Canine models of copper toxicosis for understanding mammalian copper metabolism

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Abstract Hereditary forms of copper toxicosis exist in man and dogs. In man, Wilson’s 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 the work that has been done on 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 (de Romana et al. 2011). The importance of proper functioning of its homeostatic regulation is illustrated by the genetic disorders Menkes disease (OMIM #309400) and Wilson’s 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 (Mason 1979), where CTR1 (Zhou and Gitschier 1997) and possibly CTR2 (van den Berghe et al. 2007) and DMT1 (Gunshin et al. 1997) 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 (Pase et al. 2004). In the blood, copper is bound to small molecules such as histidine and to serum proteins like α2-macroglobulin and albumin (Moriya et al. 2008) for transport to the liver, the primary site of copper storage (Liu et al. 2007; McArdle et al. 1990; Weiss and Linder 1985).

Copper enters the hepatocytes via CTR1 (Kim et al. 2009) and is sequestered by small molecules like metallothionein (Coyle et al. 2002) and glutathione (Freedman et al. 1989) in the cytosol. Specialized copper chaperones shuttle copper to their destination molecules. CCS shuttles copper to SOD1, which participates in oxidative stress defense (Culotta et al. 1997). 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 (Amaravadi et al. 1997).

The copper chaperone ATOX1 (Klomp et al. 1997) delivers copper to ATP7B that is located in the trans-Golgi compartment (Hamza et al. 1999; Larin et al. 1999; van Dongen et al. 2004). Here, copper is necessary for the formation of holo-ceruloplasmin, which is subsequently secreted into the blood (Yanagimoto et al. 2011). In addition, ATP7B facilitates the excretion of excess copper into the bile (Cater et al. 2006) (Fig. 1).

Mutations in ATP7B can result in Wilson’s disease (WD), an autosomal recessive disorder (Bull et al. 1993; Tanzi et al. 1993) characterized by copper accumulation in the liver, brain, and cornea (Gitlin 2003). Clinical signs manifest in the form of hepatic, neurologic, or psychiatric impairment and often become evident in people in the second or third decades of life (Merle et al. 2007) (Table 1). The disease incidence is estimated to be 1 in 30,000 (Schilsky 1996). A wide variety of mutations in ATP7B have been described (http://www.wilsondisease.med.ualberta.ca/database.asp) and most patients are compound heterozygotes (Kenney and Cox 2007). 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 (Riordan and Williams 2001; Senzolo et al. 2007). Other genes or environmental influences are thought to modify clinical expression of the disease but have not yet been identified (de Bie et al. 2007a).

Non-Wilsonian forms of hepatic copper toxicosis in man that often occur early in childhood include Indian

Fig. 1 Model of hepatocyte copper metabolism. Copper (diamonds) 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. 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.
Table 1: Comparison of phenotypes between different forms of copper toxicosis in man and dogs

| Gene   | WD | ICC | ETIC | ICT | Bedlington terrier | Dalmatian | Doberman | WHW terrier | Labrador |
|--------|----|-----|------|-----|---------------------|-----------|----------|-------------|----------|
| Mode of inheritance | Autosomal recessive | Autosomal recessive | Autosomal recessive | Autosomal recessive | Autosomal recessive | Autosomal recessive | Autosomal recessive | Autosomal recessive | Autosomal recessive |
| Sex predisposition | Male | Male | Male | Male | Male | Male | Male | Male | Male |
| Age of onset | Adolescence | Adolescence | Adolescence | Adolescence | Adolescence | Adolescence | Adolescence | Adolescence | Adolescence |
| Liver pathology | Cirrhosis | Cirrhosis | Cirrhosis | Cirrhosis | Cirrhosis | Cirrhosis | Cirrhosis | Cirrhosis | Cirrhosis |
| Dietary influence | Minor | Minor | Minor | Minor | Minor | Minor | Minor | Minor | Minor |

Normal values in liver copper for humans are <50 μg/g dwl and for dogs <400 μg/g dwl.

childhood cirrhosis (ICC) (Tanner 1998), endemic Tyrolean infantile cirrhosis (ETIC) (Muller et al. 1996), and idiopathic copper toxicosis (ICT) (Scheinberg and Sternlieb 1996) (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 toward 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 (Hardy et al. 1975), Skye terrier (Haywood et al. 1988), West Highland White terrier (Thorburn et al. 1986), Dalmatian (Webb et al. 2002), Doberman (Mandigers et al. 2004), and Labrador retriever (Hoffmann et al. 2006). 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 studied copper storage disorders in dogs is Bedlington terrier copper toxicosis (BTCT). The identification of the causal mutation in COMMD1 in this breed (van De Sluis et al. 2002) 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 because of the unique population structure (Tsai et al. 2007). 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 make copper toxicosis a promising phenotype for genetic studies in the dog.

Here we review the unraveling of the genetics of Bedlington terrier copper toxicosis and how it contributed to the gain in knowledge of the functional aspects of COMMD1. Furthermore, we 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 detection 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 liver copper values in the Bedlington terrier was first described in the United States (Hardy et al. 1975). Subsequently, Bedlington terrier copper toxicosis (BTCT) was recognized in Australia (Studdert 1982) and Europe (Eriksson 1983; Kelly et al. 1984; Meulenaar et al. 1983; Sewelius 1986; Wilsdorf et al. 1985). The prevalence was very high, ranging from 25 to 46% in different Bedlington terrier populations (Herrtage et al. 1987; Ubbink et al. 2000).

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 (Johnson et al. 1980; Owen and Ludwig 1982). Selective breeding by excluding affected dogs that 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% (Ubbink et al. 2000). However, because carrier dogs could not be identified by a liver biopsy, there was a need for a DNA test for eradication of the disease.

A search for the causal gene was initiated by several research groups, resulting in the exclusion of genes coding for the copper transporter ATP7B (Dagenais et al. 1999; van de Sluis et al. 1999), metal transporter ATP6H (Nanji et al. 2001), copper transporters CTR1 and CTR2 (van de Sluis et al. 1999) and the copper chaperone ATOX1 (Dagenais et al. 1999; Nanji and Cox 1999) 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 (Yuzbasiyan-Gurkan et al. 1997). Two alleles were present in the Bedlington terrier population, and allele 2 cosegregated 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 (Holmes et al. 1998; Lee et al. 2007; Rothuizen et al. 1999; Yuzbasiyan-Gurkan et al. 1997). Whereas implementation of the microsatellite marker test in the breeding programs was an important step forward in decreasing the disease incidence within the Bedlington terrier populations, the search for the causal gene continued. A positional cloning strategy identified a large genomic deletion of 39.7 kb encompassing exon 2 of the originally named MURR1 gene (Forman et al. 2005; van De Sluis et al. 2002). This MURR1 gene was previously not known to be involved in copper metabolism, and copper-binding motifs in the predicted protein product were not recognized in this stage. Upon discovery of nine other proteins related to the MURR1 protein, the gene was renamed into Copper Metabolism gene MURR1 containing Domain 1 (COMMD1) (Burstein et al. 2005). All ten proteins are characteristic for the COMMD domain, which seems to be necessary for the interaction among the COMMD proteins as well as for the interaction with other proteins (Burstein et al. 2005; Chang et al. 2011; de Bie et al. 2007b; Drevillon et al. 2011; Maine et al. 2007; Narindrasorasak et al. 2007; Thoms et al. 2010; van de Sluis et al. 2007a, 2009).

Neither full-length nor truncated COMMD1 protein was detectable in liver homogenates of affected Bedlington terriers, suggesting that COMMD1 exon 2 deletion results in a complete loss of function of COMMD1 (Klomp et al. 2003). In retrospect, the C04107 microsatellite marker was positioned within the COMMD1 gene in intron 1, 13.5 kb upstream of the exon 2 deletion (Forman et al. 2005).

Although in the majority of cases C04107 allele 2 was linked to copper toxicosis, linkage of allele 1 to the disease phenotype was reported as well (Haywood et al. 2001; Holmes et al. 1998; Yuzbasiyan-Gurkan et al. 1997). In an American Bedlington terrier pedigree, it was confirmed that C04107 allele 1 was linked to the exon 2 deletion, implying that direct analysis for the exon 2 deletion would be the only reliable genetic test for copper toxicosis in the Bedlington (Favier et al. 2005; Lee et al. 2007; van de Sluis et al. 2003). The presence of the new haplotype in the American Bedlington terriers raised the question whether this haplotype had a different genetic origin, or occurred due to a recombination event between the microsatellite marker and the exon 2 deletion (van de Sluis et al. 2003).

Remarkably, affected Bedlington terriers that were heterozygous for the exon 2 deletion or had two copies of the normal exon 2 were identified in Finnish and Australian populations. In these populations, a transition near the 5′ splice site mutation of COMMD1 exon 2 was found. However, no effect of this C-to-A transition on splicing was noted by analysis of cDNA, and no association between this mutation and BTCT could be established (Coronado et al. 2003; Hyun et al. 2004). In these dogs, another mutation may be responsible for the observed disease phenotype.

In the search for modifier genes of BTCT, the gene coding for the copper transporter ATP7B was investigated by DNA sequencing in a pedigree that did not show complete cosegregation between the COMMD1 deletion and BTCT. Eleven polymorphisms were identified, two of which affected the encoded protein. One missense mutation in exon 21 resulted in an amino acid change from arginine to glutamine at a highly conserved position. However, all investigated Bedlington terriers were homozygous for this
mutation and therefore no correlation with BTCT in this pedigree could be established (Coronado et al. 2008).

In conclusion, careful evaluation of the copper toxicosis phenotype in the Bedlington terrier and genetic mapping studies led to the discovery of the COMMD1 gene, which was previously unknown to be involved in copper metabolism. This illustrates the relevance of studying spontaneous disease phenotypes to unravel important gene functions. The homozygous state for the exon 2 deletion in COMMD1 causes copper toxicosis in Bedlington terriers. Identification of the causal mutation led to an enormous decrease in the disease frequency in Bedlington terrier populations and was very beneficial for the breed. However, the presence of one or two normal copies of COMMD1 exon 2 does not exclude copper toxicosis in subpopulations of Bedlington terriers. In these dogs copper toxicosis may be explained by unidentified mutations in regulatory elements of COMMD1 or in an unidentified gene.

**Molecular function of COMMD1**

COMMD1 specifically binds Cu(II), for which the binding site is located in the exon 2 product that is deleted in affected Bedlington terriers (Narindrasorasak et al. 2007). Direct biochemical evidence for involvement of COMMD1 in cellular copper metabolism was provided by the observation of copper accumulation after RNAi knockdown of COMMD1 in canine, human, and murine cell lines (Burstein et al. 2004; Miyayama et al. 2010; Spee et al. 2007). The deficient copper excretion into the bile in the Bedlington terrier (Su et al. 1982a) suggests a function for COMMD1 in copper excretion. Affected Bedlington terriers show massive copper accumulation in the hepatocytic lysosomes, which, in combination with the observation that COMMD1 localizes to a vesicular compartment, led to the hypothesis that COMMD1 facilitates degranulation of the lysosomal content into the bile (de Bie et al. 2005; Klomp et al. 2003) (Fig. 1).

Interestingly, COMMD1 was found to interact with the amino terminus of ATP7B (Lutsenko and Petris 2003; Voskoboinik et al. 2002), suggesting that COMMD1 may cooperate with ATP7B by facilitating copper transport from the trans-Golgi network (TGN) to the canalicular membrane of the hepatocytes for biliary excretion. As ceruloplasmin levels in affected Bedlington terriers are normal (Su et al. 1982b), copper transport to the Golgi compartment seems to be unaltered. Copper-induced translocation of ATP7B from the TGN to dispersed vesicles was not impaired by depletion of COMMD1, which indicates indeed that COMMD1 plays a role later in the process of copper excretion (Weiss et al. 2008). Facilitation of recruitment of ATP7B from the vesicles back to the TGN in low-copper conditions may be another role of COMMD1 in the regulation of efficient copper efflux by ATP7B (Miyayama et al. 2010).

In addition, COMMD1 was found to stabilize the ATP7B protein and may be involved in its quality control by promoting degradation of newly synthesized and incorrectly folded ATP7B proteins (de Bie et al. 2007b). Intriguingly, this interaction increased when ATP7B was mutated, indicating that COMMD1 may contribute to the molecular basis of WD.

Apart from its role in ATP7B functioning, COMMD1 has several other roles in copper homeostasis. It also binds ATP7A, the homolog of ATP7B which is defective in copper deficiency disorders in man (Kaler 2011). Interestingly, binding of COMMD1 to mutant ATP7A partially restored protein expression, subcellular localization, and copper-exporting activities (Vonk et al. 2011).

Recently, it was discovered that COMMD1 can bind to SOD1 and plays a role in the maturation and activation of this protein (Fig. 1). RNAi-mediated knockdown of COMMD1 expression resulted in a significant induction of SOD1 activity and a consequent decrease in superoxide anion concentrations, whereas overexpression of COMMD1 exerts the opposite effect (Vonk et al. 2010).

As is shown above, COMMD1 has many functions in the regulation of copper metabolism, but the regulation of COMMD1 itself is not yet completely understood.

Intracellular trafficking of many copper-binding proteins is regulated by intracellular copper levels (van den Berghe and Klomp 2010); however, this does not seem to be the case for COMMD1, as subcellular localization of COMMD1 is not influenced by intracellular copper levels (Klomp et al. 2003).

Besides copper-dependent transcriptional regulation (Muller et al. 2007), different forms of ubiquitination were found to be important for COMMD1 regulation. XIAP (Burstein et al. 2004; Maine et al. 2009), HSCARG (Lian and Zheng 2009), Clusterin (Zoubeidi et al. 2010), and ARF (Huang et al. 2008) were identified as COMMD1 interacting proteins regulating several components of this process.

A physiological role for the interaction between COMMD1 and XIAP was supported by the fact that increased levels of XIAP expression induced copper accumulation in several cell models, and XIAP deficiency in mice led to decreased hepatic copper concentration (Burstein et al. 2004). Interestingly, copper itself specifically binds to the cysteine residues of the XIAP protein and is delivered to XIAP via CCS (Brady et al. 2010) (Fig. 1). The binding of copper results in a conformational change of XIAP that induces an increased intracellular degradation and impairs the ability to inhibit caspases, thus lowering the apoptotic threshold (Mufti et al. 2006). This phenomenon
sheds new light on the pathogenesis of copper-associated hepatitis, which starts with copper accumulation followed by hepatocellular apoptosis. Oxidative stress induced by free copper may not be the only trigger, as has been the general belief; this may have implications for therapeutic interventions.

Ubiquitous expression of COMMD1 in a number of different cell types indicates a more pleiotropic function of COMMD1 than copper metabolism alone (Klomp et al. 2003). Indeed, COMMD1 was found to be involved in many different cellular processes, including sodium metabolism (Biasio et al. 2004; Chang et al. 2011; Ke et al. 2010), regulation of NfκB (Burstein et al. 2005; Maine and Burstein 2007), and HIF1α-mediated transcription (van de Sluis et al. 2007b, 2009, 2010).

Upon identification of COMMD1 in the Bedlington terrier, many new functions of COMMD1 were discovered and more knowledge has been gained about the function and regulation of this interesting protein. Although the entire function of COMMD1 in copper homeostasis is not completely resolved yet, recent data indicate that it at least plays a role in the functioning and stability of ATP7B. This may indicate that human Wilson’s disease and canine COMMD1-deficient copper toxicosis partly share their disease mechanism through disturbance of ATP7B-mediated copper export from hepatocytes.

COMMD1 in human copper toxicosis

The non-Wilsonian forms of copper toxicosis—ICC, ETIC, and ICT—resemble the hepatic form of Wilson’s disease, but in contrast there is no neurological involvement and the age of onset is often early in childhood (Table 1). In these diseases, consanguinity and high dietary copper intake are suggested to play a role in the pathogenesis (Muller et al. 1996; Scheinberg and Sternlieb 1996; Tanner 1998). Since a direct role of ATP7B mutations had been excluded and the phenotype of humans with ICC, ETIC, or ICT resembles that of BTCT, COMMD1 was tested as a candidate gene. In two small studies of 23 and 3 cases, respectively, no correlation between mutations and phenotype could be established (Coronado et al. 2005; Muller et al. 2003).

In Wilson’s disease there is a wide variety of mutations in ATP7B. The clinical presentation in its hepatic or neurological form is highly variable, even among patients with the same mutation, which led several research groups to propose that this variation may be subject to other modulating genes (Riordan and Williams 2001; Schaefer et al. 1999; Thomas et al. 1995). As COMMD1 is known to interact with ATP7B and both proteins work in conjunction copper excretion, COMMD1 is an interesting candidate modifier in patients with Wilson’s disease with an atypical presentation or in whom no or only one mutation was detected. Several research groups screened their WD patient cohorts for mutations in COMMD1. Heterozygosity of a silent missense mutation c.492 GAT > GAC (Asp164Asp) in COMMD1 was reported to be possibly associated with an earlier onset of neurological manifestation of Wilson’s disease (Stuehler et al. 2004); however, whereas this mutation was observed in other cohorts as well, an association with the phenotype could not be confirmed (Gupta et al. 2010; Lovicu et al. 2006; Weiss et al. 2006). Several other mutations in COMMD1 were detected, but none of them was significantly correlated with variations of the disease phenotype (Coronado et al. 2005; Hayashi et al. 2007; Lovicu et al. 2006; Weiss et al. 2006; Wu et al. 2006).

Recently, a new mutation in COMMD1 was described in a patient with Wilson’s disease. This nonsynonymous change, c.521 ACG → ATG; Thr174Met, resided in the recently identified NES (Nuclear Export Signal) region. The patient carrying this mutation was a compound heterozygote for WD mutations and exhibited extremely high urinary copper levels. In this case, the COMMD1 mutation may have contributed to exaggeration of the disease phenotype (Gupta et al. 2010).

Studies aiming to find COMMD1 mutations to explain the variability in WD are, in general, difficult to perform for two reasons. First, the disease is rare and therefore recruitment of a large enough cohort is a challenge. Secondly, WD cohorts are heterogeneous with respect to mutations in the ATP7B gene and the clinical presentation. This makes it difficult to establish a relationship between variations in COMMD1 and clinical manifestation of WD.

In conclusion, although there are indications that COMMD1 may be a modifying factor in human disorders of copper metabolism, it does not seem to have a major role. Thus far, unknown genes active in copper homeostasis may be responsible for the observed disease phenotypes.

Copper storage diseases in dogs

In addition to copper toxicosis in the Bedlington terrier, hereditary copper-associated liver disease has also been described in other dog breeds such as the Dobermann (Mandigers et al. 2004), the West Highland White terrier (Thornburg et al. 1986), and the Dalmatian (Webb et al. 2002). More incidental reports of copper-storage-related hepatitis are available for the Anatolian shepherd (Bosje et al. 2003) and the Skye terrier (Haywood et al. 1988). Recently, the Labrador retriever, which forms one of the largest purebred dog populations worldwide, was documented to have an inherited form of copper-associated hepatitis (Hoffmann et al. 2006). In addition, results from a large survey of liver copper concentrations in dogs (Thornburg et al. 1990) and results from a retrospective
review on dogs diagnosed with primary hepatitis (Polder-vaart et al. 2009) suggest that there may be more dog breeds in which high liver copper levels and copper-associated hepatitis are present.

Histologically, copper toxicosis in different dog breeds shows many similarities. Accumulation of copper precedes inflammatory changes in the liver and always starts in the centrilobular regions of the liver lobules (zone 3). Around the central vein branches, multifocal regions with increased copper develop, first in the hepatocytes which then become apoptotic and are phagocytized, after which part of the copper is concentrated in the Kupffer cells. The disease is characterized by progressive inflammation, necrosis, and bridging fibrosis between centrilobular areas, eventually leading to irreversible liver cirrhosis (Fig. 2). Cholestasis can be present in very advanced stages of the disease but is never the main histological finding. This is also underscored by blood tests which show that in copper toxicosis the liver enzyme alanine-aminotransferase is often much more increased than alkaline phosphatase, indicating hepatocellular rather than cholestatic liver disease. Clinical signs can result from acute severe liver failure or end-stage cirrhosis and include lethargy, anorexia, vomiting, icterus, ascites, and hepatoencephalopathy. In some breeds, acute hemolytic crisis due to a massive release of copper into the circulation is recognized. As in humans, treatment with the copper chelators D-penicillamine and 2,3,2-tetramine is effective in decreasing liver copper levels in dogs (Allen et al. 1987; Hoffmann et al. 2009; Mandigers et al. 2005; Twedt et al. 1988). Administration of zinc acetate or zinc gluconate is described to have beneficial effects in decoppering and in maintenance therapy (Brewer et al. 1992; Hoffmann 2009; Hoffmann et al. 2009; Hoogenraad and Rothuizen 1986).

Although there are many similarities in copper toxicosis phenotypes between breeds, differences exist in clinical

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**Fig. 2** Histological appearance of copper-associated hepatitis in different dog breeds. Slides are stained with rubeanic acid and hematoxylin countersaint. 

- **A** Liver biopsy of a female Bedlington terrier clearly showing centrilobular distribution of copper. 
- **B** Liver biopsy of a female Bedlington terrier, 3 years of age with a liver copper value of 11,500 μg/g dwl copper. Massive amounts 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 female Labrador, 5 years of age with a liver copper concentration of 2,360 μg/g dwl. Copper granules are present in hepatocytes and macrophages in the centrilobular area. The centrilobular region is characterized by loss of hepatocytes, mild fibrosis, and moderate numbers of lymphocytes and plasma cells. 
- **D** Liver biopsy of a female Dobermann, 6 years of age with a liver copper value of 1,700 μg/g dwl. The centrilobular area (bottom right of the picture) is characterized by mild fibrosis with multifocal accumulation of macrophages containing lipofuscin pigment and copper granules. Furthermore, this area shows moderate infiltration with lymphocytes. Hepatocytes in the centrilobular region contain moderate amounts of copper granules.
presentation and liver copper levels, as outlined in the following subsections and summarized in Table 1.

**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 (van De Sluis et al. 2002). 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 levels 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 (Su et al. 1982b; Twedt et al. 1979). 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 level tends to increase over time and can reach values of 5,000 µg/g dwl. In some cases extremely high liver copper levels of 15,000 µg/g dwl have been reported. A tendency toward a decrease in liver copper levels is present in old animals or in advanced stages of liver cirrhosis (Twedt et al. 1979).

**West Highland White terrier**

Hepatitis associated with hepatic copper accumulation was first reported in this breed in the Unites States (Thornburg et al. 1986). Later, the same authors reported on a larger group of 71 dogs, of which many were related (Thornburg et al. 1996). The disease had a clear familial distribution, and when two affected dogs were mated, all dogs in the offspring showed increased liver copper values, 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 levels do not reach the extremely high values seen in the Bedlington terrier (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 seen almost exclusively in females and often has a fatal course within weeks or a few months after diagnosis. Reports from the U.S. (Thornburg 1998), Finland (Speeti et al. 1998), and the Netherlands (Mandigers et al. 2004, 2007; Spee et al. 2005; van den Ingh et al. 1988) 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 3-year-old Dobermanns was followed over time. In 6% of these dogs, copper-associated subclinical hepatitis was present and liver copper levels increased to 1,000 µg/g dwl over time. The etiologic role of copper was demonstrated by the dramatic improvement upon treatment with D-penicillamine and the associated normalization of copper concentrations (Mandigers et al. 2005). Mandigers et al. (2007) also demonstrated that the biliary excretion of intravenously injected 64Cu tends to be decreased in affected Dobermanns.

MHC class II antigen expression was detected in hepatocytes in cases of Dobermann hepatitis, but not in control tissue (Speeti et al. 2003). Therefore, Dobermann hepatitis was suggested to be an autoimmune disease. Induction of MHC class II antigen expression in nonlymphatic cells can also be induced by toxins, like copper. Homozygosity for DLA-DRB1*00601 of the dog leukocyte antigen (DLA) system genotype was found to be associated with an increased risk for Dobermann hepatitis in Finnish Dobermanns (Dyggve et al. 2011). Dobermann hepatitis behaves quite differently compared with other copper storage diseases with respect to the very severe prognosis when left untreated, the strong female predisposition, and the relatively mild increase in hepatic copper levels associated with severe disease. Female predisposition is a common feature for autoimmune diseases both in humans and in dogs. Possibly, there is a combined role for copper accumulation and autoimmune deregulation in the pathogenesis of Dobermann hepatitis.

Currently, genome-wide association studies followed by next-generation sequencing of associated regions and RNA sequencing efforts are being performed in the combined Dutch and Finnish cohorts of Dobermann hepatitis liver samples within the LUPA initiative (Lequarré et al. 2011).

**Dalmatian**

One report from the U.S. (Webb et al. 2002) has convincingly demonstrated that the Dalmatian has an inherited copper storage disease causing hepatitis and liver cirrhosis. Early case reports (Cooper et al. 1997; Noaker et al. 1999) already had indicated the presence of a copper storage disease in the American population. The mean hepatic copper concentrations that were reported ranged from 650 to 9,424 µg/g dwl. In the liver biopsies, necroinflammatory alterations were present in regions with copper-laden hepatocytes and Kupffer cells. Several cases in the Netherlands and Germany have been diagnosed (J. Rothuizen, personal communication), so that this disease is also present in European Dalmatians. However, currently reliable incidence estimates are lacking. There seems to be no sex predisposition in this breed.
Labrador retriever

An increased incidence of chronic hepatitis was reported in the Labrador retriever previously (Andersson and Sevelius 1991). However, Hoffmann et al. (2006) were the first to demonstrate an association of increased liver copper levels and hepatitis in this breed in Dutch Labrador retrievers. Soon thereafter, copper-associated hepatitis in the American Labrador retriever population was recognized as well (Shih et al. 2007; Smedley et al. 2009). There is a strong female predisposition and breeding bitches in the postpartum period have an increased risk for clinical illness. Hormones or an increased stress on the liver during pregnancy and lactation may influence deterioration of the liver function; however, no evidence for this hypothesis currently exists. Copper-accumulating traits in the Labrador retriever show a heritability of up to 85% (Hoffmann et al. 2008). Involvement of environmental factors in the disease pathogenesis was proven by the fact that dietary management with a low-copper diet was effective in preventing progression of the disease (Hoffmann et al. 2009). Unpublished results demonstrated that the disease is polygenic and the Labrador form of copper storage disease might become a good example of the power of canine populations to resolve complex genetic diseases (J. Rothuizen, personal communication).

Opportunities and pitfalls in genetic studies into canine copper storage disorders

Discovery of the COMMD1 gene in the Bedlington terrier was an enormous step forward in the diagnosis of affected and carrier dogs by use of a DNA test. The implementation of this test in the selection of breeding dogs led to dramatic decrease in the number of affected puppies that were born. In addition, the subsequent functional studies have shed a new light on the regulation of mammalian copper metabolism.

However, several questions remain unanswered. A minority of Bedlington terriers is affected with copper toxicosis but do not have the homozygous COMMD1 exon 2 deletion. In addition, the role of COMMD1 as a modifier gene in Wilson’s disease was not clearly established, and no causal mutations for non-Wilsonian forms of copper toxicosis have been detected thus far. These phenomena are a reflection of the complex regulation of copper metabolism and it is likely that other as yet unidentified genes may be at play.

In the preceding subsections we summarized the forms of copper accumulation that are well documented in different dog breeds. The phenotypes in the dogs have some resemblance with those of human copper storage disorders. For example, copper accumulation in the liver and response to D-penicillamine therapy are features that are both shared among all human and canine forms of copper toxicosis. The age of onset of the clinical signs in dogs is comparable with the general age of onset of Wilson’s disease, i.e., adolescence or middle age.

In non-Wilsonian forms of copper toxicosis, a strong influence of dietary copper intake on the expression of the disease phenotype is noticed; the same strong effect is seen in Labrador retrievers. On the other hand, a change to a low-copper diet did not halt disease progression in Bedlington terriers (R. Favier, personal communication).

In dogs, no neurological impairments have been noticed, although behavioral changes have been seen in Dobermanns, Labradors, and Bedlington terriers. More research is needed in order to conclude if copper accumulation in the brain may influence these behavioral changes.

In the search for new genes involved in copper metabolism, genome-wide association studies in dog breeds with a high prevalence of copper toxicosis could make a valuable contribution. Unlike the heterogeneity of most human populations, the structure of dog breed populations is homogeneous, which is advantageous for unraveling the molecular genetics of complex diseases (Karlsson and Lindblad-Toh 2008; Wilbe et al. 2010). For this reason, the dog was one of the first mammals whose genome was sequenced to a high-quality level (Lindblad-Toh et al. 2005). As a consequence of breeding practices and population bottlenecks, linkage disequilibrium (LD) in the dog genome extends over distances that are up to 100 times longer than in the human genome and the number of haplotype variants in a breed is small (Lindblad-Toh et al. 2005; Sutter et al. 2004). This means that relative to human studies, genotyping of a limited number of SNPs in small patient and control groups suffices for a genome-wide association study. For this purpose, a 170K SNP array has been developed by Illumina (San Diego, CA, USA) in collaboration with the LUPA consortium (Lequarre et al. 2011). Large LD blocks in dogs may be a drawback in pinpointing the location of the gene of interest; however, fine mapping across breeds is one way to overcome this problem (Karlsson et al. 2007).

In addition to genetic homogeneity, the copper toxicosis phenotype within breeds is also much more homogeneous compared to, for example, WD phenotypes, which make a correct diagnosis more feasible. No biomarkers for copper status exist in dogs; therefore, a liver biopsy is always needed to establish the diagnosis of copper toxicosis. This is beneficial for genetic studies because a precise copper quantification as well as a careful histological description of the liver biopsy is often available. In addition, the availability of liver tissue opens the opportunity for transcriptomics and proteomics studies in order to gain insight.
into disease pathogenesis and the effect of gene deregulations. Another important factor that can be controlled for in dog populations is dietary copper intake. Most privately kept dogs are fed a kibble diet in which copper concentrations are relatively stable and copper intake can be estimated more precisely than in humans.

There are some pitfalls when applying genome-wide association studies in canine copper toxicosis and they have to be taken into account in study design and data analysis. As stressed before, correct phenotyping is of utmost importance in the design of a genetic study. Phenocopies can occur as a result of liver copper accumulation due to reduced bile flow and this has to be distinguished from primary copper accumulation resulting from a genetic defect. In primary forms of copper accumulation, copper is localized around the central veins in the liver lobule, whereas copper accumulation due to cholestasis is present in hepatocytes in the periportal areas. In advanced stages of copper toxicosis, when liver cirrhosis is present, the architecture of the liver is disturbed and localization of copper within the liver lobe becomes a challenge. Also, in advanced, untreated cases, liver copper levels may actually decrease due to replacement of hepatocytes with fibrotic tissue and regenerative nodules that have not yet accumulated copper. In these cases, it may become difficult to distinguish between chronic hepatitis due to primary copper toxicosis and idiopathic chronic hepatitis. In conclusion, for correct phenotyping an experienced veterinary pathologist and a reliable method for quantitative copper determination are indispensable.

In the data analysis of a genome-wide association study, it is important to look for population substructuring and encrypted relatedness in the dog sample as this can cause false positive association signals. The use of mixed models in the data analyses, for example, as implemented in the software GenABEL (Aulchenko et al. 2007), can elegantly correct for underlying population or family structure. In addition, the use of this kind of model has the advantage that traits, e.g., liver copper level, can be analyzed quantitatively and that modifying factors such as age of onset, sex, and dietary copper intake can be implemented in the analysis.

There is a high level of conservation of copper metabolism genes over species; therefore, it is likely that genetic studies into canine copper toxicosis will contribute to an increased knowledge into mammalian copper metabolism and human copper storage diseases. It is clear that upon identification of new copper metabolism-associated genes in purebred dog populations, the translational step to human disease phenotypes needs to be made. Functional studies to test the implications of mutations are indispensable and cohorts of human patients will need to be tested for involvement of the new genes. Therefore, a good collaboration between canine and human research groups is of utmost importance.

Concluding remarks

The discovery of the COMMD1 gene through genetic studies in Bedlington terrier copper toxicosis has led to a great increase in knowledge about the regulation of mammalian copper metabolism. However, several questions with respect to the etiology of copper toxicosis in both man and dogs remain to be answered. The treasury of purebred dog populations for genetic studies is expected to reveal many new details of copper homeostasis in the coming years and will be beneficial to both man and dog.

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