

Review

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## Animal models of copper-associated liver disease

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### Abstract

Recent advances in molecular biology have made possible the identification of genetic defects responsible for Wilson's disease, Indian childhood cirrhosis and copper toxicosis in Long Evans Cinnamon rats, toxic milk mice, and Bedlington terriers. The Wilson's disease gene is localized on human chromosome 13 and codes for ATP7B, a copper transporting P-type ATPase. A genetic defect similar to that of Wilson's disease occurs in Long Evans Cinnamon rats and toxic milk mice. Familial copper storage disorders in Bedlington and West Highland white terriers are associated with early subclinical disease, and copper accumulation with subsequent liver injury culminating in cirrhosis. The canine copper toxicosis locus in Bedlington terriers has been mapped to canine chromosome region CFA 10q26. Recently, a mutated MURRI gene was discovered in Bedlington terriers affected with the disease. Idiopathic childhood cirrhosis is biochemically similar to copper toxicosis in Bedlington terriers, but clinically much more severe. Both conditions are characterized by the absence of neurologic damage and Kayser-Fleisher rings, and normal ceruloplasmin levels. A recent study added North Ronaldsay sheep to the list of promising animal models to study Indian childhood cirrhosis. Morphologic similarities between the two conditions include periportal to panlobular copper retention and liver changes varying from active hepatitis to panlobular pericellular fibrosis, and cirrhosis. Certain copper-associated disorders, such as chronic active hepatitis in Doberman pinschers and Skye terrier hepatitis are characterized by copper retention secondary to the underlying disease, thus resembling primary biliary cirrhosis in humans. Copper-associated liver disease has increasingly being recognized in Dalmatians. Copper-associated liver diseases in Dalmatians and Long Evans Cinnamon rats share many morphologic features. Fulminant hepatic failure in Dalmatians is characterized by high serum activities of alanine aminotransferase and aspartate aminotransferase, and severe necrosis of centrilobular areas (periportal, zone 3) hepatocytes. Macrophages and surviving hepatocytes contain copper-positive material. Liver disease associated with periportal copper accumulation has also been described in Siamese cats. Many questions regarding copper metabolism in mammals, genetic background, pathogenesis and treatment of copper-associated liver diseases remain to be answered. This review describes the similarities between the clinico-pathological features of spontaneous copper-associated diseases in humans and domestic animals.

## Introduction

Copper-associated diseases are increasingly being reported in both man and animals [1–6]. Wilson's disease is an autosomal recessive disorder that results from pathological accumulation of copper predominantly in the liver and brain [1]. Copper also has a role in fatal, non-Wilson's liver diseases affecting young children with a genetic abnormality of copper metabolism [3,7]. Excess accumulation of copper also occurs as a consequence of chronic liver diseases such as primary biliary cirrhosis, and chronic hepatitis in both humans [8] and animals [9]. A genetic defect similar to that of Wilson's disease has been discovered in the Long Evans Cinnamon (LEC) rat [10], and the toxic milk mouse [11]. Animal models play an important role in the study of copper homeostasis, mechanisms (pathogenesis) of copper-associated liver diseases, and in the implementation of new therapeutic approaches such as gene therapy [12].

## Copper homeostasis in mammals

The proximal small intestine is recognized as the main site of dietary copper absorption in mammals [13]. Transport from the intestinal lumen into intestinal mucosa is a carrier-mediated process involving a saturable transport component [14]. The overall intestinal copper uptake is influenced by amino acids, ascorbic acid, and other dietary factors [14]. Once in mucosal cells, approximately 80% of the newly absorbed copper is in the cytosol, mainly bound to metallothioneins (MT). These are low-molecular weight inducible proteins with many functions including homeostasis, storage, transport and detoxification of metals [15,16]. Metallothioneins bind to many metals, but in normal circumstances only Zn, Cu and Cd binding is significant [17]. After passage through the enterocytes, copper enters the portal circulation where it is bound to carrier proteins (primarily albumin), peptides and amino acids and is transported to the liver [18], with lesser amounts entering the kidney [17].

Copper transport in hepatocytes can be divided into three discernible but interrelated steps: copper uptake, intracellular copper distribution and utilization, and copper export. At the hepatocellular cellular level copper uptake is likely mediated by hCtr1, a copper transporter [19]. Uptake of copper is competitively inhibited by divalent metal ions such as cadmium, manganese, zinc, and cobalt [17]. Once within the hepatocyte, cytoplasmic copper chaperones (hCOX17, HAH1/Atox1, hCCS) distribute the metal to specific cellular compartments for its incorporation into copper-requiring proteins. HAH1/Atox1 [20] may function to bind copper and supply it to the Wilson's disease protein (ATP7B) in the trans-Golgi network. The ATP7B gene encodes the Wilson's disease p-type ATPase [21]. The ATP7B protein is required for incorporation of copper into ceruloplasmin in the liver and for biliary ex-

cretion of copper [22–24]. ATP7B may also be involved in the transport of copper to a vesicular compartment [25]. Copper from these vesicles may be delivered to lysosomes [26]. Within hepatocytes, free copper would likely be toxic to cells. However, it appears that copper is complexed by reduced glutathione (GSH) soon after the metal enters the cell [27]. The importance of GSH in metal detoxification is supported by its role in the removal of toxic oxygen species [28]. Following entry to the hepatocyte, in addition to GSH copper interacts with MT, and ceruloplasmin [17]. The copper chaperone for MT has not been identified [27]. Copper is secreted into plasma as a complex with ceruloplasmin [29,30]. This complex accounts for 90% to 95% of plasma copper [31]. In most mammals, copper is excreted easily, and the main route of excretion of copper is the bile [13,32]. Urinary copper excretion is minimal under normal conditions since most of the copper in circulating blood is bound to ceruloplasmin or confined within the erythrocytes and very little copper crosses the glomerular capillaries [14,33]. The process of hepatobiliary copper secretion is still poorly understood. Two independent pathways have been identified for the elimination of copper from hepatocytes into bile [34]. The first appears to be a vesicular pathway that involves the delivery of lysosomal contents, including copper into bile [32]. Reduction of copper excretion by microtubular disruption from colchicine administration supports the notion of a vesicular pathway [34]. A second pathway may involve canalicular membrane transport of copper-glutathione, and it functions when copper loads beyond physiological levels are presented to liver cells [34]. Copper transport into bile correlates well with the biliary excretion of glutathione [28], and the canalicular multispecific organic anion transporter (cMOAT) may contribute to biliary copper excretion [26], but the mechanism of normal biliary copper excretion is poorly understood.

Disruption of the normal copper homeostasis or accumulation of copper in excess of metabolic requirements can lead to copper toxicity. Copper toxicosis can be classified as primary when it results from an inherited metabolic defect, and as secondary when it is the consequence of an abnormally high intake, increased absorption, or reduced excretion of copper due to underlying pathologic processes.

## Spontaneous Copper toxicosis in humans and animals

Familial copper storage disorders occur in Wilson disease in humans [35], LEC rats [36], toxic milk mouse [11,37], Bedlington terriers [38,39], and in West Highland White Terrier dogs [40]. Excess copper can accumulate within the liver as a consequence of chronic cholestatic liver diseases [8], particularly in diseases such as primary biliary

cirrhosis [8,41], and chronic hepatitis [42]. Similar conditions occur in certain breeds of dogs [5,9,43,44].

### **Wilson's disease**

Wilson's disease is an autosomal recessive inherited disorder of copper metabolism [35,45,46]. Wilson's disease results in copper accumulation in the liver, cornea and brain [1]. The worldwide incidence of Wilson's disease, independent of ethnic and geographic origin, is approximately 1 in 30,000 [47]. However, it has been noted that the disease may be more common than previously expected, because most incidence estimations are based on adolescent or adults presenting with neurologic symptoms, which occur only in about half of the patients [48]. The Wilson's disease gene is localized on human chromosome 13 and codes for ATP7B, a copper transporting P-type ATPase [21]. The Wilson's disease mutations occur throughout the whole gene and include missense and nonsense mutations, deletions and insertions [49]. Most of the more than 80 mutations are present at a low frequency, and mutations differ between ethnic groups [50]. Thus, diagnosis of Wilson's disease is challenging and requires a battery of tests, including morphologic evaluation and copper analysis of liver tissue [51,52]. Liver disease may mimic various forms of common liver conditions, ranging from fulminant hepatic failure, chronic hepatitis, and cirrhosis [1].

### **Animal models of Wilson's disease**

The LEC rat [36] and the toxic milk mouse are the only known valid animal models of Wilson's disease [11,37].

### **Animal models of Wilson's disease – LEC rat**

The LEC rat with a hooded dilute agouti coat is a mutant inbred strain, which was established from a closed colony of randomly bred Long-Evans rats [36]. Long Evans Cinnamon rats suffer from fulminant hepatitis and severe jaundice at about 4 months of age and show similarities to Wilson's disease in many clinical and biochemical features [10,36]. This mutant has a deletion in the copper transporting ATPase gene (*Atp7b*) homologous to the human Wilson's disease gene (*ATP7B*) [10,53], and the mode of inheritance of hepatitis is also autosomal recessive [54].

Similar to the condition in Wilson's disease, LEC rats manifest elevated hepatic copper levels, defective incorporation of copper into ceruloplasmin, and reduced biliary excretion of copper [55]. LEC rats develop intravascular haemolysis secondary to the release of large amounts of non-ceruloplasmin copper into the bloodstream [56], as it has been described in patients with Wilson's disease [14].

The hepatic copper concentration can rise to 2,126 ppm dry weight [57]. It is also known that LEC rats may accumulate as much iron as copper in the liver as a result of hemolysis [56]. This mutant strain also possesses reduced hepatic selenium [58]. Both accumulation of iron and depletion of selenium in the liver may contribute to the development of fulminant hepatitis, hepatic fibrosis, and subsequent hepatocarcinogenesis in LEC rats by increasing the process of oxidative damage with copper, and a reduction in the antioxidant capacity against copper-induced free-radical damage [56,58].

It has been suggested that an immune-mediated mechanism may play a role in the development of acute lethal hepatitis in LEC rats. Autoimmune antibodies to liver microsomal proteins have been demonstrated 3–7 weeks before death in these rats [59]. Protein disulfide isomerase and calreticulin have been identified as antigens in liver microsomes of this mutant [60], and treatment with immunosuppressant drugs such as cyclosporin-A reduced the mortality in LEC rats [61]. However, a recent study showed that the development of antimicrosomal antibodies does not precede the development of severe liver damage in the LEC rat model [62].

The clinical signs of hepatitis include severe jaundice, a bleeding tendency, oliguria, lethargy, and loss of body weight. During this period, activities of serum enzymes, lactate dehydrogenase (LDH), alanine aminotransferase (ALT) [63], aspartate aminotransferase (AST), and  $\gamma$ -glutamyltransferase (GGT), as well as bilirubin levels, are increased significantly [36,54,62]. While the serum levels of ceruloplasmin remain reduced all the time [60], the copper concentration in serum increases mainly after the onset of jaundice [63]. About half of the animals die within a week of the onset of jaundice [36].

Histological changes of acute hepatitis in LEC rats occur prior to 8 weeks of age, and the most drastic changes occur from 17 to 20 weeks of age [36]. A recent study utilizing female LEC rats reports biochemical and morphological evidence of severe liver damage at 12 weeks of age [62]. Histological changes are characterized by hepatocellular karyomegaly, large numbers of Councilman bodies, submassive necrosis [54], mitosis of hepatocytes [36], and apoptosis [56]. After this stage, surviving rats develop chronic hepatitis, cholangiofibrosis, preneoplastic foci and nodules, and hepatocellular carcinomas [46,54,56]. Histochemical examination of LEC rat liver for copper reveals that copper accumulates preferentially in hepatocytes and distributes diffusely throughout the cytoplasm. Copper accumulates in virtually all hepatocytes throughout the entire liver lobule, but shows a tendency to localize in the periportal areas [64]. LEC rats that survive the stage of fulminant hepatitis develop cirrhosis [65], and

are highly susceptible to the development of hepatocellular carcinoma [64]. This is an interesting feature of this particular animal model because hepatocellular carcinoma is rarely diagnosed in Wilson's disease patients [66].

#### **Animal models of Wilson's disease – Toxic milk mice**

Toxic milk is an autosomal recessive mutation which alters copper homeostasis in mice [37]. Offspring of mutant females are born copper-deficient and since their mother's milk is also low in copper, babies die at 2 weeks of age. The progeny of affected dams fostered to lactating normal females survive but with age copper accumulate in their livers [67]. By 6 months of age, liver changes are characterized by nodular fibrosis, bile duct hyperplasia and portal lymphocytic inflammatory cell infiltration [67]. Toxic milk mice share some biochemical abnormalities with Wilson's disease for example concentration of serum copper and ceruloplasmin are decreased. The genetic defect in toxic milk mice is similar to that of Wilson's disease [11]. Although gross and histologic changes in the liver in both rodent models (i.e. LEC rat and toxic milk mice) resemble Wilson's disease [67], same differences have been noted. Neither of the rodent models has neurologic symptoms, and in the toxic milk mice model affected dams produce Cu-deficient milk, whereas there are no reports of Cu-deficient milk in humans mothers with Wilson's disease [68].

#### **Indian childhood cirrhosis**

Indian childhood cirrhosis and its analogues endemic Tyrolean infantile cirrhosis, and idiopathic copper toxicosis, are fatal liver diseases seen in young children due to genetic susceptibility to minimal excess in dietary copper [2–4,7]. The gene for Indian childhood cirrhosis diagnosed in North America has recently been identified [69].

#### **North Ronaldsay sheep**

This primitive breed has adapted to copper impoverished environment and display an abnormal sensitivity to copper toxicity when transferred to copper adequate location [70]. A recent study reports the remarkable similarities between the condition in North Ronaldsay sheep and Indian childhood cirrhosis, whereby affected sheep exhibited liver changes varying from active hepatitis to panlobular pericellular fibrosis, and cirrhosis. Histochemical stains demonstrated periportal to panlobular histochemical copper retention [6].

#### **Primary biliary cirrhosis**

Primary biliary cirrhosis is a chronic progressive, often fatal liver disease, characterized by the eventual development of cirrhosis and liver failure [31,41]. Middle-aged females are predisposed to the condition [71]. The immunological abnormalities and morphologic features observed in primary biliary cirrhosis, favour the hypothesis

of an immune-mediated mechanism [72,73]. However, the nature of the initiating factor(s) and of the sensitizing antigen is unknown. The only effective treatment for this disease is liver transplantation [74]. Indeed, primary biliary cirrhosis is one of the five most frequent causes of liver transplantation [75]. Although copper accumulation is a secondary event [76], therapeutic approaches have included attempts to remove excess hepatic copper in order to avoid possible synergism between the initiating factor(s) and release of copper into the liver [77].

#### **Animal models of primary biliary cirrhosis**

Certain disorders, such as chronic active hepatitis in Doberman pinschers and Skye terrier hepatitis are characterized by copper retention secondary to the underlying disease, thus resembling primary biliary cirrhosis in humans [44]. Copper values are never so elevated as in the familial storage diseases.

#### **Animal models of primary biliary cirrhosis – Chronic hepatitis in Doberman Pinschers**

Doberman hepatitis is a disorder associated with histologic features of chronic active hepatitis, cholestasis, and cirrhosis. Middle aged, spayed female dogs are predisposed to the disease [9,43,78]. The cause of the disease remains undetermined, but histopathological changes support the idea of immune-mediated disorder [79]. Copper accumulates in centroacinar (portal) areas [9,80]. The present knowledge on the role of copper in this disorder is incomplete; however, the presence of copper in liver biopsies constitutes one of the essential diagnostic criteria for sub-clinical Doberman hepatitis [80].

#### **Animal models of primary biliary cirrhosis – Skye terrier hepatitis**

This condition appears to be an unusual lesion characterized by intracanalicular cholestasis, with copper accumulation and hepatocellular degeneration culminating in cirrhosis [44]. Copper accumulation occurs primarily in the periacinar area, which is inconsistent with other disorders associated with cholestasis and subsequent tissue copper retention [81]. The cause is unknown, but an inheritable metabolic defect involving membrane transfer and transport systems in the periacinar zone, resulting in disturbed bile secretion and excessive copper accumulation have been suggested [44].

#### **Animal models of primary biliary cirrhosis – Feline cholangiohepatitis complex**

This condition has three well-characterized histopathologic lesions, but the etiology and pathogenesis of the condition is poorly understood [82]. The non-suppurative type has been compared to primary biliary cirrhosis in humans [83,84]. Non-suppurative cholangitis in cats is characterized by lymphocytic and plasmacytic portal

infiltration, bile duct hyperplasia, and portal fibrosis. Histochemical stains demonstrate copper positive granules within portal hepatocytes (ICF personal observation). The value of this model in the study of primary biliary cirrhosis has not been thoroughly assessed.

#### **Other copper associated disease in dogs**

In general, naturally occurring canine genetic diseases resemble human diseases more faithfully than their rodent counterpart, as there is a higher degree of DNA sequence identity between humans and dogs than between humans and rodents [85]. Familial copper storage disorders in Bedlington and West Highland white terriers are usually associated with early subclinical disease during which copper accumulates with subsequent liver injury culminating in cirrhosis [40,86]. Occasionally however, there may be a copper-induced hemolytic crisis in Bedlington terriers [86,87]. Bedlington Terrier copper toxicosis has generated much interest as a possible animal model for Wilson's disease. However, it has been proven that Wilson's disease and Bedlington Terrier copper toxicosis do not share the same genetic defect [88]. The canine copper toxicosis locus in Bedlington terriers has been mapped to canine chromosome region CFA 10q26 [88] and in addition to ATPB, ATOX 1 [89,90], ATP6H [91] have been excluded as candidate genes underlying copper toxicosis in Bedlington terriers [89,90]. Recently, a mutated MURR1 gene was discovered in Bedlington terriers affected with the disease [85].

Some similarities have been noted between Bedlington terriers and idiopathic childhood cirrhosis. Idiopathic childhood cirrhosis is biochemically similar to copper toxicosis in Bedlington terriers [85], but clinically much more severe [7]. Both conditions are characterized by the absence of neurologic damage and Kayser-Fleisher rings [92,93], and normal ceruloplasmin levels [47].

Liver copper values can be as high as 12,000 ppm dry weight in Bedlington terriers [94] whereas the highest copper value recorded in West Highland white terriers is 6,800 ppm dry weight [95]. In Bedlington terriers older than 1 year of age, there is a progressive increase in the accumulation of tissue copper until 8 years of age [81]. There is no relationship between age, histomorphological changes and hepatic copper concentration in West Highland white terriers. The clinical signs vary widely, depending on the stage of the disease. Affected animals in the early stages usually are asymptomatic. Depression, anorexia, lethargy, vomiting, and increased ALT are usually associated with an acute onset of hepatic necrosis. Microscopically, there is a spectrum of recognizable changes. In the least affected or subclinical cases, accumulation of intracytoplasmic refractile, light-brown granules in vacuolated parenchymal cells of the periportal zones

are observed which stain histochemically positive for copper. This is followed by the development of foci of hepatocellular degeneration and necrosis with scattered inflammatory response of neutrophils, lymphocytes and plasma cells. Fine fibrous septa extend from the portal areas into the lobules. More advanced stages are characterized by periportal infiltrates of inflammatory cells and prominent piecemeal necrosis (resembling chronic active hepatitis). Piecemeal necrosis has not been described in West Highland white terriers toxicosis. Finally, there is a complete architectural disorganization of the liver with variable sized nodules of hepatocytes (with focal degeneration and aggregates of neutrophils) separated by bands of fibrous connective tissue with portal-central bridging [94,96]. Contrary to Wilson's disease, hepatocytes with Mallory bodies have not been identified [97].

Copper-associated liver disease has increasingly being recognized in Dalmatians [98,99] (Figs. 1,2). Fulminant hepatic failure characterized by high serum activities of ALT and AST. Histologically, there is severe necrosis of hepatocytes involving the centrilobular areas. Macrophages and surviving hepatocytes contain copper-positive material [98]. Primary copper storage disease was suspected on the basis of histologic findings and high copper concentration in the liver [99]. Morphologic changes observed in Dalmatians are strikingly similar to those observed in LEC rats (ICF personal observation) suggesting that copper toxicosis in Dalmatians is a promising spontaneous animal model of Wilson's disease.

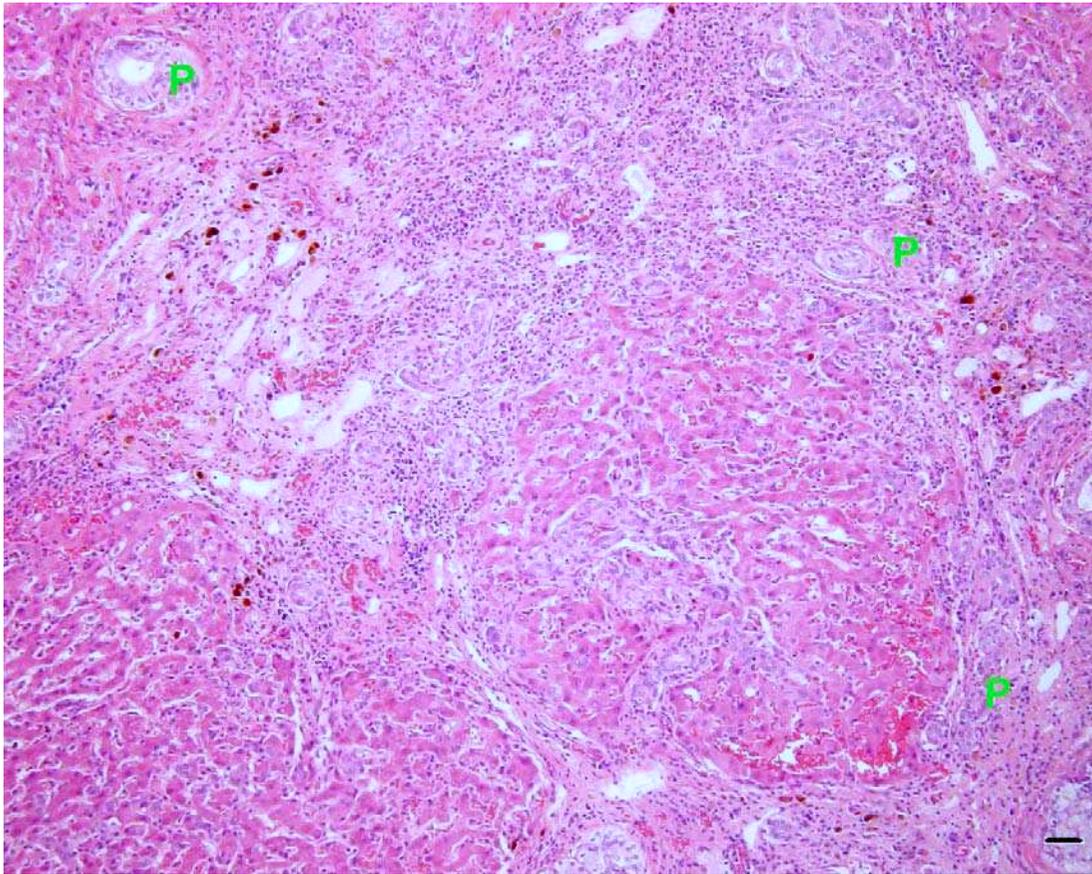
Excess copper accumulation occurs as a consequence of chronic liver disease in other canine breeds dogs [5,9,43,44], particularly Cocker Spaniels and Poodles [9]. Similarly, secondary copper accumulation has been described in chronic hepatitis in humans [42].

#### **Spontaneous copper-associated liver disease in other mammalian species**

Sheep are particularly susceptible to copper poisoning [100,101]. The condition occurs when sheep are accidentally fed rations prepared for other species (i.e. bovine or swine) [100,102]. Copper poisoning in sheep is commonly diagnosed. In contrast, copper toxicosis in other farm animals is rarely reported [103].

#### **Spontaneous copper-associated liver disease in other mammalian species – Copper toxicosis in sheep**

Chronic copper poisoning in sheep results from the accumulation of copper in hepatic tissue over a period of a few weeks to more than a year [100,101], and is considered to have two distinct phases [104]. During the accumulation or pre-hemolytic phase, animals may be clinically normal, even with liver copper concentrations of 1,000 ppm, so long as increasing mitotic rate produces enough new



**Figure 1**  
Dalmatian dog liver. Dissecting hepatitis with marked mononuclear inflammation. P = portal. HE stain. Bar = 50  $\mu$ m.

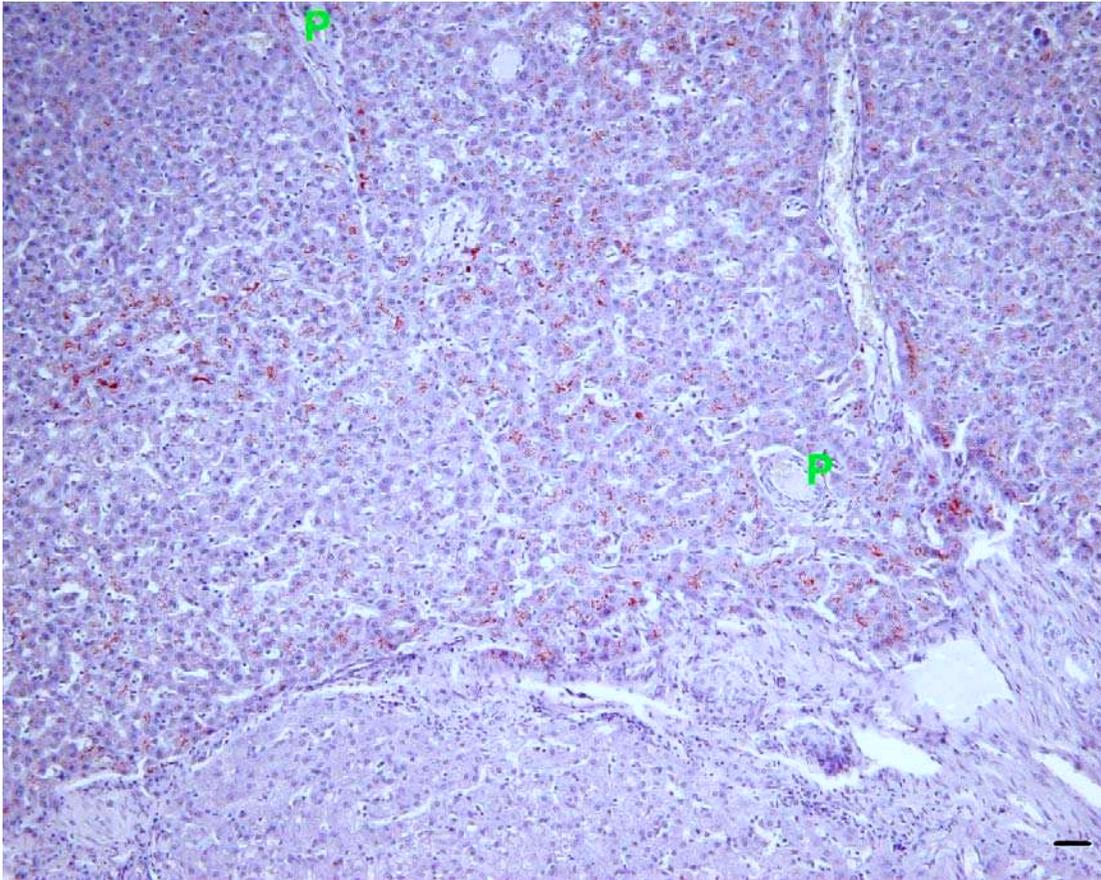
hepatocytes to take up the copper released by dying cells [94]. However, liver damage does occur during this period as indicated by increased levels of lactic dehydrogenase and AST [100]. The second phase, or hemolytic crisis, lasts from hours to days and is characterized by the sudden onset of severe intravascular hemolysis and hemoglobine-mia associated with increased blood copper levels, with resulting liver [104,105], kidney, and brain damage [106].

During the hemolytic phase, elevated serum values of AST, GGT, ALP, copper, urea nitrogen (BUN), bilirubin [100,107], and ceruloplasmin [108] have been observed. Elevation of blood copper occurs prior to and during the hemolysis; the ceruloplasmin levels tend to fluctuate but in most instances there is a two-fold or more increase immediately before and during the hemolytic crisis [108]. The histologic changes are present in the liver in the pre-clinical stages and can be somewhat obscured by the peri-

acinar (centrilobular) necrosis of hypoxemia and the bile accumulations of hemolytic disease [94]. Copper can be demonstrated histochemically with rubeanic acid or rhodanine as fine granules within the cytoplasm of parenchymal and Kupffer cells [104,108]. Copper in hepatocytes is mostly located within lysosomes [109]. It has been demonstrated that copper deposition begins in the periacinar areas extending to the mid and periportal zones with progressive copper-loading [109].

**Spontaneous copper-associated liver disease in other mammalian species – Copper-associated liver disease in cats**

Liver disease associated with centrilobular (periacinar, zone 3) copper accumulation was described in a Siamese cat [110]. One of the authors (ICF) has examined cases of copper-associated liver disease in cats, with morphologic characteristics similar to those reported in Siamese cats



**Figure 2**  
Dalmatian dog liver. Prominent copper-positive granules. P = Portal. Rhodanine stain. Bar = 50  $\mu$ m.

(Figs. 3,4), suggesting that metabolic defects of copper metabolism occur with relative frequency in the feline specie and may have been overlooked in the past.

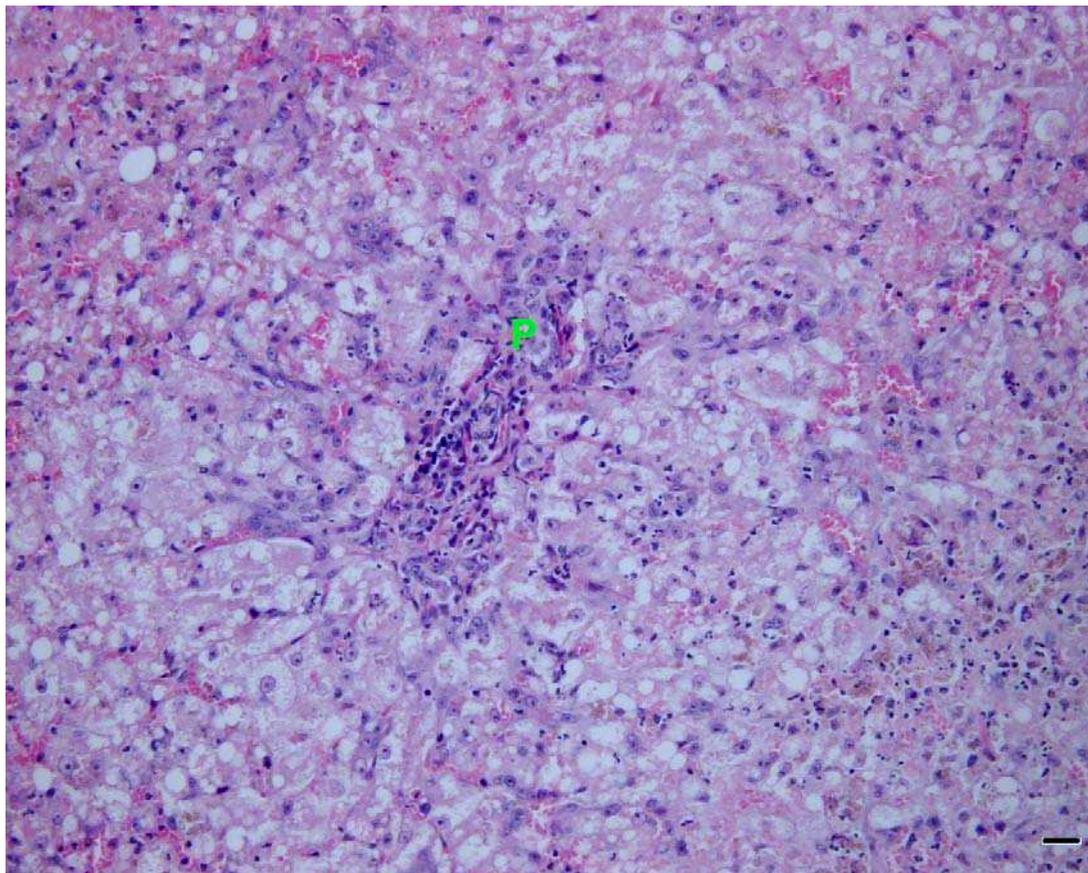
**Spontaneous copper-associated liver disease in other mammalian species – Copper-associated liver disease in ferrets**

Two cases have been described in ferrets [111]. One case was characterized by chronic hepatopathy, with diffuse hepatocellular vacuolation, and the second case had centrilobular degeneration and necrosis. In both ferrets, liver copper concentration was markedly elevated and special staining revealed copper pigment in hepatocytes and macrophages. An inherited defect of copper metabolism was suspected in these ferrets based on the lack of related illness in 11 other ferrets housed in the same environment and receiving the same diet.

**Treatment of Cu-associated disease in animal models**

D-penicillamine has been effective in the treatment of Bedlington Terrier toxicosis [81]. However, copper is only slowly removed from the liver and clinical improvement generally requires years of treatment [112]. Side effects seen with D-penicillamine therapy in humans include fever, anorexia, pyridoxine deficiency, leukopenia, thrombocytopenia [113,114], rashes and proteinuria [47]. In dogs, treatment with D-penicillamine often is associated with anorexia, nausea and vomiting [92,112,115].

Alternate agents such as trientine [116], tetrathiomolybdate (TTM) [117,118], and zinc [119,120] have been employed in the treatment of Wilson's disease. Zinc [121] have been used for the treatment of copper toxicosis in sheep and dogs [119]. In sheep, ammonium tetrathiomolybdate lowers liver concentrations and prevents the de-



**Figure 3**  
Liver from a cat with severe hepatic lipidosis. Diffuse severe hepatocellular vacuolation. P = Portal. HE stain. Bar = 50  $\mu$ m.

velopment of hemolysis, however, the kidneys accumulate large amounts of copper [122,123]. It also appears that TTM is not fully excreted after treatment but molybdenum is widely distributed and retained in many organs including brain and pituitary [124].

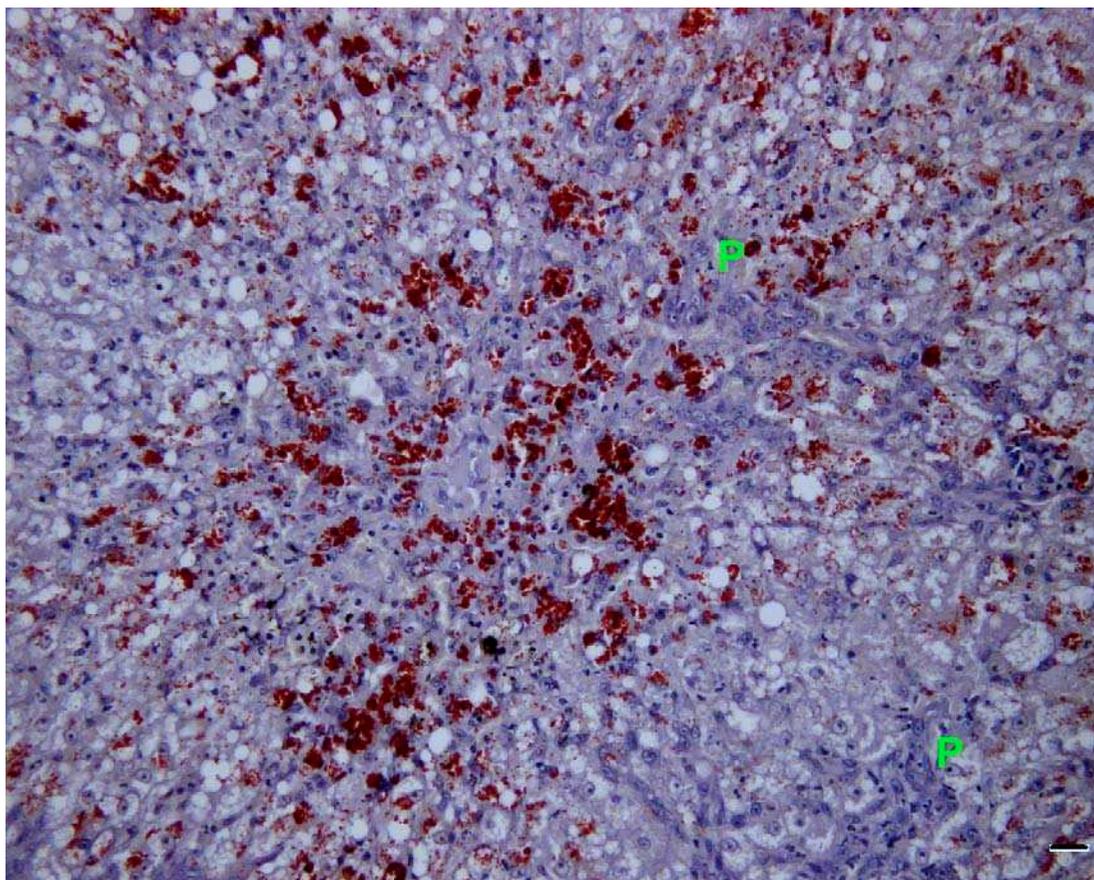
### Conclusions

Recent advances in molecular biology have made possible the identification of genetic defects responsible for Wilson's disease, Indian childhood cirrhosis, and copper toxicosis in LEC rats, toxic milk mice, and Bedlington terriers. However, many questions regarding copper metabolism in mammals, and pathogenesis and treatment of copper-associated liver diseases remain to be answered. Studies designed to identify genetic defects of copper metabolism and to implement new therapeutic approaches to cure these conditions will greatly contribute to the knowledge of the pathogenesis of copper-associated diseases. This re-

view demonstrates that there are numerous spontaneous animal models of copper-associated liver diseases. Based on the clinical presentation and morphological features, copper-associated liver disease in Dalmatian dogs has the potential to be a good model of Wilson's disease. North Ronaldsay sheep is a promising model to study Indian Childhood cirrhosis and its analogues, and Bedlington Terrier copper toxicosis may share many similarities with Indian childhood cirrhosis. In the future, increased research collaboration between basic and applied scientists will be needed to link molecular defects to their morphologic and clinical implications.

### Methods

The following methods are routinely used in our laboratory for qualitative and quantitative detection of copper.



**Figure 4**  
Liver from a cat with severe hepatic lipidosis. Prominent copper-positive granules. P = Portal. Rhodanine stain. Bar = 50  $\mu$ m.

#### **Rhodanine method for histochemical detection of copper**

Liver sections are dewaxed and hydrated to distilled water. Then, sections are placed in rhodanine working solution at 37°C for 18 hours. Rhodanine working solution is prepared using 3.0 ml of 0.2% Rhodanine stock solution (0.2 g rhodanine in 100 ml 100% ethanol) and 50 ml buffer acetate (5.0 ml 40% formalin, 20 g sodium acetate in 1000 ml distilled water).

Slides are rinsed in 3 changes of acetate buffer solution, counterstained in Mayer's hematoxylin solution, rinsed in acetate buffer, dehydrated, cleared and mounted.

#### **Measurement of copper in tissue by flame atomic absorption spectroscopy**

Liver samples are placed in plastic bags, frozen at -80°C and stored for 24 hours. Tissues are then processed for atomic absorption spectrophotometry. Briefly, approxi-

mately 1 gram of tissue from each sample is weighed and placed in a Teflon container for microwave digestion. All samples are weighed in duplicate. Control samples with known amounts of copper are also included. To each vessel, including a blank containing no tissue, 1.0 ml of deionized, distilled water and 2.5 ml full strength, trace metal free nitric acid (10.95 M HNO<sub>3</sub>) is added. Vessels are capped and placed in a MDS-200 microwave oven and digested. The digested contents are poured off and diluted to 10 ml with distilled water and thoroughly mixed. Copper concentrations are measured using a spectrophotometer equipped with a copper hollow cathode lamp, with the following instrument settings: wavelength 324.8 nm, slit width 0.7 nm, lamp current 17 mA. Standards used are 3, 6 and 12 ppm prepared from 1000 ppm stock standard in 0.2 M HNO<sub>3</sub>. Peak area is read with a read delay of 0 s, and a read time of 5 s. Standard reference materials are used as quality control substances, and are processed with

each batch of liver samples. Samples are not corrected for recovery if the recovery of the quality control samples is 95% or more. Cu concentrations are recorded in µg/g wet weight tissue.

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