy (**Chapter 4**). The resultant of the studies described in **Chapter 4** and **Chapter 5** prompted us to evaluate the utility of a low copper, high zinc diet in the long-term management of Labrador retrievers affected by hereditary copper-associated hepatitis that previously underwent chelation therapy with D-penicillamine (**Chapter 6**). The positive results from this study encouraged us to further explore the application of this diet for use in an early, pre-clinical stage of the disease in order to prevent copper accumulation and possibly clinical illness (**Chapter 7**).

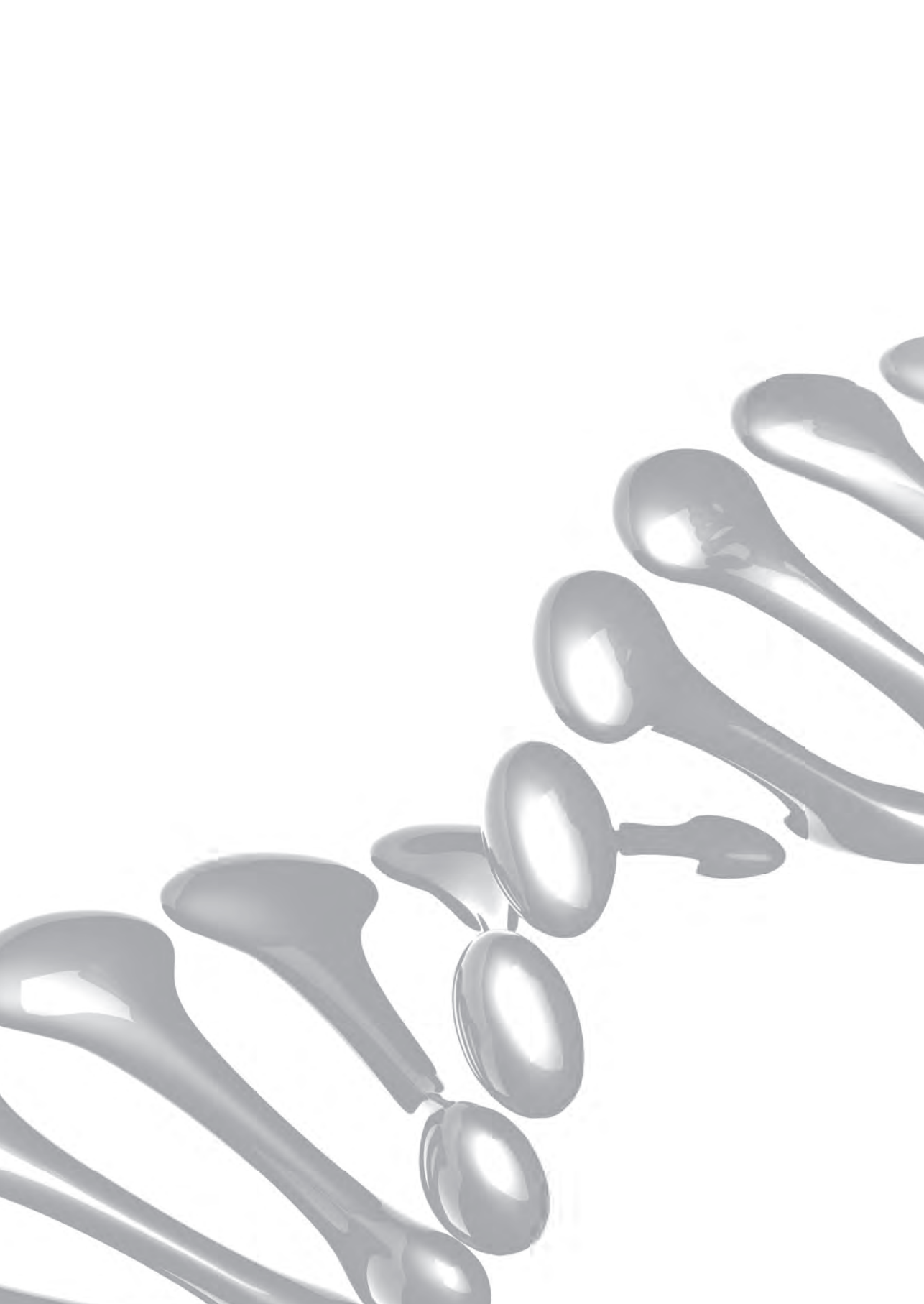
The final part of this thesis (**Part III Genetics**) contains the genetics study in which we identified gene variants involved in copper-associated hepatitis in the Labrador retriever and provided biological evidence for their involvement in the disease (**Chapter 8**). In this study we exploited the limited genetic and phenotypic variation within the Labrador retriever breed for a genome

wide association analysis with hepatic copper level as a quantitative trait. Translation of our findings in the Labrador retrievers, may be of importance for the understanding of mammalian copper metabolism, and in addition, may have direct implications for human patients suffering from a variety of copper metabolism disorders.



Part III

Genetics



Chapter 8

The Menkes and Wilson disease genes counteract in copper toxicosis in Labrador retrievers: a new canine model for copper metabolism disorders

Hille Fieten, Yadvinder Gill, Alan J. Martin, Karen Dirksen, Frank G. van Steenbeek , Ted S.G.A.M. van den Ingh, Ellen C.C.P. Martens, Paola Festa, Giancarlo Chesi, Mafalda Concilli, Bart J.A. van de Sluis, Roderick H.J.H. Houwen, Adrian L. Watson, Yurii S. Aulchenko, Roman S. Polishchuk, Peter A.J. Leegwater, Jan Rothuizen

Disease Models and Mechanisms, under review

Abstract

The deleterious effects of a disrupted copper metabolism are illustrated by hereditary diseases caused by mutations in the copper transporters ATP7A and ATP7B. Menkes disease, involving *ATP7A*, is a fatal neurodegenerative disorder of copper deficiency. Mutations in *ATP7B* lead to Wilson disease, which is characterized by a predominantly hepatic copper accumulation. The low incidence and the phenotypic variability of human copper toxicosis hampers identification of causal genes or modifier genes involved in the disease pathogenesis. The Labrador retriever was recently characterized as a new mammalian model for copper toxicosis. Purebred dogs have reduced genetic variability which facilitates identification of genes involved in complex heritable traits that may influence phenotype in both humans and dogs.

We performed a genome wide association study in 235 Labrador retrievers and identified two chromosome regions containing *ATP7A* and *ATP7B* that were associated with variation in hepatic copper levels. DNA sequence analysis identified missense mutations in each gene. The amino-acid substitution ATP7B:p.Arg1453Gln was associated with copper accumulation, whereas the amino-acid substitution ATP7A:p.Thr327Ile partly protected against copper accumulation. Confocal microscopy indicated that aberrant copper metabolism upon expression of the ATP7B variant occurred due to mis-localization of the protein in the endoplasmic reticulum. Dermal fibroblasts derived from ATP7A:p.Thr327Ile dogs showed copper accumulation and delayed excretion.

We identified the Labrador retriever as the first natural, non-rodent model for *ATP7B* associated copper toxicosis (Wilson disease). Attenuation of copper accumulation by the *ATP7A* mutation, sheds an interesting light on the interplay of copper transporters in body copper homeostasis and warrants a thorough investigation of *ATP7A* as a modifier gene in copper metabolism disorders. The identification of two new functional variants in ATP7A and ATP7B contribute to the biological understanding of protein function, with relevance for future therapy development.

Introduction

Copper is an essential trace element for a wide range of biochemical processes in the body. The P-type copper-transporting ATPases ATP7A[1] and ATP7B[2] are crucial for copper homeostasis. On a cellular level, these proteins have a biosynthetic role in the *trans*-Golgi network, where they facilitate incorporation of copper into proteins. Further, they prevent toxic accumulation of cellular copper by redistribution to a vesicular compartment resulting in excretion of copper through either the apical membrane (ATP7B) or the baso-lateral membrane (ATP7A). This redistribution involves metal binding domains (MBD) in cytoplasmic domains of the proteins and is in part regulated by their phosphorylation state[3].

Body copper homeostasis is maintained by balancing the rates of dietary copper absorption and biliary copper excretion. ATP7A is highly expressed in enterocytes where it is involved in copper uptake across the baso-lateral membrane into the portal circulation[4]. Excretion of excess copper into the bile requires expression of functional ATP7B in hepatocytes[5].

The severe effects of copper metabolism imbalance are illustrated by inherited disorders resulting from mutations in *ATP7A* and *ATP7B*. Mutations in *ATP7A* result in a fatal, X-linked copper deficiency disorder in infants known as Menkes disease. The disease is characterized by cerebral and cerebellar degeneration, failure to thrive, coarse hair and connective tissue abnormalities[6]. Wilson disease is caused by mutations in *ATP7B*[5] and is associated with copper accumulation in the liver and secondarily in the brain resulting in liver cirrhosis and neuronal degeneration. The age of onset and the clinical manifestations vary greatly between Wilson disease patients. This lack of genotype-phenotype correlation may be influenced by currently unidentified genetic modifiers. Other hereditary diseases leading to hepatic copper accumulation in infants include Indian Childhood Cirrhosis (ICC)[7] and Endemic Tyrolean Infantile Cirrhosis (ETIC)[8]. In these diseases, the causal genes are currently unknown and dietary copper intake is believed to contribute significantly to disease progression. In order to develop new treatment strategies for copper metabolism disorders, several rodent models were investigated, including natural models like the mottled mouse[9], the toxic milk mouse[10] and the Long Evans Cinnamon rat[11] as well

100 µl 10.3 M HNO_3 and subsequently neutralized with 0.5 M NaOH. DMEM medium was added to give a final concentration of 10 mM copper.

Fibroblast cells were plated in triplicate in 6 well plates in DMEM + 10% FBS. Cells were incubated with 3 µM ^{64}Cu for 6, 22 or 30 hours. Culture medium was removed after treatment, and the cells were washed four times with Hanks containing 3 µM $CuCl_2$. Following wash steps, 325 µl of 0.2% SDS was added to lyse the cells. Cellular copper loading was determined from counts of the cell lysate (450 and 800 keV for 1 min, Packard B5003 gamma counter, Packard BioScience Benelux, Groningen, Netherlands). For efflux studies, following copper treatment for 22 hours as described above, cells were washed four times with Hanks containing 3 µM $CuCl_2$ prior to incubation in fresh medium containing no additional copper for 8 hours. Cellular copper loading was assessed at 0, 0.5, 1.5, 3, 6 and 8 hours as described in the previous paragraph. Copper loading was normalized to the protein concentration of the cell lysate (Bio Rad Protein Assay Kit, Bio Rad, the Netherlands). ^{65}Zn (Perkin Elmer, 1mCi chloride solution) was used as a control and was prepared as described for ^{64}Cu experiments.

Statistical analysis

Counts per minute normalized to the protein concentration of the cell lysate (cpm/mg) were analyzed using linear mixed-effect modeling with the R package "nlme". The outcome variable was transformed by taking its natural logarithm in order to fulfill the criteria of normality and constant variance of residuals. Restricted maximum likelihood was used to estimate the fixed effects of ^{64}Cu treatment (hours, modeled as a categorical factor), (presence or absence of the ATP7A:p.Thr327Ile substitution), sex (male, female) and their interactions. A stepwise backward method was used to determine the model of best fit, based on Akaike's information criterion. Estimates were reported with their 95% confidence intervals. For the efflux studies, the amount of ^{64}Cu at the start of the experiment was set to 100% and at the time points 0.5, 1.5, 3, 6 and 8 hours the amount of ^{64}Cu was calculated as a percentage of the initial value. Per time-interval the efflux rate of copper out of the cells was calculated in % efflux/hour. The average efflux rates between fibroblasts derived from dogs with ATP7A wild type and dogs with the ATP7A:p.Thr327Ile substitution were compared by a Wilcoxon signed rank test.

Results

Dogs

The initial GWAS cohort consisted of 235 Labrador retrievers. The independent replication cohort consisted of 59 Labrador retrievers. The median copper score in the 294 Labrador retrievers included in the study was 1.5 (range, 0-5). A summary of characteristics of the dogs, including age, sex and hepatic copper scores is provided in Table 1.

Table 1 Study sample of Labrador retrievers

		Number of dogs	Copper score	Age (years)
GWAS	**Female dogs**	71	<2	6.7 ± 2.8
N = 235	***N* = 154**	45	2-3	5.3 ± 2.5
		38	≥3	5.7 ± 2.4
	Male dogs	46	<2	5.6 ± 2.3
	***N* = 81**	23	2-3	5.7 ± 2.3
		12	≥3	5.6 ± 2.8
Replication set	**Female dogs**	21	<2	7.2 ± 3.6
N = 59	***N* = 37**	11	2-3	8.2 ± 3.4
		5	≥3	6.3 ± 3.8
	Male dogs			
	***N* = 22**	15	<2	7.9 ± 2.3
		7	2-3	7.0 ± 3.7

Data are grouped by dataset (GWAS and independent replication set), sex (male, female) and hepatic copper score: normal hepatic copper (score <2), increased hepatic copper (score 2-3) and high hepatic copper (score ≥ 3). Age is reported as average ± standard deviation.

Electron microscopy evaluation of copper toxicosis in Labrador retrievers

To complement the phenotype description of copper toxicosis in Labrador retrieves, liver biopsies of two Labrador retrievers affected with copper toxicosis and a control dog were assessed by electron microscopy. Ultrastructural changes in the biopsies of the affected dogs corresponded to copper-laden lysosomes comparable to what is recognized by electron microscopy of the liver of patients with Wilson disease (Figure 1).

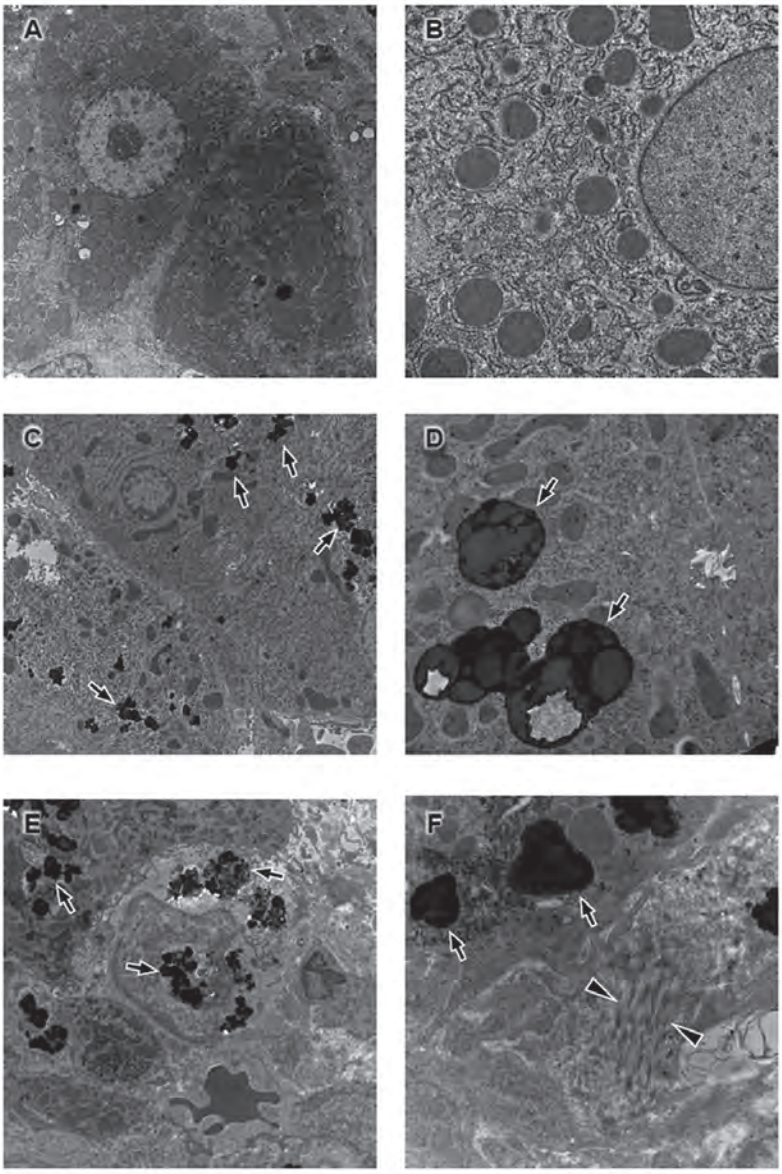


Figure 1 Hepatic ultrastructure in Labrador retrievers with increased hepatic copper concentration. (A,B) Low (A) and high (B) magnification of thin sections from the liver reveal regular organization of the hepatic tissue in a 1 year old male cross breed (control dog, hepatic copper: 250 mg/kg dry weight liver). (C,D) Ultrastructure of an 11 year old, neutered, female Labrador retriever (affected dog, hepatic copper 756 mg/kg dwl) exhibits wide areas with accumulation of electron dense material (C; arrows) which at higher magnification appear as copper-laden lysosomes comparable to what is observed in Wilson disease patients (D; arrows). (E,F) Ultrastructure of a liver biopsy of a 12 year old, neutered, female Labrador retriever (Affected, hepatic copper 1445 mg/kg dwl): some areas of the liver show severe disorganization of the tissue architecture (E) with massive electron dense inclusions (E,F; arrows). Arrowheads in F indicate bundles of collagen fibers as a sign of extensive fibrosis.

Genome wide association study of copper toxicosis in Labrador retrievers

After quality control of genotype data used for the GWAS, 35.7% of SNPs were removed due to a low minor allele frequency and 0.7% of SNPs had a call rate of less than 98%, leaving 109,496 SNPs and 235 dogs for analysis. Population stratification was determined by a multidimensional scaling plot (see supplementary material Figure S1A) and inflation of the test statistic was inspected by a QQ-plot (see supplementary material Figure S1B). The heritability of hepatic copper score was estimated to be 0.48. The highest association signal using the liver copper level as a quantitative trait in a linear mixed model was identified on chromosome 22 with a p-value of $2.4 * 10^{-6}$ and a beta of 0.65 (SE 0.14) (Figure 2A). Inspection of the region showed an LD-block at the first 10 Mb of the chromosome. The critical interval was determined to be at 22:152034-10753911 which contained 83 genes, including the candidate gene *ATP7B* located at position 22:162769-225266 (Canfam 3.1) (Figure 2B). A stratified analysis for males (Figure 2C) and females (Figure 2D) was performed. In the male subset, the critical region was located at X:54322875-65713151 and contained 77 genes. The highest association signal ($p= 1.1 * 10^{-4}$ and a beta of -0.57 (SE 0.15)) was caused by 6 SNPs that formed an LD-block of 8.2 Mb including the candidate gene *ATP7A* at position X:60203319-60356690 (Figure 2C). Because *ATP7A* and *ATP7B* are copper transporters, and strong candidate genes for involvement in copper toxicosis, we focused on these genes by in-depth DNA sequence analysis. A list of observed DNA variants and their association with hepatic copper score were presented in Table 2.

The 5 nominally significant variations (p-value ≤0.05) were genotyped in an independent cohort of 59 Labrador retrievers (Table 1&2). The association with hepatic copper score was replicated for the non-synonymous nucleotide substitution of *ATP7A* at X-chromosomal position 60279238 (ENSCAFT00000049745 *ATP7A*:c.980C>T), the intronic variation in *ATP7A* (position X:60338569) and the non-synonymous nucleotide substitution of *ATP7B* at chromosome 22, position 225112 (ENSCAFT00000006859 *ATP7B*:c.4358G>A). When analyzed in the complete dataset (n=294) the p-values were similar to or lower than initially derived in the GWAS; $1.6* 10^{-4}$ and $4.9*10^{-7}$ for *ATP7A*:c.980C>T and *ATP7B*:c.4358G>A, respectively (Table 2).

Table 2 Mutations and effect estimates with regard to hepatic copper scores

									Sequence set of 96 dogs				Replication in 59 dogs				Total 294 dogs			
Gene	**Variant**	**Location**	**Chr**	**Position (CF 3.1)**	**allele ref**	**allele_nonref**	**AA ref**	**AA non ref**	**All freq non-ref**	**Effect estimate**	**SE**	**p-value**	**All freq non-ref**	**Effect estimate**	**SE**	**p-value**	**All freq non-ref**	**Effect estimate**	**SE**	**p-value**
ATP7A	SNP	Exon 4	X	60279238	C	T	T	I	0.26	-0.44	0.18	0.02	0.22	-0.45	0.21	0.038	0.22	-0.36	0.09	$1.6*10^{-4}$
ATP7A	SNP	Intron 5	X	60289928	C	A			0.38	0.059	0.17	0.73								
ATP7A	SNP	Intron 14	X	60326659	G	T			0.51	0.069	0.15	0.65								
ATP7A	SNP	Exon 15	X	60327032	C	T			0.39	0.056	0.17	0.74								
ATP7A	SNP	Intron 18	X	60338569	C	T			0.25	-0.52	0.18	0.005	0.22	-0.62	0.2	0.004	0.22	-0.38	0.09	$7.4*10^{-5}$
ATP7A	SNP	Intron 19	X	60348365	C	T			0.39	0.003	0.17	0.98								
ATP7A	SNP	Intron 20	X	60348994	A	G			0.69	-0.63	0.18	0.00079	0.19	0.029	0.23	0.9				
ATP7A	SNP	Intron 21	X	60351656	G	A			0.40	0.01	0.17	0.95								
ATP7B	SNP	Intron 1	22	191461	C	T			0.62	-0.06	0.2	0.76								
ATP7B	indel	Intron 1	22	191502	_	Ins AGCTCAG			0.62	-0.022	0.2	0.91								
ATP7B	SNP	Exon 2	22	192722	G	A	A	T	0.19	-0.52	0.22	0.019	0.09	-0.45	0.36	0.21				
ATP7B	indel	Exon 2	22	192865	gccccgccccgcccc	gccccgccccgccccgcccc	(AP)3	(AP)4	0.09	0.61	0.31	0.05	0.09	-0.12	0.38	0.74				
ATP7B	SNP	Exon 2	22	192970	C	T	I	I	0.01	-0.67	0.91	0.46								
ATP7B	SNP	Intron 2	22	193128	T	C			0.01	-0.67	0.91	0.46								
ATP7B	SNP	Exon 3	22	199368	A	G	A	A	0.38	0.034	0.19	0.86								
ATP7B	SNP	Exon 3	22	199395	T	C	A	A	0.38	0.034	0.19	0.86								
ATP7B	SNP	Exon 4	22	201943	C	T	I	I	0.01	0.052	0.19	0.79								
ATP7B	SNP	Intron 4	22	203200	A	G			0.39	0.056	0.19	0.77								
ATP7B	SNP	Intron 6	22	204445	G	A			0.375	0.065	0.19	0.74								
ATP7B	SNP	Intron 8	22	207706	C	T			0.85	-0.021	0.19	0.91								
ATP7B	indel	Intron 11	22	213592		delT			0.01	-0.67	0.91	0.46								
ATP7B	SNP	Intron 11	22	213626	G	A			0.38	0.096	0.19	0.62								
ATP7B	SNP	Intron 14	22	218433	C	T			0.37	0.052	0.19	0.79								
ATP7B	SNP	Intron 16	22	220886	C	T			0.62	0.014	0.19	0.94								
ATP7B	SNP	Intron 16	22	222014	G	A			0.01	-0.62	0.9	0.49								
ATP7B	indel	Intron 19	22	223949		ins C			0.48	-0.047	0.18	0.8								
ATP7B	indel	Intron 20	22	224511		ins C			0.61	0.0091	0.19	0.96								
ATP7B	SNP	Exon 21	22	225112	G	A	R	Q	0.29	0.41	0.2	0.039	0.27	0.48	0.23	0.046	0.31	0.51	0.1	$4.9*10^{-7}$

Summary of identified mutations in ATP7A and ATP7B in 96 Labrador retrievers that were randomly selected from the cohort of 235 Labrador retrievers used for the GWAS. Effect estimates, standard errors and p-values for the non-reference allele on copper score, corrected for sex. Significant mutations ($P \leq 0.05$) were replicated in an independent cohort of 59 Labrador retrievers. Significantly replicated mutations were genotyped in the entire cohort of 294 Labrador retrievers and allele frequencies, effect estimates and *P*-values were exported in the last column. AA, amino acid; All freq, allele frequency; Chr, Chromosome; CF 3.1, CanFam 3.1 genome build; non-ref, non-reference; ref, reference; SE, standard error.

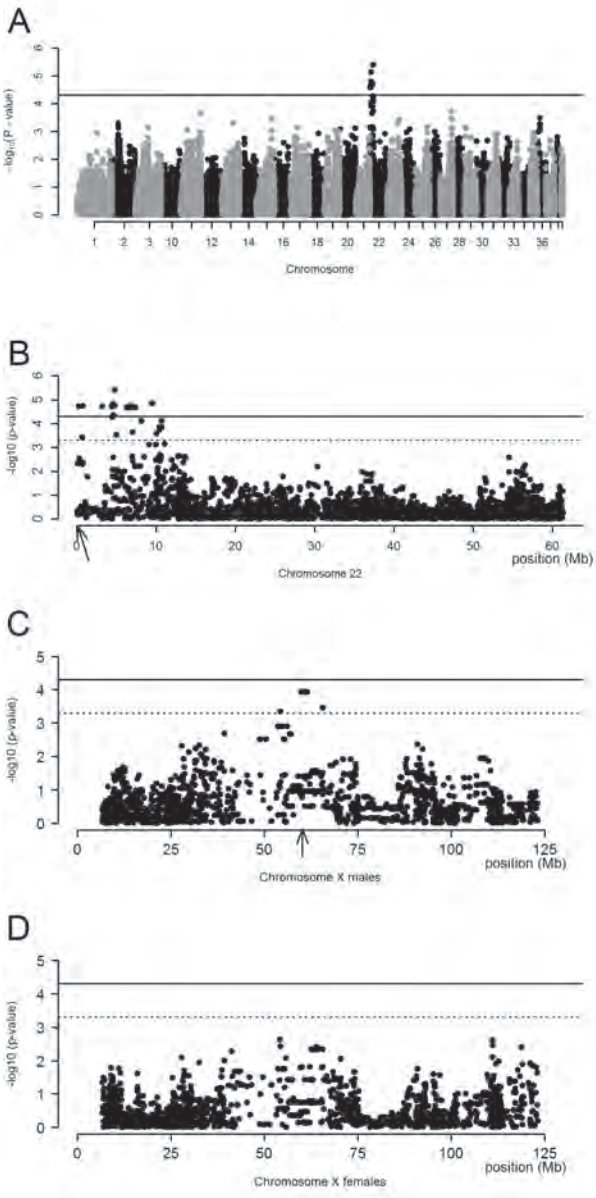
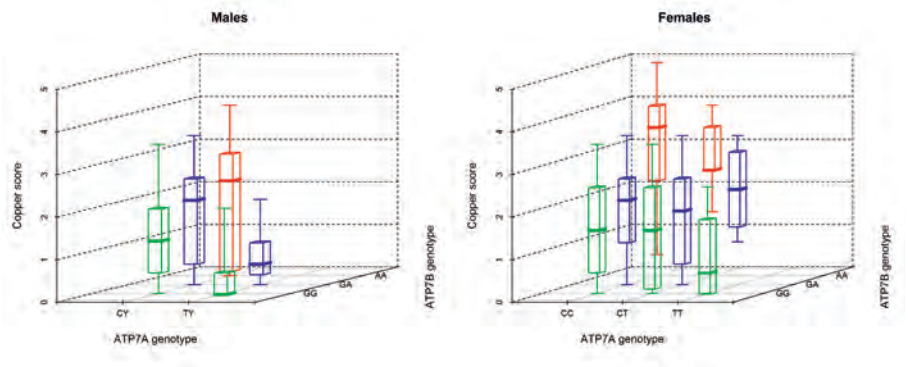


Figure 2 Manhattan plots for hepatic copper score in Labrador retrievers. **2A** Manhattan plot of hepatic copper score in 235 Labrador retrievers, shows a genome wide association signal at chromosome 22. **2B** Manhattan plot of chromosome 22 shows that the signal includes an LD block comprising the first 10 Mb of the chromosome. The arrow indicates the location of ATP7B. **2C** Mapping results for hepatic copper score at the X-chromosome in male dogs show an association signal at position X:60203319-60356690 (CanFam 3.1). The arrows indicate the location of ATP7A. **2D** Mapping results for hepatic copper score at the X-chromosome in females shows no substantial association. The solid line indicates the boundary for suggestive genome wide association at a significance level of $P = 5*10^{-5}$. The dotted line indicates the boundary used for determination of the critical regions at $P = 5* 10^{-4}$.



		Males			Females	
		ATP7A:c.980C>T Genotypes				
		CY	**TY**	**CC**	**CT**	**TT**
***ATP7B*:c.4358G>A genotypes**	**AA**	N=10 2.25 (0-4) a	N=0	N=7 3.5 (0.5-5) y	N=9 2.5 (1.5-4) y	N=0
	AG	N=41 2 (0-3.5) a	N=11 0.5 (0-2) b,c	N=53 2 (0-3.5) z	N=24 1.75 (0-3.5) z	N=4 2.25 (1-3.5)
	GG	N=28 1.25 (0-3.5) a,b	N=11 0 (0-2) c	N=55 1.5 (0-3.5) z	N=34 1.5 (0-3.5) z	N=7 0.5 (0-2.5) Z

Figure 3 Hepatic histological copper score in relation to ATP7A and ATP7B genotype in male and female Labrador retrievers. Genotypes of individual dogs for ATP7A:c.980C>T and ATP7B:c.4358G>A are indicated. The table summarizes the number of dogs (N) and median and range (in brackets) for hepatic copper score per genotype category. Significant differences between genotype categories tested by the Wilcoxon rank-sum test are indicated by letters in males (a,b,c) and females (y,z). Genotypes of which <5 animals were present, were omitted from the statistical analysis.

When inspecting the effect of the combined genotypes for ATP7A and ATP7B on hepatic histological copper levels (Figure 3), we appreciated that for both sexes the ATP7B:c.4358A is associated with increased hepatic copper levels in an additive way. This effect is most clear in the female dogs, leading to a significant difference between copper levels of dogs homozygous for ATP7B:c.4358A and any of the other female dogs. Concurrent presence of ATP7A:c.980T, attenuated the copper accumulation effect in both sexes. In males, this effect was most notable, leading to a significant difference between dogs with and without the mutant allele of ATP7A (Figure 3). The heritability for hepatic

copper score in the model including the co-variates sex, ATP7A:c.980C>T and ATP7B:c.4358G>A was calculated to be 0.42. Thus the explained genetic variability by the two non-synonymous mutations is ((0.48-0.42)/0.48) 12.5%, of which 4.3% could be attributed to ATP7A:c.980C>T and 8.2% could be attributed to ATP7B:c.4358G>A.

ATP7A:c.980C>T resulted in an amino-acid substitution from a threonine to an isoleucine in the 3rd MBD of ATP7A at position 327 (Ensembl: ENSCAFT00000049745, ATP7A:p.Thr327Ile). *ATP7B*:c.4358G>A caused an amino acid substitution from an arginine to a glutamine in the C-terminus of the ATP7B gene at position 1453 (Ensembl:ENSCAFT00000006859, ATP7B:p.Arg1453Gln). Alignment of the amino-acid sequence adjacent to canine ATP7A:p.Thr327Ile and ATP7B:p.Arg1453Gln, indicated strong conservation of the domains in mammals (see supplementary material, Figure S2).

Functional evaluation of ATP7B:p.Arg1415Gln

ATP7B:p.Arg1415Gln is partially mis-localized to the endoplasmic reticulum
To analyze how the canine ATP7B:p.Arg1453Gln may affect copper homeostasis at the cellular level, we expressed the mutant protein exogenously in a cell line. To investigate the consequences of canine ATP7B:p.Arg1453Gln substitution on the subcellular localization of ATP7B, the conserved arginine (Arg) at position 1415 (Ensembl: ENST00000242839) of human GF-tagged *ATP7B* was replaced by a glutamine (Gln). Both human ATP7B:p.Arg1415-GFP ($ATP7B^{WT}$) and ATP7B:Gln1415-GFP ($ATP7B^{R1415Q}$) were expressed in HeLa cells and treated with either bathocuproine disulphonate (BCS) or 200 μM CuSO4. Confocal microscopy revealed that in low Cu^{2+} conditions ATP7B:p.Arg1415-GFP exhibited significant overlap with the trans-Golgi marker TGN46 as reported before[34], while stimulation with CuSO4 induced ATP7B:p. Arg1415-GFP trafficking to the cell surface and numerous cytosolic vesicular structures (Figure 4A, upper row). In contrast, a significant amount of ATP7B:Gln1415-GFP was detected in the ER network-like membranes both in low and high Cu conditions (Figure 4A, bottom row) although substantial amounts of the protein were delivered to the Golgi and post-Golgi compartments.

Morphometric analysis confirmed an increase in percentage of cells that exhibit GFP signal in the ER upon expression of ATP7B:p.Gln1415, while the number of cells with ATP7B:p.Arg1415-GFP in the ER was very low (Figure 4C). Partial mis-localization of ATP7B:p.Gln1415 mutant to the ER compartment was also observed in a HepG2 cell line (Figure 4B&C). This indicated that the substitution of arginine to glutamine impairs (at least partially) appropriate compartmentalization of ATP7B.

ATP7B:p.Arg1415Gln fails to reach the canalicular domain of polarized HepG2 cells

To verify whether ATP7B:p.Gln1415 can be targeted to the apical (canalicular) domain of hepatic cells, which is necessary to remove excess copper from hepatocytes into bile, we grew HepG2 cells at the conditions that allow their maximal polarization[30]. Then, the cells were transfected with either ATP7B:p.Arg1415-GFP ($ATP7B^{WT}$-GFP) or ATP7B:p.Gln1415-GFP ($ATP7B^{R1415Q}$-GFP) and subsequently incubated with 200 μM $CuSO_4$. Stimulation with copper resulted in delivery of $ATP7B^{WT}$-GFP to the apical surface domains, which were labelled with the canalicular marker MRP2 (Figure 4B, arrows in upper row). On the contrary, $ATP7B^{R1415Q}$-GFP failed to arrive at the canalicular surface of the cells (Figure 4B, arrows in bottom row), and remained mainly in the intracellular cytoplasmic structures. These results indicated that the mutant cannot be delivered to regular copper-excretion sites in polarized hepatocytes.

ATP7B:p.Arg1415Gln exhibits copper-transporting activity

To assess the ability of ATP7B:p.Gln1415 to transport copper we transfected HeLa cells with the Metal Responsive Element (MRE)-Luciferase reporter, a copper sensor based on the metallothionein-1 promoter that responds to bioavailable cytosolic copper and that was previously used to characterize cellular copper import[31]. Co-expression of MRE-Luciferase reporter with empty GFP vector resulted in strong induction of luciferase activity (Figure 4D) upon exposure of the cells to $CuSO_4$. The induction of this copper-dependent reporter was significantly reduced when $ATP7B^{WT}$ was co-expressed in the cells, while transfection of the partially active $ATP7B^{H1069Q}$ mutant only modestly inhibited MRE-Luciferase activation (Figure 4D). Cells expressing ATP7B:p.Gln1415 exhibited substantial reduction of reporter activity indicating that the mutant is still capable of transporting copper out of the cytosol.

Taken together, our findings indicated that accumulation of copper upon ATP7B:p.Gln1415 expression occurs mainly due to failure of the mutant to reach the appropriate copper- excretion compartments rather that to the reduction of its copper-translocating activity.

Fibroblasts from dogs with ATP7A:p.Thr327Ile accumulate more copper than wild type fibroblasts

To ascertain whether ATP7A:p.Ile327 influenced cellular copper accumulation, a copper uptake and retention assay[35] was performed using copper 64 isotope (^{64}Cu) in dermal fibroblasts. Fibroblasts with ATP7A:p.Thr327 were derived from 5 females and 3 males and fibroblasts with the variant ATP7A:p.Ile327derived from 4 females and 3 males, and were used for the ^{64}Cu experiments. For the ^{65}Zinc experiments fibroblasts from 3 ATP7A:p.Thr327 dogs (2 female, 1 male) and 3 ATP7A:p.Ile327 dogs (1 female, 2 males) were used. All dogs used for the fibroblast studies that were homo- or hemizygous for *ATP7A*:c.980C were homo- or hemizygous for the reference allele for the SNP in intron 18 (position 60338569) and all dogs that were homo- or hemizygous for *ATP7A*:c.980T were homo- or hemizygous for the non-reference allele. The model for ^{64}Cu accumulation in fibroblasts including fixed factors time and mutation, had the lowest Akaike's information criterion (AIC). Overall, a significant increase of copper at time points 22 and 30 hours was present. The fibroblasts derived from the ATP7A:p.Ile327 dogs accumulated significantly more copper than fibroblasts derived from ATP7A:p.Thr327 dogs (Figure 5A). To establish whether the differences in ^{64}Cu levels between fibroblasts were due to abrogated efflux, a chase method was employed, following pre-loading of cells with copper for 22 hours. The average export rate of fibroblasts with the ATP7A:p.Ile327 variant was significantly lower compared to the average export rate in fibroblasts derived from ATP7A:p.Thr327 (Figure 5B). As a control, fibroblasts from a human fibroblast cell line (ATP7A wild type) and a Menkes patient (Menkes) (both n=1) were included in the experiments. Fibroblasts derived from a Menkes patient accumulated considerably more ^{64}Cu than the ATP7A wild type fibroblasts (Figure 5C) and showed abrogated efflux (Figure 5D).

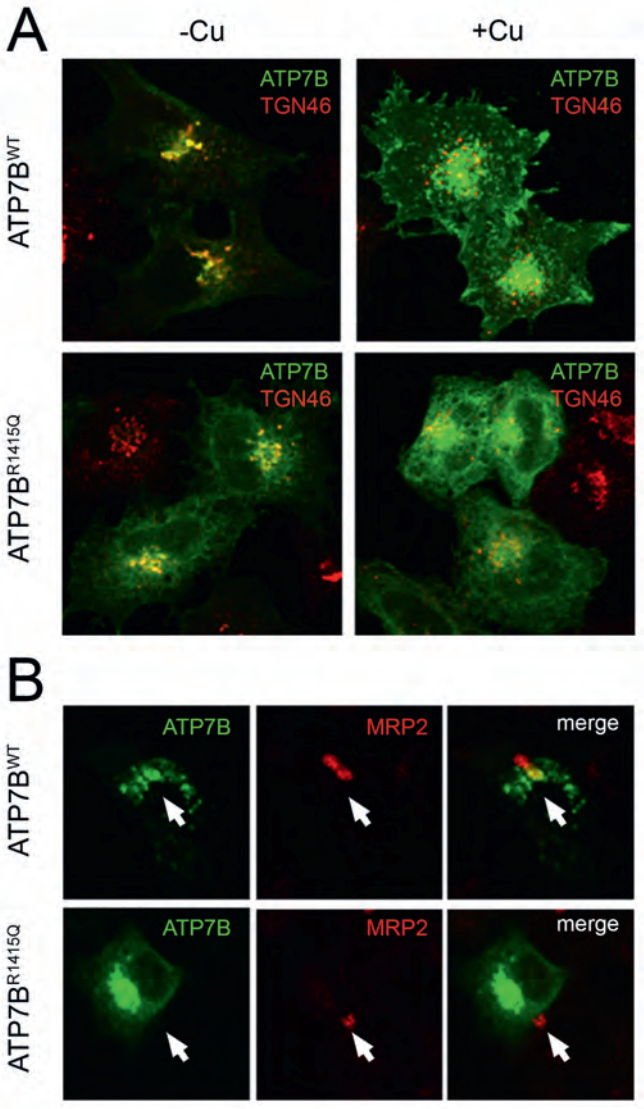
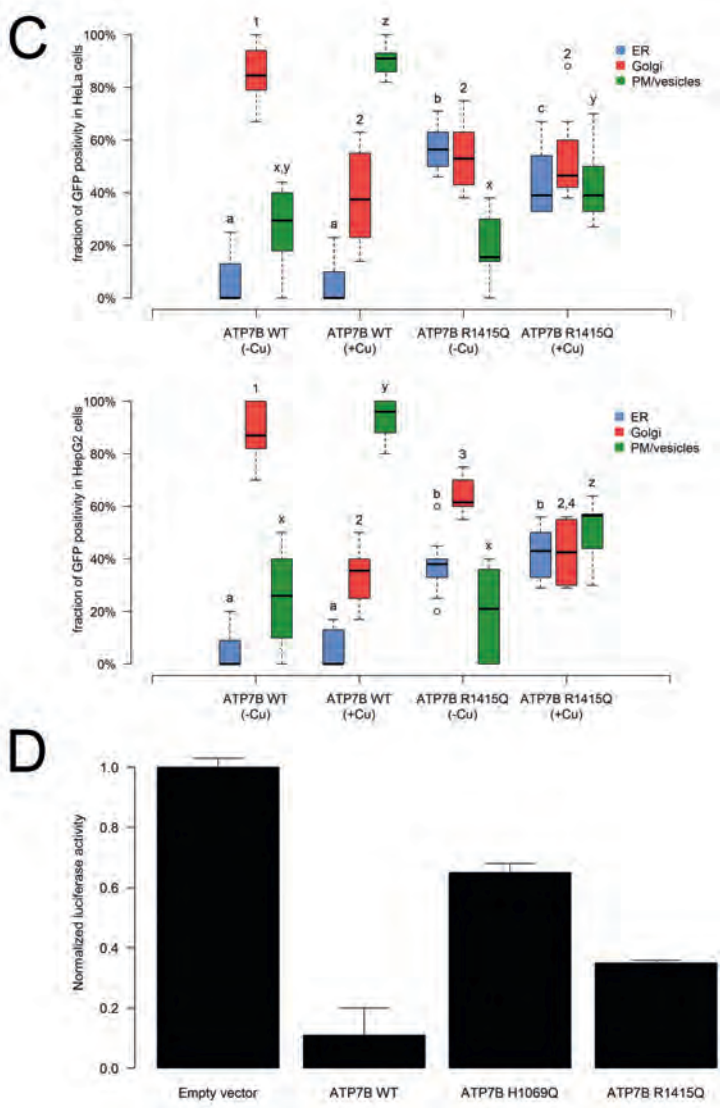


Figure 4 Functional evaluation of ATP7B:p.Arg1415Gln

A) HeLa cells were transfected with either ATP7B:p.Arg1415 ($ATP7B^{WT}$, upper row) or ATP7B:p.Arg1415Gln ($ATP7B^{R1415Q}$, lower row) and treated with either 500 μM of the copper chelator BCS (Cu^{-}) or were incubated for 2 h with 200 μM $CuSO_4$ (Cu^{+}). In low copper conditions, the localization of $ATP7B^{WT}$ overlapped with the trans Golgi marker TGN46, while copper stimulation induced $ATP7B^{WT}$ trafficking to cytosolic vesicular structures and the cell surface (**upper row**). A significant cohort of $ATP7B^{R1415Q}$ was detected in the endoplasmic reticulum network-like membranes both in low and high copper conditions (**lower row**). **B)** HepG2 cells were polarized before transfection with either $ATP7B^{WT}$ (**upper row**) or $ATP7B^{R1415Q}$ (**lower row**) and were incubated for 2 h with 200 μM $CuSO_4$. $ATP7B^{WT}$ was delivered to the apical surface domains, indicated by co-localization of the canalicular membrane marker MRP2 (**upper row**), whereas $ATP7B^{R1415Q}$ failed to arrive at the canalicular surface of the cells. **C)** The intracellular distribution of GFP-



tagged ATP7B^{WT} and ATP7B^{R1415Q} protein was analyzed in HeLa (**upper graph**) and HepG2 (**lower graph**) cells in low and high copper circumstances. Significant differences (P<0.05) in the distribution of GFP within the cellular compartments were indicated with a,b,c (ER); 1,2,3 (Golgi) or x,y,z (PM/vesicles). There was an increase in the percentage of cells that exhibit GFP signal in the endoplasmic reticulum upon expression of ATP7B^{R1415Q}, while the number of cells with ATP7B^{WT}-GFP in the endoplasmic reticulum was very low in both HeLa and HepG2 cells. **D)** HeLa cells were transfected with empty vector, ATP7B^{WT}, ATP7B^{H1069Q} or ATP7B ^{R1415Q} and the Metal Responsive Element (MRE)-luciferase reporter for 24 hours and treated with 200 µM CuSO4 for 24 hours. Induction of the MRE-luciferase reporter was reduced when ATP7B^{WT} was co-expressed in the cells, while transfection of the partially active ATP7B^{H1069Q} mutant only modestly inhibited MRE-Luciferase activation. Cells expressing ATP7B^{R1415Q} exhibited substantial reduction of reporter activity, indicating that ATP7B^{R1415Q} is still capable of transporting copper away from the cytosol. Bars represent the mean values and standard deviation is depicted by the error bars.

Handling of an alternative trace metal in canine fibroblasts was not influenced by the *ATP7A* genotype. Incubation of fibroblasts with ^{65}Zn resulted in similar patterns of accumulation of isotope over time (see supplementary material, Figure S3A) and also release kinetics of newly accumulated ^{65}Zn were comparable between the fibroblasts derived from ATP7A:p.Thr327 dogs and dogs with ATP7A:p.Ile327 (see supplementary material, Figure S3B).

In summary, this experiment showed that fibroblasts derived from dogs with the ATP7A:p.Ile327 accumulate more copper than fibroblasts derived from dogs with ATP7A:p.Thr327 and that copper accumulation may be the result of decreased copper excretion by ATP7A:p.Ile327 fibroblasts.

Discussion

We performed a GWAS in 235 Labrador retrievers to identify causal genes for copper toxicosis and identified two chromosomal loci that were positively and negatively associated with hepatic copper levels. The 2 loci respectively harbored the genes coding for the copper transporters ATP7B and ATP7A. Subsequent Sanger sequencing in 96 Labrador retrievers and validation of mutations in an independent cohort of 59 Labrador retrievers identified a non-synonymous SNP in *ATP7B* associated with an increase, and a non-synonymous and an intronic SNP in *ATP7A* associated with a decrease in hepatic copper levels. Functional follow-up studies for the non-synonymous mutations provided biological evidence for their involvement in disturbed copper metabolism.

Copper toxicosis in Labrador retrievers and Wilson disease are similar with regard to the amount of hepatic copper accumulation, presence of iron accumulation and progression to liver cirrhosis. Electron microscopy of liver sections of affected Labrador retrievers revealed ultra-structural changes that are comparable to those in patients with Wilson disease. We now show for the first time that Labrador retrievers and Wilson disease patients also share *ATP7B* as the causal gene. However, histo-pathological differences between copper toxicosis in Labrador retrievers and Wilson disease are present and include predominant centrolobular copper accumulation in Labrador retrievers versus a peri-portal distribution in WD patients. Further, fatty degeneration and Mallory

bodies are not recognized in liver histopathology from Labrador retrievers. In both diseases, age of onset is usually in middle or older age, but neurological symptoms and Kaiser-Fleisher rings have not been recognized in Labrador retrievers. Dietary intake of copper seems important in disease progression in the Labrador retrievers[36;37] and in this regard, Labrador retriever copper toxicosis is comparable to ETIC and ICC. The observed differences among copper toxicosis diseases in man and the Labrador retriever are intriguing and further studies are needed to identify whether they can be attributed to general species differences in copper metabolism, or to other yet unidentified gene mutations in either species.

ATP7B is highly expressed in hepatocytes, where it is located in the trans-Golgi network in low copper conditions. With increasing intracellular copper levels, ATP7B traffics to vesicles or to the apical cell surface[38;39] to facilitate copper excretion into the bile. The C-terminus has an important role in regulating copper responsive trafficking. Mutations of residues in this region are suggested to contribute to aberrant Golgi retention of ATP7B under high copper circumstances[40]. Our fluorescence studies did not show a significant difference in GFP signals in both HeLa and polarized HepG2 cells between the wild type and ATP7B:p.Arg1415Gln after copper treatment (Figure 4C). However, ATP7B:p.Arg1415Gln showed increased retention in the ER in both low and high copper conditions, which may indicate mis-folding of the protein. Mis-folding and aberrant retention in the ER has been previously reported in Wilson disease associated ATP7B mutations[41;42]. The luciferase assay demonstrated that the copper transport capacity of ATP7B:p.Arg1415Gln was only partly impaired, indicating that the functional effect of ATP7B:p.Arg1453Gln in hepatic copper accumulation in Labrador retrievers results from mis-localization of the protein, rather than from severely decreased copper transport capacity (Figure 4D).

In Labrador retrievers, there is a strong female predisposition for disease[18]. X-chromosomal mapping in a combined dataset can be hampered by hemizygosity in males, and thereby possibly cause disturbance of association signals. Therefore we analyzed males and females separately. In the region harboring *ATP7A*, we identified a suggestive signal of association with lower hepatic copper levels in male dogs. We hypothesized that mutations in *ATP7A* may modify copper accumulation and this gene was therefore analyzed by

DNA sequence analysis. The identified missense mutation *ATP7A*:c.980C>T results in the amino acid substitution ATP7A:p.Thr327Ile in MBD3 of ATP7A and was negatively associated with hepatic copper levels. MBD3 is located in the cytoplasm and has the ability to receive copper from the Atox1 chaperone[43]. MBD3 of ATP7A is only metallated under elevated copper conditions[44] and is thought to be an important domain for regulating ATP7A activity via cell signaling pathways[45]. It has been shown that ATP7A:p.T327 in MBD3 in the human protein (equivalent to canine ATP7A:p.T327) can become phosphorylated in response to increased copper concentration[46] and is predicted to up-regulate copper-ATPase function via stabilization of the domain[45]. To ascertain whether ATP7A:p.Thr327Ile exerted influence on cellular copper accumulation, we studied copper uptake and retention in dermal fibroblasts derived from Labrador retrievers hemi- or homozygous for either ATP7A:p.Thr327 or ATP7A:p.Ile327. Mutant fibroblasts accumulated and retained significantly more copper, indicating a functional impairment of ATP7A. ATP7A has an important function in enterocytes, where it facilitates copper transport across the basal membrane into the portal circulation. The consequence of an aberrant ATP7A function may be a decrease in copper uptake from the intestinal tract. Another single base-pair substitution C>T, located in intron 18 (position 60338569, CF 3.1) was inversely related to hepatic copper score. In the dogs from which the fibroblasts were derived, the SNP in intron 18 and *ATP7A*:c.980C>T were in complete LD, and therefore we cannot rule out that the intronic variation exerts an effect on *ATP7A* gene function, however we consider it less likely. The alleles are both pyrimidines within a stretch of pyrimidines, are not situated close to an intron-exon junction and the mutation is not expected to activate a cryptic splice site. In the total data set, the intronic SNP was in high LD (r^2 0.92, D'0.97) with *ATP7A:*c.980C>T making it likely that the observed association is due to the fact that this SNP is in LD with *ATP7A:*c.980C>T.

ATP7B:c.4358G>A showed an additive effect on hepatic copper levels, which was most significant in female dogs (Figure 3). Copper accumulating effects of *ATP7B*:c.4358G>A were modified by the presence of *ATP7A:*c.980C>T and this effect was most clear in male dogs (Figure 3). We suggest that in Labrador retrievers with the ATP7A:p.Thr327Ile substitution, the lower hepatic copper concentration reflects a decrease in intestinal copper uptake, thereby attenuating hepatic copper accumulation due to ATP7B:p.Arg1453Gln (Figure 6).

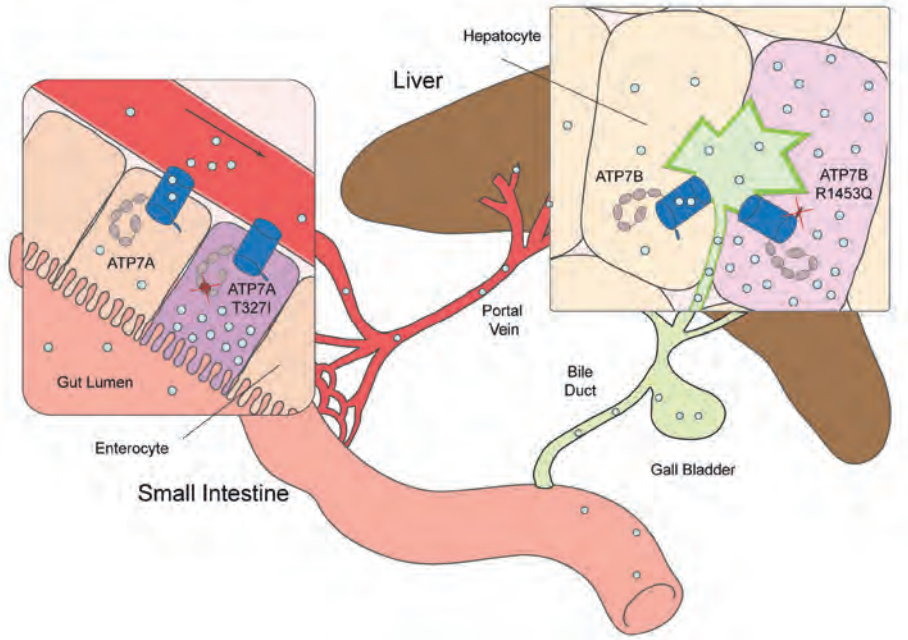


Figure 6 Model for a combination of mutations in *ATP7A* and *ATP7B* influencing body copper homeostasis. Copper translocation from enterocytes into the portal circulation for transport to the liver is facilitated by ATP7A in the basolateral membrane of enterocytes. Upon uptake within hepatocytes, copper is metabolized and incorporated in proteins. Excretion of excess copper via the bile is facilitated by ATP7B. We propose a model in which copper accumulation in hepatocytes results from functional impairment of ATP7B by the presence of the ATP7B:p.Arg1415Gln amino acid substitution. An attenuation of this effect will occur when ATP7A function is hampered due to the presence of ATP7A:p. Thr327Ile, by accumulation of copper in enterocytes resulting in increased fecal copper excretion and subsequent decrease of copper uptake in the portal circulation. Light blue dots: copper particles.

In Wilson disease patients a wide variation in clinical symptoms and hepatic copper accumulation is noticed which is currently unexplained[47-50]. When we translate our observations in the Labrador retrievers to the human disease, we may think of *ATP7A* as a possible modifier gene in Wilson disease. Variations in *ATP7A* with a small effect on protein function may reduce the rate of copper accumulation in Wilson disease patients, thereby modifying the disease phenotype, for example by delaying the age of onset of clinical symptoms. In fact, part of the apparent discrepancy between the genetic incidence of Wilson disease and the actual number of patients diagnosed[51] might be due to a slower rate of copper accumulation that does not reach toxic levels in the lifespan of some individuals.

This study illustrates the potential of canine inbred populations for the unravelling of complex hereditary diseases. We identified the involvement of the copper transporter ATP7B in copper toxicosis in the Labrador retriever, identifying this breed as the first naturally occurring non-rodent mammalian model for Wilson disease. The concurrent identification of a functional mutation in *ATP7A*, suggests a role for the Menkes genes as a disease modifier in copper metabolism disorders. Due to its long life span and large body size, the Labrador retriever will be an invaluable large animal model for the development of new therapies, including gene therapy, beneficial to both human and canine patients.

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Competing interests
Patents for mutations described in this manuscript are filed by Mars Inc. under publication numbers WO2009044152A2 and WO2013083988A3. YG, AJM and ALW were employed by the Waltham Centre of Pet nutrition.

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Supplementary material

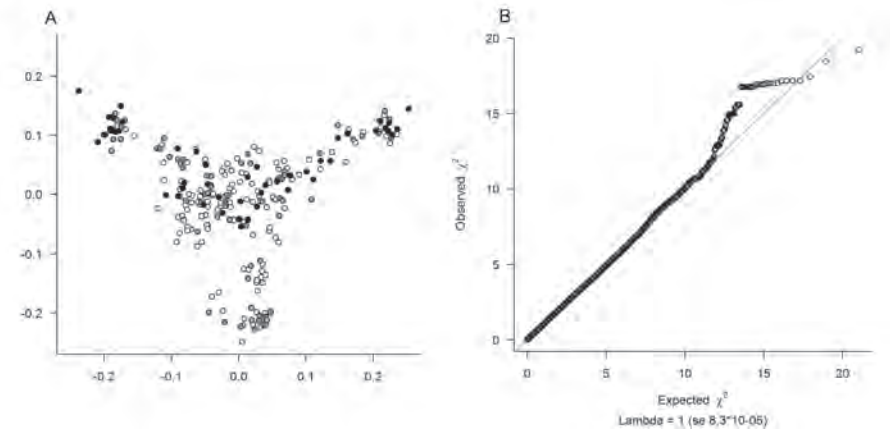

Figure S1 Multidimensional scaling plot and QQ-plot **A)** Multidimensional scaling plot prior to correction. Population stratification was visualized by plotting the components C1 and C2 from the multidimensional scaling analysis in 235 Labrador retrievers from the genome wide association study. Labrador retrievers with hepatic copper score <2 (white), copper scores between 2 and 3 (grey) and copper scores >3 (black) are depicted. **B)** QQ-plot for the "mmscore" analysis for hepatic copper score in 235 Labrador retrievers.

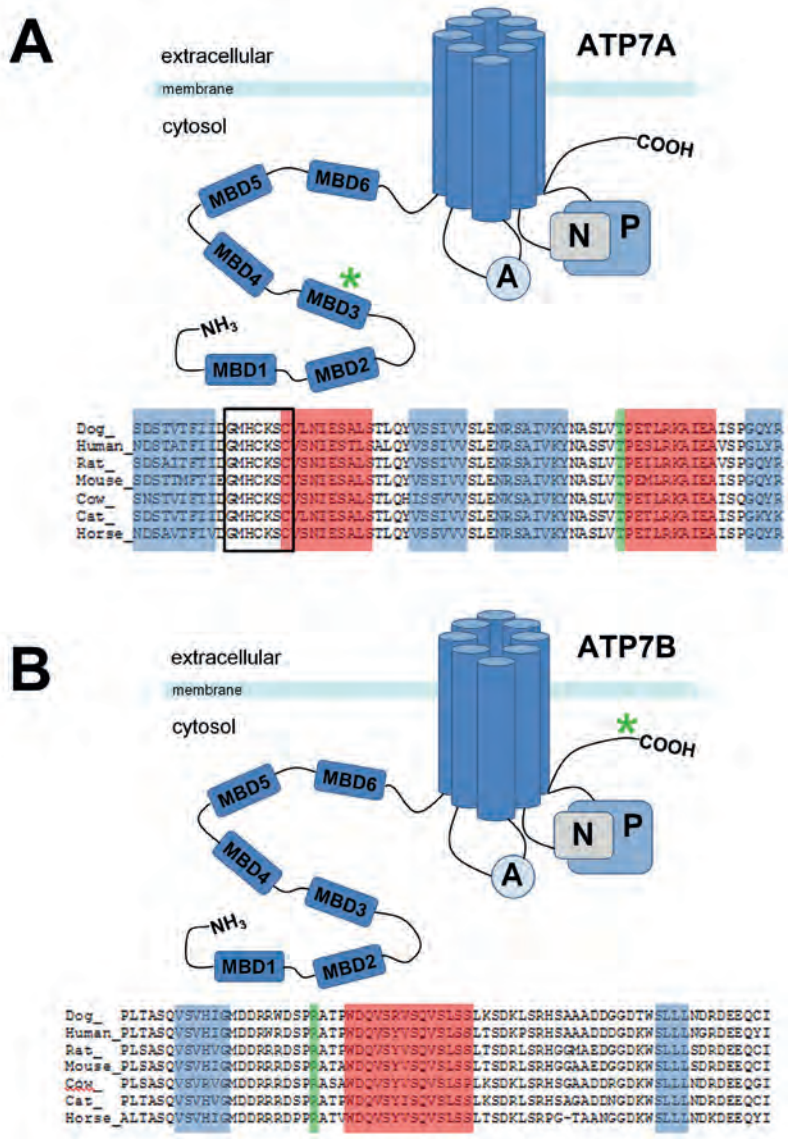

Figure S2 Protein domains of ATP7A and ATP7B involved in copper toxicosis in Labrador retrievers. Overview of the ATP7A (A) and ATP7B (B) proteins with the N-terminus, metal binding domains (MBD), actuator domain (A), nucleotide binding domain (N), phosphorylation domain (P) and the C- terminus. The green asterisk indicates the position of the mutations. **A**) Alignment of the region containing ATP7A:p.Thr327Ile (in green) shows a strong conservation of this amino-acid position in human, rat, mouse, cow, cat and horse. The copper binding site XMXCXXC (boxed), predicted alpha-helix (red) and beta-sheets (blue) are indicated. **B**) Alignment of the region containing ATP7B:p.Arg1453Gln (in green) shows a strong conservation of this amino-acid position in human, rat, mouse, cow, cat and horse. The predicted alpha-helix (red) and beta-sheets (blue) are indicated.

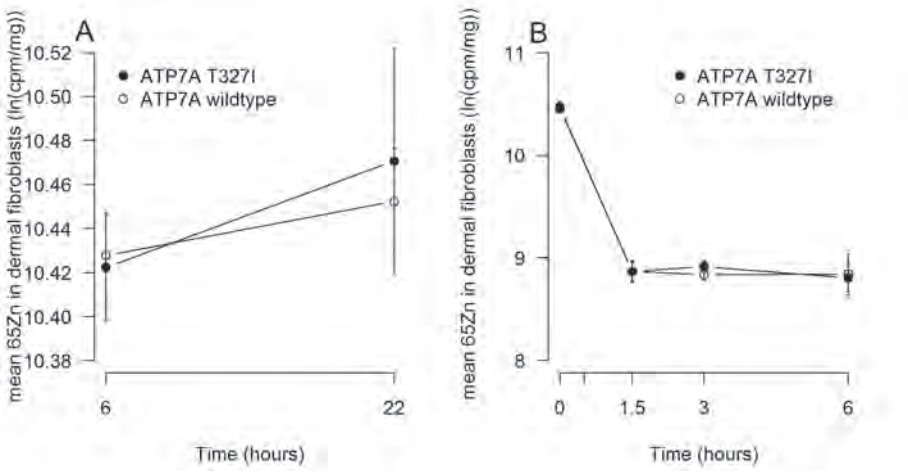

Figure S3 Zinc accumulation and retention in canine dermal fibroblasts. **A)** Dermal fibroblasts derived from ATP7A:p.Ile327 dogs show similar zinc uptake compared to dermal fibroblasts derived from dogs with ATP7A:p.Thr327 (wild type). Dots represent mean values and standard deviations, were indicated by the error bars. **B)** Dermal fibroblasts derived from ATP7A:p.Ile327 dogs show similar zinc efflux compared to dermal fibroblasts derived from dogs with ATP7A:p.Thr327 (wild type). Dots represent mean values and standard deviations were indicated by the error bars.

References

1. Vulpe C., B. Levinson, S. Whitney *et al*. 1993. Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. Nat. Genet. 3: 7-13.
2. Bull P.C., G.R. Thomas, J. M. Rommens *et al*. 1993. The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. Nat. Genet. 5: 327-337.
3. Voskoboinik I., J. Camakaris & J.F. Mercer. 2002. Understanding the mechanism and function of copper P-type ATPases. Adv. Protein Chem. 60: 123-150.
4. Linder M.C. & M. Hazegh-Azam. 1996. Copper biochemistry and molecular biology. Am. J. Clin. Nutr. 63.
5. Gitlin J. D. 2003. Wilson disease. Gastroenterology. 125: 1868-1877.
6. Kaler S.G. 2011. ATP7A-related copper transport diseases-emerging concepts and future trends. Nat. Rev. Neurol. 7: 15-29.
7. Tanner M.S. 1998. Role of copper in Indian childhood cirrhosis. Am. J. Clin. Nutr. 67: 1074S-1081S.
8. Muller T., H. Feichtinger, H. Berger *et al*. 1996. Endemic Tyrolean infantile cirrhosis: an ecogenetic disorder. Lancet. 347: 877-880.
9. Grimes A., C.J. Hearn, P. Lockhart *et al*. 1997. Molecular basis of the brindled mouse mutant (Mo(br)): a murine model of Menkes disease. Hum. Mol. Genet. 6: 1037-1042.
10. Theophilos M.B., D.W. Cox & J.F. Mercer. 1996. The toxic milk mouse is a murine model of Wilson disease. Hum. Mol. Genet. 5: 1619-1624.
11. Li Y., Y. Togashi, S. Sato *et al*. 1991. Spontaneous hepatic copper accumulation in Long-Evans Cinnamon rats with hereditary hepatitis. A model of Wilson's disease. J. Clin. Invest. 87: 1858-1861.
12. Wang Y., S. Zhu, G.A. Weisman *et al*. 2012. Conditional knockout of the Menkes disease copper transporter demonstrates its critical role in embryogenesis. PLoS One. 7: e43039.
13. Buiakova O.I., J. Xu, S. Lutsenko *et al*. 1999. Null mutation of the murine ATP7B (Wilson disease) gene results in intracellular copper accumulation and late-onset hepatic nodular transformation. Hum. Mol. Genet. 8: 1665-1671.
14. Van de Sluis B., J. Rothuizen, P.L. Pearson *et al*. 2002. Identification of a new copper metabolism gene by positional cloning in a purebred dog population. Hum. Mol. Genet. 11: 165-173.
15. Muller T., B. van de Sluis, A. Zhernakova *et al*. 2003. The canine copper toxicosis gene MURR1 does not cause non-Wilsonian hepatic copper toxicosis. J. Hepatol. 38: 164-168.
16. Lovicu M., V. Dessi, M.B. Lepori *et al*. 2006. The canine copper toxicosis gene MURR1 is not implicated in the pathogenesis of Wilson disease. J. Gastroenterol. 41: 582-587.
17. Coronado V.A., J.A. Bonneville, H. Nazer *et al*. 2005. COMMD1 (MURR1) as a candidate in patients with copper storage disease of undefined etiology. Clin. Genet. 68: 548-551.
18. Fieten H., P.A. Leegwater, A.L. Watson *et al*. 2012. Canine models of copper toxicosis for understanding mammalian copper metabolism. Mamm. Genome. 23: 62-75.
19. Boyko A.R. 2011. The domestic dog: man's best friend in the genomic era. Genome Biol. 12: 216-2011-12-2-216.
20. Miller S.A., D.D. Dykes & H.F. Polesky. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16: 1215.
21. Rothuizen J., V.J. Desmet, T.S.G.A.M. Van Den Ingh *et al*. 2006. Sampling and handling of liver tissue. *In* WSAVA Standards for Clinical and Histological Diagnosis of Canine and Feline Liver Disease - WSAVA Liver Standardization Group. Rothuizen J., Ed.: 5-14. Elsevier. Philadelphia.
22. Uzman L.L. 1956. Histochemical localization of copper with rubeanic acid. Lab. Invest. 5: 299-305.
23. Gordon H. & H.H. Sweets. 1936. A Simple Method for the Silver Impregnation of Reticulum. Am. J. Pathol. 12: 545-552.1.
24. Van Den Ingh T.S.G.A.M., T. Van Winkle, J.M. Cullen *et al*. 2006. Morphological classification of parenchymal disorders of the canine and feline liver, 2. Hepatocellular death, hepatitis and cirrhosis. *In* WSAVA Standards for Clinical and Histological Diagnosis of Canine Liver Diseases. WSAVA Liver Standardization Group., Ed.: 85-101. Elsevier. Philadelphia.
25. Fieten H. & J. Rothuizen. 2014. Web Chapter 48: Copper-associated hepatitis. *In* Kirk's Current Veterinary Therapy. Bonagura J. D. and D. C. Twedt, Eds.: e231-e236. Elsevier. Missouri.
26. R Development Core Team. 2011. R: A Language and Environment for Statistical Computing. Vienna, Austria.

27. Aulchenko Y.S., S. Ripke, A. Isaacs *et al*. 2007. GenABEL: an R library for genome-wide association analysis. Bioinformatics. 23: 1294-1296.
28. Chen W.M. & G.R. Abecasis. 2007. Family-based association tests for genomewide association scans. Am. J. Hum. Genet. 81: 913-926.
29. Ronnegard L., X. Shen & M. Alam. 2010. Fitting hierarchical generalized linear models. The R Journal. 2: 20-28.
30. Slimane T.A., G. Trugnan, S.C. Van IJzendoorn *et al*. 2003. Raft-mediated trafficking of apical resident proteins occurs in both direct and transcytotic pathways in polarized hepatic cells: role of distinct lipid microdomains. Mol. Biol. Cell. 14: 611-624.
31. van den Berghe P.V., D.E. Folmer, H.E. Malingre *et al*. 2007. Human copper transporter 2 is localized in late endosomes and lysosomes and facilitates cellular copper uptake. Biochem. J. 407: 49-59.
32. Collins T.J. 2007. ImageJ for microscopy. BioTechniques. 43: 25-30.
33. Spee B., B. Arends, A.M. van Wees *et al*. 2007. Functional consequences of RNA interference targeting COMMD1 in a canine hepatic cell line in relation to copper toxicosis. Anim. Genet. 38: 168-170.
34. Barnes N., M.Y. Bartee, L. Braiterman *et al*. 2009. Cell-specific trafficking suggests a new role for renal ATP7B in the intracellular copper storage. Traffic. 10: 767-779.
35. Moller L.B., J.D. Hicks, C.S. Holmes *et al*. 2011. Diagnosis of copper transport disorders. Curr. Protoc. Hum. Genet. Chapter 17: Unit17.9.
36. Fieten H., B.D. Hooijer-Nouwens, V.C. Biourge *et al*. 2012. Association of Dietary Copper and Zinc Levels with Hepatic Copper and Zinc Concentration in Labrador Retrievers. J. Vet. Intern. Med. 26: 1274-1280.
37. Fieten H., V. Biourge, A. Watson *et al*. 2014. Nutritional management of inherited copper-associated hepatitis in the Labrador retriever. Vet. J. 199: 429-433.
38. Guo Y., L. Nyasae, L.T. Braiterman *et al*. 2005. NH2-terminal signals in ATP7B Cu-ATPase mediate its Cu-dependent anterograde traffic in polarized hepatic cells. Am. J. Physiol. Gastrointest. Liver Physiol. 289: G904-16.
39. Schaefer M., R.G. Hopkins, M.L. Failla *et al*. 1999. Hepatocyte-specific localization and copper-dependent trafficking of the Wilson's disease protein in the liver. Am. J. Physiol. 276: G639-46.
40. Braiterman L., L. Nyasae, F. Leves *et al*. 2011. Critical roles for the COOH terminus of the Cu-ATPase ATP7B in protein stability, trans-Golgi network retention, copper sensing, and retrograde trafficking. Am. J. Physiol. Gastrointest. Liver Physiol. 301: G69-81.
41. Gupta A., A. Bhattacharjee, O.Y. Dmitriev *et al*. 2011. Cellular copper levels determine the phenotype of the Arg875 variant of ATP7B/Wilson disease protein. Proc. Natl. Acad. Sci. U. S. A. 108: 5390-5395.
42. van den Berghe P.V., J.M. Stapelbroek, E. Krieger *et al*. 2009. Reduced expression of ATP7B affected by Wilson disease-causing mutations is rescued by pharmacological folding chaperones 4-phenylbutyrate and curcumin. Hepatology. 50: 1783-1795.
43. Strausak D., M.K. Howie, S.D. Firth *et al*. 2003. Kinetic analysis of the interaction of the copper chaperone Atox1 with the metal binding sites of the Menkes protein. J. Biol. Chem. 278: 20821-20827.
44. Banci L., I. Bertini, S. Ciofi-Baffoni *et al*. 2010. Affinity gradients drive copper to cellular destinations. Nature. 465: 645-648.
45. Veldhuis N.A., M.J. Kuiper, R.C. Dobson *et al*. 2011. In silico modeling of the Menkes copper-translocating P-type ATPase 3rd metal binding domain predicts that phosphorylation regulates copper-binding. Biometals. 24: 477-487.
46. Veldhuis N.A., V.A. Valova, A.P. Gaeth *et al*. 2009. Phosphorylation regulates copper-responsive trafficking of the Menkes copper transporting P-type ATPase. Int. J. Biochem. Cell Biol. 41: 2403-2412.
47. Shah A.B., I. Chernov, H.T. Zhang *et al*. 1997. Identification and analysis of mutations in the Wilson disease gene (ATP7B): population frequencies, genotype-phenotype correlation, and functional analyses. Am. J. Hum. Genet. 61: 317-328.
48. Riordan S.M. & R. Williams. 2001. The Wilson's disease gene and phenotypic diversity. J. Hepatol. 34: 165-171.
49. de Bie P., P. Muller, C. Wijmenga *et al*. 2007. Molecular pathogenesis of Wilson and Menkes disease: correlation of mutations with molecular defects and disease phenotypes. J. Med. Genet. 44: 673-688.
50. Lee B.H., J. H. Kim, S.Y. Lee *et al*. 2011. Distinct clinical courses according to presenting phenotypes and their correlations to ATP7B mutations in a large Wilson's disease cohort. Liver Int. 31: 831-839.
51. Coffey A.J., M. Durkie, S. Hague *et al*. 2013. A genetic study of Wilson's disease in the United Kingdom. Brain. 136: 1476-1487.

Chapter 9

General discussion

Adapted from:

New canine models of copper toxicosis: Diagnosis, treatment and genetics

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Rationale

Copper-associated hepatitis is one of the most common forms of primary hepatitis in dogs[1] and is detected with increased incidence in a number of dog breeds, including the Labrador retriever[2]. The studies presented in this thesis were aimed at 1) improving diagnosis and therapeutic protocols for copper-associated hepatitis in dogs, 2) elucidating the role of diet in the disease pathogenesis and long-term treatment and 3) identifying causal mutations involved in copper-associated hepatitis in the Labrador retriever.

In this chapter the main findings of the different studies will be discussed and evidence based recommendations for diagnosis and clinical management of Labrador retrievers with copper-associated hepatitis will be provided. Furthermore, follow-up studies and future perspectives will be elaborated.

Diagnosis

Copper-associated hepatitis is a progressive disease, which can have a long subclinical phase. The age at which clinical signs become apparent can range from 3 to 12 years of age (median 7 years). This is illustrated in the studies described in **Chapter 5** and **Chapter 7**, for which clinically healthy dogs were recruited that were related to affected dogs. All dogs in the study in **Chapter 7** and approximately 75% of the dogs in the study in **Chapter 5** had increased hepatic copper levels, despite appearing clinically healthy. The length of this subclinical phase may be influenced by genetic predisposition and/or dietary intake of copper and zinc.

Liver biopsies from dogs in an early disease stage may contain significant copper accumulation within hepatocytes, with or without an apparent inflammatory component. Presumably, copper is initially stored in a cellular compartment where it is harmless to the cell. However, progression of copper accumulation leads to hepatocyte damage, activation of an inflammatory response and initiation of liver fibrosis eventually leading to liver cirrhosis. This may indicate that defense mechanisms preventing cellular damage ultimately become exhausted.

An earlier diagnosis of the disease would be ideal for two reasons. Firstly, early treatment may prevent further hepatic copper accumulation, hepatocellular damage, inflammation and progression to liver cirrhosis. Secondly, early diagnosis may facilitate the decision to exclude affected dogs from breeding programs. In the section "genetics" we will address this second point in more detail.

Currently, the only way to screen for copper-associated hepatitis is through the evaluation of a liver biopsy, which is a relatively invasive procedure, and therefore not ideal for routine screening purposes. To identify affected dogs in an early phase, biomarkers for hepatocellular damage and copper status are required that can be measured in body fluids and can be obtained in a relatively non-invasive way.

Blood

Currently, ALT is considered the most sensitive and specific blood parameter for hepatocellular damage[3]. In the study population described in **Chapter 7** the dogs had average hepatic copper concentrations of 919 ± 477 mg/kg dwl, and only 1 out of 28 dogs had an increased ALT. An ALT within reference limits can therefore mean three things. Firstly, the reference limit for ALT is set too high; reference limits are usually determined in a group of clinically healthy dogs, however usually no liver biopsies are evaluated for copper concentration or hepatocellular damage. Secondly, in the subclinical phase of copper accumulation, hepatocellular damage may be limited and therefore ALT levels are not increased. And thirdly, ALT may not be sensitive enough for detecting copper induced hepatocellular damage. Overall, we can conclude that ALT cannot be used to identify Labrador retrievers with subclinical hepatic copper accumulation, and clinicians and owners should be aware that an ALT value within the reference range does not necessarily preclude hepatic copper accumulation.

Urine

In **Chapter 3** we investigated a non-invasive alternative to estimate hepatic copper concentration in dogs. We evaluated several urinary parameters that are effectively being used in the diagnostic protocol for Wilson disease in humans, including 24 h basal copper excretion and D-penicillamine induced

copper excretion. Since 24 h urine collection in dogs at home is not practical, we explored the use of urinary copper/creatinine and copper/zinc from single urine samples. We found that urinary copper/zinc ratio in these samples was correlated with hepatic copper concentration in Labrador retrievers. However, we observed overlapping values for dogs with normal, increased and high hepatic copper concentrations. Therefore urinary copper/zinc ratio as a single estimate for hepatic copper concentration is of limited use for identifying dogs at risk.

Diet

Dietary intake of copper and zinc is associated with hepatic copper concentration (**Chapter 5**) and the rate of hepatic copper accumulation varies between individual dogs (**Chapter 5, 6, 7**). Diet can thus influence hepatic copper accumulation, and most likely the age at which clinical disease will become apparent. As shown in our study in **Chapter 5,** it is important to realize that the copper and zinc levels presented on package labels often indicate the amount of copper and zinc that is added to the diet as a pre-mix and do not reflect the copper or zinc levels that were already present in the raw ingredients. In fact, the majority of packages did not have information with regard to copper or zinc levels at all. Analysis of a diet sample may provide more reliable information or, alternatively, the pet food company could be contacted and questioned about their diet analysis reports. In conclusion, knowledge of dietary intake of copper and zinc can be valuable for interpreting results from a liver biopsy during the diagnostic process, i.e., feeding a copper restricted diet may mask copper accumulating traits in some dogs.

Continuing research

Currently, several research efforts are being made in the biomarkers domain, which could lead to both improved and early diagnosis of the disease. One promising new candidate method is the use of hepatocyte-derived micro-RNAs. Micro-RNAs are small RNA molecules that are very stable and are thought to play a key role in regulation of gene expression[4]. Quantification of micro-RNAs with qPCR is a very sensitive measure for detecting hepatocellular damage in humans[5;6]. Our future studies will explore the possibilities of using micro-RNAs for early detection of hepatocellular damage in dogs. Beside markers for hepatocellular damage, we would also need relatively non-invasive markers that could be used to predict hepatic copper status. Evidence from rodent

models and studies in human patients indicated that protein concentrations of the copper chaperone CCS and its target protein SOD1 in erythrocytes could be valid candidates for predicting both copper overload and copper deficiency[7-9]. Currently, we are evaluating these proteins as indicators for hepatic copper concentration in Labrador retrievers.

Recommendations

The following practical recommendations regarding diagnosis and treatment of copper-associated hepatitis in the Labrador retrievers were derived from the results of our studies:

- The consideration of taking a liver biopsy from a clinically healthy dog for screening purposes should take into account the family history of this dog, as family members of affected dogs may have increased hepatic copper concentrations and varying stages of hepatitis without clinical signs.

- An ALT value within reference range should not preclude a clinician from taking a liver biopsy for screening for copper-associated hepatitis.

- D-penicillamine induced 24 h urinary copper excretion, urinary copper/creatinine ratio and urinary copper/zinc ratio are not recommended as single parameter screening methods for increased hepatic copper concentrations.

- Information on pet food packaging should not only contain information about copper and zinc in the added premix, but should also provide information about the concentrations in the end product.

- Previous dietary intake of copper and zinc should be considered when interpreting the results from a liver biopsy with regard to copper-associated hepatitis.

Treatment

The overall therapeutic approach for the treatment of copper toxicosis in humans and dogs is to create a negative copper balance. This can be achieved by decreasing copper uptake and/or by promoting copper excretion using copper chelators.

D-penicillamine

D-penicillamine is a highly soluble degradation product of penicillin, which binds copper and other metals at its SH-group and then promotes urinary excretion[10]. D-penicillamine is an effective copper chelator both in humans and in dogs. Oral administration leads to a rise in urinary copper excretion within 2 hours in dogs and the effect lasts for no longer than 18 hours (**Chapter 3**). Besides copper, D-penicillamine also promotes urinary zinc excretion[11]. Lifelong continuous chelation therapy is usually necessary in COMMD1-deficient dogs, which are at risk for very high hepatic copper concentrations[12]. In dogs with complex forms of copper-associated hepatitis, copper accumulation does not reach very high concentrations, and guidelines for necessary treatment duration are lacking. With the results presented in **Chapter 4** we developed a prediction model for the necessary treatment duration with D-penicillamine based on the initial hepatic copper concentration. In our dataset, there was no evidence that factors such as age, sex, occurrence of side effects and type of medication used (compounded D-penicillamine, or enteric coated Metalcaptase® tablets), influenced the rate of de-coppering. Our prediction model allows the estimation of the necessary treatment duration for de-coppering and the determination of the optimal moment for doing a control liver biopsy. Although the dogs described in **Chapter 4** had relatively short treatment durations with a median of 4.8 months (range, 1.8 – 15.7), a significant decrease in hepatic zinc levels was already noted, indicating that zinc deficiency during D-penicillamine treatment is a realistic risk. Furthermore, the grading score for inflammatory lesions decreased significantly after D-penicillamine therapy, indicating that by chelating hepatic copper, the trigger for inflammation is removed with a subsequent decrease in inflammatory activity as a consequence. It is to be noted that from our results it cannot completely be ruled out that immunomodulating effects of D-penicillamine contributed to a decrease in grading score.

Diet

Among veterinary internal medicine specialists there has been an ongoing debate, as to whether copper-associated hepatitis in Labrador retrievers has resulted from an increase in bio-available copper levels in pet food over the last decade rather than from an inherited predisposition[13]. The results obtained in the studies described in **Chapter 5, 6, 7** and **8** allow an evidence- based answer to this question. In **Chapter 8** we describe the identification of mutations in *ATP7A* and *ATP7B* that influence hepatic copper levels, showing that copper-associated hepatitis in the Labrador retriever is indeed a genetic disease with at least 2 genes influencing the disease phenotype. We will elaborate on these findings in more detail in the final part of the general discussion. Data obtained from the diet studies in subclinical Labrador retrievers provided evidence for a major influence of diet on hepatic copper accumulation.

We identified a wide variety in copper and zinc levels in commercial kibble diets (**Chapter 5**). The recommendations for minimum and maximum copper and zinc levels in diets according to the AAFCO Dog and Cat Food Nutrient Profiles for 2014 are 2.1-34 mg/Mcal for copper and 71-286 mg/Mcal for zinc. The mean dietary copper level obtained in the investigated kibble samples (4.2 ± 1.4 mg/Mcal) was almost 2 times higher than the minimally required levels for dietary copper. The mean zinc level (52.4 ± 17.8 mg/Mcal) was below the recommended levels for dietary zinc. Fourteen out of 32 diets in this study contained zinc levels below the recommended minimum level. The Royal Canin Labrador diet contained the lowest copper level of all diets that were investigated: a copper level of 1.9 ± 0.3 mg/Mcal and a zinc level of 62.1 ± 4.8 mg/Mcal. Interestingly, the Labrador retrievers using this diet had a significantly lower hepatic copper level than the dogs fed any of the other diets (**Chapter 5).** In comparison, the Royal Canin Hepatic dry diet that was used for the diet trials described in **Chapter 6** and **7**, is a diet which is only available via veterinary clinics and contains 1.3 ± 0.3 mg copper/Mcal and 64.3 ± 5.9 mg zinc/Mcal). Results from **Chapter 5** indicate that copper and zinc levels that are currently present in over the counter commercial kibble diets are not suitable for maintaining a normal hepatic copper level in dogs with a genetic predisposition to copper toxicosis.

In the studies presented in **Chapter 6** and **7** we used Royal Canin Hepatic dry diet, because this was the kibble diet with the lowest copper concentration

available. One of our aims was to develop a long-term treatment protocol for dogs with complex forms of copper-associated hepatitis. Because lifelong continuous D-penicillamine therapy may not be an appropriate long-term treatment (**Chapter 4)**, an alternative management strategy using a low-copper, high-zinc diet was investigated in **Chapter 6**. We studied Labrador retrievers with copper toxicosis that had been treated with D-penicillamine until their hepatic copper concentrations normalized. We then continued to treat them with Royal Canin Hepatic diet only. We distinguished three main group of responders: Dogs that could be maintained on a hepatic copper concentration below 400 mg/kg dwl (group 1), dogs that showed re-accumulation of hepatic copper above 400 mg/kg dwl at the time of the first follow-up, but remained relatively stable with their hepatic copper around 500 mg/kg dwl (group 2) and dogs that showed a relatively quick accumulation of hepatic copper reaching a copper concentration of 800 mg/kg dwl within the study period (group 3). Currently it is not possible to predict the response to a low-copper, high-zinc diet in these Labrador retrievers. Therefore, it remains necessary to check hepatic copper levels in dogs treated with diet in control liver biopsies. A safe interval for the follow-up would be 6 months, as we did not observe extreme and possibly dangerous hepatic copper accumulation while feeding the Royal Canin Hepatic diet after 6 months. Depending on the results obtained, an individual control biopsy scheme for a dog may be developed, based on the re-accumulation rate observed.

After identification of the mutations in *ATP7A* and *ATP7B* (**Chapter 8)** we genotyped the dogs from the diet study described in **Chapter 6** in which the enrolled dogs all suffered from copper-associated hepatitis. The allele frequency of the *ATP7A* mutation *(ATP7A:c.980T)* was with 0.11 much lower than the allele frequency of 0.22 present in the total group of Labrador retrievers described in **Chapter 8**. This finding was in line with our hypothesis that *ATP7A:c.980T* prevents hepatic copper accumulation. The allele frequency of the causal *ATP7B* mutant (*ATP7B:c.4358A*) in this study group was 0.50, higher than the 0.31 observed in the total population of Labrador retrievers described in **Chapter 8.** The allele frequency of the *ATP7B* mutant was 0.63 in group 3, which was with the most severely affected group, versus an allele frequency of 0.43 in groups 1 and 2. It was not possible to predict the response to diet in this relatively small group of Labrador retrievers based on the presence of mutations in *ATP7A* and *ATP7B*.

In **Chapter 7** we studied a group of 28 Labrador retrievers diagnosed with subclinical copper toxicosis that were treated with Royal Canin Hepatic dry diet alone and were followed longitudinally with repeated liver biopsies. In 15 out of 28 dogs, hepatic copper levels decreased to <400 mg/kg during dietary management. However, individual variation in response to diet was noted, and 6/28 of the dogs accumulated hepatic copper despite being fed the diet. We used logistic regression analysis to investigate the effect of sex, age, initial copper concentrations as well as being part of a high risk pedigree on the ability to normalize hepatic copper concentration. We found that dogs that were part of the high risk pedigree were less likely to normalize their hepatic copper levels.

After finalizing the genetic studies and the identification of the mutations in *ATP7A* and *ATP7B* **(Chapter 8)**, we genotyped the original 28 dogs and re-analyzed the data to investigate whether the presence of mutations in *ATP7A* and *ATP7B* could account for the observed differences between dogs from the high risk pedigree and dogs from the other pedigrees. The allele frequency of the protective mutation *(ATP7A:c.980T)* in the high risk pedigree vs the other group was 0.18 vs 0.24. In contrast, the allele frequency of the *ATP7B:c.4358A* mutant was 0.58 in the high risk pedigree vs 0.27 in the other group. Logistic regression analysis showed that the presence of the *ATP7B:c.4358A* mutant had a significant negative effect on the chance to normalize hepatic copper concentration. All dogs that were homozygous for the risk genotype, were not able to reach a hepatic copper concentration <400 mg/kg dwl through dietary management (Figure 1). The opposite effect was observed for the presence of the *ATP7A:c.980T* mutant. However, this effect did not reach significance. Although the study group was relatively small, results from re-analyzing the data suggest that the *ATP7B:c.4358A* allele may influence response to diet and that the observed pedigree effect was actually caused by *ATP7B:c.4358A*. However, based on these observations, we cannot exclude the possibility that other (genetic) factors that may influence the response to diet were present in the high risk pedigree.

Continuing research

D-penicillamine proved to be an effective and reliable copper chelator for treatment of copper-associated hepatitis in dogs. Drawbacks associated with the use of this chelator are the relatively frequently encountered side effects including anorexia and vomiting. Furthermore, the necessary treatment period

is quite long (usually a minimum of 6 months). To improve chelation, studies should focus on the development of copper chelators that target toxic (free) copper or copper in mitochondria[14]. Also, copper chelators that promote copper excretion through the bile (which is the natural route), rather than through urinary excretion should be further explored for their utility and safety[15]. Finally, there is a need for developing copper chelators that work faster and induce a much quicker de-coppering effect.

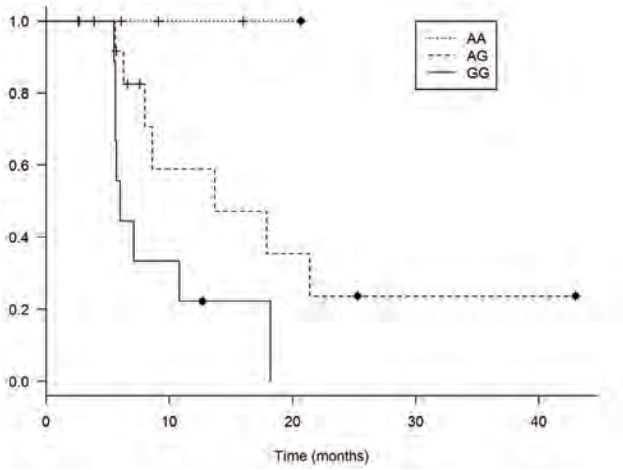


Figure 1 Kaplan-Meier curve for normalization in hepatic copper in 28 subclinical Labrador retrievers treated with Royal Canin Hepatic dry diet by *ATP7B:c.4358* genotype. Black dots: dogs that were lost to follow-up. Vertical lines: dogs that were removed from the study due to accumulation of copper or worsening histology.

Dog food can be easily standardized in kibble or canned food on which the animal can be maintained for a long period. Therefore, diet is a potentially valuable intervention tool in copper toxicosis. In order to define a safe diet for dogs, it will be necessary to continue the search for optimal copper and zinc concentrations. (Long-term) diet trials with stable isotopes and varied levels of copper and zinc are recommended to investigate the relative effect of copper, zinc and possibly the ratio of copper and zinc in the diet on intestinal copper absorption and body copper homeostasis. Results from our current studies indicate that the response to diet can be variable, and may be influenced by the *ATP7B* genotype. Our observations should be confirmed in a prospective diet trial using a larger group of dogs selected on the basis of *ATP7A* and *ATP7B* genotype.

Although we did not observe copper deficiency in *ATP7B* wild-type dogs that

were homo- or hemizygous for the *ATP7A* mutation, we might expect that these dogs have an increased risk of becoming copper deficient when fed a low copper diet. Stable isotope studies for copper uptake should be performed in *ATP7A* mutant dogs to investigate this effect and to predict the risk for copper deficiency in these dogs.
In the nearby future, we may be able to proceed towards personalized dietary management strategies for dogs based on genetic background.

Recommendations
Regarding treatment and long-term management of copper-associated hepatitis in Labrador retrievers we propose the following practical guidelines that could potentially be extended to other dog breeds with complex forms of copper-associated hepatitis.

- A quantitative copper measurement should be performed in the initial liver biopsy in order to determine optimal treatment duration and biopsy interval based on the estimates provided in the model as presented in **Chapter 4**.

- The clinician should monitor owner compliancy, efficacy of D-penicillamine uptake, and side effects in the dog.

- The clinician should perform a control liver biopsy after D-penicillamine therapy to check whether copper chelation was effective.

- When control liver biopsies become negative for copper using rubeanic acid staining, an additional biopsy for a quantitative measurement of copper and zinc should be obtained to evaluate possible deficiencies.

- After normalization of hepatic copper, a low-copper, high-zinc diet can be used for long-term management.

- Follow-up biopsies should initially be scheduled every 6 months. Subsequently, the clinician should determine an alternate schedule (D-penicillamine/diet) for individual dogs aimed at control biopsies at a frequency of maximally once a year.

Genetics

In humans, the copper metabolism disorder Wilson disease[16;17] is caused by mutations in the copper transporter ATP7B. A wide variety in age of onset and clinical presentation is recognized and there is often a lack of genotype-phenotype correlation, which implies the existence of modifier genes influencing the clinical presentation. Ecogenetic disorders of copper metabolism in humans, in which genetically predisposed individuals exposed to high levels of exogenous copper develop clinical disease, include Endemic Tyrolean Infantile Cirrhosis (ETIC)[18], Indian Childhood Cirrhosis (ICC)[19] and idiopathic copper toxicosis[20]. In these disorders copper accumulation is restricted to the liver and the manifestation of clinical symptoms is likely to be influenced by copper uptake via diet or drinking water. At present the genetic background of these diseases is unresolved. The wide variability of the human genome, the rarity of the copper metabolism disorders and the variation in clinical phenotypes hamper genetic mapping studies of these diseases in humans.

Since their domestication, dogs have undergone artificial selection through breeding, resulting in the development of effectively isolated populations of dog breeds. Through inbreeding and selection based on specific phenotypic characteristics, genetic diseases are frequently present in pure bred dog populations, and many breeds are at increased risk for developing specific disorders with a complex inheritance pattern[21].

The reduced genetic variability within inbred dog populations effectively works as a magnifying glass and may be instrumental for discovering causal as well as modifier genes involved in complex genetic diseases that are of interest for both dogs and humans. Therefore, the final aim of this thesis was to study the genetic background of copper-associated hepatitis in the Labrador retriever in order to identify genes that may influence phenotype in both humans and dogs.

Methodological considerations

For mapping a complex disease that is influenced by multiple mutations each with a small effect, a genome-wide association study (GWAS) is currently considered to be the preferred approach[22]. Mapping of disease genes in inbred dog populations has several advantages compared to mapping studies in

human individuals. Dog breeds are closed populations and display limited locus and disease heterogeneity[23] thereby reducing the number of dogs and DNA markers needed for successful gene-mapping in a complex genetic disease[24]. While utilizing the unique genetic structure of inbred dog populations, one must carefully evaluate cryptic relatedness among dogs used in the genome wide association study in order to prevent false positive association signals. When evaluating the pedigrees from the dogs included in our study, it proved impossible to select dogs that were unrelated at the grand-parental level, as all dogs were related to each other within a few generations. Therefore, we decided to use a sophisticated mixed-model based GWAS analysis method implemented in the R-package GenABEL[25]. In short, the GenABEL program utilizes a relationship matrix, based on SNP-marker information of all individual dogs for a first phenotype fit. Subsequently, the residuals from this analysis are tested for association with the phenotype. Additional corrections for genomic control were applied[26]. A consequence of the use of related individuals in GWAS studies is the fact that relatively large regions in LD are identified, hampering the pinpointing of individual genes in initial GWAS analysis. In our study, the large associated region at chromosome 22 was an illustration of this phenomenon. In this phase, one can either choose a fine-mapping strategy or a candidate gene approach. We identified 2 strong candidate genes and therefore deployed the strategy to first focus on these genes. Possible causal mutations were replicated in an independent cohort and showed very strong *P*-values in the total dataset.

Phenotyping

One of the most important steps at the start of a GWAS analysis is the collection of cases and controls combined with careful phenotyping of each individual. Copper-associated hepatitis in the Labrador retrievers is not a simple, binary trait. Variation in observed histological changes is present in liver biopsies, dependent on the phase of the disease in which the first liver biopsy is taken. From our understanding of disease progression, hepatic copper accumulation precedes inflammatory changes in the liver which eventually progresses to liver cirrhosis. However, not all cases of chronic hepatitis in the Labrador retrievers are copper-associated and it might be that two different diseases (copper-associated hepatitis and idiopathic or possibly immune mediated hepatitis) exist within the Labrador retriever population. In addition,

the presence of increased hepatic copper concentrations may exacerbate inflammation and accelerate progression to liver cirrhosis. We considered copper to be an important trigger for hepatic inflammation and therefore in our GWAS analysis, we focused on hepatic copper levels as the underlying trait for the development of copper-associated hepatitis. In order to approach the phenotype as unbiased as possible and to gain maximum power for the analyses, we utilized the hepatic copper scores as quantitative trait in a mixed-model association analysis (**Chapter 8**). For the GWAS we used 235 carefully phenotyped Labrador retrievers. The phenotypic analysis included signalment (age, sex) and histological copper score.

Sex difference

A strong female predisposition for copper-associated hepatitis prompted us to pay extra attention to the X-chromosome in our analysis. We analyzed the pseudo-autosomal region of the X-chromosome in the genome wide analysis. The rest of the X-chromosome was analyzed for males and females separately. Because males are hemizygous, a bias in the analysis of SNP data was possible. We identified an association signal in males, harboring the gene *ATP7A*, which is involved in the human copper deficiency disorder Menkes disease in humans. This was an unexpected, but very interesting finding. Sanger sequencing and replication of mutations in an independent cohort of Labrador retrievers resulted in the identification of *ATP7A*:c.980C>T, which results in the amino-acid change ATP7A:p.Thr327Ile at a presumed phosphorylation site. Dogs homozygous for the mutation showed lower hepatic copper levels. Therefore, we postulated that this mutation decreased the efficiency of copper transport capacity over the baso-lateral membrane in enterocytes, thereby decreasing enteral copper uptake. The allele-frequency of this mutation in our dog cohort was estimated to be 0.22. Extrapolation of this number to the population would mean that 22% of male dogs are hemizygous for the *ATP7A* mutation, compared to 5% homozygous female dogs. The frequency difference for this `protective´ mutation could explain part of the observed prevalence difference of copper-associated hepatitis between male and female Labrador retrievers.

Explained genetic variability

With the identified mutations in *ATP7A* and *ATP7B* we were able to explain approximately 12.5% of the total estimated genetic variability of 48%. This is a

substantial percentage for a complex genetic disease. The mutations in *ATP7A* and *ATP7B* are both present with a high frequency in our study population (0.22 and 0.31 respectively). Other mutations may play a role in the disease pathogenesis in copper-associated hepatitis in the Labrador retrievers, which are rarer and may have major effects in only a limited number of families, making them difficult to identify in genome wide association studies. Furthermore, the mutations that we have identified both have a major effect on hepatic copper scores. The *ATP7A* allele decreases the hepatic copper scores with 0.36 (SD, 0.09) and the *ATP7B* allele increases the hepatic copper scores with 0.51 (SD, 0.1), representing substantial shift based on a copper score ranging from 0-5. Other mutations with a possible smaller effect would require much larger data-sets for detection.

Translational aspects

Copper toxicosis in Labrador retrievers and Wilson disease in humans have similarities in clinical presentation (i.e., the wide range in age of onset with a peak at middle age), amount of hepatic copper accumulation, concurrent presence of iron accumulation and progression to liver cirrhosis. We have shown for the first time that Labrador retrievers and Wilson disease patients also share *ATP7B* as a causal gene. However, there are also striking differences between both diseases. For instance clinical presentation, clinical pathology and histopathology of liver biopsies. In Labrador retrievers neurological symptoms and Kaiser-Fleisher rings have not been found. Furthermore, differences in urinary copper excretion between Labrador retrievers and human patients are present (**Chapter 3**). Ceruloplasmin status in Labrador retrievers with and without the *ATP7B* mutation is currently under investigation. Histopathological differences in liver biopsies include predominant centrolobular copper accumulation in Labrador retrievers, versus a peri-portal distribution in Wilson disease patients. Further, fatty degeneration and Mallory bodies are not recognized in Labrador retrievers. Dietary intake of copper seems important in disease progression in the Labrador retrievers (**Chapter 5, 6 and 7**) and in view of this, Labrador retriever copper toxicosis is more similar to the ecogenetic disorders of copper metabolism: ETIC and ICC. The observed differences among copper toxicosis diseases in man and the Labrador retriever are intriguing and further studies are needed to identify whether they can be attributed to general species differences in copper metabolism or as yet unidentified genetic modifiers.

Interestingly, dogs in general have higher hepatic copper levels than humans; 400 mg/kg dwl in dogs is considered normal, versus 50 mg/kg dwl in humans. Nonetheless, the dogs can be an important translational model for generation of new hypotheses and may provide new insights on how copper homeostasis can be influenced. In addition, further genetic studies carried out in other breeds may lead to identification of new genes and modifiers of the disease phenotype. The new mutations identified in *ATP7A* and *ATP7B* in the Labrador retrievers may provide insights in specific functions of the protein regions involved. Finally, gene-correction strategies could be first employed in canine patients, before implementing them in treatment protocols for human patients.

Continuing research

Identification of more underlying genes influencing (copper-associated) hepatitis in the Labrador retriever.

In order to tackle missing heritability and in an attempt to explain more of the phenotype by gene mutations, we performed a second genome wide association study in a follow-up project. As mentioned earlier, there could be two forms of hepatitis in the Labrador retriever population (i.e., copper-associated and idiopathic/immune mediated). In order to map the genes for the idiopathic form of chronic hepatitis, we analyzed our data-set phenotyped in a binary fashion (chronic hepatitis vs normal histology), corrected for the covariates: hepatic copper levels, *ATP7A* and *ATP7B* genotype. This analysis resulted in a new genome wide significant signal. In order to identify more copper-associated mutations, we repeated the original GWAS analysis and added the *ATP7A* and *ATP7B* mutations as covariates. As expected, the association signals at chromosome 22 and X completely disappeared, while other regions (in total 9) appeared. We designed a next generation sequencing strategy for the analysis of all exons in these chromosomal regions. With Solid 5500 technology, these regions were sequenced with on average 100 x coverage in 95 individual Labrador retrievers that were thoroughly phenotyped. Currently, the replication of identified mutations in an independent cohort is being performed and functional studies will be designed based on the specific functions of identified genes.

Genetic risk prediction and implementation of breeding strategies in the Labrador retriever.

Although there is still unexplained heritability, based on the identification of two mutations influencing hepatic copper levels in the Labrador retrievers genetic risk prediction for individual dogs becomes an opportunity. We recently started a project based on this approach in close collaboration with the Labrador retriever breed club. Owners of Labrador retrievers can submit cheek-swabs for DNA isolation and SNP-typing. Based on the genotype of the dogs, a risk prediction can be made with the currently available dataset and owners can then decide, whether they wish to test their dog for the presence of copper-associated hepatitis by conducting a liver biopsy. With this approach, we will get a more unbiased estimate of the allele frequency of the two mutations in the general Labrador retriever population. In the present dataset, there is a bias towards certain severely affected pedigrees. Moreover, liver biopsies will provide extra evidence for the predictive value of the DNA test based on the two mutations.

There is a general consensus that affected dogs should be excluded from breeding programs, not only for their own safety (i.e. breeding bitches are at increased risk for developing clinical disease when bred), but also for decreasing the disease burden in the population. Implementing DNA tests in the breeding strategy has the benefit of identifying carriers as well as affected dogs in the subclinical phase. At this point in time however, it may be too early to implement the DNA test in breeding strategies in the Labrador retriever population as excluding dogs with the *ATP7B* mutation from the breeding program could bring unwanted side effects due to the high frequency of the disease allele. A positive selection for dogs with the *ATP7A* mutation could be an option to explore. However more functional studies into this specific mutation on cellular level should be conducted. The copper-transport ATPA-ses may also have a role in drug transport and may be involved in multi-drug resistance[27]. A full understanding of how the *ATP7A*:c.980C>T mutation influences gene function not only for copper metabolism, but also for its other functions is needed, before implementing promotion of the *ATP7A*:c.980C>T mutation in the population.

Other breeds

Identification of *COMMD1* as the causal gene in the Bedlington terrier was an important step in the field of copper genetics. Since its discovery, many more interesting functions of this protein have been discovered, of which the most recent findings include a role in the inflammatory response control[28] and cholesterol metabolism. The Bedlington terrier shows a unique phenotype which is quite different in clinical presentation compared to other dog breeds with copper toxicosis including the Dobermann, Labrador retriever, West Highland white terrier, Airedale terrier, Norfolk terrier, Cavalier King Charles spaniels, Dalmatians and more. The involvement of the deletion of exon 2 of COMMD1 in copper toxicosis in Labrador retrievers and Dobermanns was excluded in our lab. Over the years we have collected DNA and liver samples from other dog breeds affected with copper-associated hepatitis. One of the best represented breeds was the Dobermann, of which we have approximately 200 samples available. For this breed, a genome wide association study will be performed in the nearby future. For the other breeds, a next generation sequencing approach of candidate genes and new genes identified in the new GWAS analysis in Labrador retrievers and Dobermanns can be performed.

Recommendations:

- Do not breed with dogs affected by copper-associated hepatitis.
- Screen breeding animals by taking a liver biopsy between 2 to 3 years of age.
- A DNA test for *ATP7A* and *ATP7B* mutation can be implemented in a screening protocol for the general population, dogs with a high genetic burden can be admitted for a liver biopsy. The full advantage of genotyping for screening will be achieved when remaining underlying genes have been identified.

References

1. Poldervaart J.H., R.P. Favier, L.C. Penning *et al*. 2009. Primary hepatitis in dogs: a retrospective review (2002-2006). J. Vet. Intern. Med. 23: 72-80.
2. Hoffmann G., T.S.G.A.M. Van Den Ingh, P. Bode *et al*. 2006. Copper-associated chronic hepatitis in Labrador Retrievers. J. Vet. Intern. Med. 20: 856-861.
3. Center S.A. 2007. Interpretation of liver enzymes. Vet. Clin. North Am. Small Anim. Pract. 37: 297-333, vii.
4. Krol J., I. Loedige & W. Filipowicz. 2010. The widespread regulation of microRNA biogenesis, function and decay. Nat. Rev. Genet. 11: 597-610.
5. van der Meer A.J., W.R. Farid, M.J. Sonneveld *et al*. 2013. Sensitive detection of hepatocellular injury in chronic hepatitis C patients with circulating hepatocyte-derived microRNA-122. J. Viral Hepat. 20: 158-166.
6. Farid W.R., Q. Pan, A.J. van der Meer *et al*. 2012. Hepatocyte-derived microRNAs as serum biomarkers of hepatic injury and rejection after liver transplantation. Liver Transpl. 18: 290-297.
7. Suazo M., F. Olivares, M.A. Mendez *et al*. 2008. CCS and SOD1 mRNA are reduced after copper supplementation in peripheral mononuclear cells of individuals with high serum ceruloplasmin concentration. J. Nutr. Biochem. 19: 269-274.
8. Bertinato J., M. Iskandar & M.R. L'Abbe. 2003. Copper deficiency induces the upregulation of the copper chaperone for Cu/Zn superoxide dismutase in weanling male rats. J. Nutr. 133: 28-31.
9. Iskandar M., E. Swist, K.D. Trick *et al*. 2005. Copper chaperone for Cu/Zn superoxide dismutase is a sensitive biomarker of mild copper deficiency induced by moderately high intakes of zinc. Nutr. J. 4: 35.
10. Walshe J.M. 1956. Penicillamine, a new oral therapy for Wilson's disease. Am. J. Med. 21: 487-495.
11. Lenz G.R. & A.E. Martell. 1964. Metal Chelates of some Sulfur-Containing Amino Acids. Biochemistry. 3: 745-750.
12. Favier R.P., B. Spee, H. Fieten *et al*. 2015. Aberrant expression of copper-associated genes after copper accumulation in COMMD1-deficient dogs. J. Trace Elem. Med. Biol. 29: 347-353.
13. Center S.A. 2009. Copper-associated Liver Disease in the Dog: an Emerging Problem or Chronic Confusion? American Animal Hospital Association: 273-277.
14. Zischka H., J. Lichtmannegger, S. Schmitt *et al*. 2011. Liver mitochondrial membrane crosslinking and destruction in a rat model of Wilson disease. J. Clin. Invest. 121: 1508-1518.
15. Gateau C. & P. Delangle. 2014. Design of intrahepatocyte copper(I) chelators as drug candidates for Wilson's disease. Ann. N. Y. Acad. Sci. 1315: 30-36.
16. Tanzi R.E., K. Petrukhin, I. Chernov *et al*. 1993. The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. Nat. Genet. 5: 344-350.
17. Bull P.C., G.R. Thomas, J.M. Rommens *et al*. 1993. The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. Nat. Genet. 5: 327-337.
18. Muller T., H. Feichtinger, H. Berger *et al*. 1996. Endemic Tyrolean infantile cirrhosis: an ecogenetic disorder. Lancet. 347: 877-880.
19. Tanner M.S. 1998. Role of copper in Indian childhood cirrhosis. Am. J. Clin. Nutr. 67: 1074S-1081S.
20. Scheinberg I.H. & I. Sternlieb. 1994. Is non-Indian childhood cirrhosis caused by excess dietary copper? Lancet. 344: 1002-1004.
21. Ostrander E.A. 2012. Franklin H. Epstein Lecture. Both ends of the leash--the human links to good dogs with bad genes. N. Engl. J. Med. 367: 636-646.
22. Cordell H.J. & D.G. Clayton. 2005. Genetic association studies. Lancet. 366: 1121-1131.
23. Shearin A.L. & E.A. Ostrander. 2010. Leading the way: canine models of genomics and disease. Dis. Model. Mech. 3: 27-34.
24. Lindblad-Toh K., C.M. Wade, T.S. Mikkelsen *et al*. 2005. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature. 438: 803-819.

25. Aulchenko Y.S., S. Ripke, A. Isaacs *et al*. 2007. GenABEL: an R library for genome-wide association analysis. Bioinformatics. 23: 1294-1296.
26. Chen W.M. & G.R. Abecasis. 2007. Family-based association tests for genomewide association scans. Am. J. Hum. Genet. 81: 913-926.
27. Tadini-Buoninsegni F., G. Bartolommei, M.R. Moncelli *et al*. 2014. Translocation of platinum anticancer drugs by human copper ATPases ATP7A and ATP7B. Angew. Chem. Int. Ed Engl. 53: 1297-1301.
28. Bartuzi P., T. Wijshake, D.C. Dekker *et al*. 2014. A cell-type-specific role for murine Commd1 in liver inflammation. Biochim. Biophys. Acta. 1842: 2257-2265.

Chapter 10

Summary

Copper is an essential trace element and is indispensable for a large number of important biological processes in the body. Both copper deficiency and copper overload may lead to deleterious conditions. Therefore, copper metabolism is tightly regulated. The liver is involved in regulation of copper metabolism and is the first organ to be affected in copper overload disorders. Accumulation of copper in the liver may result from excessive intake of copper via diet or drinking water, and/or from inherited aberrant copper metabolism. Hereditary disorders of copper metabolism are observed in humans, dogs and other mammals including rats, mice, sheep and cows.

Chapter 1 provides an overview of the regulation of cellular copper metabolism. An introduction of the most important copper transporters (ATP7A, ATP7B, CTR1), sequestering proteins (MT, GSH), copper chaperones (CCS, ATOX1, COX17) and target proteins (CcO, SOD1, XIAP) is given. Furthermore, hereditary disorders of copper metabolism that are recognized in humans and dogs are introduced. In humans, Wilson disease is caused by mutations in the gene coding for the copper transporter ATP7B, and results in copper accumulation in the liver and neuronal tissues. A variable clinical presentation is observed in Wilson disease patients, including a predominantly hepatic, neurologic or psychiatric presentation. Ecogenetic disorders of copper overload include Indian Childhood Cirrhosis, Endemic Tyrolean Infantile Cirrhosis and Idiopathic Copper Toxicosis and are characterized by hepatic copper accumulation, likely due to a genetic predisposition in combination with high dietary copper intake. One of the best studied copper metabolism disorders in dogs is Bedlington terrier copper toxicosis, which is an autosomal recessive disease caused by a deletion in the gene coding for COMMD1. The identification of this gene was a breakthrough in the understanding of mammalian copper homeostasis and a good example of the power of using purebred dogs in genetic studies for identification of disease-causing genes. Upon the identification of COMMD1 involvement in copper metabolism (likely through interaction with the copper transporter ATP7B), many more functions of this protein have been elucidated, including hypoxia adaptation and mediation of the cellular localization of numerous proteins. A convincing role for COMMD1 in the modulation of disease phenotype in Wilson disease or for involvement in ecogenetic disorders of copper metabolism could not yet be demonstrated.

In other dog breeds including the Dobermann, West Highland white terrier, Dalmatian and Labrador retriever, other forms of copper toxicosis are recognized that that show a complex mode of inheritance. Genetic studies in these breeds offer the possibility to identify new genes involved in disturbed copper metabolism. Newly identified mutations may have implications for human patients, as the genes involved in copper metabolism are highly conserved over species.

The Labrador retriever is the most popular dog breed worldwide, and copper-associated hepatitis is diagnosed with increasing frequency in this breed. The disease is characterized by a long subclinical phase, as clinical symptoms often only appear in an end stage of the disease where treatment is less effective. Dietary intake of copper and zinc may influence disease progression, as such the disease shows similarities to ecogenetic disorders of copper metabolism in humans. **Chapter 2** summarizes the main aims of this thesis. The first aim was to optimize the diagnosis and medical treatment of copper-associated hepatitis in the Labrador retriever (**Part I, Chapter 3 and 4**). Moreover, the role of diet in the disease pathogenesis was investigated, and possible therapeutic opportunities for long-term management strategies were evaluated (**Part II, Chapter 5, 6 and 7**). The final aim of this thesis was to discover the causal gene mutations involved in copper-associated hepatitis in the Labrador retriever and to provide functional evidence for their causality in the disease (**Part III, Chapter 8**).

Chapter 3 describes a study in which urinary excretion patterns of copper, zinc and iron with and without previous D-penicillamine administration was investigated for possible use as a non-invasive screening tool for copper-associated hepatitis in Labrador retrievers, Beagles and Beagletons (a cross between Beagle and Bedlington terrier, homozygous for the *COMMD1* mutation). Twenty-four h D-penicillamine induced copper excretion and urinary copper/creatinine levels were not predictive for hepatic concentration in dogs, in contrast to what is observed in Wilson disease patients. Urinary copper/zinc ratio in a single urine sample in Labrador retrievers was significantly associated with hepatic copper concentration in Labrador retrievers. However, there was an overlap between dogs with normal- and dogs with increased hepatic copper concentrations. Consequently, the urine copper/zinc ratio cannot be used as a single test to predict hepatic copper concentration in Labrador retrievers.

In **Chapter 4** the development of an optimal protocol for D-penicillamine treatment of Labrador retrievers diagnosed with copper-associated hepatitis is described. D-penicillamine was effective in decreasing hepatic copper concentrations. In parallel, a concomitant significant decrease in inflammatory grading scores in the liver was observed after treatment. Hepatic copper concentration before the start of D-penicillamine therapy had a significant impact on necessary treatment duration. Sex, age, presence of side effects or formulation of medication (enteric coated tablets vs compounded D-penicillamine) did not have a significant effect. The estimates derived from the dogs in the dataset can now be used to predict necessary treatment duration, based on initial hepatic copper concentration. Hepatic iron concentrations were unaffected by D-penicillamine therapy, whereas zinc concentrations decreased significantly. To monitor treatment effectivity, as well as presence of copper and/or zinc deficiency follow-up biopsies are advised.

In **Part II** of the thesis the focus was mainly on dietary intake of copper and zinc and the role of diet in the development of copper-associated hepatitis and possible implementation in long-term management strategies.

The main question addressed in **Chapter 5** was whether copper and zinc concentration in the diet significantly influences hepatic copper concentration in Labrador retrievers. This question was addressed in a cohort of 55 Labrador retriever, of which the majority (n = 44) was related to dogs which had been previously diagnosed with copper-associated hepatitis. Hepatic copper concentrations were determined in a liver biopsy, and analysis of kibble of each dog was performed. Hepatic copper concentrations were respectively positively and negatively correlated to dietary copper and zinc intake. Although 51 out of 55 Labrador retrievers in this study appeared clinically healthy, approximately 75% of dogs had an increased copper concentration, indicating that copper and zinc content in current commercial dry food may not be suitable for dogs with a genetic predisposition for copper-associated hepatitis.

Results from the study in **Chapter 5** indicate that diet may have a major impact on hepatic copper accumulation. Therefore, the use of an adjusted diet (low-copper, high-zinc) in the management of Labrador retrievers affected with copper-associated hepatitis was investigated in the study described in **Chapter 6**.

Sixteen Labrador retrievers with copper-associated hepatitis, that had been treated with D-penicillamine until hepatic copper concentrations normalized, were recruited for this study. The dogs were treated with a low copper, high-zinc diet (Royal Canin Hepatic dry diet). Every 6 months liver biopsies were taken for histological evaluation and measurement of hepatic copper concentration. Substantial variability in response to diet was noted and 3 groups of responders were recognized: 1) dogs from group 1 could be maintained on a hepatic copper concentration below 400 mg/kg dwl. In these dogs, no significant re-accumulation of hepatic copper occurred, and the diet alone may be sufficient for long term management; 2) dogs from group 2 showed re-accumulation of hepatic copper concentration above 400 mg/kg dwl at the first follow-up but were then relatively stable with their hepatic copper around 500 mg/kg dwl; 3) the last group showed a relatively quick re-accumulation, and higher concentrations of hepatic copper were associated with histological changes in the liver. It was concluded that diet adaptation may be a valuable tool for long-term management of dogs with copper-associated hepatitis. Nevertheless, follow-up biopsies should be scheduled since response to diet cannot be predicted beforehand.

Chapter 7 describes a study in which we investigated the possible use of a low-copper, high-zinc diet in order to decrease hepatic copper concentrations in Labrador retrievers with subclinical copper toxicosis. Twenty-eight Labrador retrievers with increased hepatic copper concentration were recruited for this study. Thirteen dogs were recruited from a pedigree with a high disease burden, whereas the other 15 dogs were derived from seven other pedigrees. In 15 out of 28 dogs hepatic copper concentrations decreased to values <400 mg/kg dwl upon dietary treatment. Variation in response was however present. This was illustrated by the fact that 6 out of 28 dogs actually accumulated copper during the study period. Being a member of the high risk pedigree significantly affected the possibility to be able to normalize in hepatic copper, indicating that hereditary factors might influence response to diet.

The final part of this thesis (**Part III, Chapter 8**) was dedicated to identify causal gene mutations underlying copper-associated hepatitis in the Labrador retriever. Given the genetic complexity of the disease, a genome wide association approach was chosen for this study. Overall, liver biopsies and DNA samples of 294 Labrador retrievers were collected. Liver biopsies were

stained for rubeanic acid, and hepatic copper score was used in the analysis as underlying quantitative trait for the development of copper-associated hepatitis. Approximately 100.000 DNA markers were analyzed over the entire genome. A sophisticated analysis method was used for mapping the genes influencing hepatic copper, taking into account the relatedness amongst individual dogs. Two chromosome regions containing the genes coding for copper-transporters ATP7A and ATP7B were associated with variation in hepatic copper levels. Subsequent DNA sequence analysis identified missense mutations in each gene. The mutations were validated in an independent cohort of 59 Labrador retrievers, leaving 2 missense mutation for functional follow-up. The amino-acid substitution ATP7B:p.Arg1453Gln was associated with copper accumulation, whereas the amino-acid substitution ATP7A:p.Thr327Ile partly protected against copper accumulation. Confocal microscopy indicated that aberrant copper metabolism upon expression of the ATP7B variant occurred due to mis-localization of the protein in the endoplasmic reticulum. Dermal fibroblasts derived from ATP7A:p.Thr327Ile dogs showed copper accumulation and delayed excretion. The identification of a mutation in *ATP7B* involved in copper toxicosis in Labrador retrievers identifies this breed as the first naturally occurring non-rodent model for Wilson disease. The unexpected observation that a mutation in *ATP7A* attenuates the copper accumulation phenotype sheds an interesting light on the interplay of copper transporters in body copper homeostasis and proposes *ATP7A* as a possible modifier gene in different forms of copper-associated hepatitis.

The results presented in this thesis are discussed in **Chapter 9**, comprising three sections: diagnosis, treatment and genetics. Practical recommendations for clinical management of Labrador retrievers with copper-associated hepatitis were provided and suggestions for future studies were made.

In conclusion, results described in this thesis contributed to better diagnostic and therapeutic protocols for copper-associated hepatitis in Labrador retrievers. The role of diet in the disease progression and the long-term management of Labrador retrievers with copper-associated hepatitis was established. Finally, the discovery of involvement of *ATP7A* and *ATP7B* in copper-associated hepatitis in the Labrador retrievers will have major implications for understanding the biological background of the disease, and may be valuable for development of new therapies for both humans and dogs.

Nederlandse samenvatting voor niet-ingewijden

Koper is een essentieel sporenelement dat erg belangrijk is voor een groot aantal biologische processen in het lichaam. Zowel een tekort aan koper als een overmaat aan koper kunnen schadelijke gevolgen hebben, daarom is de koperstofwisseling in het lichaam strak gereguleerd. De lever speelt hierin een belangrijke rol. Koper dat via het dieet en drinkwater wordt opgenomen in de dunne darm, wordt eerst via de poortader naar de lever getransporteerd, alwaar het wordt verwerkt. Koper wordt in de lever ingebouwd in eiwitten en vervolgens verder getransporteerd naar andere organen. Een deel wordt opgeslagen in de levercellen. Het teveel aan koper wordt normaliter via de gal uitgescheiden met de ontlasting. Ophoping of stapeling van koper in de lever kan plaatsvinden wanneer de opname van koper via voeding en drinkwater zo hoog is, dat normale reguleringsmechanismen voor uitscheiding overschreden worden. Tevens kan koperstapeling in de lever veroorzaakt worden door een (erfelijk) defect in eiwitten die de uitscheiding of opname van koper regelen. Erfelijke koperstapelingsziekten komen voor bij de mens, de hond en andere zoogdieren waaronder knaagdieren (ratten en muizen) en herkauwers (schapen en koeien).

In **Hoofdstuk 1** wordt een overzicht gegeven van de belangrijkste eiwitten die de koperstofwisseling reguleren. Hierbij zijn van belang de kopertransport eiwitten die het koper vanuit het darmlumen de darmcel in transporteren (CTR1), doorpompen vanuit de darmcel de bloedbaan in (ATP7A), vanuit het bloed de levercellen in (CTR1) en vervolgens vanuit de levercel naar de gal uitscheiden (ATP7B). Binnen in de cel moet voorkomen worden dat koper "vrij" aanwezig is, omdat het schadelijk kan zijn door de productie van zuurstofradicalen die membranen, vetten, erfelijk materiaal (DNA) en eiwitten kunnen beschadigen. Daarom bestaan er een aantal kleine eiwitten die het koper binden en daardoor onschadelijk maken (MT en GSH). Verder zijn er nog gespecialiseerde chaperone eiwitten (CCS, ATOX1, COX17) die het koper kunnen binden en afleveren bij de eindgebruikers van koper in de cel (CcO, SOD1, XIAP, ATP7B). De best beschreven erfelijke koperstapelingsziekte bij de mens is de ziekte van Wilson, die wordt veroorzaakt door foutjes (mutaties) in het gen dat codeert voor het koper transport eiwit ATP7B. In mensen met de ziekte van Wilson stapelt het koper zich op in de lever, maar ook in zenuwweefsel en bijvoorbeeld in het hoornvlies van het oog. Deze mensen kunnen een breed scala aan symptomen vertonen, waaronder levercirrose,

neurologische verschijnselen, maar ook psychiatrische stoornissen. Omdat de ziekte vrij zeldzaam is, is het een uitdaging voor artsen om de diagnose correct te stellen, dit heeft soms grote consequenties voor patiënten, bijvoorbeeld in verband met het uitblijven van een correcte behandeling. Het is tot op heden onduidelijk hoe het kan dat er zo'n grote verscheidenheid aan symptomen voorkomt bij mensen met de ziekte van Wilson. Het kan zijn dat mutaties in andere genen, die tot op heden nog niet bekend zijn, het ziektebeeld kunnen beïnvloeden. Door de zeldzaamheid van de ziekte is dit in mensen vaak moeilijk te onderzoeken.

Andere vormen van koperstapeling die bij de mens voorkomen zijn koper-geassocieerde levercirrose bij pasgeborenen of jonge kinderen. Deze vormen werden waargenomen in kinderen geboren uit bloedverwante ouders uit bepaalde gebieden in Tirol en India. Deze kinderen hadden als zuigeling melk te drinken gekregen die verwarmd was in koperen of messing potten, waarin de koperconcentratie erg hoog was. De genen die verantwoordelijk zijn voor het ontstaan van deze ziekten zijn tot op heden niet opgehelderd.

In de hond worden verschillende vormen van koperstapeling waargenomen. Het best beschreven voorbeeld is de Bedlington terriër, waarin een mutatie in het *COMMD1* gen zorgt voor extreem hoge koperwaardes in de lever die uiteindelijk leiden tot koperstapeling. De ontdekking van dit gen was een doorbraak in het doorgronden van de biologische achtergrond van de koperstofwisseling. *COMMD1* was een nieuw gen, waarvan initieel de functie nog niet bekend was. In de loop der jaren is gebleken dat *COMMD1* niet alleen een rol speelt in de koperstofwisseling, door interactie met het koper transport eiwit ATP7B, maar dat het ook een rol heeft in zeer veel andere processen in de cel waaronder cholesterol metabolisme. Tot op heden is nog niet gebleken dat *COMMD1* een belangrijke rol speelt in humane koperstofwisselingsziekten.

In andere hondenrassen waaronder de Dobermann, West Highland White terriër, Dalmatiër en de Labrador retriever komt ook koperstapeling voor. In deze rassen wordt de ziekte niet veroorzaakt door een mutatie in *COMMD1*. Het ziektebeeld dat deze honden vertonen verschilt ook enigszins van het ziektebeeld bij de Bedlington terriër. Het kopergehalte in de lever behaalt meestal niet de extreme waarden die bij de Bedlington terriër gezien worden.

Bij stamboomonderzoek is gebleken dat er bij deze dieren geen sprake is van een simpele Mendeliaanse overerving, maar van een gecombineerd effect van mutaties in verschillende genen (complexe overerving). Genetische studies in deze hondenrassen bieden de mogelijkheid voor het opsporen van nieuwe genen die de koperstofwisseling beïnvloeden. Hondenrassen zijn ideaal voor genetische studies, omdat ze door inteelt minder variatie in hun genetisch materiaal hebben dan mensen, hierdoor zijn minder DNA markers nodig. Verder is het ziektebeeld dat ze vertonen ook vaak homogener dan vergelijkbare ziektebeelden bij de mens, hierdoor zijn minder dieren nodig om een studie te kunnen uitvoeren. Omdat de koperstofwisseling voor een groot deel hetzelfde is bij mensen en honden, kunnen nieuwe genen die geïdentificeerd worden in hondenstudies ook van belang zijn voor de mens.

De Labrador retriever is een zeer populair hondenras en koper-geassocieerde leverontsteking (hepatitis) wordt wereldwijd steeds vaker gediagnosticeerd in dit ras. De ziekte bij de Labrador retriever wordt gekenmerkt door een lange periode waarin koper opstapelt in de lever, maar waarin de honden nog geen verschijnselen vertonen. Dit komt omdat de lever een grote reservecapaciteit heeft, waardoor pas verschijnselen optreden als een zeer groot deel van de lever is aangetast. In zo'n stadium is behandeling helaas vaak minder effectief. Koperopname via dieet en drinkwater lijkt een invloed te hebben op het ontwikkelen van de ziekte en hierin lijkt de ziekte bij de Labradors op de kinder-cirrose uit Tirol en India. De diagnose kan alleen worden gesteld door het laten onderzoeken van een leverbiopt (klein stukje weefsel dat kan worden weggenomen met een dikke naald). Hierop wordt een koperkleuring uitgevoerd om de hoeveelheid koper in te schatten. Tevens wordt door een patholoog onderzocht hoe ernstig de ontsteking (hepatitis) is en hoeveel littekenweefsel (fibrose) er gevormd is in de lever. De behandeling bestaat uit het verwijderen van koper uit de lever middels medicatie en het beperken van koperinname via dieet en drinkwater.

In **Hoofdstuk 2** worden de belangrijkste doelstellingen van dit proefschrift samengevat. Ten eerste het verbeteren van de diagnose en behandeling van koper-geassocieerde hepatitis bij de Labrador retriever (**Deel I, Hoofdstuk 3** en **4**). Ten tweede de rol van voeding in het ontstaan van de ziekte beter in kaart brengen en onderzoeken of dieetaanpassing gebruikt zou kunnen worden in

de lange termijn behandeling van koper-geassocieerde hepatitis in Labrador retrievers (**Deel II, Hoofdstuk 5, 6** en **7**). Het derde en laatste doel was om de gen mutaties te identificeren die ten grondslag liggen aan koper-geassocieerde hepatitis in de Labrador retriever en te bewijzen dat deze mutaties ook daadwerkelijk een functionele rol hebben in de koperstofwisseling (**Deel III, Hoofdstuk 8**).

Verhoogde koperuitscheiding in een 24-uurs urinemonster is een belangrijk diagnostisch criterium dat vaak gebruik wordt om de diagnose te stellen en om therapie effect te monitoren bij mensen met de ziekte van Wilson. Om een nog duidelijker onderscheid te kunnen maken, wordt de uitscheiding van koper in de urine gestimuleerd door het toedienen van het medicijn D-penicillamine, dit is een derivaat van penicilline dat koper in het lichaam bindt en zorgt voor een verhoogde uitscheiding van koper in de urine. In **Hoofdstuk 3** hebben we al dan niet D-penicillamine geïnduceerde uitscheiding van koper, ijzer en zink gemeten in de urine van honden met verschillende leverkoperconcentraties. Verder is geïnventariseerd of koper- en zinkmeting in urine zou kunnen dienen als een non-invasieve methode om het kopergehalte in de lever van Labrador retrievers te kunnen voorspellen. Uit de resultaten bleek dat D-penicillamine binnen 2 uur na orale opname leidt tot uitscheiding van koper en zink, maar niet van ijzer via de urine. De mate van koperuitscheiding alleen kon niet differentiëren tussen honden met verschillende koperwaardes in de lever. De koper/zink ratio in de urine was significant geassocieerd met de concentratie van koper in de lever bij Labrador retrievers. Er bestond overlap in deze waarde tussen Labrador retrievers met normale dan wel verhoogde lever koperconcentraties. De urine koper/zink ratio is daarom niet als enkele parameter te gebruiken om te voorspellen wat het leverkopergehalte is in een individuele hond. Mogelijk kan het in de toekomst wel toegepast worden in een protocol waarbij meerdere diagnostische parameters naast elkaar gemeten worden.

In **Hoofdstuk 4** wordt verder ingegaan op de behandeling van koper-geassocieerde hepatitis met het medicijn D-penicillamine. Normaal gesproken worden honden met koperstapeling, zoals de Bedlington terriër, levenslang behandeld met medicijnen die koperuitscheiding bevorderen, zoals D-penicillamine. Labrador retrievers hebben echter een veel minder

hoog kopergehalte in de lever, in vergelijking tot Bedlington terriërs. Verder werd in **Hoofdstuk 3** gezien dat D-penicillamine niet alleen zorgt voor een verhoogde urinaire koperuitscheiding, maar dat ook zink in sterke mate werd uitgescheiden. De vraag was daarom of een levenslange, continue therapie met D-penicillamine wel de optimale behandelingsstrategie is voor Labrador retrievers. In deze retrospectieve studie is gekeken naar de effecten van D-penicillamine op het leverkopergehalte en de ontstekingsveranderingen. Tevens hebben we onderzocht welke factoren van invloed zijn op de benodigde behandelingsduur in Labrador retrievers. In alle onderzochte gevallen was D-penicillamine effectief in het verlagen van het kopergehalte in de lever. Bovendien trad er een verbetering op in de ontstekingsveranderingen die te zien waren in de leverbiopten. Als neveneffect bleek ook dat de zinkgehaltes in de lever significant verlaagd waren aan het einde van de therapie, de ijzergehaltes bleven onveranderd. De benodigde behandelingsperiode was alleen afhankelijk van de hoogte van het kopergehalte in de lever voorafgaand aan de therapie. Geslacht, leeftijd, de aanwezigheid van bijwerkingen of de vorm van de tabletten (capsules gemaakt door de apotheek of maagsapresistente tabletten) hadden hierop geen invloed. Met de parameters die zijn geschat op basis van onze onderzoeksgroep is het nu mogelijk om, indien het leverkopergehalte voorafgaand aan de therapie bekend is, de benodigde therapieduur voor een individuele hond te bepalen. Dit is van belang omdat de eigenaar niet te vroeg wil komen voor een controlebiopt, dit betekent namelijk dat de hond dan opnieuw behandeld en opnieuw gebiopteerd moet worden. De meeste honden waren tussen 6 maanden en 1.5 jaar na behandeling genormaliseerd in leverkopergehalte. Een levenslange, continue therapie met D-penicillamine is dus niet geïndiceerd in de Labrador retrievers en kan zelfs het risico inhouden van een koper- of zinktekort. In **Hoofdstuk 6** zal verder worden ingegaan op de mogelijkheden van lange termijn management van deze honden, nadat de D-penicillamine therapie is afgerond.

In **Deel II** werd ingegaan op de rol van dieet in het ontstaan en de behandeling van koper-geassocieerde hepatitis in de Labrador retriever. Dat de opname van overmatig veel koper via het dieet zal leiden tot koperstapeling in de lever klinkt logisch. Echter, de belangrijkste vraag die gesteld werd in **Hoofdstuk 5** was, of gehaltes in koper en zink die aanwezig zijn in normale, commercieel verkrijgbare hondenvoeders van invloed kunnen zijn op de

kopergehaltes in de lever van Labrador retrievers. Om deze vraag te kunnen beantwoorden werden leverbiopten van 55 Labrador retrievers en een sample van hun voer onderzocht. In totaal waren 44 van de 55 honden gerelateerd aan een Labrador retriever die al eerder was gediagnosticeerd met koper-geassocieerde hepatitis en zouden mogelijk een erfelijke predispositie voor deze ziekte kunnen hebben. Ondanks het feit dat 51 van de 55 Labrador retrievers geen ziekteverschijnselen vertoonden, had driekwart van de honden een verhoogd kopergehalte in de lever. Koperconcentraties in de lever van deze groep honden waren respectievelijk positief en negatief gecorreleerd met koper- en zinkconcentraties in de verschillende diëten. Uit deze studie kan worden geconcludeerd dat de huidige samenstelling van commerciële hondenvoeren over het algemeen niet geschikt is voor honden met een mogelijke erfelijke predispositie voor koperstapeling. Het feit dat er een sterk verband werd aangetoond tussen het koper- en zinkgehalte in het dieet en het leverkopergehalte in de Labrador retrievers, was aanleiding om te onderzoeken of een aangepast dieet van waarde zou kunnen zijn in de behandeling van Labrador retrievers op langere termijn (**Hoofdstuk 6**).

Voor de studie in **Hoofdstuk 6** werden 16 Labrador retrievers uitgenodigd die eerder waren gediagnosticeerd met koper-geassocieerde hepatitis en die inmiddels succesvol behandeld waren met D-penicillamine. Op het moment van de start van de studie hadden deze honden een normale koperconcentratie in de lever. In **Hoofdstuk 3** werd geconcludeerd dat levenslange, continue therapie met D-penicillamine mogelijk niet de meest ideale lange termijn behandelingsstrategie was. Dat was de reden om uit te zoeken of het mogelijk was om deze honden gezond te kunnen houden met een laag-koper, hoog-zink dieet. De honden kregen strikt Royal Canin Hepatic brokken te eten en kwamen iedere 6 maanden terug naar de kliniek voor een leverbiopt, waarin het kopergehalte gemeten werd. Er was een grote mate van variatie in de respons op het dieet in de individuele honden. Drie groepen konden worden onderscheiden. In de eerste groep bleef het kopergehalte normaal met alleen het dieet. Deze honden hebben waarschijnlijk geen medicinale behandeling meer nodig om het kopergehalte in de lever onder controle te houden. In groep twee was een duidelijke stijging zichtbaar, echter de koperconcentraties in de lever stabiliseerden zich daarna op een waarde die net boven de normaalwaarde lag. De honden van groep 3 toonden een snelle re-accumulatie

van het koper in de lever en dit ging ook gepaard met het ontstaan van ontstekingsveranderingen in de lever. Samenvattend kan gesteld worden dat een laag- koper, hoog-zink dieet een waardevolle lange termijn behandeling kan zijn voor een groot aantal honden met koper-geassocieerde hepatitis. Echter, omdat de respons op het dieet niet op voorhand te voorspellen is, blijft het nodig om het effect te controleren middels controle biopten.

In **Hoofdstuk 7** is geïnventariseerd of een laag-koper, hoog-zink dieet gebruikt zou kunnen worden in de behandeling van Labrador retrievers die al wel een verhoogd kopergehalte in de lever hebben, maar nog geen symptomen van de ziekte vertonen. Hiertoe werden 28 Labrador retrievers met een verhoogd leverkopergehalte behandeld met het Royal Canin Hepatic brok dieet. Ieder half jaar werden leverbiopten genomen om de koperconcentratie in de lever te monitoren, evenals eventuele ontstekingsactiviteit. Van deze groep van 28 honden, waren 13 honden afkomstig uit een familie van Labradors waarin een hele hoge ziekte incidentie voorkwam. De andere 15 honden waren afkomstig uit 7 andere families. Het eindpunt van de studie was gedefinieerd als het bereiken van een normale lever koperconcentratie. Honden werden uit de studie verwijderd wanneer de concentratie koper in de lever steeg ondanks het dieet of wanneer de ontstekingsveranderingen verergerden. In deze gevallen werd een behandeling met D-penicillamine ingesteld. Een grote variatie in respons op het dieet werd opnieuw gezien. In ongeveer de helft van de honden (15/28) kon een complete normalisatie van de koperconcentratie in de lever worden bereikt met enkel het dieet. In 6 van de 28 honden steeg het leverkopergehalte ondanks het dieet. In andere honden daalde het koper in een langzaam tempo, echter ontstekingsveranderingen in de lever verergerden, waardoor deze honden toch uit de studie zijn verwijderd. Een opvallende observatie was het feit dat de honden die afkomstig waren uit de ernstig aangedane familie een kleinere kans hadden om te normaliseren in hun leverkopergehalte ten opzichte van de andere honden. Deze honden hadden over het algemeen ook een hogere concentratie koper in hun lever, echter de stamboom invloed was groter dan de invloed van de koperconcentratie in de lever op het wel of niet normaliseren in leverkopergehalte. Deze observatie wijst erop dat een positieve, dan wel negatieve respons op dieet therapie mogelijk erfelijk bepaald kan zijn.

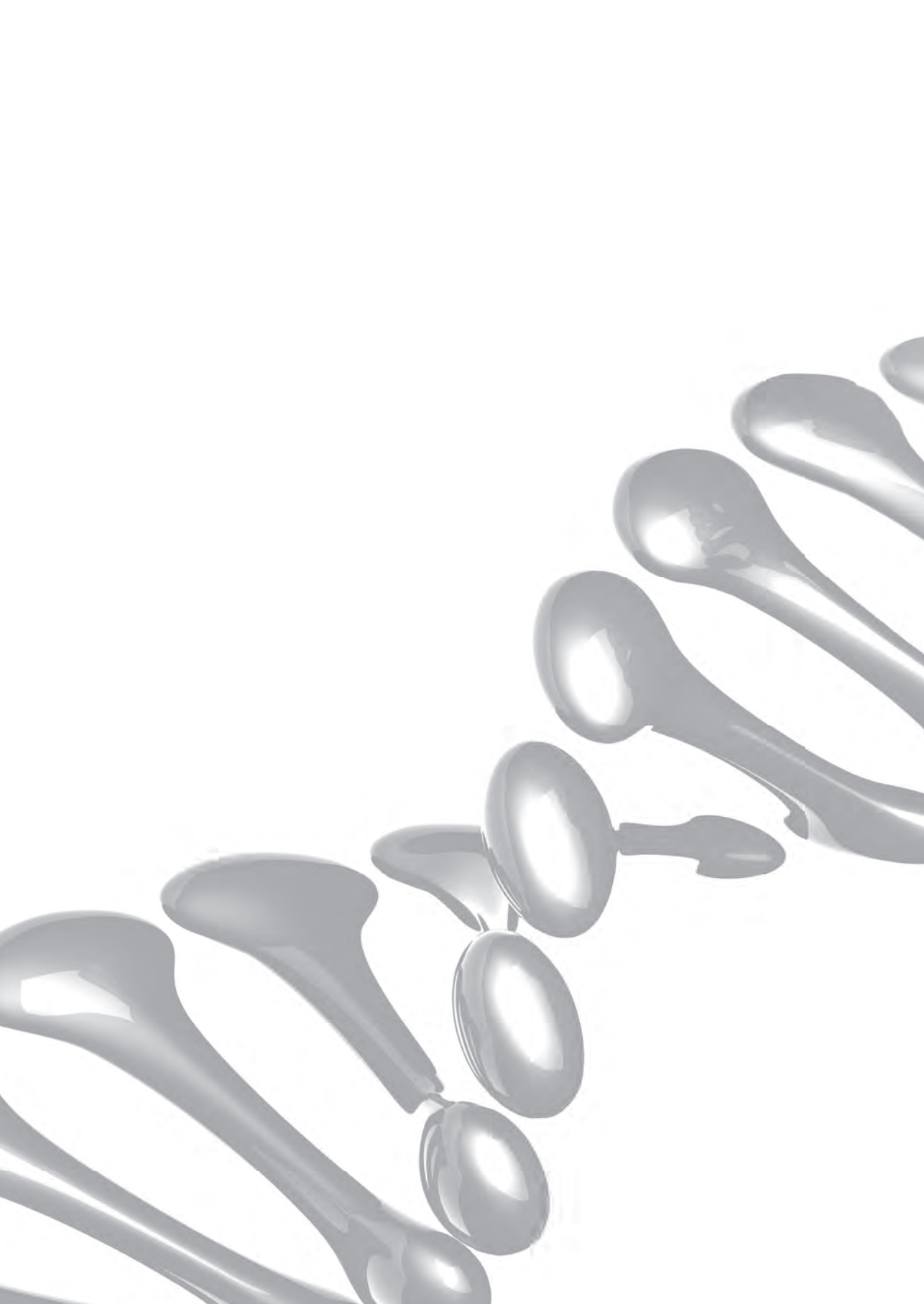
Het laatste gedeelte van het proefschrift (**Deel III, Hoofdstuk 8**) beschrijft een genetische studie, waarin de erfelijke achtergrond van koper-geassocieerde hepatitis in de Labrador retrievers wordt onderzocht. Uit eerder stamboomonderzoek bleek dat er geen sprake was van een simpele, mono-genetische overerving, maar dat er waarschijnlijk meerdere gen mutaties elk met een klein effect op het uiteindelijke ziektebeeld een rol spelen in het ontstaan van de ziekte. De meest geschikte aanpak om deze genen op te sporen is een genoom brede associaties studie. Hiertoe werden in totaal leverbiopten en DNA samples verzameld van 294 Labrador retrievers. De mate van stapeling van koper in de lever werd gezien als een onderliggende oorzaak voor het uiteindelijke ontwikkelen van koper-geassocieerde hepatitis. Honden in verschillende stadia van de ziekte werden verzameld; bijvoorbeeld dieren met alleen nog een hoog kopergehalte in de lever, maar nog geen ernstige ontstekingsveranderingen tot aan honden met eind stadium levercirrose waarbij het koper ten gevolge van de bindweefselvorming in de lever weer gedaald was. Om een zo objectief mogelijke maat voor de ernst van de ziekte te krijgen werd de score van koper in de lever genomen als uitgangswaarde in de berekeningsmethodiek. Verder werd gecorrigeerd voor geslacht (vrouwelijke dieren zijn vaker aangedaan en hebben vaak een hogere concentratie koper in de lever) en leeftijd (hoe ouder de dieren zijn, des te langer hebben ze kans gehad om koper te stapelen. Het DNA van 235 honden werd in kaart gebracht met behulp van ongeveer 100.000 DNA markers, waarvan de locatie op de verschillende chromosomen exact bekend is. Vervolgens werd uitgerekend of er een verband was tussen variatie in koperwaarde in de lever en variatie in de DNA markers. Tijdens deze berekeningen werd gecorrigeerd voor het feit dat honden die meer aan elkaar gerelateerd waren ook meer dezelfde DNA markers met elkaar delen. Uit deze berekeningen bleek dat 2 chromosomale gebieden significant geassocieerd waren met variatie in koperconcentratie in de lever. Het eerste gebied was gelokaliseerd op chromosoom 22 en was sterk geassocieerd met een hogere leverkoperscore in de totale groep honden. Het tweede gebied was gelegen op het X-chromosoom en was in mannelijke dieren verrassend genoeg geassocieerd met een lagere leverkoperscore. Nadere inspectie van deze gebieden toonde aan dat er twee belangrijke kandidaat-genen aanwezig waren, te weten *ATP7B* in het gebied op chromosoom 22 en *ATP7A* in het gebied op het X-chromosoom. Omdat dit beide kopertransport eiwitten zijn, werd eerst in meer detail op deze 2

genen gefocusseerd. De exacte base-volgorde van de coderende gedeeltes van deze genen werd bepaald in 96 Labrador retrievers. Dit leverde een aantal mutaties op die opnieuw significant geassocieerd waren met koperscores in de lever. De significante mutaties werden gevalideerd in een onafhankelijk cohort van 59 Labrador retrievers. Dit leverde een coderende mutatie op in elk gen. Om te onderzoeken of de mutaties ook een daadwerkelijk biologisch effect hadden op de koperstofwisseling, werden vervolgstudies uitgevoerd. Voor de mutatie in *ATP7B* werd een construct gemaakt van het wildtype (normale) gen en een construct van het gen met daarin de mutatie. Studies in cellijnen toonden aan dat de mutatie zorgde voor het achterblijven van een deel van het mutante gen in een verkeerd cellulair compartiment. Hiermee kan verklaard worden dat het ATP7B door de mutatie gestoord wordt in zijn functie en dit kan leiden tot opstapeling van koper in levercellen. Voor *ATP7A* werd een andere aanpak gekozen. Hiertoe werden huidcellen (fibroblasten) gekweekt van honden met en zonder de gevonden mutatie. Deze fibroblasten werden vervolgens behandeld met radioactief koper. De fibroblasten met de mutatie stapelden koper op en hadden moeite met het verwijderen van koper uit de cel in vergelijking met de fibroblasten van dieren zonder de mutatie. Omdat ATP7A met name een belangrijke rol speelt in de koperopname in de darm, zal door ophoping van koper in de darmcellen, die vervolgens afsterven en uitgescheiden worden met de ontlasting, juist een verminderde opname van koper plaats vinden. Hiermee kunnen de lagere lever koperwaardes in de honden met de *ATP7A* mutatie verklaard worden. In deze studie werd een deel van de erfelijke achtergrond van koper-geassocieerde hepatitis bij de Labrador retriever opgehelderd. Het feit dat zowel bij de Labrador retriever als in mensen met de ziekte van Wilson de ziekte wordt veroorzaakt door een fout in *ATP7B* biedt interessante mogelijkheden voor onderzoek in de toekomst. Informatie die wordt verkregen door onderzoek in de Labrador retrievers kan van belang zijn voor de mens. Het uitzoeken van een mogelijke rol van *ATP7A* in de variatie in symptomen die voorkomen bij mensen met de ziekte van Wilson is daar een eerste voorbeeld van. Verder bestaat nu ook de mogelijkheid voor de ontwikkeling voor nieuwe therapieën, waaronder gen correctie therapie, die voor zowel de hond als de mens waardevol kunnen zijn.

In **Hoofdstuk 9** worden de resultaten behaald in dit proefschrift bediscussieerd, worden praktische aanbevelingen gemaakt voor diagnostiek en behandelingen

van Labrador retrievers met koper-geassocieerde hepatitis en worden de mogelijkheden voor verder onderzoek verder toegelicht.

Concluderend kan gesteld worden dat de resultaten die in dit proefschrift worden beschreven bijdragen aan betere diagnostische en therapeutische mogelijkheden voor Labrador retrievers met koper-geassocieerde hepatitis. De identificatie van de betrokkenheid van *ATP7A* en *ATP7B* in de ziekteontwikkeling heeft belangrijke implicaties voor het begrijpen van de biologische achtergrond van de ziekte en is van waarde voor ontwikkeling van nieuwe therapieën voor zowel de hond als de mens.



About the author

Curriculum Vitae

Hille Fieten was born on September 14, 1980 in Visvliet (municipality Grijpskerk). She started her studies in veterinary medicine in 1999 at the Faculty of Veterinary Medicine, Utrecht University. During her studies she fulfilled the Honours Program of the College of Veterinary Medicine (Excellent Tracé) into the expression of Hepatocyte Growth Factor and its receptor c-Met in canine osteosarcoma, and obtained her Master in Veterinary Research at the Department of Clinical Sciences of Companion Animals in 2004. In 2005 she attended the Veterinary Leadership Program at Cornell University, Ithaca NY, USA where she performed a 3 month research project on 'Expression of the Rb family genes in the developing mouse pituitary gland'. Her participation in the program was rewarded with the 'Innovative biology prize'. After obtaining her DVM degree (with honours) in 2006 she started a 1-year rotating internship at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University. After successful completion she started her PhD studies into the clinical and genetic aspects of copper-associated hepatitis in the Labrador retriever under supervision of Prof.Dr. Jan Rothuizen and Dr. Peter Leegwater. As part of the PhD project she obtained a Master of Science degree in Genetic Epidemiology in August 2011 from the Netherlands Institutes for Health Sciences (NIHES). The results of these studies are described in this thesis which will be defended on June 11, 2015. She participated in the European LUPA consortium in 2009, aiming at the unravelling of complex genetic diseases in both dogs and humans. Furthermore, since 2014 she is the secretary of the Society of Comparative Hepatology. Since 2014 she is a member of the Genetics Expertise Center where she is involved in methodology development for decreasing the incidence of heritable diseases in companion animal populations. In June 2013 she started the Residency Program in Veterinary Internal Medicine, at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University.

PhD portfolio

PhD training

Master in Health Sciences, specialization: Genetic Epidemiology	Year	Workload (ECTS)/ grade
Research skills		
Principles of Research in Medicine and Epidemiology	2008	0.7
Genetics and Genomics	2008	0.7
Study Design	2009	4.3 / 8.0
Classical Methods for Data-Analysis	2009	5.7 / 8.8
Modern Statistical Methods	2010	4.3 / 8.7
Case-control Studies	2010	0.7
Survival Analysis	2010	1.9 / 9.3
Research Period (proposal, presentation, report)	2011	33.1/ excellent
In-depth courses		
Genetic Linkage Analysis: Model-free analysis	2008	1.4
Advances in Population-based Studies of Complex Genetic Disorders	2008	1.4
Genome Wide Association Analysis	2008	1.4
Large-scale Multicenter Studies	2008	0.4
Advances in Genomics Research	2008	0.4
Genomics in Molecular Medicine	2008	1.4
Principles of Genetic Epidemiology	2008	0.7
Genetic-Epidemiologic Research Methods	2008	5.7 / 7.7
Planning and Evaluation of Screening	2009	1.4
Mendelian Randomization & Bayesian Modelling in Genetic Epidemiology	2010	1.1
SNP's and Human Diseases	2010	1.4
Other attended courses and training programmes		
Genomics and DNA technology	2007	
Management for PhD Students	2008	
Micro array data analysis	2010	
Next generation sequencing data analysis	2011	
Female leadership	2012	

Congress attendance and presentations

Invited presentations

"Copper-associated chronic hepatitis in the Labrador retriever" 20th ECVIM congress, Toulouse, France	2010
"GenABEL: R-package for GWAS of binary and quantitative traits" LUPA genetics meeting, Barcelona, Spain	2010
"Role of nutrition in copper-associated chronic hepatitis in the Labrador retriever" Royal Canin Veterinary Managers meeting, Lisbon, Portugal	2011
"Copper-associated liver disease in the dog" 58th Annual Conference of GSAVA, Dusseldorf, Germany	2012
"Portosystemic vascular anomalies in dogs: genetics, diagnosis and therapeutic options" 58th Annual Conference of GSAVA, Dusseldorf, Germany	2012
"New canine models of copper toxicity" Workshop: Human disorders of copper metabolism: recent advantages and main challenges, Baltimore, USA	2013
"Chronic hepatitis in the dog" 59th Annual Conference of GSAVA, Berlin, Germany	2013
"Copper-associated hepatitis in dogs and the role of diet" Congress of the European Society of Veterinary Clinical Nutrition, Utrecht, the Netherlands	2014
"What's new: hereditary liver diseases in the dog" Voorjaarsdagen congress, Amsterdam, the Netherlands	2014
"Diagnosis and long-term management of copper-associated hepatitis in the dog" Voorjaarsdagen congress, Amsterdam, the Netherlands	2014

Oral presentations

"Koperstapeling bij de Labrador retriever" In Praktijk Wintercongres, Utrecht, the Netherlands	2009
"Copper-associated chronic hepatitis in Labradors: a worldwide disease" 18th ECVIM congress, Ghent, Belgium	2009
"Optimizing diagnosis and treatment of copper-associated hepatitis in the Labrador" 21st ECVIM congress, Sevilla, Spain	2011
"Long-term management of copper-associated hepatitis in the Labrador retriever" 23rd ECVIM congress, Liverpool, UK	2013

Poster presentations

"Gene expression profiles in canine hepatic cell lines during copper overload' Advances in canine and feline genomics and inherited diseases, St Malo, France	2008
"New canine model for copper toxicosis: A genome wide association study in the Labrador retriever" 8th International copper meeting: Copper in Biology, Alghero, Italy **1st prize: best poster**	2012
"Hepatic copper concentration and blood parameters in subclinical Labrador retrievers and association with diet." 22nd ECVIM congress, Maastricht, the Netherlands **1st prize: best poster**	2012
"Dietary management of hereditary copper-associated hepatitis in the Labrador retriever." The Waltham International Nutritional Sciences Symposium, Portland, USA	2013

Other attended conferences

KNAW conference: The role of DNA polymorphisms in complex traits and diseases, Amsterdam, the Netherlands	2008
Genetics retraite, Rolduc, the Netherlands	2009
Lupa genetics meeting, Uppsala, Sweden	2009
Advances in canine and feline genomics and inherited diseases, Baltimore, USA	2010
7th International copper meeting: Copper in biology, Alghero, Italy	2010
Congress of the European Society of Human Genetics, Amsterdam, the Netherlands	2011
Voorjaarsdagen congress, Amsterdam, the Netherlands	2011

International exchange

Short term visit to the Biomedical center (genetics lab of professor Hannes Lohi) and Faculty of Veterinary medicine, Helsinki, Finland	2011

List of publications

Publications in this thesis

Fieten H, Leegwater PAJ, Watson AL, Rothuizen J. Canine models of copper toxicosis for understanding mammalian copper metabolism. Mamm Genome. 2012 Feb;23(1-2):62-75

Fieten H, Hooijer-Nouwens BD, Biourge VC, Leegwater PAJ, Watson AL, van den Ingh TSGAM, Rothuizen J. Association of dietary copper and zinc levels with hepatic copper and zinc concentration in Labrador Retrievers. J Vet Intern Med. 2012 Nov-Dec;26(6):1274-80

Fieten H, Dirksen K, van den Ingh TSGAM, Winter EA, Watson AL, Leegwater PAJ, Rothuizen J. D-penicillamine treatment of copper-associated hepatitis in Labrador retrievers. Vet J. 2013 Jun;196(3):522-7

Fieten H, Hugen S, van den Ingh TSGAM, Hendriks WH, Vernooij JC, Bode P, Watson AL, Leegwater PAJ, Rothuizen J. Urinary excretion of copper, zinc and iron with and without D-penicillamine administration in relation to hepatic copper concentration in dogs. Vet J. 2013 Aug;197(2):468-73

Fieten H, Biourge VC, Watson AL, Leegwater PAJ, van den Ingh TSGAM, Rothuizen J. Nutritional management of inherited copper-associated hepatitis in the Labrador retriever
Vet J. 2014 Mar;199(3):429-33

Fieten H, Penning LC, Leegwater PAJ, Rothuizen J. New canine models of copper toxicosis: diagnosis, treatment, and genetics. Ann N Y Acad Sci. 2014 May;1314:42-8

Fieten H, Biourge VC, Watson AL, Leegwater PAJ, van den Ingh, TSGAM, Rothuizen J. Dietary management of Labrador retrievers with subclinical hepatic copper accumulation. J Vet Intern Med. 2015 Mar 16

Publications not in this thesis

Fieten H, Spee B, Ijzer J, Kik MJ, Penning LC, Kirpensteijn J. Expression of hepatocyte growth factor and the proto-oncogenic receptor c-Met in canine osteosarcoma. Vet Pathol. 2009 Sep;46(5):869-77

Selvarajah GT, Kirpensteijn J, van Wolferen ME, Rao NA, **Fieten H**, Mol JA. Gene expression profiling of canine osteosarcoma reveals genes associated with short and long survival times. Mol Cancer. 2009 Sep 7;8:72

Identification of genomic regions associated with phenotypic variation between dog breeds using selection mapping. Vaysse A, Ratnakumar A, Derrien T, Axelsson E, Rosengren Pielberg G, Sigurdsson S, Fall T, Seppälä EH, Hansen MS, Lawley CT, Karlsson EK; **LUPA Consortium**, Bannasch D, Vilà C, Lohi H, Galibert F, Fredholm M, Häggström J, Hedhammar A, André C, Lindblad-Toh K, Hitte C, Webster MT. PLoS Genet. 2011 Oct;7(10)

Favier RP, Spee B, Schotanus BA, van den Ingh TSGAM, **Fieten H**, Brinkhof B, Viebahn CS, Penning LC, Rothuizen J. COMMD1-deficient dogs accumulate copper in hepatocytes and provide a good model for chronic hepatitis and fibrosis. PLoS One. 2012;7(8)

van Straten G, van Steenbeek FG, Grinwis GC, Favier RP, Kummeling A, van Gils IH, **Fieten H**, Groot Koerkamp MJ, Holstege FC, Rothuizen J, Spee B. Aberrant expression and distribution of enzymes of the urea cycle and other ammonia metabolizing pathways in dogs with congenital portosystemic shunts. PLoS One. 2014 Jun 19;9(6)

van der Laan LJ, de Ruiter PE, van Gils IM, **Fieten H**, Spee B, Pan Q, Rothuizen J, Penning LC. Canine hepacivirus and idiopathic hepatitis in dogs from a Dutch cohort. J Viral Hepat. 2014 Dec; 21(12):894-896

Fieten H, Rothuizen J. Web Chapter 48: Copper-associated hepatitis.
In Kirk's Current Veterinary Therapy XV, 2014: e231-e236

Favier RP, Spee B, **Fieten H**, van den Ingh TSGAM, Schotanus BA, Brinkhof B, Rothuizen J, Penning LC. Aberrant expression of copper-associated genes after copper accumulation in COMMD1-deficient dogs. J Trace Elem Med Biol. 2015 Jan;29:347-353

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Promoveren doe je niet alleen…

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