Canine hepatitis and the pathomechanisms of copper-induced hepatitis in COMMD1 deficient dogs

Robert Paul Favier

2011

Canine hepatitis and the pathomechanisms of copper-induced hepatitis in COMMD1 deficient dogs

Hepatitis bij de hond en de pathomechanismen van koper-geïnduceerde hepatitis in COMMD1 deficiënte honden.

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op dinsdag 15 februari 2011
des middags te 4.15 uur

door

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geboren op 14 oktober 1973 te Leiden

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The studies described in this thesis were performed at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, the Netherlands.

Publication of this thesis was made possible by the financial support of:

- Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, the Netherlands
- AUV Dierenartsencoöperatie, Cuijk, the Netherlands
- Vétoquinol, ‘s-Hertogenbosch, the Netherlands
- Boehringer Ingelheim, Alkmaar, the Netherlands
- Novartis Animal Health, Breda, the Netherlands

R.P. Favier, 2010
Canine hepatitis and the pathomechanisms of copper-induced hepatitis in COMMD1 deficient dogs

Dissertation Utrecht University, Faculty of Veterinary Medicine
(With summaries in English and Dutch)

ISBN/EAN: 978-90-393-5500-8

Cover Anjolieke Dertien, Division Multimedia
Lay out Harry Otter, Division Multimedia
Printing Drukkerij Ridderprint, Ridderkerk

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1

Aims and scope of the thesis

Chronic inflammatory liver disease regardless of aetiology leads to failing regeneration and fibrosis, ending in cirrhosis. Both in man and in animals this worldwide health problem has no definitive cure. Chronic liver injury causes the hepatic stellate cells to proliferate and differentiate into matrix-producing cells. New therapeutic options may be developed upon detailed understanding of the molecular mechanisms driving liver fibrosis [1]. This may lead to new anti-fibrotic therapies which need to be tested in suitable large animals before application in the veterinary and human clinic. On the other hand, to restore the failing regenerative capacity of the diseased liver, adult progenitor cell directed therapies are of interest, as alternative to whole organ transplantation [2]. In the near future new anti-fibrotic and pro-regenerative therapies are to be expected.

Pathways of regeneration and fibrosis can be studied *in vitro* in cell culture or tissue slices, and *in vivo* in zebrafish, mouse, or rat models. Proof of principle for new strategies of intervention can be obtained in rodent models. However, it is still a giant step from these fundamental animal model studies to the clinical application. The availability of a suitable large animal model for such studies will greatly enhance the selection and validation of effective new strategies before they enter the expensive and time consuming clinical phase.

In order to find the most suitable large animal model it is important to recognise that the typical histopathological reaction pattern of the liver is different between mammalian species. The reaction pattern of the liver in cats and dogs, for example, is very different leading to entirely different hepatobiliary diseases [3]. It is therefore important that specialists in veterinary internal medicine and pathology, being familiar with the diseases and pathologies of the liver in different animal species, are involved in finding the best large animal species in which models for human liver diseases can be identified. In cooperation with prof. Roskams' research group, our institute has compared different forms of hepatitis and primary liver tumours in dogs and humans. These studies have shown that pathways of fibrosis, regeneration, adult stem cell activation and tumour formation are very similar in dogs and humans [4-8]. It seems that the patterns of progression and the molecular pathways affected are to a high degree independent of the initial trigger, e.g. excessive alcohol consumption, DNA- or RNA viruses [9,10].

Based on our observations that man and dog share the same hepatopathies and have identical clinical, pathological and pathogenetic reaction patterns during the development of liver disease, the dog seems to be a properly suited species to test new therapeutic strategies.

With this recognition the need for a well controlled and reproducible form of canine hepatitis became apparent. The copper-induced chronic hepatitis with simple recessive autosomal inheritance in Bedlington terriers may fulfil this role [11]. A mutation within the *COMMD1* gene, discovered in these Bedlington terriers, most likely causes the disease [11]. A solid genetic screen was hampered for a long time due to a large deletion (over 13 kb) [12,13]. *In vitro* gene-silencing experiments proved the causative effect of COMMD1 depletion and copper accumulation [14,15]. Mice lacking COMMD1 die *in utero* at day 9 p.c., again emphasizing species differences and stress the urgency to develop good animal models [16]. We have crossed dogs with the mutated *COMMD1* gene with healthy beagles and now have a colony of COMMD1 deficient dogs available. The COMMD1 mutation affects the function of ATP7B. Mutations in this gene cause different forms of Wilson's disease in man with a pathogenesis highly comparable to the dog model. The molecular events leading to Wilson's disease are not completely understood and this canine model can be helpful in unravelling the underlying processes.

The central aim of this thesis is to analyse hepatitis in dogs in general and specifically the pathogenesis of hepatitis in COMMD1 deficient dogs. Detailed evaluation of the latter disease to evaluate may reveal its potential as future model for pre-clinical studies. Emphasis has been put on three main processes: fibrogenesis, regeneration, and copper metabolism including oxidative stress.

The hypotheses studied were**:**

1) In a clinical referral population canine chronic hepatitis is the most frequent diagnosed form of hepatitis (chapter 3&4).

2) Copper as an aetiology for canine chronic hepatitis is of major importance (chapter 3&4).

3) Prednisone treatment is of benefit in the treatment of dogs with idiopathic chronic hepatitis (chapter 5).

4) Liver fibrogenesis in COMMD1 deficient dogs is activated before the onset of hepatic inflammation (chapter 7).

5) Liver regeneration in chronic hepatitis of COMMD1 deficient dogs occurs through proliferation of mature hepatocytes; activation of the liver progenitor cell compartment is of minor importance (chapter 7).

6) In copper-associated hepatitis, oxidative stress is the initial pathogenetic trigger; inflammation starts when anti oxidant defence mechanisms are overwhelmed (chapter 8).

7) COMMD1 deficiency disturbs the copper excretion function of the ATP7B protein by preventing its canalicular membrane localization (chapter 8).
8) COMMD1 deficient dogs nicely mimic the human Wilson's disease patient (chapter 8).

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Chapter

2

General introduction

The liver, one of the largest organs in the body, has an enormous reserve capacity and plays a key role in many metabolic processes and maintaining homeostasis of the body. It consists of epithelial cell types (hepatocytes, cholangiocytes and progenitor cells), mesenchymal cell types (hepatic stellate cells), and sinusoidal cells (Kuppfer cells, endothelial cells). There is a continuous interplay between these different cell types in the liver and their surrounding stroma. Adult liver parenchyma is composed of hundreds of thousands of lobules that can be considered as the smallest structural and functional units. From the portal areas blood is supplied from the smallest portal vein and hepatic artery branches to the sinusoids, which are oriented towards the central veins of the lobules. Going from periportal to pericentral, hepatocytes have different functions, which is called "metabolic zonation". The Wnt/β-catenin pathway plays a key role in controlling this "metabolic zonation" [1]. The huge adaptive capacity of the liver implies that signs of disease and concomitant dysfunction of the liver often become clinically apparent in the chronic stage when the repair mechanisms are hampered.

Chronic hepatic injury activates a general aetiology-independent process characterized by fibrogenesis and liver regeneration. The sequence of events starts with the primary insult (e.g. toxins, viruses), followed by regenerating hepatocytes, activation of non-hepatic cells, and fibrosis. When mature hepatocytes are damaged or hampered in their replication, the liver progenitor cell (LPC) compartment may be activated [2]. Hepatic fibrosis, a common manifestation of chronic liver diseases, is the result of accumulation of extracellular matrix (ECM) and can progressively lead to cirrhosis [3]. Liver cirrhosis represents a worldwide health problem and no definitive cure other than transplantation is available [4]. In human medicine, a short supply of donor organs is a serious problem and for people in need for a new liver who are on the waiting list a suitable donor liver often comes too late. A strong pressure has been put to evaluate alternatives for whole organ transplantation since the last two decades. One of these alternatives is liver cell transplantation. This technique has been studied for more than four decades, mainly in mice and rats. Although liver cell transplantation has also been used in over 200 human beings with different kind of liver diseases [5], the step from principle to practice still remains full with technical and theoretical hurdles. The availability of a suitable large animal model in which the pathophysiology of chronic liver disease closely resembles that in man is lacking [6]. A dog model with an inducible, controllable and reproducible form of chronic hepatitis with a well understood cause might be a valuable tool for evaluating new therapeutic strategies. Spee et al. [7], IJzer et al. [8] and Schotanus et al. [9] demonstrated that the molecular pathophysiology of canine fibrotic liver diseases is highly comparable to the pathophysiology of their human counterparts.

In human medicine the molecular pathogenesis of diseases associated with copper excess (Wilson disease, WD) is of great interest [10]. Several animal models resembling WD have been described [11]. The Long Evans Cinnamon (LEC) rat and (Jackson) toxic milk mouse are naturally occurring models for Wilson disease. LEC rats have a deletion in the ATP7B gene resulting in the total loss of the protein [12] and toxic milk and Jackson toxic milk mice have missense mutations in ATP7B that affects ATP7B function leading to an incomplete activation [13-15]. Another interesting animal model for studying the molecular pathophysiology of copper accumulation, is the Bedlington terrier with copper toxicosis (CT). CT in the Bedlington terrier is caused by a deletion of exon-2 of the *COMMD1* gene which results in the absence of a functional COMMD1 protein [16]. The result is a progressive accumulation of copper in the hepatocellular lysosomes in the centrolobular region around the central vein in the liver, histologically distinguishable from the normal situation at one year of age, which leads to progressive hepatitis and cirrhosis. At the department of Clinical Sciences of Companion Animals of the University of Utrecht a COMMD1 deficient dog population has been created for longitudinal follow-up studies on the development of copper-associated chronic hepatitis. This unique animal model allows an in-depth characterization of the fibrosis, regeneration, copper, and oxidative stress pathways.

Liver regeneration

Many growth factors and cytokines have been implicated in regulating liver regeneration. Hepatocyte growth factor (HGF), a ligand for the c-Met proto-oncogene product (MET), is one the most potent stimulators of hepatocyte proliferation [17,18]. In liver, HGF is mainly produced by hepatic stellate cells, sinusoidal endothelial cells, and Kupffer cells [19,20]. For the regulation of the release and the activation of HGF during regeneration metalloproteinases, tissue inhibitor of metalloproteinases (TIMP) levels, urokinase-type plasminogen activator (uPA), and HGF-activator are important [21,22]. HGF is secreted as a single-chain, 83-kDa precursor without biological activity (pro-HGF) and proteolytic cleavage into HGF is necessary for activation. Maturation of pro-HGF into the bioactive dimer takes place in the extracellular environment. Naldine et al. showed that uPA activates pro-HGF *in vitro*, although at a 1000-fold lower activity as HGF-activator. Thus, a crucial limiting step occurs in the HGF signaling pathway after its secretion [22,23]. After binding of HGF to c-Met, signalling to multiple transducers is mediated, including the Akt/PKB, ERK1/2 and STAT3 (signal transducer and activator of transcription 3) (Figure 1) [24,25]. Other cytokines important in activation of the STAT pathway include

transforming growth factor (TGF) alpha and interleukin (IL)-6 [26,27]. Recently, the idea came up that the activation of multiple pathways is required for liver regeneration instead of one single humoral agent such as HGF [28]. Taub proposed an essential circuitry required for liver regeneration compassed of three types of pathways: cytokine, growth factor, and metabolic networks that link liver function with cell growth and proliferation [28-30].

When mature hepatocytes are damaged or inhibited in their replication, a reserve compartment, in humans called the liver progenitor cell (LPC) compartment and in rodents the oval cell compartment, is activated. This compartment resides in the smallest and most peripheral branches of the biliary tree, the ductules and canals of Hering. K7 and K19 are generally accepted as immunohistochemical markers for the identification for LPCs [31-34], but cholangiocytes also stain positive. Unfortunately, no universal LPC marker specific to this compartment has been identified to date [35]. Recently two interesting potential candidates were described; TWEAK [36] and FoxoL1 [37]. Progenitor cell activation is seen in the majority of subacute to chronic human liver diseases and the degree of activation increases with the severity of the disease [34,35]. A general trigger for progenitor cell activation is a lack of the mature cell compartments ability to proliferate [2]. Progenitor cells are able to survive when hepatocytes are lost due to toxic damage or viral infections [34] and, in view of their bipotent differentiation potential towards hepatocytes or cholangiocytes, progenitor cells would be interesting cells to use for cell therapy [38]. The mechanisms controlling LPC activation and differentiation are under intense investigation [39] and these processes could be driven by different signals acting onto the LPC niche as result of the stage (acute or chronic) and type of disease (parenchymal or biliary) [40-42]. Interestingly, Wnt signalling is involved in the LPC response in mice [43], rats [44], and humans [42,45] and activation of the Wnt pathway plays a significant role in LPC expansion.

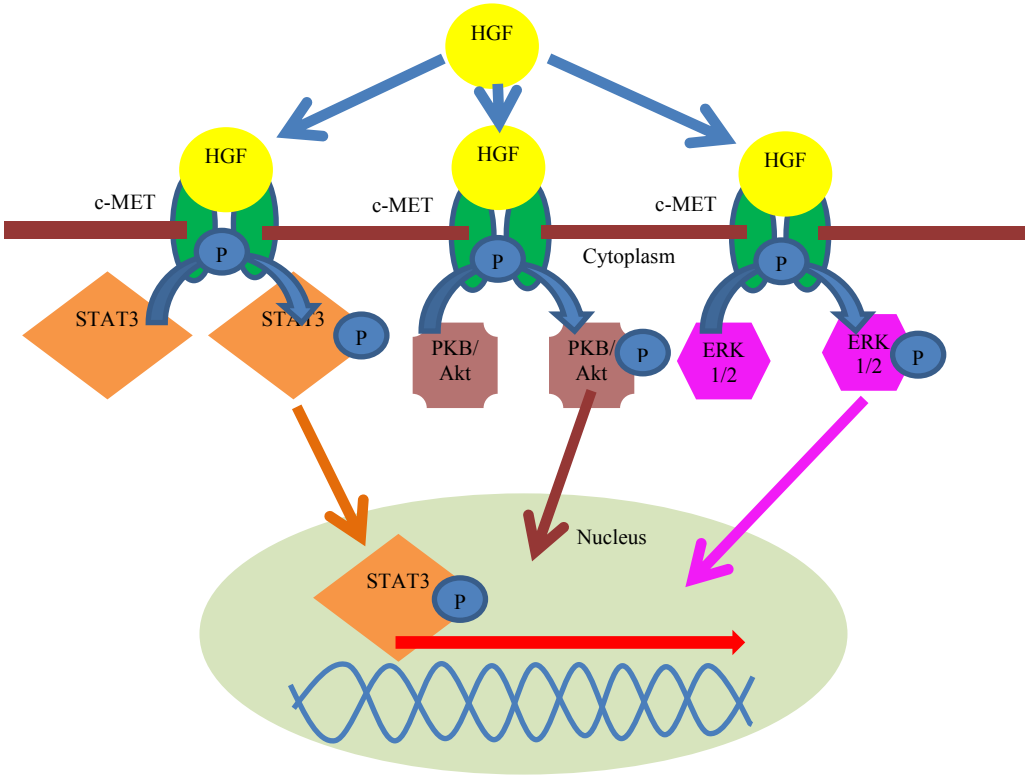

Figure 1: HGF signalling
After activation, HGF binds to its receptor c-MET. Autophosphorylation of c-MET induces multiple intra-cellular pathways (STAT3, PKB/Akt, and ERK1/2).
HGF: hepatocyte growth factor; c-MET: c-MET tyrosine kinase receptor; STAT3: signal transducer and activator of transcription 3; PKB: protein kinase B; ERK: one of the MAP (mitogen activated protein) kinases.

Liver fibrosis

Hepatic fibrosis results of an accumulation of extracellular matrix (ECM) and when it progresses it can lead to cirrhosis [3]. ECM deposition is the result of increased synthesis and/or decreased breakdown. The bulk of ECM in the fibrotic liver is produced by myofibroblast (MF)-like cells. Three different MF-like cells have been described in rat and man based on location and immunohistochemical profile [8,46]. These three types comprise portal or septal MF, interface MF, and perisinusoidally located hepatic stellate cells (HSCs). HSCs are non-parenchymal, quiescent cells that, when activated, show a cytoplasmic alpha-smooth muscle actin (α-SMA) immunoreactivity. It has already been

established that a transient increase of transforming growth factor β1 (TGF-β1) in the liver, mainly produced by the HSC, promotes fibrosis with the formation of extracellular matrix (ECM) components and suppresses hepatocyte proliferation [47]. The signalling responses to TGF-β1 are mediated by two receptors, TGF-β receptor type I (TGF-β RI) and TGF-β receptor type II (TGF-β RII) at the cell surface and their intracellular substrates, the Smad proteins (Figure 2). Recently is has been shown that β-catenin dependent Wnt signaling is involved in keeping HSCs quiescent [48]. Sawitza et al. proposed that the space of Disse represents a newly discovered stem cell niche of the vertebrate liver, in addition to the earlier described stellate cell niche in the canal of Hering [49]. Important regulators of proteolytic activity which determine ECM turnover are plasminogen activator-plasmin system components. Plasmin, generated from circulating plasminogen by proteolytic cleavage by uPA, is capable of degrading ECM components directly by proteolysis, and indirectly by inhibiting deposition of ECM by the activation of matrix metalloproteinases (MMPs). In this way, an up regulation of uPA in the liver might inhibit the deposition of ECM and reverse hepatic fibrosis [50]. Matrix degrading proteases are inhibited in their activity by the tissue inhibitor of metalloproteinase (TIMP). Overall, matrix remodelling is an important component of liver regeneration and the point at which cirrhosis or extensive fibrosis becomes irreversible has not been well defined [51,52].

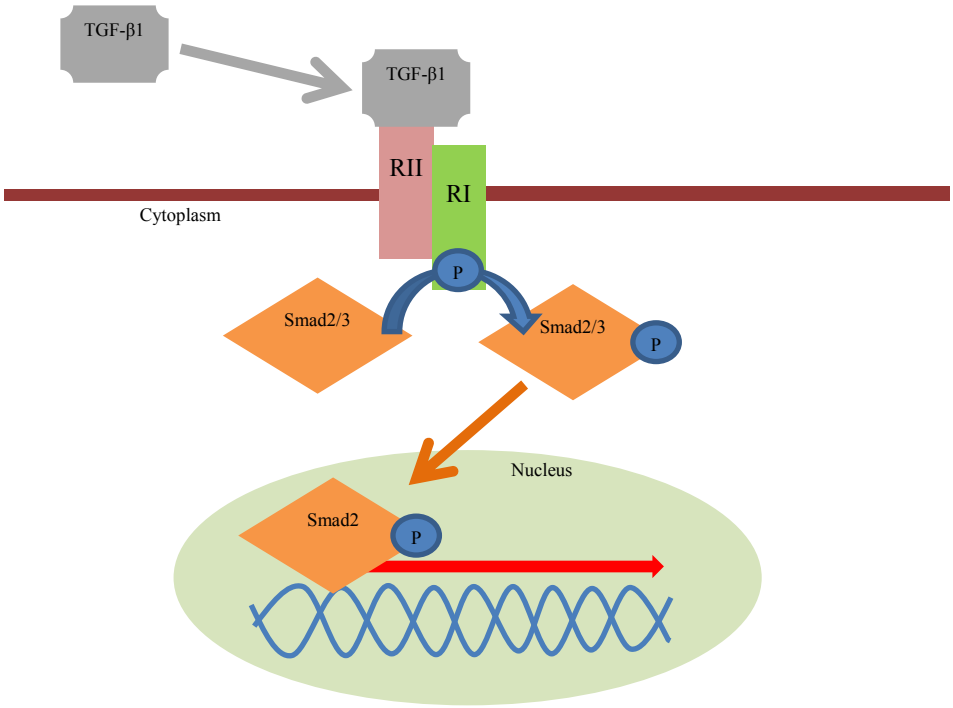

Figure 2: TGF-β / Smad pathway
The signalling response to TGF-β1 is mediated by its two receptors TGF-β RII for binding and TGF-β RI for phosphorylation at the cell surface and the intracellular substrates, the Smad proteins.
TGF-β: transforming growth factor β; RI: transforming growth factor β receptor 1; RII: transforming growth factor β receptor 2.

Apoptosis and necrosis

Several modes of cell death have been classified, apoptosis and necrosis being the most important ones [53]. Apoptosis, a regulated form of cell death, can be initiated through two fundamental pathways: the intracellular organelle-based intrinsic pathway and the death receptor or extrinsic pathway. Both pathways usually interact with each other [54,55]. The mechanisms involved in apoptosis connect cell death to inflammation and fibrosis [55]. Regulation of the apoptotic cascades in liver cells is complex, but appears to be commonly triggered via the extrinsic pathway through activation of death receptors (Fas, TNF receptor 1, TRAIL-R1 and -R2) [56]. Mitochondrial dysfunction plays an important role in augmenting the apoptotic processes and integrating death receptor initiated and stress signals into a common final pathway. Mitochondrial release of

cytochrome C is a common event in apoptosis and triggers a final caspase-dependent apoptosis cascade resulting in cellular fragmentation [57,58]. Depending on the balance between the production and removal by antioxidant systems, reactive oxygen species (ROS) may function as signalling molecules to induce damage to mitochondria and disrupt cellular function and integrity, finally resulting in apoptosis [59]. ROS includes the superoxide anion ($O_2\bullet^-$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (OH●) [60]. Necrosis represents a form of cell death with simultaneous disruption of multiple pathways and is accompanied by complete release of cellular constituents into the extracellular space, which can initiate an inflammatory response. The mode of cell death by toxic stimuli, e.g. ROS, seems to be concentration dependent, with low concentrations inducing apoptosis and high concentrations necrosis [53].

Regulation of Copper Homeostasis

Copper, an essential micronutrient, plays an important function as a cofactor for a number of cellular processes [61]. Copper homeostasis is regulated by copper uptake in the gastro-intestinal tract, distribution through the body, and excretion mainly into the bile. At the cellular level, the copper transporters 1 and 2 (CTR1,2) regulate the intake, after which the distribution in the cell is mediated via the so called copper chaperones (COX17, CCS, ATOX1). ATOX1 delivers copper to the copper transporting ATPases (ATP7A (Menkes disease gene) and ATP7B (Wilson's disease gene)) in the secretory pathway, CCS distributes copper to Cu/Zn superoxide dismutase (SOD1), and COX17 delivers copper to cytochrome c oxidase in the mitochondria. Copper efflux occurs via the copper ATPase pumps encoded by ATP7A and ATP7B. Both of these proteins exhibit copper-induced trafficking and redistribution in response to changes in copper abundance [62,63]. In intestinal epithelial cells, copper is transported across the basolateral membrane by ATP7A, where it is transported via the portal circulation to the liver. Excess liver copper is removed by biliary excretion via the ATP7B copper pump and ATP7B deficiency results in a loss of biliary excretion of copper as well as a lack of copper incorporation into secreted proteins produced in the liver, with ceruloplasmin (CP) being the most notable example [64]. CP is a metalloprotein that binds copper during synthesis and is secreted into the blood. In plasma 90% of copper is bound to CP. Free intracellular copper is sequestered by metallothioneins. Metallothionein (MT1A) is known to be regulated by intracellular copper levels [65]. MT1A mRNA as well as protein concentrations are known to increase after acute copper administration [66]. Spee et al. found MT1A mRNA concentrations to be significantly decreased in dogs with primary copper toxicosis [67].

Free copper is highly toxic due to its ability to generate hydroxyl radicals and high hepatic levels of copper induce oxidative stress [59]. Recent data suggest that both hepatocellular necrosis and apoptosis may be triggered by copper-induced cell damage [68,69].

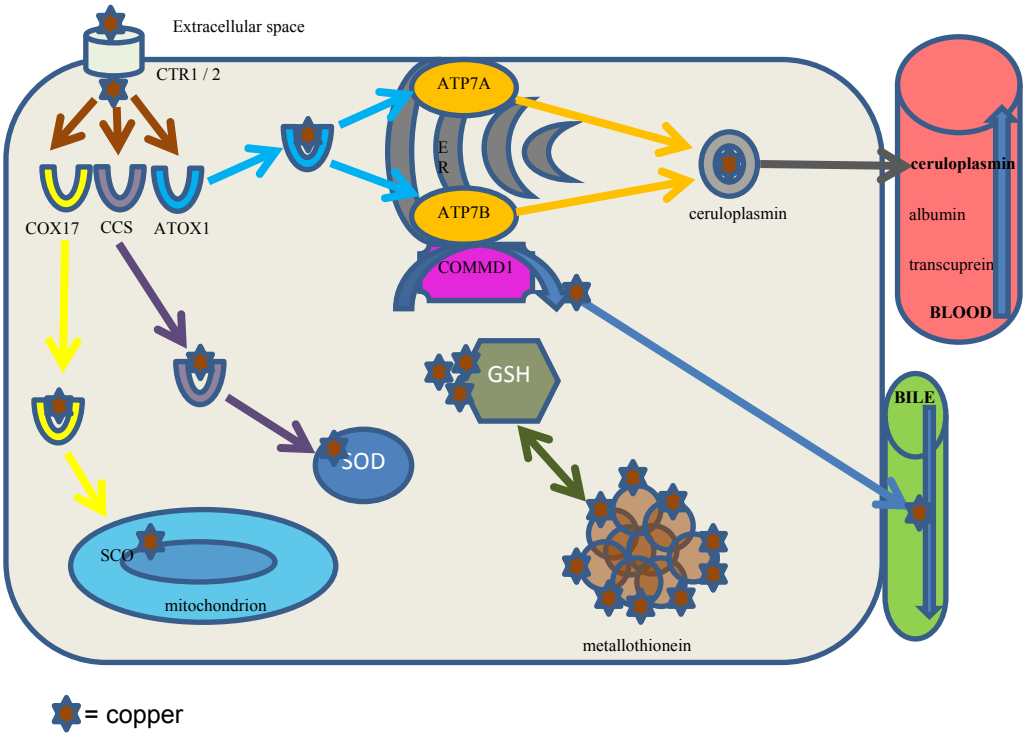

Figure 3: copper trafficking within the cell
Copper enters the cell via copper transporters, after which the distribution in the cell is mediated via the copper chaperones to the endoplasmatic reticulum (ER), mitochondrion or metallothionein. Copper efflux to bile or blood occurs via the copper ATPase pumps.
CRT: copper transporter; COX17 (cytochrome c oxidase assembly protein), CCS (copper transporter for superoxide dismutase), ATOX1 (anti-oxidant protein 1), SCO: target-specific copper transporters; SOD: superoxide dismutase; COMMD1: copper metabolism murr1 domain containing protein 1; GSH: glutathione; ATP7A: Menkes disease protein; ATP7B: Wilson's disease protein.

Oxidative stress

Free copper ions are capable to form hydroxyl radicals via the *Haber-Weiss* reaction [70]. The final outcome of this reaction is a toxic hydroxyl radical which can damage DNA, proteins, and lipids and can create other ROS [60].

$O_2\bullet^- + Cu^{2+} \rightarrow O_2 + Cu^+$

$Cu^+ + H_2O_2 \rightarrow O_2 + OH^- + OH\bullet$

$O_2\bullet^- + H_2O_2 \rightarrow O_2 + OH^- + OH\bullet$

To be protected against these toxic radicals, cells possess several important proteins and molecules involved in the defence against oxidative stress. Most of the antioxidants can be grouped into either enzymatic defences or non-enzymatic defences [71].

The non-enzymatic defences

Glutathione (GSH) is part of the cellular non-enzymatic antioxidant system, which also includes vitamins C and E, caretenoids and flavonoids. GSH is present in high concentrations in the cytosol, nucleus and mitochondria and is very important in the protection against oxidative damage, both by direct reaction with ROS and as an electron donor for peroxidases [59]. GSH is oxidized to GSSG by ROS and glutathione peroxidise (GPX). Glutathione Reductase (GR) continually recycles GSSG back to GSH [72], keeping the GSH/GSSG ratio high and under normal circumstance 99% of GSH is reduced [73].

The enzymatic defences: Cu-Zn Superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX)

The superoxide dismutase family, SOD-1 (Cu/ZnSOD), -2 (MnSOD), and 3 (ECSOD), is specialized in eliminating $O_2\bullet^-$ from external sources and those produced within the mitochondrial matrix as byproducts of oxygen metabolism through the electron transport chain [74]. SOD is a metalloenzyme, essential for the dismutation of $O_2\bullet^-$ to H_2O_2 in the cytosol:

$O_2\bullet^- + O_2\bullet^- + 2H^+ \rightarrow H_2O_2 + O_2$ (SOD)

Regulation of SOD genes plays an important role in balancing the concentration of ROS. Diverse transcriptional factors like Nuclear Factor-KappaB (NK-κB), Activator Protein 1 and 2 (AP-1 and AP-2), and Specificity Protein 1 (Sp1) have been shown to play important roles in regulating expression levels of SOD [75].

In most mammalian cell types H_2O_2 is broken down by catalase and GPX:

$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$ (catalase)

H_2O_2 + 2GSH → 2H_2O + GSSG (GPX)

Copper-associated liver diseases

Hepatic copper accumulation can result from increased copper intake, inherited metabolic defects in the hepatic copper metabolism or from a reduced biliary excretion of copper. In dogs with inherited metabolic copper storage diseases, copper always starts to accumulate in the centrolobular area, which differs from secondary copper loading due to cholestasis or increased intake which is localized to the periportal area [76-78].

Wilson's disease in man

Wilson's disease (WD) is an autosomal recessive inherited copper storage disorder which was first described in 1912 [79,80]. The diseased appeared to be caused by mutations or reduced expression of the ATP7B gene. An impaired function of ATP7B reduces the excretion of copper into the bile or incorporation into CP [81]. In the blood CP concentration is reduced and the non-CP copper is increased [80]. The therapeutic options of patients with WD include medical treatment, dietary restrictions and orthotopic liver transplantation (OLT). Pharmacological treatments for WD include chelating agents (penicillamine, trientine) and zinc salts [82,83]. OLT is reserved for patients with end stage liver failure. After successful OLT patients require no further therapy specific to WD. The prognosis for patients who comply with pharmacotherapy is excellent, even if chronic hepatitis or cirrhosis is present at the time of diagnosis [84]. Although the affected gene for WD is known, and progress has been made into the molecular events leading to WD [10] further molecular characterization is feasible and necessary. Several inbred rodent strains are available to study the consequences and to dissect the involved disease mechanisms of copper overload in the liver [12-15].

The toxic milk (tx) mouse and the Jackson toxic (tx^j) mouse

There are two versions of tx mouse (toxic milk and the Jackson toxic milk mouse), both having missense mutations in *ATP7B* that do not disrupt ATP7B synthesis but affect the function of the protein [13-15]. The tx mouse, although an accepted animal model for WD, shows differences with WD besides clear similarities: liver morphology of adult tx mice show significant differences form WD livers and neurological defects have not been observed [13, 85,86].

The Long-Evans Cinnamon (LEC) rat

LEC rats have a naturally occurring deletion in the *ATP7B* gene, resulting in the loss of the protein and they share many clinical and biochemical features with WD, but neurological

defects have not been observed [12,85,86]. Hepatic copper accumulation occurs prior to the development of hepatitis and hepatitis can be prevented by treatment with penicillamine. When left untreated they develop chronic hepatitis and often progress into hepatocellular carcinoma [87,88].

ATP7B$^{-/-}$ mice

Huster et al. have evaluated *ATP7B$^{-/-}$* mice, genetically engineered by targeted inactivation of the WD gene (spliced mRNA is present but the ATP7B protein is not produced), as a model for analysis for copper toxicity in the liver [89-91]. They also compared these mice with tx mice and LEC rats and found besides clear similarities also differences: LEC rats developed carcinomas which was not observed in the *ATP7B$^{-/-}$* mice and tx mice had milder liver pathology compared with the *ATP7B$^{-/-}$* mice [90]. A remarkable finding was the time-dependent decrease in hepatic copper content, most likely due to age-dependent up-regulation of copper-handling mechanisms independent of ATP7B [89]. Overall it was concluded that *ATP7B$^{-/-}$* mice represent a valuable model to study hepatic WD [92].

Canine copper-associated chronic hepatitis

Canine chronic hepatitis (CH) is a complex disease which occurs frequently in dogs [93]. Clinical signs can develop at any age, and the history of a CH patient indicates illness present for several weeks to months. It is morphologically characterized by fibrosis, hepatocellular necrosis and apoptosis, a mononuclear or mixed inflammatory cell infiltrate, ductular proliferation and eventually nodular regeneration and cirrhosis. Copper storage is a well known cause for the development of CH in dogs. For the diagnosis of copper-associated CH (CACH), a histochemical rubeanic acid copper stain (graded from 0-5) demonstrating semi-quantitative copper grades of three or more in liver, is sufficient [67,78]. The treatment of CACH patients exists of either reduced intake of copper via the diet or by oral zinc administration, or increased copper excretion from the body using a copper chelating agent, e.g. penicillamine, or a combination of both. For only one dog breed, the Bedlington Terrier (BT), the genetic defect has been found [16]. In this breed a lack of exon-2 of the COMMD1 gene is correlated with copper toxicosis. The precise role of this protein is not clear yet, but increased evidence indicates the involvement of COMMD1 in cellular trafficking and ubiquitination processes [94]. An increased number of breeds have been discovered with primary copper toxicosis. Hoffmann et al. demonstrated a form of CACH in the Labrador Retriever [95] and a retrospective review of CH cases referred to our university clinic revealed that one third of the CH cases was copper related. In addition, primary copper toxicosis was overrepresented in English and

American cocker spaniels, Cavalier King Charles spaniels, Labrador and Golden retrievers, West Highland white terriers, and German pointers [93].

Functional aspects of COMMD1

COMMD1 is the founding member of a recently discovered family of COMMD. This protein family consists of ten members which are widely conserved throughout evolution and share certain functional properties [96]. COMMD1 stands for Copper Metabolism MURR1 Domain protein1 and plays an important role in the regulation of ATP7B, NF-κB, and delta epithelial sodium channels [94,97,98]. In addition, COMMD1 restricts HIV-1 replication in resting T lymphocytes by inhibiting basal and cytokine-stimulated NF-κB activity [99]. COMMD1 was shown to bind copper *in vitro*, but whether this binding represents an *in vivo* property is uncertain [100]. A role of COMMD1 in copper export was demonstrated by an increased retention of copper in different cell cultures in which COMMD1 was down regulated with small interfering RNA [101,102]. However, the precise function of COMMD and the mechanism through which COMMD1 performs its multiple roles are not yet understood. COMMD1 appears to be involved in the proteolysis of ATP7B [96] and Maine et al. demonstrated that XIAP (X-linked IAP (inhibitor of apoptosis)) functions as the ubiquitin ligase of COMMD1 [103]. Van den Berghe et al. demonstrated that ATP7B protein misfolding leads to the induction of an increased COMMD1 binding, consistent with a proposed general role of COMMD1 in protein folding, maturation, and degradation [104]. Burkhead et al. proposed COMMD1 as a scaffold protein in a distinct sub-compartment of the endocytic pathway and that it functions as a regulator of structurally unrelated membrane transporters [94]. Weiss et al. concluded that the COMMD1 protein plays a role in the copper excretion pathway but is not involved in the copper mediated translocation of ATP7B [105]. Vonk et al. showed COMMD1 to be a regulator of SOD maturation and activity and might be involved in the defense against toxic superoxide anions [106]. To understand the *in vivo* role of COMMD1 van der Sluis et al. created *COMMD1* knockout mice, but the mutation was embryonically lethal [107]. Gene expression analysis revealed that the HIF (hypoxia inducible factor) pathway was up regulated when COMMD1 was deficient. It was unclear, however, whether this was a direct effect of lack of COMMD1 or due to changes in the placental development. In another paper, van der Sluis et al. demonstrated that COMMD1 promotes the proteolysis of HIF-1α [108]. Overall, the COMMD1 protein seems to be a kind of traffic agent in escorting target proteins to the proteasome for further breakdown or incorporation in (apical) membranes.

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Part

1

Canine hepatitis: the veterinary clinic

Chapter

3

Idiopathic hepatitis and cirrhosis in dogs

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Adapted from *Vet Clin North Am Small Anim Pract* 2009; 39: 481-488

INCIDENCE AND PATHOGENESIS

Primary hepatitis (PH) is the most frequent occurring liver disease in dogs and should be distinguished from non-specific reactive hepatitis (NSRH). A previous study demonstrated that 1% of all referred patients presented to our university clinic had a form with primary canine hepatitis. In contrast to human hepatology, the diagnosis of canine hepatitis is mainly based on histological morphology, and the term hepatitis is often used regardless of the etiology. Regularly encountered forms of PH in dogs include acute (AH) and chronic hepatitis (CH, with or without cirrhosis); less frequently encountered are lobular dissecting (LDH), granulomatous (GH), and eosinophilic hepatitis (EH). For each of these forms the World Small Animal Veterinary Association (WSAVA) Liver Standardization Group has published standards for the diagnosis [1]. From 101 cases with PH referred to our clinic (2002-2006, Department of Clinical Sciences for Companion Animals, University Utrecht), 21 had AH (21%) from which al least 5 became CH at a later date, 67 CH (66%), 7 LDH (7%), 1 GH (1%) and 1 EH (1%). From the CH cases, after reevaluation of the biopsies with a copper staining, 36% appeared to be copper-associated (CH(ca)), which was to our surprise much higher than expected and 64% of the CH cases had an unknown cause and where considered as idiopathic (CH(i)). From dogs with CH(i) and CH(ca) about 50% had cirrhosis at initial diagnosis and both groups contained a relatively large number of female Labrador Retriever dogs (7 and 5, respectively).

In different publications and case reports a wide variety of causes for hepatopathy in general has been documented, including micro-organisms, toxins and drugs, immune mediated reactions, and breed associated metabolic errors [2]. Especially the inherited disorders of copper metabolism received much attention the last few decades [3-9]. However, in spite of large effort the majority of PH cases remain of idiopathic origin. Although hepatitis in dogs has been extensively characterized, there are neither data published on the occurrence of the various WSAVA classified forms of hepatitis in a clinical population, on progression between those forms, nor on the occurrence of idiopathic and copper-associated forms of hepatitis [10-12].

ACUTE HEPATITIS

In the field, probably most cases of acute hepatitis will be missed. These dogs are ill for a couple of days after which they will recover spontaneously with or without supportive care without knowing what has happened. The most aggressive form of AH, fulminant hepatitis, is rapidly progressive within hours or days. In our referral clinic these patients are not often seen (alive), probably due to the fact that the time to get to the clinic via the referring veterinarian is too long. CAV-1 is a known cause for the development of acute,

sometimes fulminant hepatitis. Because of vaccination AH caused by CAV-1 has been effectively controlled and practically eliminated from the domestic dog population.

We have the impression that, although initially diagnosed as acute, some cases remain acute for months on histopathology and might be considered, although fibrosis is lacking, as a more or less chronic hepatitis. This form of hepatitis, although not a WSAVA classified form, might be considered as a more or less subacute hepatitis (SAH). When starting with prednisone treatment of SAH in the clinically chronic stage, it does not seem to respond, neither clinically nor histopathologically.

Twenty-five % of the dogs diagnosed with AH were copper-associated. Hepatitis due to primary copper accumulation starts somewhere in the process of developing hepatitis and depending on the stage when the diagnosis is made can be either acute or chronic. This implies that even when we are dealing with a dog with hepatitis with a history of sudden onset, suggesting an acute inflammation, that copper still might be the cause and it is advisable to ask for a routine copper staining in this type of patients. From 21 dogs initially diagnosed with AH, at least 5 became CH when a second liver biopsy was taken six weeks later.

CHRONIC HEPATITIS AND CIRRHOSIS

CH is a frequently occurring disease in dogs. Approximately 2/3 of the patients with primary hepatitis referred to our university clinic had CH at initial diagnosis and several more patients with acute hepatitis histologically progressed to CH in due time. In humans, the diagnosis of CH is based on both patients' history and the results of histopathological examination of liver biopsies. Since the diagnosis is etiology-based a specific etiology-focused treatment approach is possible. In contrast, the diagnosis in dogs is mainly morphology-based, which severity is based on the type and distribution of inflammatory cells, hepatocellular apoptosis and necrosis, and the abundance and localization of fibrosis [1]. In most cases there is no evidence for an etiology, resulting in a large proportion of idiopathic chronic hepatitis (CH(i)) cases [2]. This poor understanding of the etiology of CH(i) results in limited options for adequate treatment and also in variable results. Although probably not the initial cause, oxidative stress plays an important role in the maintenance and progression of disease and for this reason antioxidants might play a beneficial role in the treatment of at least chronic hepatitis. For decades canine CH(i) patients are treated mainly with orally administered immunosuppressive medication, of which prednisone is most commonly applied. The efficacy of prednisone has been described in one publication [10]. In this retrospective study a prolonged survival time was demonstrated for prednisone treated canine CH patients upon comparison with untreated patients. These results indicate a positive long-term effect of prednisone in the treatment of

CH(i). Besides anti-inflammatory effects corticosteroids also have (weak) anti-fibrotic properties. A retrospective histopathologic evaluation of the inflammatory activity and fibrosis formation in dogs with CH(i) before and after a six-week treatment with prednisone, although not double-blind, placebo-controlled, revealed a reduced inflammatory activity and a stable fibrotic situation, the latter suggesting a fibrosis inhibitory effect of prednisone (Poldervaart, in preparation).

CIRRHOSIS

Fibrosis, a hallmark of CH, is defined as detectable deposit of extracellular matrix (ECM). Cirrhosis is the end-stage of chronic hepatitis and is defined as a diffuse process characterized by fibrosis of the liver and the conversion of normal liver architecture into structurally abnormal nodules, micro- or macronodular [1]. Cirrhosis is the result of an accumulation of ECM materials, which is the resultant of increased synthesis and/or decreased breakdown. The bulk of the ECM in the fibrotic liver is produced by myofibroblast (MF)-like cells. Three different MF-like cells have been described based on location and immunohistochemical profile [13]. These comprise portal or septal MF, interface MF, and perisinusoidally located hepatic stellate cells (HSC). HSCs are non-parenchymal, quiescent cells that are activated by hepatic injury and produce most of the factors that lead to hepatic fibrosis. One of the most important of these factors is Transforming Growth Factor (TGF-β), which acts via its two receptors TGF-β receptor type I (TGF-β RI) and TGF-β receptor type II (TGF-β RII) at the cell surface and the intracellular substrates, the Smad proteins. TGF-β stimulates fibrosis by inducing the up regulation and the release of many of the ECM components (collagens, glycoaminoglycans) as well as inhibitors of the metalloproteinases, preventing the breakdown of the ECM.

Important regulators of ECM turnover and breakdown are plasminogen activator-plasmin system components. Urokinase-type plasminogen activator (uPA) generates plasmin from circulating plasminogen by proteolytic cleavage. This plasmin is capable of degrading ECM components directly by proteolysis, and indirectly by inhibiting deposition of ECM by activation of matrix metalloproteinases (MMPs). In this way, an upregulation of uPA in the liver might inhibit the deposition of ECM and reverse hepatic fibrosis. Overall, matrix remodelling is an important component of liver regeneration.

Cirrhosis is considered to be irreversible and the point at which this happens has not well been defined. Up till now no anti-fibrotic therapy is clinically available and in man liver transplantation remains the only treatment option in case of hepatic dysfunction due to cirrhosis. Recently some evidence is coming up that the process of fibrogenesis might

be reversible [14,15], opening possibilities for evaluation of newly designed anti-fibrotic therapies.

LOBULAR DISSECTING HEPATITIS

Dogs with LDH are clinically more acute, but on histopathology diagnosed having a chronic hepatitis with cirrhosis based on a massive deposition of fibrous tissue around individual or small groups of hepatocytes. The cause of this form of hepatitis is unknown. In most cases, patients with LDH are young animals (average age of 2.3 years in our clinic) and die shortly after diagnosis, with an estimated median survival time (ESMT) of 0.7±01 months (n=7, 2002-2006), despite treatment, mostly consisting of prednisone and sometimes diuretics because of the development of severe ascites due to portal hypertension.

NON SPECIFIC REACTIVE HEPATITIS

NSRH presents the a-specific response to extrahepatic disease processes, especially inflammation somewhere in the splanchnic bed (gastro-intestinal tract, pancreas) or a systemic illness with fever. It can also be found as a residual lesion of a previous inflammatory primary intrahepatic disease. NSRH, which is a secondary problem, does not have to be treated. It is essential to look for the primary cause (GI tract, systemic illnesses) and to treat this underlying cause.

CLINICAL PRESENTATION

Patients with PH seen in our clinic will present in most cases with non-specific clinical signs. The most noticed symptoms mentioned by the owners are decreased appetite (50%), vomiting (48%), PU/PD (47%), reduced activity (39%), weight loss (28%), jaundice (24%), diarrhea (23%), and abdominal distension (21%). Episodic neurologic symptoms due to hepatic encephalopathy are rarely mentioned. Acholic faeces, a specific indicator for extrahepatic biliary obstruction, is not seen in cases of primary hepatitis. Depending on the form of hepatitis present, signs can be obvious from hours (acute fulminant hepatitis) to months (chronic hepatitis).

DIAGNOSTIC EVALUATION

Physical examination

At general appearance dogs with CH, but not with AH, will have a slight to moderate muscle atrophy. Dogs with ascites due to portal hypertension will show abdominal distension.

Physical examination related to liver diseases concentrates on the mucous membranes and abdominal palpation. In case of hepatomegaly, examination of the circulation is indicated to detect or exclude cardiac disease. The mucous membranes are normal in most cases of patients with liver disease. Abnormalities may include icterus, pallor, and indications for coagulopathy. Very pale mucous membranes in the presence of icterus indicates that the liver dysfunction is secondary to haemolytic anemia and a further work-up should focus on this problem. Petechiae, an indicator for thrombocytopenia or thrombocytopathy, although rarely seen in patients with PH, can be found due to disseminated intravascular coagulopathy (DIC).

Hepatomegaly as a finding at abdominal palpation is rare in dogs with PH. Ascites, resulting in abdominal distention, due to portal hypertension and/or hypoproteinaemia may also be an indication for liver disease, but be aware of the fact that there are many diseases of other than hepatic origin which may induce ascites formation. Abdominal palpation may also reveal splenomegaly in case of portal hypertension.

In most dogs with hepatitis, physical examination reveals no specific information. Therefore, in the majority of cases with symptoms as described above, laboratory investigation is required to detect or exclude a liver disease.

Blood

Blood work can be used for 2 reasons. First, in order to detect or to exclude a (primary) liver problem. Second, to screen for the overall status of the patient. For answering the first part, blood work should consist of at least serum bile acids, AP, and ALT. In almost all cases of primary hepatitis one of these parameters will be out of the upper reference range. Liver enzymes are indicators for cellular damage, whereas bile acids are a functional parameter. Of the liver enzymes, ALT will be the first to increase when a primary hepatitis is present. Adding more (liver) enzymes to the diagnostic panel will not give more information. When a patient with jaundice presents to your clinic, this part of the blood work up is not necessary. For the second part a CBC, a biochemistry and a coagulation profile are necessary. The biochemistry profile should include at least, besides the serum bile acids, AP, and ALT, urea, creatinine, total protein, albumin, sodium, potassium. Because for further work-up liver biopsies are needed, also a coagulation

profile has to be determined. It should be performed shortly before the biopsy procedure because coagulation parameters may change quickly in patients with hepatitis (inadequate vitamin K absorption, reduced production of coagulation factors or increased consumption (DIC)). In our clinic we measure prothrombin time (PT), activated thromboplastin time (APTT), fibrinogen, and platelet count. We have experienced that especially the fibrinogen concentration is a critical indicator and that a concentration < 1g/l is a contraindication for taking a liver biopsy, which happens in about 8% of the cases with PH. This lowered fibrinogen concentration increases in more than 90 % of these cases above the critical level after a one-week treatment with prednisone/prednisolone so that a liver biopsy can be safely taken at that time.

There is some debate on what type of liver function tests to use for a functional evalutation. World wide the serum bile acid tolerance test (comparison of pre- and post prandial serum bile acids) is commonly used. A major reason for this is that it is easily accessible for private clinics because samples can be sent to laboratories for measurement. The serum bile acid tolerance test does not give much additional information regarding the liver function above only pre-prandial serum bile acid concentration and for screening for portosystemic shunting determination of the basal plasma NH_3 concentration is a better test [16]. When the basal plasma NH_3 measurement is not informative, the rectally applied NH_3 tolerance test will confirm or exclude the presence of portosystemic shunting. Measurement of plasma NH_3, which should be done immediately after sampling can be more problematic in private clinics, although in recent years equipment for ammonia measurement has become more accessible for private clinics.

Ultrasound

For a further diagnostic work-up ultrasound is needed. With ultrasonography the liver parenchyma, gallbladder / biliary tree, the portal vein, acquired shunting, when present, and ascites can be evaluated. In a recently performed evaluation of patients with PH in 20% of the cases no abnormalities were found at abdominal ultrasonography. In about 25% of the cases the liver was enlarged, irrespective of the type of hepatitis. Ascites, in case of liver disease a result of portal hypertension, often combined with a slightly to moderate decreased plasma albumin concentration, was found mainly in dogs with CH and LDH. In the cases where ascites due to liver failure can be seen, there is also a high change of finding acquired portosystemic collaterals. The best place to look for is the region caudal to the left kidney. The finding of enlarged portal lymph nodes, ascites and/or a decreased liver size has a negative prognostic value.

Last but not least, ultrasonography is necessary for the guidance of taking liver biopsies with True-cut needles.

CT/MRI

For a further work-up of a patient with hepatitis CT or MRI is normally not necessary. In cases where a primary liver tumour is suspected based on ultrasonography and a surgical intervention is needed, a pre-operative screening with CT or MRI with contrast is very helpful in estimating the size and localization of the tumour and visualizing the presence of tumour metastasis.

Liver biopsy: pathology

A liver biopsy is considered to be the gold standard for establishing a diagnosis of PH and to differentiate, when necessary, PH from NRSH. Fine needle aspiration (FNA) is not sufficient for diagnosing any form of hepatitis (primary or secondary). Liver biopsies can either be taken ultrasound guided with a True-cut needle or blind by aspiration using a syringe attached to the needle (Menghini technique). At least 2 or more samples are advisable in order to minimise sampling errors. As we noticed that about 1/3 of the PH referred to our clinic are copper-associated, we advocate routine staining for copper, e.g. with rubeanic acid, besides HE staining, as a standard procedure for liver histology in dogs.

TREATMENT AND PROGNOSIS

Most cases of idiopathic AH do not need treatment, but depending on the severity of vomiting and presence of dehydration, anti-emetic treatment and fluid therapy are indicated. Most dogs with AH will recover after several days without medical interference. However, progression from (initial) acute hepatitis to its chronic counterpart may occur, resulting in recurrence of clinical signs [2]. It is advisable to repeat the liver biopsy 6 to 8 weeks after the initial diagnosis to control if the hepatitis has been solved or has progressed to CH. Idiopathic CH is treated as an immune mediated disease with oral submission of prednisone or prednisolone, combined with supportive therapy (e.g. anti-emetics, diuretics, fluid therapy, and dietary adjustments). As mentioned earlier, only one publication (a retrospective evaluation) is available on the efficacy of prednisone in the treatment of CH in dogs, which showed a prolonged survival time for dogs with CH when treated with prednisone (0.6-1.1 mg/kg/day) [10]. The response to prednisone therapy is controlled on a regular basis by liver biopsy, mostly at a six-week interval, and therapy is continued until histologically no hepatocellular death and inflammation is observed. In humans the application of glucocorticoid treatment is indicated in both alcohol-induced

cirrhosis and auto-immune hepatitis, in contrast to virally induced hepatitis, where it is contra-indicated. Histological similarities between human virally induced hepatitis and canine CH could indicate a reversed effect of prednisone efficacy. The majority of dogs with CH(i) referred to our clinic (2002-2006, n=36) treated with prednisone (1 mg/kg/day), initially aiming at a 6 week treatment period showed an estimated median survival time (MST) of 9.9 months. When only the CH(i) with cirrhosis cases treated with prednisone (n=19) were included the MST was 1.3 months, stressing the fact that the presence of cirrhosis is a strong negative prognostic indicator. In the past, when unacceptable side effects (extreme PU/PD, severely increased appetite, reduced exercise tolerance) due to prednisone/prednisolone medication occurred, we tapered the dosage of prednisone/prednisolone and started a combined therapy with azathioprine (1mg/kg/day) for 6 weeks. But due to increased awareness of toxic side effects of cytostatic drugs in households (young children, pregnant women) and in the treatment of CH(i) no proven benefit, we do not advocate this combination therapy any longer.

Other proposed medicinal options, mainly based on extrapolated human data and personal experiences, for treatment of CH(i) other than immunosuppressive medication are ursodeoxycholic acid (UDCA) (7.5 mg/kg BID), anti-oxidants like S-Adenosyl-L-Methionine (SAM-e) (10 mg/kg BID) (17), silymarin (100-200 mg/dog single oral administration), vitamin E (100-400 IU/day), and the antifibrotic drug colchicine (0.025 mg/kg/day). UDCA is a synthetic non-toxic hydrophilic bile acid that provides a couple of positive actions. First, it enhances the bile flow and in this way it stimulates the excretion of inflammatory products. Second, UDCA decreases by dilution the concentration of the endogenous more toxic bile acids. Third, it modulates the immune system, resulting in a reduction of the immune response and fourth, there is proof that UDCA has anti-oxidative properties. In our clinic we recently started a trial with UDCA to evaluate if this drug might be a fair alternative for prednisone as a treatment of CH(i). SAM-e is a natural metabolite in hepatocytes and is a precursor of glutathione (GSH). It is important in the defence against oxidative stress and exhaustion might occur due to exposure to toxic substances in patients with CH. Silymarin appears to be a strong free-radical scavenger by increasing cellular levels of superoxidedismutase (SOD), important in enzymatic defences against oxidative stress, it regulates cell membrane permeability and it has been shown to inhibit leukotriene synthesis and the effects of tumour necrosis factor (TNF)-α. Evidence from many human and veterinary reports underlines the protective effects of silymarin in patients with mushroom or acetaminophen intoxications [18,19]. Vitamin E is a nutritional antioxidant that protects against different routes of membrane peroxidation.

Colchicine has been proposed for treating chronic hepatic fibrosis presumably by decreasing the formation and increasing the breakdown of collagen, but benefit is

unproven and there is very little experience with colchicine in dogs. It can be a very toxic drug after relatively small overdoses. It is not advisable to use this drug in dogs until it has been proven effective.

Many of the above mentioned medications are generally accepted and clinically used for the treatment of liver diseases, mostly as part of a multidrug therapy. Unfortunately, up till now for most of these drugs critical scientific evaluation of their effectiveness is lacking.

If there is clinical evidence of portal hypertension (ascites, hepatic encephalopathy) a treatment with spironolactone, a potassium sparing diuretic (1-2 mg/kg bid), lactulose (0.5 ml/kg BID-TID) and dietary adjustments (high quality protein diets, moderately restricted) can be started. Spironolactone is preferred above furosemide due to the underlying pathophysiology of portal hypertension in which the renin-angiotensin-aldosterone system is activated. In case of severe ascites, a combination of spironolactone and furosemide might be very effective. Lactulose, a synthetic disaccharide fermented by colonic bacteria into short chain fatty acids, helps acidifying the colonic environment to trap NH_3 (NH_4^+) so that it remains mainly in the faeces and doesn't enter the portal circulation, reducing clinical signs of HE. When the portosystemic collaterals are optimally activated and plasma albumin concentration is above the oedema border (>15 g/l), ascites can disappear and diuretic treatment might be stopped. This activation of collaterals normally will take 2 to 3 weeks. Symptomatic treatment of gastric erosions and ulceration consists of sucralfate (1 gram po TID) and a H2 blocker (ranitidine (1mg/kg BID), famotidine, avoid cimetidine) or omeprazole (1 mg/kg once daily).

In case of CH(ca) or AH(ca), an etiology-based specific therapeutic approach is applied by feeding a low-copper diet, submission of a copper-chelator (e.g. D-penicillamine (10-15 mg/kg BID)), or submission of exogenous zinc (10 mg elemental zinc/kg BID). Penicillamine has, besides metal chelating properties, also an immunomodulatory effect and it possesses antifibrotic activity via inhibition of collagen cross linking, causing collagen to be more susceptible to degradation.

CONCLUSION

It can be stated that the poor understanding of the etiology of PH and especially CH(i) results in limited options for adequate treatment and also in variable results. Elucidating the etiologies, besides the copper-associated form of hepatitis is of utmost importance in order to find etiology-based treatments for canine (chronic) hepatitis, when possible, most likely resulting in a better prognosis. The prognosis for patients with CH(i) which have

developed cirrhosis is very poor. As a large part of AH and CH cases is concluded to be copper-associated (25%-30%) it is advisable to ask for a copper staining (e.g. rubeanic acid) besides routine HE staining when sending liver materials to a pathology department, otherwise this diagnosis can be missed and the patient doesn't get the appropriate treatment with copper-binding agents.

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Chapter

4

Primary hepatitis in dogs: a retrospective review (2002-2006)

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Adapted from *the Journal of Veterinary Internal Medicine* 2009; 23: 72-80

Abstract

Background: Little is known about etiology, disease progression, treatment outcome, survival time, and factors affecting prognosis in dogs with primary hepatitis. Objectives: To retrospectively review different forms of hepatitis in a referral population, using the WSAVA standardization criteria.
Animals: 101 dogs examined for histologically confirmed primary hepatitis between 2002-2006. Dogs with nonspecific reactive hepatitis were excluded.
Methods: Retrospective study. Medical records were reviewed for prevalence, signalment, clinical and clinicopathological manifestation, outcome, survival time, and prognostic factors for shortened survival.
Results: Primary hepatitis occurred in 0.5% of dogs in this referral population. Acute and chronic hepatitis were diagnosed in 21 and 67 dogs, respectively. Progression from acute to chronic hepatitis occurred in 5/12 of the repeatedly sampled dogs. Chronic hepatitis was idiopathic in 43 (64%) dogs, and was associated with copper accumulation in 24 (36%) dogs. Median survival time was longer in dogs with acute hepatitis than in dogs with chronic hepatitis (either idiopathic or copper-associated), and dogs with lobular dissecting hepatitis had the shortest survival time. Prognostic factors predicting shortened survival were associated with decompensated liver function and cirrhosis at initial examination.
Conclusions and clinical importance: The majority of primary hepatitis in dogs is chronic hepatitis. Previous studies appear to have underestimated the etiologic role of copper in both acute and chronic hepatitis. Prognosis is reduced in dogs with hepatic cirrhosis or cirrhosis-related clinical findings. Further research into etiology and treatment effectiveness in all primary hepatitis forms is needed.

Introduction

Primary hepatitis (PH) is the most frequently occurring group of liver diseases in dogs, and comprises all inflammatory hepatic diseases that are not characterized by nonspecific changes, as observed in nonspecific reactive hepatitis [1]. In contrast to humans, the diagnosis of hepatitis in dogs is mainly based on histological morphology, and the term is often used regardless of the etiology. Regularly encountered forms of PH in dogs include acute (AH) and chronic hepatitis (CH, with or without cirrhosis); less frequently encountered are lobular dissecting (LDH), granulomatous (GH), and eosinophilic hepatitis (EH). The World Small Animal Veterinary Association (WSAVA) Liver Standardization Group has published standards for the diagnosis for each of these forms [1].

A range of causes of hepatopathy have been documented in different publications and case reports, including micro-organisms [2-14], toxins and drugs [15-22], immune mediated reactions [23-26], and breed associated metabolic errors [27-29]. The inherited disorders of copper metabolism received particular attention in the last few decades [30-35]. In spite of significant research efforts, the causes remain elusive [36]. No published data are available on (1) the occurrence of the various forms of the disease in a clinical population, (2) its progression from acute to chronic forms, (3) the occurrence of idiopathic and copper-associated forms of hepatitis, nor (4) on survival and prognostic factors for all forms of PH, including AH, CH, and LDH [37-39].

The purpose of this study was four-fold. First to describe different forms of hepatitis in our clinical referral population, including clinical, laboratory and pathological findings. Secondly, to assess the frequency of copper accumulation as a potential cause in all these forms of hepatitis. Thirdly, to evaluate progression of acute to chronic hepatitis, and of chronic hepatitis to cirrhosis. And fourthly, to assess the clinical outcome and survival time after diagnosis, and prognostic factors for shortened survival time.

Material and Methods

Study design – Retrospective review of medical records.
Dogs – All dogs in this study were referred between 2002-2006 to the Department of Clinical Sciences of Companion Animals, University of Utrecht, the Netherlands. The dogs were identified from the records of the diagnostic pathology service at the Faculty of Veterinary Medicine. Included were dogs with a form of histology-proven primary hepatitis as described by the WSAVA liver standardization group [1]. Excluded were dogs if they had secondary hepatic inflammation, such as nonspecific reactive hepatitis or

ischaemic injury with secondary inflammatory reactions, based on histopathology of liver biopsy samples. Data concerning signalment, medical history, clinical, laboratory and histopathological findings were obtained from the medical records. Histopathology was reviewed as described below. All procedures were approved by the university's ethical committee as required under Dutch legislation.

Histopathology – At the time of original diagnosis, at least two liver biopsy samples per occasion were obtained from each dog by percutaneous biopsy with a 14G needle (blind Menghini biopsy or true cut biopsy under ultrasonographic guidance) [40]. Histology of all identified cases was reviewed by one board-certified veterinary pathologist according to the WSAVA-criteria [1]. Slices of formalin-fixed, paraffin-embedded specimen were stained with Hematoxylin and Eosin (HE), reticulin according to Gordon and Sweet, and rubeanic acid. Several variables were evaluated semi-quantitatively, including necro-inflammatory activity and presence of apoptosis (0=none, 1=slight, 2=mild, 3=moderate, 4=marked, 5=severe), fibrosis (0=none, 1=focal, 2=bridging, 3=bridging with architectural distortion or cirrhosis), and copper content and distribution, using a previously reported copper grading system [41,42]. Copper scores above 2 are considered abnormal and a potential etiological factor [43]. Presence of steroid-induced hepatopathy (SIH) was also evaluated. Consequently, dogs were categorized into AH, CH, LDH, GH, or EH. A group of miscellaneous hepatic disorders was included, which had not been diagnosed with hepatitis initially, but progressed to CH later, as proven by repeated histopathological exams. All dogs in the AH and CH groups were judged as either idiopathic (i) or copper-associated (ca) according to the semi-quantitative evaluation for copper (copper $\leq$ or >2, respectively). No quantitative copper analysis was performed. Necropsy examination was rarely performed; thus those results are not reported.

Follow-up – Data were collected during February 2007 by telephone interviews with the owners or referring veterinarians, regarding clinical progress with or without continuation of medication. Data included clinical outcome, including residual disease, remission or recurrence of hepatitis-related clinical signs, and death, survival time after diagnosis, and the presumed cause of death, where applicable.

Evaluated variables – The variables included for description and statistical analysis are presented in *Table 1*. Not all blood variables are described for the study sample, because some values for some variables were missing.

Statistical analysis – Analysis was performed using a commercially available software package (SPSS version 15.0, Benelux BV, Gorinchem, the Netherlands). The chi-square test was performed for goodness-of-fit of the study group compared with the total canine clinical population (2002-2006) for breed and sex distribution. A one-sample Kolmogorov-Smirnov test was used to assess normality of all data. A non-parametric

Kruskal-Wallis *H* test was performed for comparison of ordinal mean values in case of three groups or more. A one-way ANOVA test with a posthoc Bonferroni correction was performed for comparison of continuous values (age and blood measurements) in case of three groups or more, and a Levine's test was used for determining the homogeneity of variances.

Table 1 *– Evaluated variables that were collected from medical records and histopathology reviews (n=101, 2002-2006).*

Data categories	Collected variables
Signalment and history	Age, sex, clinical signs and duration, medical history.
Biochemistry markers	Total alkaline phosphatase (ALP), steroid-induced alkaline phosphatase (ALP-65), alanine aminotransferase (ALT), fasting total serum bile acids (sBA), urea, creatinin, glucose, total serum protein (TP), albumin, serum ammonia (NH_3), sodium, potassium, calcium, anorganic phosphate, prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen.
Hematological markers	Hematocrit (Ht), leukocytes and differentiation, thrombocytes.
Ultrasonographic findings	Hepatic size, surface regularity, and structure regularity and echodensity; presence of portosystemic collateral shunts and ascites, gallbladder changes, and portal lymph node size.
Histopathological findings	Semi-quantitation of necro-inflammatory activity, fibrosis and cirrhosis, grading for copper content and distribution; and presence of steroid-induced hepatopathy.
Treatment	Medical therapy; type and duration.
Follow-up	Outcome after treatment (complete remission or recurrence of clinical signs, or residual disease), date of death, and presumable cause of death (clinical signs prior to death related to hepatitis or not).

The log-values of hematological and serum biochemical values were used due to non-normal distributions and frequent outliers. Survival fractions were calculated according to the Kaplan-Meier procedure. The survival time was defined as the interval between the date of initial diagnosis and the date of death. Dogs that died of hepatitis-related causes were counted as events. Dogs that died of unrelated causes or that were alive at follow-up were censored. Differences between Kaplan-Meier curves were tested for significance by a log-rank test. Prognostic factors for shortened survival time were identified by combined

analysis of survival time and variables retrieved from the medical records and histopathological reviews using Cox regression models. These variables were screened with a univariate Cox's proportional-hazard analysis to assess which variables were suitable for further analysis. All variables that were significant or approached significance ($P<0.10$) were entered into a stepwise multivariate Cox's proportional-hazard analysis with backward removal of variables (likelihood ratio). Various multivariate analyses were executed, since not all analyzed variables were available for all dogs included in the study. Those with lower numbers of cases and events were each entered separately for maximal output, and to prevent an unnecessary reduction of cases [44]. Due to small group size, variable analysis was performed for the entire study population, and not for each form of hepatitis. Descriptive and group comparative data are presented as mean ± SD (range) when normally distributed, and median (range) when not normally distributed; survival data as estimated median survival times (EMST) ± SD (95% confidence interval, 95%CI); and Cox regression analysis as hazard rates (HR), and 95%CI. All statistical tests were considered significant at the 5% level ($P<0.05$), unless stated otherwise.

Results

Prevalence and progression – 101 dogs with primary hepatitis (PH) were identified, which represented 0.5% of the entire number of first visits of our clinic population in the same period. Most frequently observed was CH (67/101), consisting of 43/67 dogs with idiopathic CH (CH(i)) and 24/67 dogs with copper-associated CH (CH(ca)) (*Table 2*). Hepatic cirrhosis was found in all chronic groups; CH(i) (24/43), CH(ca) (13/24), and LDH (7/7). Four dogs did not have an initial diagnosis of PH, but developed PH at a later stage. Therefore these cases are marked 'miscellaneous'. Excessive hepatic copper was found in 29/88 dogs with AH (5/21) and CH (24/67).

Forty-eight dogs returned to the university clinic for a repeated liver biopsy approximately six weeks after the initial biopsy. Of the 21 AH dogs, 12 returned for a second biopsy; histopathology revealed complete remission of inflammatory and degenerative changes in 6 dogs, no changes in one dog, and progression from AH to CH in 5 dogs. Of the 37 initially cirrhosis-free CH dogs, 2 showed progression to cirrhosis upon examination of follow-up biopsies. All 4 dogs diagnosed with miscellaneous hepatitis progressed to CH; one of them also developed cirrhosis.

Etiology – Of the 21 AH and 67 CH dogs, 5 and 24 – respectively – had copper scores higher than 2 (reference ≤2), and were considered copper-associated. *Leishmania*-induced

hepatic lesions were found in the repeated liver biopsy sample of one CH dog, which was therefore diagnosed with *Leishmania*-associated CH. The remaining 16 AH and 42 CH dogs were considered idiopathic. Eight dogs, mainly in the CH group, were treated with potentially hepatotoxic drugs prior to referral or for treatment of concurrent disease, including short-term administration of antibiotics (TMP/S, n=1) and NSAIDs (n=5), and chronic administration of phenobarbital (n=2); no idiosyncratic hepatic changes were found upon histopathological examination. None of the other dogs have been treated with potentially hepatotoxic drugs in the months prior to presentation. Leptospirosis was suspected in 5 dogs, but only 1 dog had high IgG titers (1:160) and normal IgM titers (1:20) upon serological examination. In the LDH and GH groups no potential etiologies were found with histology or clinical pathology. In the single dog with EH, histology revealed parasitic migrating tracks in close proximity to eosinophilic changes.

Breed, sex, and age – Forty-three purebred (n=91) and crossbred (n=10) breeds were encountered (*Table 2*). The breed distribution of the study population was significantly ($P<0.001$) different from the total clinic population. Overrepresented were English and American cocker spaniels, Labrador and Golden retrievers, West Highland white and Jack Russell terriers, and German pointers. The sex distribution in the entire study population was significantly ($0.001<P<0.01$) different from the clinic population, with an overrepresentation of females (25 intact and 43 neutered females; 22 intact and 11 castrated males). Individual forms of PH showed a sex difference in the AH(i) group ($P<0.001$), with an overrepresentation of neutered females (n=11), and in the LDH group ($P<0.025$), with an overrepresentation of intact females (n=5). Age (years, mean ± SD) was distributed as follows: AH(i) 7.8±4.1 (range, 2.4-14.5), AH(ca) 5.9±3.2 (range, 1.4-9.0), CH(i) 7.7±3.4 (range, 0.4-14.2), and CH(ca) 6.5±3.4 (range, 0.9-13.8). Mean age at presentation was lower for LDH (2.3±2.5, range, 0.5-7.2), GH (3.4), and EH (0.6). The LDH group had a significantly lower mean age in comparison with the AH and CH groups ($P<0.05$).

Clinical signs and medical history – Clinical signs and physical examination findings in the 101 dogs were lethargy (56/101), anorexia (56/101), vomiting (48/101), polyuria and polydipsia (47/101), weight loss (28/101), coffee colored urine (27/101), jaundice (24/101), diarrhea (23/101), abdominal distention (21/101), signs of hepato-encephalopathy (HE, 22/101), hepatomegaly (17/101), urinary incontinence (15/101), abdominal fluid wave (9/101), pruritis (9/101), and abdominal pain (8/101). Signs related to portal hypertension (HE and ascites) occurred only in dogs with histology-proven cirrhosis.

Table 2 *– Dog breeds and frequency of primary hepatitis of differing form.*

Dog breeds	AH(i)	AH(ca)	CH(i)	CH(ca)	LDH	GH	EH	Misc.
Airdale Terrier		1						
American Cocker Spaniel			2	1				
Bedlington Terrier		1		1				
Bernese Mountain Dog			1		1			
Border Terrier			1				1	
Boxer	1		1					
Cairn Terrier				2				
Cavalier King Charles Spaniel			1	1				
Dobermann Pincher	1			1				
German Pointer			3*					
English Cocker Spaniel			2	2	1			1
Fox Terrier	1				1			
Golden Retriever			3	1		1		
Jack Russell Terrier	3				1			
Crossbreeds	2		5	3				
Labrador Retriever	2		11	5				1
Scottish Terrier			2					
Stabyhound			1		1			
West Highland White Terrier	1		2	2				
Incidental cases in other breeds (n = 25)	5	3	8	5	2	0	0	2
Total number of dogs	16	5	43	24	7	1	1	4

* = In one dog Leishmania-associated lesions were found.

Fifty-four of all dogs had elevated liver enzymes and/or bile acid concentrations prior to referral, and chronically elevated liver enzymes were the only reason for referral in 2 dogs that were free of clinical signs. Clinical signs were present during 10.0±15.0 weeks (range, 0.5-52) for AH, 9.6±18.9 weeks (range, 0.5-104) for CH(i), 4.3±3.8 weeks (range, 0.5-14.0) for CH(ca), and 5.1±6.3 weeks (range, 1-19) for LDH. No significant difference was found between the groups for the duration of clinical signs. Twenty-five dogs had a history of concurrent disease and were treated accordingly. They presented with epilepsy (4/25), diabetes mellitus (2/25), pituitary-dependent hyperadrenocorticism (3/25), hypothyroidism (1/25), hyperthyroidism (1/25), arthritis/arthrosis (2/25), chronic kidney insufficiency (1/25), signs of chronic large or small bowel diarrhea (4/25), keratoconjunctivitis sicca

(2/25), signs of unidentified heart disease (2/25), atopic dermatitis (2/25), and pseudopregnancy (1/25).

Hematological and serum biochemistry findings – Several first consultation biochemical and hematological markers measured are summarized in *Table 3*. The markers not presented did not differ significantly between the analyzed groups. Bilirubin levels were rarely measured in the study population, and were therefore not included. Blood coagulation was abnormal in 18 dogs, resulting in postponement of their liver biopsy procedure. All of these dogs were treated with prednisone during one week, which normalized coagulation variables, permitting harvesting of biopsies. Values of all variables were statistically compared between the groups. The LDH group had a lower mean total serum protein value than the other three groups ($P<0.01$). The CH(i) group had a lower glucose concentration than dogs with CH(ca) ($P=0.016$). No significant differences were found upon comparison of the mean values above or below the reference range of each variable.

Ultrasonographical examination findings – Of the 97 dogs that were ultrasonographically examined, 2/21 AH, 15/67 CH, and 2/7 LDH cases revealed no abnormalities. Hepatic size was assessed as normal (AH–7/21; CH–26/67), enlarged (AH–8/21; CH–15/67), or too small (AH–6/21; CH–26/67). Hepatic surface was mostly assessed as normal (AH–19/21; CH–54/67). Hepatic structure was found to be either normal (AH–7/21; CH–27/67), irregular (AH–11/21; CH–31/67), or with increased echodensity (AH–3/21; CH–11/67). Nodular processes were observed mostly in chronic forms of PH (AH–2/21; CH–16/67; LDH–1/7; GH–1/1). Ascites was also predominantly found in chronic groups (AH–3/21; CH–18/67; LDH–4/7), as well as portosystemic collaterals (AH–6/67; LDH–1/7). Enlarged portal lymph nodes were infrequently observed (AH–1/21; CH–7/67; GH–1/1), as well as abnormal gallbladder walls (AH–7/21; CH–7/67; LDH–1/7).

Table 3 *— Liver-related and other selected serum biochemical and hematological markers at initial diagnosis (n=95; 2002-2006).*

				Serum biochemical markers				
Variable	**sBA**	**ALP**	**ALP-65**	**ALT**	**Glucose**	**TP**	**Albumin**	**NH_3**
RR	*<10*	*<73*	*<15% AP*	*16-69*	*4.5-5.8*	*53-66*	*28-36*	*24-45*
Unit	*µmol/l*	*U/l*	*U/l*	*U/l*	*mmol/l*	*g/l*	*g/l*	*µmol/l*
AH (total)	**13/18 (↑)**	**12/14 (↑)**	**13/13 (↑)**	**8/8 (↑)**	**2/6 (↓)**	**3/12 (↓)**	**5/12 (↓)**	**0/5 (-)**
Mean±SD	108.0±190.0	982.5±962.6	335.3±691.0	1055.5±1539.5	5.8±2.4	57.1±10.1	24.9±5.9	15.8±8.3
CH(i)	**32/35 (↑)**	**32/33 (↑)**	**26/26 (↑)**	**16/17 (↑)**	**2/8 (↓)**	**5/23 (↓)**	**15/24 (↓)**	**1/7 (↑)**
Mean±SD	75.7±79.6	660.3±728.8	238.7±450.0	403.4±340.6	4.9±1.4*	57.9±7.9	26.0±4.5	33.9±21.3
CH(ca)	**13/19 (↑)**	**15/16 (↑)**	**10/10 (↑)**	**5/5 (↑)**	**0/5 (-)**	**5/13 (↓)**	**7/14 (↓)**	**3/8 (↑)**
Mean±SD	45.6±61.7	496.1±605.0	255.9±286.8	430.0±221.6	10.4±5.1*	54.8±10.8	26.9±6.2	47.0±47.1
LDH	**6/6 (↑)**	**5/6 (↑)**	**5/5 (↑)**	**2/3 (↑)**	**0/0 (-)**	**3/3 (↓)**	**3/4 (↓)**	**3/4 (↑)**
Mean±SD	49.8±42.3	319.8±156.2	10.2±4.1	134.0±94.0	-	37.7±7.4**	20.3±8.1	56.5±33.5
			Hematological markers				**Coagulation markers**	
Variable	**Ht**	**Leukocytes**	**Monocytes**	**Segments**	**Thrombocytes**	**PT**	**APTT**	**Fibrinogen**
RR	*0.42-0.61*	*4.5-14.6*	*0-0.9*	*2.9-11*	*144-603*	*6.7-9.5*	*10-17.2*	*1-2.8*
Unit	*l/l*	*$x10^9$/l*	*$x10^9$/l*	*$x10^9$/l*	*$x10^9$/l*	*s*	*s*	*g/l*
AH (total)	**5/11(↓)**	**5/11(↑)**	**4/11(↑)**	**6/11(↑)**	**2/5 (↓)**	**4/18 (↑)**	**5/18 (↑)**	**0/18 (↓)**
Mean±SD	0.42±0.09	15.9±8.8	0.9±0.5	12.0±7.9	164.4±171.0	14.9±28.8	18.7±12.5	2.8±1.9
CH(i)	**11/21 (↓)**	**11/24 (↑)**	**8/23 (↑)**	**10/24 (↑)**	**4/10 (↓)**	**14/34 (↑)**	**12/34 (↑)**	**8/34 (↓)**
Mean±SD	0.40±0.10	14.8±10.2	0.9±0.7	11.3±9.5	201.7±105.3	10.1±3.9	17.1±7.3	2.5±2.9
CH(ca)	**5/12 (↓)**	**5/12 (↑)**	**5/12 (↑)**	**5/12 (↑)**	**1/10 (↓)**	**6/20 (↑)**	**8/20 (↑)**	**7/20 (↓)**
Mean±SD	0.44±0.09	20.2±14.5	1.2±1.0	16.6±13.6	219.9±68.9	9.9±5.9	18.7±6.4	1.8±1.3
LDH	**1/3 (↓)**	**1/2 (↑)**	**0/2 (-)**	**1/2 (↑)**	**0/2 (-)**	**5/6 (↑)**	**5/6 (↑)**	**1/6 (↓)**
Mean±SD	0.41±0.04	15.2±9.2	0.5±0.4	13.1±9.3	237.0±29.7	11.2±2.7	22.7±4.9	1.4±1.0

RR = reference range; (↑) = above reference range; (↓) = below reference range; (-) = within reference range; * = mean glucose value significantly lower (P=0.016) for CH(i) when compared to CH(ca); ** = mean serum total protein value significantly lower (P<0.01) for LDH when compared to the other groups.

Histopathological findings – The histological semi-quantitative scoring of liver samples at initial diagnosis is summarized in *Table 4*. Necro-inflammatory activity was not

significantly different (P=0.27) between the groups. Fibrosis extent was significantly higher (P=0.020) in the LDH group compared to CH(i) and CH(ca). Copper was not significantly different (P=0.27) between AH(ca) and CH(ca). Copper was distributed mainly in zone 1 (periportal) of the liver acini in the CH(i) group, and mainly in zone 3 (centrolobular) in the CH(ca) group. Cirrhosis was observed in the CH(i), CH(ca), and LDH groups (n=23, n=14, n=7, respectively), including macronodular cirrhosis (n=7) in the CH(i) group and micronodular (n=2) in the CH(ca) group. Steroid-induced hepatopathy (SIH) was observed in 39 liver samples, mainly in the dogs with CH that had been treated with glucocorticoids.

Table 4 *– Histological semi-quantitative scoring of liver samples at initial diagnosis (n=95; 2002-2006).*

Diagnosis	**AH(i)**	**AH(ca)**	**CH(i)**	**CH(ca)**	**LDH**
No. dogs	16	5	43	24	7
Inflammation:					
Mean	2.2	2.3	2.0	2.5	2.1
(range)	(0-5)	(2-3)	(0-4)	(2-4)	(2-3)
Fibrosis (extent):					
Mean	0.0	0.0	2.2	2.3	3.0*
(range)	(0-0)	(0-0)	(0-3)	(1-3)	(3-3)
Copper score:					
Mean	0.5	3.8	0.7	3.2	1.8
(range)	(0-2)	(3-5)	(0-2)	(2.5-4.5)	(0-3)

No. = number; * = significantly higher (P=0.020) semi-quantitative fibrosis scores for LDH when compared to the other groups. Please note that 6 dogs were excluded due to small group sizes.

Treatment and clinical outcome – All dogs were treated symptomatically and palliatively as indicated by their clinical status and disease. Treatment adjustments were made if histology of repeated biopsies resulted in a different diagnosis. Dogs with AH(i) were treated with antibiotics (5/16), hepatic support diet and ursodeoxycholic acid (7/16), and 5/16 dogs were not treated. Dogs with AH(ca) were treated with prednisone (3/5) or D-penicillamine (2/5), supplemented by hepatic support diet and oral zinc gluconate. Dogs with CH(i) were mainly treated with prednisone (29/43), a minority received only

antibiotics or combined with prednisone (6/43), and 12/43 dogs were not treated due to mild inflammatory activity. Dogs with CH(ca) were initially treated with prednisone (9/23), D-penicillamine (5/23), or not treated at all in case of mild activity (9/23). After a repeated biopsy, more dogs received D-penicillamine (10/23), oral zinc gluconate (2/23), and hepatic support diet (5/23). Most LDH dogs were only treated symptomatically, one additionally with antibiotics, and 3 with prednisone. Lactulose, diuretics and low protein diets were prescribed in case of clinical signs of portal hypertension in all relevant groups. The single GH and EH dogs were not treated, because the former died shortly after diagnosis, and the latter did not have clinical disease. After progression to CH, the dogs in the miscellaneous group were treated with prednisone. The clinical outcomes are summarized in *Table 5*.

Table 5 *– Clinical outcome after therapy of PH at follow-up (n=93; 2002-2006).*

	AH(i)	AH(ca)	CH(i)	CH(ca)	LDH
No. of dogs with follow-up	15/16	5/5	43/43	23/24	7/7
Clinical outcome					
Remission (recurrence)	9 (2)	3 (0)	20 (9)	11 (5)	1 (1)
Residual disease	6	2	23	13	5
Alive at follow-up	6	3	16	7	0
Death (hepatitis-related)	9 (7)	2 (2)	27 (24)	16 (13)	7 (7)
Survival time (months)					
EMST (95%CI)	34.9 (N/A)		18.3 (0.0-49.0)**	16.9 (6.8-27.0)	0.7 (0.4-1.0)
Mean (range)*	9.2 (0-35)		4.1 (0-30.6)	8.1 (0-36.8)	2.9 (0.1-11.8)

No. = number; EMST = estimated median survival time (months); N/A = data not available; * = concerning only dogs that died of hepatitis-related cause; ** = concerning n=42 dogs, excluding the one dog with *Leishmania*-induced CH. Please note that 2 dogs were lost to follow-up.

Survival time – Survival after diagnosis was calculated for the most frequently observed forms of PH (*Figure 1*). Survival times are summarized in *Table 5*, also including the proportions of hepatitis-related deaths (events). No significant difference was found between AH and CH median survival time, but LDH was significantly lower than the others (P=0.017). Survival curves of both AH groups were not presented separately, due to a low number of dogs and events in the AH(ca) group (n=5). The one dog with *Leishmania*-induced CH was not included in the survival calculations, and was

successfully treated with allopurinol, resulting in a survival time of 58 months at follow-up.

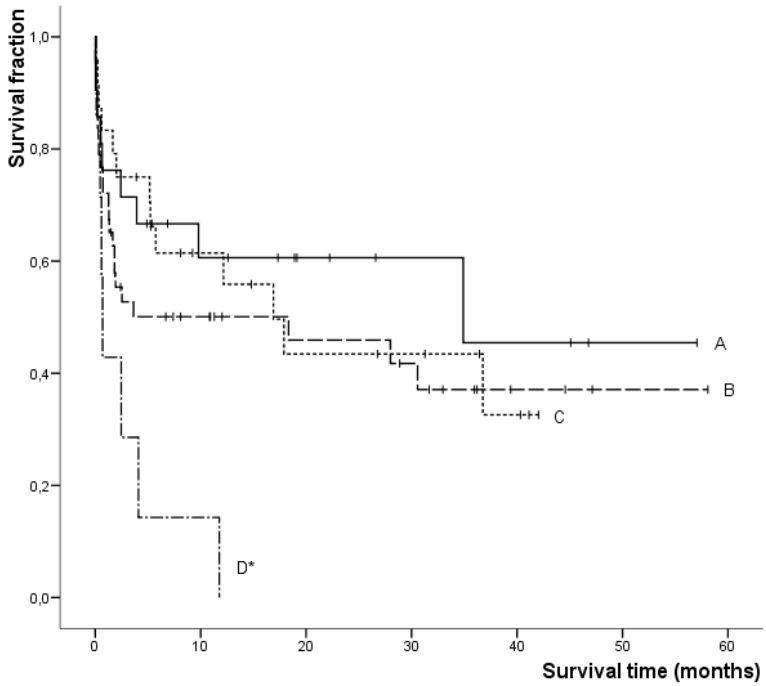

Figure 1 – *Kaplan-Meier curves for the survival time of dogs diagnosed with PH (n=94; 2002-2006). A = AH(i); B = CH(i); C = CH(ca); D = LDH.*

Prognostic factors for shortened survival time – A univariate Cox's proportional-hazard analysis (*Table 6*) was performed for AH, CH, and LDH (n=95) to screen for variables with prognostic value for shortened survival time. Significant (P<0.10) variables present at initial diagnosis are summarized in *Table 6*.

The multivariate analysis generated a model with prognostic factors of significant value for shortened survival time. The variables that are individually associated with a shortened survival time in the analyzed population (n=95) are presented in *Table 7*. Excluded were hypoproteinemia, hypoalbuminemia, PT, APTT, and leukogram left shift, due to missing data. Each of these initially excluded variables were singularly entered in new multivariate analyses, resulting in new models for shortened survival time. These additional models revealed that hypoalbuminemia (P=0.036; HR 2.30; 95%CI 1.05-5.01) and a leukogram

left shift (*P*=0.073; HR 2.41; 95%CI 0.92-6.33) prognosticated a shortened survival time in these reduced populations in addition to the variables presented in *Table 7*.

Table 6 *– Significant prognostic factors (P<0.10) for a shortened survival time in dogs with any form of PH (n=95; 2002-2006), calculated with a univariate analysis (Cox regression).*

Variable	***No.***	***Events***	***P***
Clinical signs:			
Jaundice	95	53	0.053
Lethargy	95	53	0.007
Weight loss	95	53	0.089
Abdominal distention	95	53	0.013
Abdominal fluid wave	95	53	0.007
Blood examination:			
Hypoproteinemia	51	34	0.017
Hypoalbuminemia	54	36	0.005
PT (prolonged)	78	41	0.004
APTT (prolonged)	78	41	0.011
Leukogram left shift	47	33	0.007
Ultrasonographic examination:			
Small liver size	95	53	0.077
Ascites	95	53	< 0.001
Enlarged portal lymph nodes	95	53	0.069
Histopathological examination:			
Cirrhosis	95	53	0.002

No. = number; events = all dogs with hepatitis-related death; HR = hazard ratio; CI = confidence interval.

Table 7 *– Significant prognostic factors for a shortened survival time in dogs with any form of PH (n=95; 2002-2006), calculated with a 'multivariate' analysis (Cox regression).*

No.	Events	Variable entered	Significant variables	*P*	HR	95%CI
95	53	Jaundice	Jaundice	0.013	2.19	1.18-4.06
		Lethargy	Abdominal fluid wave	0.035	2.19	1.06-4.55
		Weight loss	Small liver size	0.052	1.87	0.99-3.50
		Abdominal distention	Ascites	0.006	2.43	1.28-4.59
		Abdominal fluid wave	Enlarged portal lymph nodes	0.001	5.68	2.00-16.16
		Small liver size	Cirrhosis	0.054	1.75	0.99-3.11
		Ascites				
		Enlarged portal lymph nodes				
		Cirrhosis				

No. = number of dogs; events = all dogs with hepatitis-related death; HR = hazard ratio; CI = confidence interval.

Discussion

Primary canine hepatitis occurred in 0.5% of the canine referral population, with the highest prevalence seen for CH(i). Although we regularly encountered AH, the number of dogs referred may not reflect the true prevalence of the AH population in the Netherlands, because the majority of these dogs in our study group underwent spontaneous clinical remission either without therapy or with only short-term medical intervention, mostly resulting in long-term survival. In contrast, 5/12 of the pair-wise sampled AH dogs progressed towards CH, suggesting a considerable risk of the recurrence of clinical signs despite initial clinical remission. Administration of immunosuppressive medication, such as prednisone, prior to referral of the dogs with AH, may have stimulated progression to CH, or may have caused or compounded the deterioration of the acute condition [2,10,36].

The majority of dogs with acute and chronic hepatitis were classified as idiopathic. Etiologic factors other than accumulation of copper were rarely found. Testing for infectious agents as a potential etiology was limited to serology (for leptospirosis) and histopathology. No specific tests were performed for detection of infectious agents with a possible etiological role, because extensive investigations failed to detect any infectious agents in various forms of canine hepatitis [2,10]. No tests were performed for detection of

liver-specific auto-antibodies. A few dogs (5/101) had concurrent diseases associated with immune aberrations; however, the existence of a causal relation with hepatitis is not established [36]. Retrospective staining of all liver histology slides with rubeanic acid revealed that a remarkably large proportion (29/101) of all dogs had an increased hepatic copper concentration, suggesting a copper-associated etiology. The accumulation of copper does not automatically imply copper as the etiologic factor of hepatitis. However, it has recently been shown that copper is very unlikely to be an epiphenomenon of cholestasis when it is increased to >2 in the presently used grading system [43]. Furthermore, cholestasis causes periportal copper accumulation (zone 1) whereas all known forms of primary copper storage disease start with centrolobular accumulation (zone 3) [1]. In all dogs in the AH(ca) and CH(ca) groups, copper staining did occur in zone 3 of the liver acini, and we suspect that copper may very likely have been the underlying cause of hepatitis in most of these dogs. This assumption is supported by their favorable response to D-penicillamine treatment. D-penicillamine may also have anti-inflammatory effects that could also lead to clinical improvement independent of the effect on copper levels [45-47]. However, recent findings in copper-associated hepatitis in Labrador retrievers demonstrate that D-penicillamine effectively decreases hepatic copper concentrations (Hoffmann, in press). The authors recommend to routinely stain for copper with rubeanic acid and to grade copper semi-quantitatively as standard procedures for liver histology in dogs.

AH and CH typically affected middle-aged to older dogs, with an on average earlier onset in copper-associated forms. The other groups were affected at a younger age. Female animals were much more frequently affected than males as reported earlier [46]. This suggests a female predisposition for both idiopathic and copper-associated forms of hepatitis. The breed composition of the study population differed from the overall clinic population, in accordance with earlier studies [37,39,48]. Most predisposed dog breeds were affected by idiopathic hepatitis. Labrador retrievers and West Highland white terriers have reportedly been associated with CH(ca) [34,42], but our results suggest that these breeds are also at increased risk for the idiopathic forms. A bias for breed disposition can be caused by increased awareness of a breeder association. Our previous studies into copper-associated hepatitis in Dobermann pinchers [30,37,48] may have caused an underrepresentation in the current study, because most dogs have been included in another study and were therefore excluded from the investigated general hospital population.

The majority of dogs were presented with nonspecific clinical signs, which were also reported in earlier studies [38,39]. Sporadically, dogs were asymptomatic with only increased serum activity of liver enzymes and/or fasting bile acids. Generally, all PH groups had elevated serum liver enzyme activity and/or fasting bile acids. Other frequently

observed blood findings were hypoalbuminemia, elongated coagulation times (PT and APTT), decreased fibrinogen concentrations, and mild leukocytosis. Mean total serum protein was significantly lower in the LDH group when compared to the other groups, and mean serum glucose was significantly different between CH(i) and CH(ca). Neither the other mean blood values, nor the mean elevated or decreased values of all blood measurements were significantly different between the studied groups. This could partly be the result of underpowered statistical testing. Ultrasonographic abnormalities, if present at all, were largely nonspecific. Our findings suggest that neither blood examination nor ultrasonography should be considered valid or reliable stand-alone diagnostic tests for hepatitis or for distinguishing between subtypes, with the exception of hepatic cirrhosis.

The examination of liver biopsy samples may have been subject to sampling errors, observer variation, and specimen size [40,49]. It is routine in our clinic to take at least two and preferably three 14G liver biopsy samples of good quality for diagnostic purposes. We believe that sampling errors were an insignificant factor in this study; although they cannot be prevented altogether, our approach minimizes the incidence of such errors to a large extent. The WSAVA diagnostic criteria, however, had not been published at the time of first examination of our dogs. We therefore re-evaluated all dogs using those criteria. Subsequently, the diagnoses of 29 dogs had to be amended, including 10 dogs with CH(ca), illustrating the clinical relevance of such criteria.

Due to non-uniformity of the treatment in each group and small sample size in some groups, the median survival time (EMST) must be interpreted with care. Considerable overlap in confidence intervals and entanglement of survival curves occurred. However, the median AH survival time was much longer than that of both CH(i) and CH(ca). Dogs with severe AH (e.g. fulminant hepatitis, n=2) died during hospitalization, and LDH survival was significantly the shortest. It is likely that dogs with fulminant hepatitis and LDH do not live long enough to be referred and were underrepresented compared to given the true prevalence. No difference was found between CH(i) and CH(ca) groups in survival time, nor in mean age at presentation and prevalence of cirrhosis. This might be (partly) due to the 10 initially misdiagnosed dogs with CH(ca) that therefore did not receive anti-copper medication, which may have resulted in a shorter overall survival time in this subgroup. Regardless of the administered therapy, the EMSTs of both CH groups were comparable to those in three other retrospective studies. Two of these studies concerned mixed breed populations, without distinguishing idiopathic and copper-associated etiology, and demonstrated a mean survival time of approximately 18 months (mean of n=151, of which n=58 received prednisone treatment) [37] and 16 months (mean of n=57, range 1–87) [39], respectively. The third study concerned a Labrador CH population, which also made no distinction between idiopathic and copper-associated

cases, and found a survival time of approximately 1 year (median; range, 1 day to 8 years) [38]. We conclude that with better identification of copper-associated forms, considerable improvement of treatment effectiveness can be achieved for these forms of hepatitis. Until the etiologies underlying of idiopathic hepatitis have been elucidated, nonspecific regimens for the treatment of dogs with CH(i) need careful evaluation. However, improving therapeutic effectiveness may be reliant upon the identification of the relevant etiologies of these conditions.

The results of the multivariate analysis demonstrated that jaundice, abdominal fluid wave, hypoalbuminemia, leukogram left shift, microhepatica, ascites, enlarged portal lymph nodes, and cirrhosis are prognostic factors for shortened survival time after diagnosis of PH. All of these findings are related to decompensated liver function and chronic portal hypertension. The majority of these prognostic factors occur in chronic forms of PH, thus explaining the observed differences in survival time between the PH groups. This is also demonstrated in previous retrospective studies into prognostic factors for shorter survival time. Their results are mostly in accordance with our findings, and include the following variables with a prognostic value: low serum glucose concentration and prolonged PT (death <1 week), hypoalbuminemia and presence of bridging fibrosis (short-term survival, >1 week) [37]; anorexia, prolonged PT and APTT, and thrombocytopenia (short-term survival, <2 months) [38]; and low serum globulin concentration [29].

This study underscores the clinical importance of the WSAVA diagnostic standards, which enables comparison of our results with those of other groups when the same standards are employed [1]. We conclude that copper-associated hepatitis may be much more prevalent in the canine population than generally assumed and may account for approximately 1/3 of all dogs with acute and chronic forms of primary hepatitis. Furthermore, a considerable fraction of dogs with acute hepatitis develop chronic hepatitis which may progress to cirrhosis. Signs of liver decompensation and cirrhosis at initial presentation are predictors of shortened survival time. Results from examination of prognostic factors in this study and previous studies suggest that early recognition and appropriate therapy are likely to be of key importance for prevention of survival-shortening disease progression. To current knowledge, this especially concerns copper-associated cases of hepatitis, because etiology-specific treatment is available. Further information on the etiology of idiopathic PH is likely necessary in order to prevent progression from acute- to chronic hepatitis to cirrhosis by application of etiology-specific treatment.

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Chapter

5

Effect of prednisone treatment on chronic idiopathic canine hepatitis and improvement of associated coagulopathy

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Submitted

Abstract.

Background: Strombeck and colleagues demonstrated that prednisone treatment leads to prolonged survival of dogs with chronic hepatitis [1]. However, the experience accumulated in the past decades that the positive effect is likely marginal, if at all existent.
Objectives: To perform a retrospective study analyzing the effects of prednisone treatment on clinical performance, survival, clinicopathological parameters, and criteria of histological grading and staging in 36 dogs with idiopathic chronic hepatitis.
Results: At follow-up recurrence of clinical signs (8/36) and residual disease occurred frequently (17/36), often resulting in hepatitis-related death (20/36). Complete clinical remission occurred in a minority of cases (11/36) after prednisone treatment. Prednisone treatment caused normalization of coagulopathies associated with chronic idiopathic hepatitis within one week in all 11 dogs that had coagulopathies. Duration of prednisone administration was a time-dependent variable that was significantly associated (P=0.010) with a hazard rate <1. Median survival time was shorter than previously found by Strombeck et al., most likely caused by differences in inclusion criteria [1]. Non-cirrhotic dogs survived significantly longer than cirrhotic dogs.
Conclusions and clinical importance: Prednisone seems to have a beneficial effect on the survival time of dogs with non-cirrhotic CH.

Introduction.

Hepatitis is frequently encountered in dogs in different forms and is caused by various aetiologies. Hepatic inflammation may be secondary to exposure to (endo)toxins. This so called non-specific reactive hepatitis disappears spontaneously upon recovery of the primary disease and requires no specific treatment. In contrast, the hepatic inflammation in primary hepatitis (PH) is the cause rather than the consequence of disease, and the liver is the primary target organ. Hepatic involvement in systemic infections (e.g. Leishmaniasis) may cause severe hepatitis which is not included in the definition of PH. PH is further characterized according to aetiology, activity and chronicity. Grading systems of activity range from mild to severe based on the amount of inflammatory cells and hepatocyte necrosis/apoptosis. Staging scores (acute-chronic-cirrhosis) indicate the degree of fibrosis and the extent of disrupture of the normal lobular architecture. Known aetiologies include viruses, toxins and drugs [2-7]. Furthermore, copper accumulation is a profound cause in many breeds (Labrador retriever, Dalmatian, Dobermann, Spaniel breeds) and may explain around 35% of all forms of PH in a referral hospital population [8-15]. However, in the vast majority of dogs with PH an aetiology is unknown and therefore called idiopathic hepatitis.

Rational treatment is possible in hepatitis with known aetiology. However, for idiopathic hepatitis there is little consensus about the most effective treatment. Whereas it is generally agreed that acute hepatitis requires only supportive care to permit spontaneous recovery, the dispute focuses on the treatment of chronic idiopathic hepatitis (CH(i)). The milestone study on which prednisone treatment is based was published in 1988 [1]. The current hesitation for prednisone treatment is amongst others based on contraindicated immunosuppression and other undesirable side effects.

Ideally, the therapeutic effect of prednis(ol)one should be revisited in a randomized, placebo controlled, double-blinded, prospective study using recently assessed criteria [16]. However, if there are still good arguments that corticosteroid therapy improves survival and/or reduces hepatic inflammation of dogs with CH(i), it is unethical to withhold it in a placebo group. In that case the consequence is that the proposed most suitable therapy to be tested in a prospective trial should be compared with the effect of prednis(ol)one in a blinded, randomized way. Until now, there is no further literature supporting refraining or using corticosteroids. Expert panel and audience discussions with participation of American and European specialists at ECVIM-congresses in 2008 and 2009 concluded, although hesitant, that withholding corticosteroids to dogs with CH(i) is unethical.

Because the combined clinical experience of experts is important, nevertheless subjective, the authors decided to find more objective evidence. Therefore, we performed

a retrospective study in our University Hospital population of dogs with CH(i). We applied the WSAVA-diagnostic criteria, making the outcome verifiable and according to modern standards. In addition we have routinely performed follow-up biopsies in our cases, so that we could base conclusions on clinical performance, survival, and histological grading and staging criteria. Acknowledging the limitations of a retrospective study, we think this is the best available material until now to support a rational decision on the direction of a future prospective multicenter study on the effect of prednisone in CH(i).

Material and methods.

Study design – Retrospective review of medical records.

Dogs – All dogs in this study were referred between 2002 and 2006 to the Department of Clinical Sciences of Companion Animals, Utrecht University, the Netherlands. The dogs were identified from the records of the diagnostic pathology service at the Faculty of Veterinary Medicine. Included were dogs with histology-proven CH without any aetiology-specific changes, as described by the WSAVA liver standardization group. Copper-associated CH was excluded by histological copper grading and only dogs with a score ≤ 2 on a scale ranging from 0 to 5 were included[a] [8,12,13]. *Leptospirosis, Leishmaniasis* and *Herpesvirus* infection were excluded by histopathology and serology in cases with clinical or clinicopathological signs that were suggestive of these infectious agents. Thirty-six dogs with CH(i) were included in the study. Previous medications given by referring veterinarians were carefully recorded.

Clinicopathological data that were obtained from the medical records are listed in *Table 1*. All dogs were treated with prednisone for at least 6 weeks following histological diagnosis with 1 mg/kg/day, which was extended with an additional 6 weeks (treatment period of 12 weeks in total) when hepatitis was still present. If there were missing data, those dogs were excluded for paired data analysis of results obtained before and after prednisone treatment. Eleven patients received prednisone treatment (1 mg/kg/day) for one week before the initial biopsy was taken because abnormal coagulation tests (PT and/or APTT and/or fibrinogen) prevented the liver biopsy procedure at the first examination.

Table 1 *– Evaluated parameters that were collected from medical records and liver histopathology reviews (n=36, 2002-2006).*

Data categories	**Collected parameters**
Medical history	Concurrent diseases and current medication administration
Biochemistry markers	Total alkaline phosphatase (AP), heat-stable, steroid-induced alkaline phosphatase (AP-65), alanine aminotransferase (ALT), fasting total serum bile acids (sBA), ammonia, urea, creatinin, glucose, total serum protein (TP), albumin, prothrombin time (PT), activated partial thromboplastine time (APTT), fibrinogen
Haematological markers	Haematocrit (Ht), leukocytes and differentiation, thrombocytes
Histopathological findings	Grading of necro-inflammatory activity and fibrosis and cirrhosis, grading for copper content and distribution; and presence of steroid-induced hepatopathy
Treatment	Medical therapy; type and duration
Follow-up	Outcome after treatment (complete remission or recurrence of clinical signs, or residual disease), survival time after diagnosis, and presumable cause of death (clinical signs prior to death related to hepatitis or not)

Histopathology – At the time of original diagnosis, at least 2 liver biopsy samples per occasion were obtained from each dog by percutaneous biopsy with a 14G needle (blind Menghini biopsy or true cut biopsy under ultrasonographic guidance) [16]. Histology of all identified cases was reviewed by one board-certified veterinary pathologist (TSGAMvdI) according to the WSAVA criteria [17]. Liver tissue samples had been formalin-fixed and paraffin-embedded, and were stained with Hematoxylin and Eosin (HE), with the reticulin stain according to Gordon and Sweet to assess fibrosis, and with rubeanic acid for copper storage. All cases were semi-quantitatively evaluated for necro-inflammatory activity (0=none, 1=slight, 2=mild, 3=moderate, 4=marked, 5=severe), fibrosis (0=none, 1=focal, 2=bridging, 3=bridging with architectural distortion or cirrhosis), and copper content and distribution.[a]

[a] 0 – no copper; 1 – solitary liver cells and/or reticulohistiocytic (RHS) cells containing some copper positive granules; 2 – small groups of liver cells and/or RHS cells containing small to moderate amounts of copper positive granules; 3 – larger groups or areas of liver cells and/or RHS cells containing moderate amounts of copper positive granules; 4 – large areas of liver cells and/or RHS cells with many copper positive granules; 5 – diffuse presence of liver cells and/or RHS cells with many copper positive granules

No quantitative copper analysis was performed. The presence of hepatocytic ballooning as evidence for steroid-induced hepatopathy (SIH) was evaluated qualitatively.

Follow-up – Data were collected by one of the authors (JHP) during February 2007 by telephonic interviews with the owners or referring veterinarians for all 36 included dogs. Data included clinical outcome after diagnosis (residual disease, remission or recurrence of CH-related clinical signs), survival time after diagnosis, and the presumable cause of death.

Parameters evaluated – The parameters, all analysed with standard clinical-chemistry methods, included for description and statistical analysis are presented in *Table 1*. Not all blood parameters are included and described for the entire study sample, due to missing data.

Statistical analysis – Analysis was performed using a commercially available SPSS software package.[b] A one-sample Kolmogorov-Smirnov test was used to assess normality of all data. A paired-sample Student's t-test was performed to compare paired blood values (max. n=21). The log-values of these blood measurements were used due to incidental non-normal distributions and frequently observed outliers. A Wilcoxon paired rank test was used on all paired semi-quantitative histopathological scores. A 1-way ANOVA with Bonferroni correction was used to compare mean ages of the dogs in the three clinical outcome groups. Survival time of all dogs with CH(i), and of all cirrhotic and non-cirrhotic cases of CH(i) was calculated according to the Kaplan-Meier estimate procedure. The survival time was defined as the interval between the date of initial diagnosis and the date of death. Hepatitis-related deaths in our sample were counted as events; dogs that died of causes unrelated to hepatitis or that were alive at follow-up were censored. Differences between Kaplan-Meier curves (i.e. cirrhotic vs. non-cirrhotic dogs) were tested with a log-rank test. Duration of prednisone administration was analyzed as an extrinsic time-dependent covariate (TDC) [18].

Descriptive and group comparative data are presented as median (range); survival data as estimated median survival times (EMST) (95% confidence interval, CI); and TDC analysis as hazard rates (HR), and 95%CI. All statistical tests were considered significant at the 5% level ($P<0.05$).

[b] SPSS version 15.0, Benelux BV, Gorinchem, the Netherlands

Results.

Medical history and initial histopathology – Histology-proven CH without any detectable aetiology was observed in 36 dogs that were presented between 2002-2006 at the university clinic. Eleven patients received prednisone treatment for one week before the initial biopsy was taken because abnormal coagulation tests (PT and/or APTT and/or fibrinogen) prevented the liver biopsy procedure during the first examination. During the second examination coagulation tests had normalized in all 11 dogs (*Table 2*) and biopsies were taken. No other anti-coagulant drugs were administered to these 11 dogs. An additional 8 patients had for a short period (< 7 days) been treated with orally administered or intramuscular injected glucocorticoids by the referring veterinarian. Three dogs had a history of a single concurrent disease, including epilepsy (n=2) and keratoconjunctivitis sicca (n=1) One dog was treated for degenerative arthritis, atopic dermatitis, and hypothyroidism. Five dogs had previously been treated with potentially hepatotoxic drugs, including TMPS (n=1), NSAIDS (n=2), and phenobarbital (n=2).

Histological scoring of the liver samples at initial diagnosis is presented in *Table 3*. Cirrhosis was observed in the initial liver biopsy samples of 19 dogs.

All 36 dogs were treated and evaluated in accordance with a standard protocol. Treatment consisted of oral administration of prednisone (1 mg/kg/day) for 6 weeks, after which they were reviewed with blood examinations and hepatic histopathology. In case hepatic inflammation did not decrease sufficiently, the prednisone administration was prolonged with another 6 weeks. The mean duration of prednisone administration was 8.8 ± 5.4 weeks. The shorter than 6 weeks of treatment was caused by the death of the animals before the end of the 6 weeks (standard protocol) treatment. Additional treatments included antibiotics (n=4), low-copper diets (n=3), low-protein diets (n=3), lactulose (n=4), diuretics (n=5), and anti-emetics (n=2).

Table 2 *– Normalization of coagulation in 11 dogs with CH(i) after one week of prednisone (1 mg/kg/day) treatment.*

Dog no.	Before treatment			After treatment		
	PT	APTT	Fibrinogen	PT	APTT	Fibrinogen
Ref value	7.2-9.9 sec	13.2-18.2 sec	1.0-2.7 g/L	7.2-9.9 sec	13.2-18.2 sec	1.0-2.7 g/L
1	13	14.1	0.7	7.7	18.4	1.4
2	13.7	25.2	0.9	12	18	1.1
3	11.1	23.4	0.5	8.6	17.7	1.8
4	11.7	19.6	0.9	10.7	17.5	1.6
5	8.9	11.8	0.5	7.1	12	1.3
6	14.4	23.4	0.5	11.3	17.9	1.2
7	7.7	20	2.1	6.7	17.1	1.3
8	10	18	0.8	8	10	1.2
9	12	19	0.6	8	12	1.6
10	9	17	0.7	8	14	1.0
11	11.5	23.4	0.5	6	22.8	1.3

No., number; PT, Prothrombin time; APTT, Activated partial thromboplastine time; sec, seconds

Table 3 *– Semiquantitative fibrosis and inflammatory scores in 36 dogs with CH(i) at the time of initial diagnosis.*

fibrosis score	**No. of cases**	**necro-inflammatory activity scores (median (range))**	**No. of cases with SIH**
1 (focal)	7	1 (0-3)	6
2 (bridging)	10	1 (0-4)	5
3 (cirrhosis)	19	2 (0-4)	8

No., number; SIH, steroid-induced hepatopathy; necro-inflammatory activity: 0=none, 1=slight, 2=mild, 3=moderate, 4=marked, 5=severe

Paired biochemical and haematological findings in the prednisone-treated dogs – Twenty-one out of 36 prednisone-treated dogs returned to the university clinic for blood examination after 6 weeks of treatment. Significant changes following therapy were observed in elevated heat-stabile alkaline phosphatase (AP-65) (P=0.015), lowered prothrombin time (PT) (P=0.009), and elevated fibrinogen (P=0.004) (*Table 4*). The mean

AP-65 value was elevated above the reference range, the mean values of PT and fibrinogen remained within their respective reference ranges.

Table 4 *– Blood values before and after 6 weeks of prednisone treatment (1 mg/kg/day) (n=21; 2002-2006).*

Parameter	RR	Unit	No.	Mean ± SD				*P*
				Before		After		
Bile acids	0-10	µmol/l	10	51.2±74.4	(7↑)	24.3±20.5	(7↑)	0.295
AP	0-73	U/l	7	369.3±319.3	(7↑)	565.6±414.3	(6↑)	0.229
AP-65	<15% of AP	U/l	6	50.2±62.9	(6↑)	289.7±251.5	(5↑)	0.015
ALT	0-54	U/l	7	408.9±247.1	(5↑)	364.1±240.0	(5↑)	0.568
Total protein	55-72	g/l	2	64.0±7.1	(0↓)	56.0±1.4	(0↓)	0.276
Albumin	26-37	g/l	2	27.0±4.2	(1↓)	26.0±0.0	(0↓)	0.825
PT	6.7-9.5	sec	21	8.5±2.0	(6↑)	7.2±1.1	(0↑)	0.009
APTT	10.0-17.2	sec	21	14.3±4.7	(6↑)	14.6±3.5	(2↑)	0.450
Fibrinogen	1.0-2.8	g/l	21	2.0±1.5	(4↓)	2.7±0.8	(0↓)	0.004
Leukocytes	4.5-14.6	10-9/l	4	22.0±7.4	(4↑)	30.6±18.3	(3↑)	0.383

RR = reference range; No. = number of dogs with available paired blood values; *P* = *P*-value; In brackets the number of dogs above (↑) or below (↓) the reference range.

Paired histological findings in the prednisone-treated dogs – Follow-up biopsy samples were available for 20 out of 36 dogs after 6 weeks of prednisone treatment. None of these 20 dogs were treated with potentially hepatotoxic drugs before or during prednisone treatment. Histological scores of paired liver biopsy samples for the whole group (n=20) were compared for inflammation and fibrosis (*Table 5*).

Complete histological resolution of inflammation occurred in 6 dogs after 6 weeks of prednisone treatment, and in 3 additional dogs after 12 weeks. However, in 6 dogs necro-

inflammatory activity recurred or worsened in the same time span. Fibrosis resolved completely in 5 dogs, was reduced in 4 dogs, and progressed in 5 dogs after 6 weeks. Cirrhosis was observed in 5 dogs in the pre-treatment biopsy samples, and 3 additional dogs had developed cirrhosis after 6 weeks of prednisone treatment. On average, no progression of fibrosis was observed. Complete histological resolution of CH-associated changes occurred in 5 dogs after 6 weeks, and in 3 additional dogs after 12 weeks. At presentation, 9 dogs had steroid-induced hepatopathy (SIH); this number increased to 15 at the end of the 6 week prednisone treatment period.

Table 5 *– Paired semi-quantitative (inflammation and fibrosis) and qualitative (SIH) scoring (number of dogs with SIH) of liver histology before and after 6 weeks of prednisone treatment (1 mg/kg/day) in dogs with CH(i) (n=20; 2002-2006).*

Parameter	Median (range)		*P*
	T=0	T=6	
Inflammation	1.5 (0-4)	1 (0-3)	0.103
Fibrosis	1.5 (0-3)	1.5 (0-3)	0.361
SIH	9	15	-

P = *P*-value; SIH = steroid-induced hepatopathy; T=0, before prednisone treatment; T=6, after 6 weeks of prednisone treatment
Necro-inflammatory activity (0-5): 0=none, 1=slight, 2=mild, 3=moderate, 4=marked, 5=severe; fibrosis score (0-3): 0=none, 1=focal, 2=bridging, 3=bridging with architectural distortion or cirrhosis

Post-diagnosis clinical outcome and survival time – The clinical outcome for all 36 treated dogs with histology-confirmed CH(i) is illustrated by *Figure 1*.

Eleven out of 36 dogs (the remission group) had no hepatitis-related clinical signs at follow-up, 5 dogs were cirrhotic at initial diagnosis. No dogs died with concurrent CH-related clinical signs, 10 were alive and one died not related to hepatitis. Eight out of 36 dogs (the recurrence group) had a recurrence of hepatitis-related clinical signs at follow-up after an initial clinical sign-free period, 3 dogs were cirrhotic at initial diagnosis. Five dogs died presumably because of CH. Seventeen out of 36 dogs (the residual disease group) had hepatitis-related clinical signs despite of treatment at follow-up. Eleven dogs were cirrhotic at initial diagnosis, and 15 dogs died presumably because of CH, one was alive at

follow-up and one died not related to hepatitis. No significant differences were found for the mean age of the three clinical outcome groups.

Twenty out of 36 prednisone treated dogs died of hepatitis-related cause, and these dogs had an estimated median survival time (EMST) of 9.9 months (range, 0-32.4) (*Figure 2*). Nineteen out of 36 dogs had cirrhosis, and 17 out of 36 dogs did not have cirrhosis at initial diagnosis. The non-cirrhotic dogs survived significantly longer (P=0.016) than the cirrhotic dogs. The non-cirrhotic dogs had an EMST of 33 months (CI, not available), the cirrhotic dogs had an EMST of 1.3 months (CI, 0.9-1.8).

Effect of prednisone on survival time – The duration of prednisone administration was considered to be a time-dependent variable that was significantly associated (P=0.010) with a hazard rate <1 for hepatitis-related death (HR, 0.88; CI, 0.81-0.97), meaning that prednisone probably has a survival prolonging effect in dogs with CH(i).

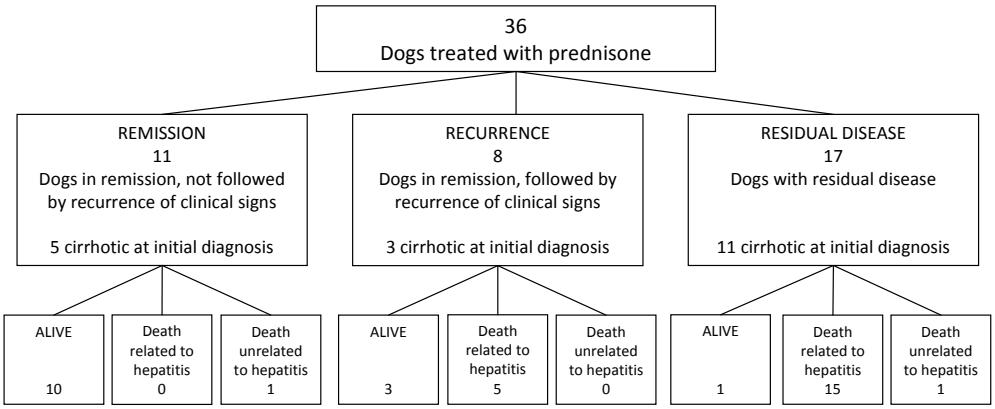

Figure 1 *– Follow-up of 36 dogs with CH(i) (2002-2006) treated with prednisone (1 mg/kg/day); mean duration of prednisone administration was 8.8 ± 5.4 weeks.*

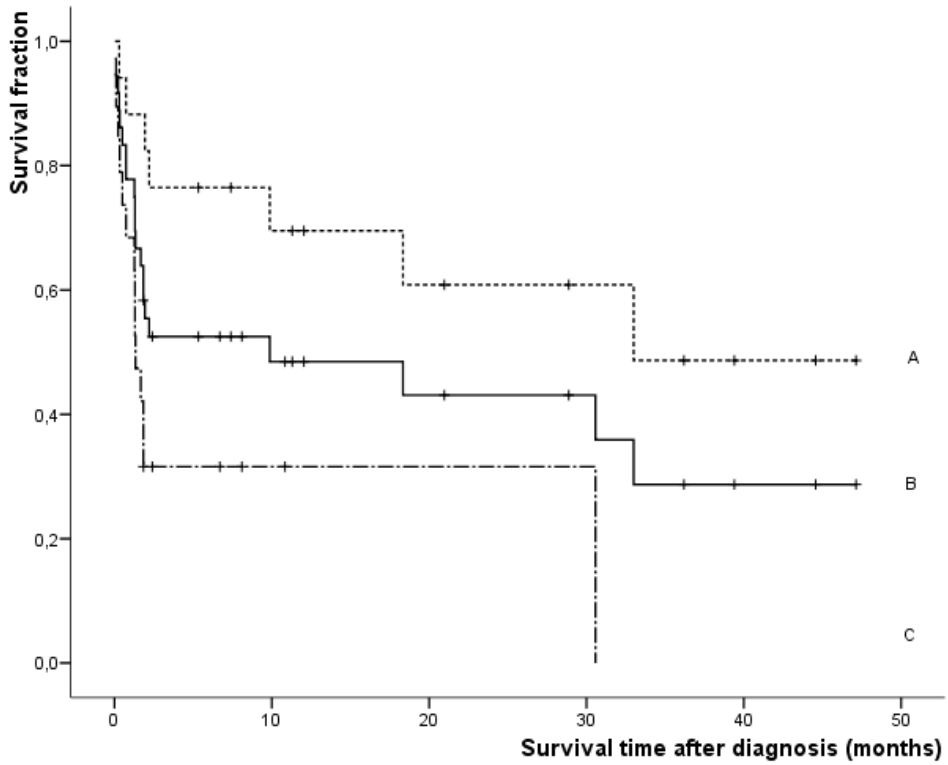

Figure 2 *– Survival curves of 36 dogs after diagnosis with CH(i) and prednisone treatment (2002-2006), as calculated with the Kaplan-Meier estimate procedure. Censored cases are represented by vertical bars. A = Dogs without cirrhosis (n=17); B = all 36 dogs with CH(i); C = Dogs with cirrhosis (n=19). Non-cirrhotic dogs survived significantly (P<0.016) longer than dogs with cirrhosis.*

Discussion

Strombeck and colleagues demonstrated a prolonged survival time in prednisone-treated patients when compared to untreated patients (mean survival time of 33 months and 19 months, respectively) [1]. However, new diagnostic methods have improved fine-tuning in both diagnosis and treatment, such as by distinguishing copper-associated from other aetiologies and applying the WSAVA standardized criteria for canine liver histology [16]. Such differentiation could not be made by Strombeck and colleagues at the time of their study [1]. In the current study, prednisone's effectiveness in treating CH(i) was assessed

by comparing blood and histopathological parameters (necro-inflammatory activity and fibrosis) before and after a standard 6 weeks protocol treatment period, by analysis of post-treatment clinical outcome, and post-diagnosis survival time.

In all eleven cases with coagulopathy at the time of referral coagulation values normalized after one week of prednisone treatment. Vitamin K supplementation was not used in any of these dogs. In addition to these findings, post-treatment values were significantly altered for PT and fibrinogen after 6 weeks of treatment with prednisone. The present results indicate that prednisone treatment facilitates to normalize PT, APTT, and fibrinogen related coagulation. To the authors' knowledge this effect has not been reported before. In humans and dogs with hyperadrenocorticism, the increased risk of thromboembolism is well known, indicating a relation between corticosteroids and the coagulation system [19-21]. Jacoby et al. suggested that the hypercoagulable state in dogs with in naturally occurring hyperadrenocorticism was caused by an elevation of procoagulant factors and decreased concentrations of antithrombin [21]. Our findings of normalizing PT, APTT, and fibrinogen plasma concentrations are in agreement with these findings.

Paired liver tissue samples were histopathologically evaluated. Both median post-treatment necro-inflammatory activity scores and median fibrosis scores were not significantly changed. Although not significantly reduced as a group, in some individual dogs fibrotic changes were partially or completely reversed. In molecular terms, glucocorticoids such as prednisone interact with transforming growth factor beta (TGF-β) signalling pathways at the transcriptional and translational level, thereby potentially reducing fibrotic progression [22,23]. Additionally, inhibition of inflammation leads to reduced activation of hepatic stellate cells (i.e. myofibroblasts), thereby inhibiting deposition of extracellular matrix and formation of additional fibrosis [2,24,25].

The initial presence of SIH may have masked part of the effect of prednisone treatment on idiopathic hepatitis. The true effects of prednisone may therefore be better than we could conclude from the present study. The number of SIH cases increased after treatment in the prednisone-treated cases with paired observations as expected [26].

The majority of dogs had a recurrence of clinical signs (8 out of 36 dogs) or residual disease (17 out of 36 dogs); the minority (11 out of 36 dogs) was free of clinical signs after prednisone treatment at follow-up. Despite treatment, 20 out of 36 dogs died with hepatitis-related clinical signs. In contrast, analysis of the duration of prednisone administration related to survival time resulted in a hepatitis-related death hazard rate of 0.88, meaning that prednisone reduced the risk of hepatitis-related death in our study population. The prednisone-treated patients in our population have an estimated median survival time (EMST) of approximately 10 months, with a significantly shorter survival

time for cirrhotic dogs (EMST, 1.3 months) when compared to non-cirrhotic dogs (EMST, 33 months). Cirrhosis is a negative prognostic factor, as is demonstrated in recent publications [15,27].

Our findings suggest that prednisone has beneficial effects on clinicopathological parameters (e.g. hepatic inflammation), and that it may at least in some cases limit fibrotic progression, confirming the importance of early diagnosis and treatment. This is underlined by the poor prognosis that comes with hepatic cirrhosis. Additionally, our results suggest that prednisone reduces the risk of a shortened survival time due to hepatitis-related death. However, the majority of patients either had a either recurrence (n=8) of hepatitis-related clinical signs, or residual disease (n=17) despite prednisone therapy. Therefore, it is still not clear whether prednisone is the best therapy for CH(i), or if it is only beneficial for particular subgroups of CH(i). Survival times for non-cirrhotic dogs are generally good, which suggests that prednisone's anti-inflammatory and fibrosis-limiting effects may be beneficial for those dogs that have not yet developed cirrhosis. Some dogs showed a reduction of fibrosis in association with prednisone medication, whereas in others fibrosis was not regressed. This may indicate a heterogeneous aetiology of idiopathic canine hepatitis. Another possibility is that there was variable representation of the disease process in the biopsy samples. Although we cannot exclude sampling bias, inclusion of at least two biopsies of larger than 1.5 cm will minimize this effect.

We conclude that prednisone treatment has beneficial effects in some dogs with chronic idiopathic hepatitis, which confirms earlier results published by Strombeck et al. [1]. Although not all of our findings were statistically significant, various important prognostic factors for the survival of dogs with hepatitis (such as fibrosis parameters) were on average beneficially altered after treatment with prednisone. Additionally, dogs that had not developed hepatic cirrhosis had a long survival time, and prednisone reduced the hazard of hepatitis-related death in the entire study group. Therefore, the main effect we can report is a better survival time associated with prednisone medication. Our results confirm that cirrhosis has a very poor prognosis, underlining the importance of an early diagnosis requiring taking biopsies without delay. Finally, we could for the first time document a very favourable effect of prednisone treatment on coagulopathy associated with canine CH(i). Together, these data argue for a multicenter trial with prednisone combined with a suitable medication in a double-blind randomized placebo controlled set-up.

Acknowledgements.

The authors wish to thank D. van Drie, J. van den Broek (Utrecht University Center for Biostatistics), and E.W. Bakker (MSc) for their respective technical assistance, statistical and linguistic advice.

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Part

2

Canine hepatitis: from clinic to translational model

Chapter

6

Quantitative PCR method to detect a 13 kb deletion in the *MURR1 (COMMD1)* gene associated with copper toxicosis and HIV-1 replication

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Adapted from *Mammalian Genome* 2005; 16: 460-463

Abstract

The recently discovered locus for copper toxicosis (CT) in Bedlington terriers (BT) has a 13 kb deletion enveloping the 187 bp exon-2 of the *MURR1* gene. This *MURR1* gene is not only involved with biliary copper excretion but also associated with HIV-1 replication. The micro-satellite C04107 lying in an intron of the *MURR1* gene is highly associated with the disease but shows haplotype diversity. The only solid molecular test for the disease is by showing the deletion in exon-2 in cDNA in liver tissue; this test is not robust on RNA from peripheral leukocytes due to their low MURR1 expression level. Because of these drawbacks, we developed a new quantitative PCR (Q-PCR) protocol. Here we show that the *MURR1* exon-2/exon-3 ratio measured by Q-PCR on genomic DNA correlates perfectly with the micro-satellite marker and with RT-PCR data from blood samples, buccal swabs, and liver biopsies. In view of the important role of *MURR1* in cells of many tissues this new test has a wide range of applications in comparative biomedical research. Furthermore, Q-PCR on DNA may be a new tool in general to analyse mutations which cannot be approached by standard methods.

Introduction

Copper toxicosis (CT) affecting the liver was identified as an inherited disease in Bedlington terriers in 1975 by Hardy et al. [1]. CT is inherited as an autosomal recessive disorder [2] characterized by an inefficient excretion of copper via the bile [3]. The result is a progressive accumulation of copper in the hepatocellular lysosomes, becoming histologically distinguishable from the normal situation at one year of age and leads to progressive hepatitis and cirrhosis [1,4,5]. The disease appeared to be caused by a 13 kb deletion in an until then unknown gene which plays a crucial role not only in copper metabolism but also in other cell functions. Recent research revealed that (i) *MURR1* (also known as *COMMD1*) restricts HIV-1 replication in resting $CD4^+$ lymphocytes by increasing NF-kB activity [6], (ii) *MURR1* as a regulator of the human δ epithelial sodium channel [7], and (iii) that there is a novel role for XIAP, an anti-apoptotic protein, in copper homeostasis through regulating MURR1 [8].

Close linkage of the micro-satellite marker C04107 to the disease was found by Yuzbasiyan-Gurkan et al. [9]. C04107 lies in an intron of the causative gene *MURR1* [10]. Due to haplotype diversity it is, however, less reliable for diagnostic testing [11-13]. It proved also impossible to develop a practical PCR reaction to show the absence or presence of the 13 kb deletion as only one side of the deletion could be identified [14]. Therefore the only PCR-based molecular test possible today is by showing the deletion of exon-2 by RT-PCR on RNA from liver tissue. *MURR1* expression level is very low in peripheral leukocytes, which makes a robust RT-PCR-based test less feasible (unpublished).

Because these drawbacks, we developed a new DNA-based method using a variation of the Q-PCR protocol recently reported by Faugère et al. [15] by measuring the ratio between exon-2 and exon-3. A clear correlation between this ratio and independent genotyping was found. We show that two tube Q-PCR on small amounts of DNA from peripheral blood leukocytes or buccal swabs provides a reliable alternative for molecular diagnostics of this mutation, providing a new tool to evaluate mutations which cannot be approached with standard methods.

Materials and Methods

DNA isolations from Whole Blood.
Genomic DNA was isolated from 4 ml blood collected in EDTA tubes by using the Qiagen QIAamp DNA Mini Kit (Hilden, Germany), Blood and Body Fluid Spin Protocol according to the manufacturer's instructions. The DNA samples were digested with Qiagen Ribonuclease A (28 U) to remove RNA. The amount of DNA was quantified spectrophotometrically by the absorbance at λ = 260 nm.

DNA isolations from Swabs.
Genomic DNA obtained from buccal swabs (Omni Swabs, Fitzco Inc., Spring Park, MN) was isolated by using the Qiagen QIAamp DNA Mini Kit (Hilden, Germany), Buccal Swab Spin Protocol according to the manufacturer's instructions. DNA samples were digested with Qiagen Ribonuclease A (28 U) to remove RNA from samples. The amount of DNA was quantified spectrophotometrically by absorbance at λ = 260 nm.

Quantitative PCR.
Q-PCR involved amplification of two exons of the *MURR1* gene with primers in combination with high affinity double-stranded (ds) DNA-binding dye SYBR green I (SYBR® green I, BMA, Rockland, ME). Reactions were performed in triplicate in a spectrofluoremetric thermal cycler (iCycler®, BioRad Laboratories, Hercules, CA). Data were collected and analysed with the provided application software. For each real-time PCR reaction, 1.67 µl of genomic DNA (1 to 100 ng/ml) was used in a 50 µl reaction volume containing 1x manufacturer's buffer (Applied Biosystems, Roche, Brandenburg, NJ), 0.5 × SYBR® green I, 200 µM dNTP's (PromegaBenelux, Leiden, the Netherlands), 20 pmol of both primers (Table 1) were designed using PrimerSelect software (DNASTAR Inc., Madison, WI), and 1.25 units of AmpliTaq Gold (Roche, Brandenburg, NJ) on 96-well iCycler iQ plates (BioRad Laboratories). All PCR protocols included a 5 minute polymerase activation step followed by 40 cycles consisting of a 95°C denaturation for 20 sec, annealing at 60°C for 30 sec, and an elongation step at 72°C for 30 sec with a final extension step for 5 min at 72°C. Melt curves (iCycler, BioRad), agarose gel electrophoresis, and standard sequencing procedures were used to examine each sample for purity and specificity (ABI PRISM 3100 Genetic Analyser, Applied Biosystems, Foster City, CA). Standard curves constructed by plotting the relative starting amount versus threshold cycles were generated using serial 4-fold dilutions of pooled DNA fractions containing DNA from healthy dogs. The amplification efficiency, E (%) = $(10^{(1/-s)}-1) \cdot 100$ (s = slope), of each standard curve was determined and appeared to be >95 %,

and < 105 %, over a large dynamic range. For each experimental sample the amount of *MURR1* exon-2, and of the endogenous reference *MURR1* exon-3, was determined from the appropriate standard curve in autonomous experiments. Results were normalised according to the average amount of the endogenous reference.

Animals

For the *MURR1* exon-2 vs. exon-3 expression experiment nine dogs from our breeding colony were used. These dogs were offspring of Bedlington terriers with copper toxicosis (proven by liver histology) and Beagles. Eight of these dogs were known heterozygous carriers; one was homozygous affected. One healthy dog, not Bedlington terrier associated, served as a control. The RT-PCR was performed on liver tissue as described by van de Sluis et al. [10].

For further validation 11 dogs from our in house breeding colony (seven from the first experiment and 4 new born affected) were used next to six healthy dogs, not Bedlington terrier associated.

In addition, we used stored DNA samples isolated from peripheral blood leukocytes from 20 purebred Bedlington terriers of which the status was assessed with the microsatellite marker C04107.

Table 1 - *Primer sets*

Gene	Primer sequence (5'-3')	Annealing (^{o}C)	Product size (bp)
MURR1 exon-2	Forward GACCAAGCTGCTGTCATTTCCAA	60	122
	Reverse TTGCCGTCAACTCTCCAACTCA		
MURR1 exon-3	Forward GTTCATGATCCCTCCCCAGTG	60	118
	Reverse AAGACAAAAGAAATCTCAGCAAGTG		

Results

The results of Q-PCR of exon-2 were expressed as fraction of the measured amount of exon-3 for which all animals are homozygous. For all eight dogs known to be heterozygous (RT-PCR) the ratio exon-2/exon-3 appeared very close to 0.5. In the single affected dog (RT-PCR) from our colony the ratio was close to zero (Table 2). Because taking buccal swabs is less invasive than blood sampling, we measured in four dogs (two carriers, one affected, one healthy) the exon-2/exon-3 ratio in buccal swab derived DNA. These ratios closely resembled the results from whole blood. To make sure that there was no confounding effect of the dogs' age with regard to the exon-2/exon-3 ratio, we measured this ratio repetitively over a wide age range. As shown in Figure 1, the exon-2/exon-3 ratio was not influenced by age. To compare this new technique with the microsatellite marker C04107, we measured blindly the exon-2/exon-3 ratio in blood samples from 20 purebred Bedlington terriers (Table 3).

Table 2 *- RT-PCR (left column) and Q-PCR on MURR1 exon-2 vs. exon-3 expression (right column) in nine mixed breed Bedlington Terrier – Beagle * Bedlington Terrier – Beagle and one healthy dog in whole blood. No. 1-8 carriers (C), 9,10 affected (A) (one dog), 11 healthy (H). Values are expressed as mean ± SE.*

Dog	**RT-PCR** **Liver tissue**	***MURR1* exon2/exon3 ratio** **Whole blood**
1	C	0.36±0.08
2	C	0.37±0.05
3	C	0.43±0.0
4	C	0.42±0.01
5	C	0.49±0.01
6	C	0.52±0.09
7	C	0.46±0.02
8	C	0.48±0.13
9	A	0.00±0.0
10	A	0.00±0.0
11	H	0.93±0.10

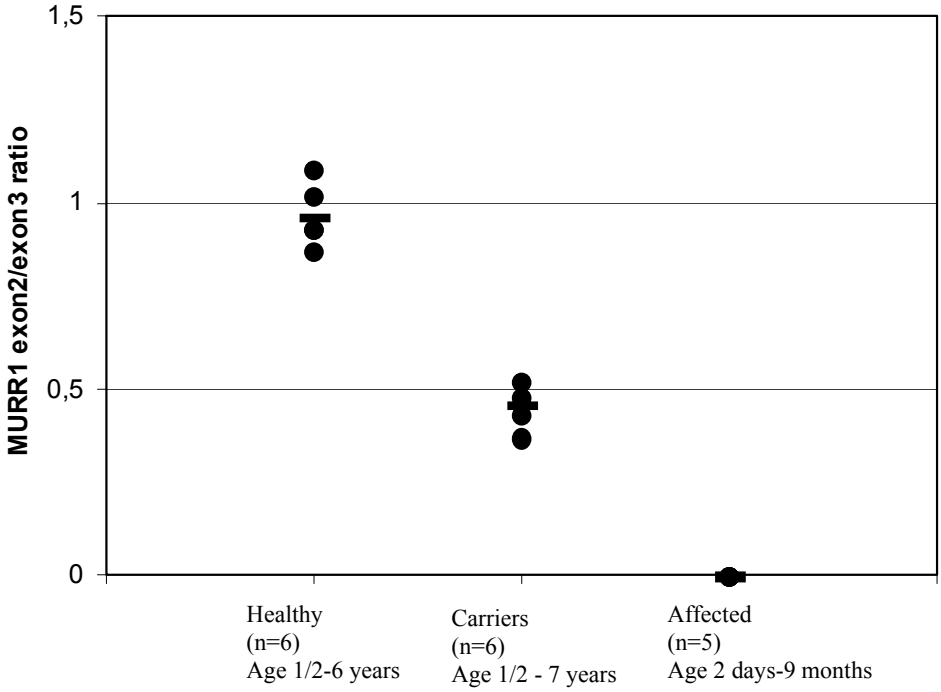

Figure 1 - *Quantitative PCR on MURR1 exon-2/exon-3 ratio in healthy, carriers and affected dogs; long-term reproducibility. In affected dogs ratio was close to zero at each time point.*
- = Mean within groups

Table 3 *- MURR1 exon-2 vs. exon-3 expression in 20 purebred Bedlington carriers in whole blood, formerly diagnosed with the C04107 microsattelite marker. Values are expressed as ranges.*

Group	**Range**	**Median**
Affected (n=3)	0.002 – 0.083	0.016
Carrier (n=11)	0.39 – 0.60	0.44
Healthy (n=6)	0.92 – 1.15	0.99

Discussion

We have found that Q-PCR on DNA isolated from blood samples and buccal swabs permits to differentiate between affected, heterozygote and healthy individuals. The accuracy is very high and it is valid, independent of age. The application of Q-PCR on genomic DNA is principally different from that in RT-PCR reaction. It should be noted that RT-PCR amplification is not a gold standard; it may be hard to read the difference between heterozygotes and healthy homozygotes for 2 reasons. First, due to organ specific splice variants [10] and second due to low expression levels, as was the case in peripheral blood leukocytes. Quantitative evaluation of PCR-products has been developed to analyse differential levels of expression of genes involved in pathways under study. Quantitative analysis at the level of DNA aims at measuring within a narrow variation; there may be 0, 1, or 2 copies of an allele of interest. Therefore, it was critical to evaluate the sensitivity and reproducibility of the measurements performed in this study.

We have validated the method described in several ways. First, we used the non-affected adjacent exon-3 as an internal standard. Whereas the possible forms of exon-2 were absent, present or double present, the normal exon-3 could only be present twice. Exon-3 could therefore serve as standardization factor for the outcome of amplification of exon-2, both PCR reactions being carried out on the same narrow region of genomic DNA. We obtained similar results if the house keeping gene *GAPDH* was used as an internal standard (data not shown), emphasising the validity to use exon-3 as an internal standard. Second, all reactions were done in triplicate to analyse the reproducibility, proving little variation in the outcomes of the measurements per sample (Table 2 and 3, Fig.1). Third, final proof of validation was by performing the quantitative genomic PCR reaction on DNA of animals of which the genetic status could be assessed independently. Our colony of cross bred dogs was obtained by mating an affected Bedlington Terrier sire to healthy Beagle dams producing offspring of obligatory heterozygote carriers. Samples of these dog could serve as independent gold standard. In freshly isolated whole blood samples, we could test the smallest possible variation 1x exon-2 compared with 2x exon-3 in nine of these dogs. There was 100% accordance between the outcome of the Q-PCR reaction and the genetic status of these nine dogs (Table 2), and also between the outcomes of this new test and those of micro-satellite marker C04107 genotyping in 20 Bedlington Terriers (Table 3).

This report emphasises the value of successful application of quantitative genomic PCR for molecular diagnostics of a genetic mutation [15]. This method should therefore be more generally applicable, especially if large genomic deletions are involved, mRNA levels are very low, or the presence of splice variants. It should also be possible to develop

a test for other mutations which cannot be evaluated with standard methods by variants of this quantitative genomic PCR reading.

The *MURR1* gene which underlies a newly discovered copper storage disease has to be analysed with respect to its function in copper homeostasis. From its phenotypical impact in dogs with the disease one may conclude that *MURR1* has a major role in the cellular handling of copper. In analogy to dogs, defects in this gene will also appear to relate to pathologies in humans and rodent models, as emphasized by recent publications. For instance, Tao et al. [16] demonstrated that the Wilson disease protein, a copper transporting ATPase, directly interacting with the human homologue of *MURR1 in vitro* and *in vivo*. Ganesh et al. [6] found that MURR1 restricts HIV-1 replication in resting $CD4^+$ lymphocytes. Furthermore, Burstein et al. [8] discovered an association between the anti-apoptotic protein XIAP and MURR1. Biasio et al. [7] showed that the MURR1 protein functions as a regulator of the human δ epithelial sodium channel. Genetic screening for *MURR1* mutations is thus expected to become of utmost importance in comparative studies on the role of *MURR1* in different cellular processes in man and animals.

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Chapter

7

The COMMD1 deficient dog provides a good model for chronic hepatitis and fibrosis.

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Manuscript in preparation

Abstract

Background/Aims: New therapeutic concepts developed in rodent models with chronic hepatitis could be evaluated in a large animal model prior to human clinical application. Detailed understanding of the pathogenesis and time course of such model is required.
Methods: Over a period of 42 months, each half year liver biopsies were taken from five COMMD1 deficient dogs. Biopsies were used for H&E, reticulin (Gordon and Sweet), and rubeanic acid (copper) staining. Immunohistochemistry was performed on hepatic stellate cell (HSC) activation marker (alpha-smooth muscle actin, α-SMA), proliferation (Ki67), apoptosis (caspase-3), and liver progenitor cell (LPC) markers keratin (K) 19 and 7. Gene expression and Western Blots were performed on gene products involved in the regenerative and fibrotic pathways.
Results: Maximum copper accumulation was reached at 12 months, which coincided with the start of hepatitis. HSCs were activated (α-SMA) from 18 months, with increasing reticulin deposition and hepatocytic proliferation in later stages. Hepatitis and caspase-3 activity (first noticed at 18 months) increased over time. Both HGF and TGF-β1 gene expression peaked at 24 months, and thereafter gradually decreased. Both STAT3 as c-MET showed an increased time-dependent activation. Smad2/3 phosphorylation was present at all time-points and coincides with an increased presence of fibrosis.
Conclusions: COMMD1 deficient dogs develop chronic liver disease and cirrhosis comparable to human chronic hepatitis, although at much higher pace. These animals are suitable large animal models to translate fundamental research into clinical reality.

Introduction

Chronic hepatic injury activates a general aetiology-independent process characterized by liver regeneration and fibrogenesis. The sequence of events starts with the primary insult, followed by proliferation of hepatocytes and activation of non-parenchymal cells, including hepatic stellate cells (HSCs), liver progenitor cells (LPCs) and macrophages, leading to fibrosis, concomitant failure of regeneration, and finally cirrhosis [1,2].

HSCs are situated in the space of Disse along the sinusoids. Quiescent HSCs contain clear vitamin-A loaded lipid droplets. Upon cytokine-induced activation they rapidly loose the lipid droplets, increase the expression of alpha-smooth muscle actin (α-SMA), and secrete extracellular matrix (ECM) components [3-5]. HSCs, together with other mesenchymal liver cells and hepatocytes, produce transforming growth factor β (TGF-β) [6]. It has been established that a transient increase of TGF-β1 in the liver promotes fibrosis with the formation of ECM components, and suppresses hepatocyte proliferation [7]. The hallmark of TGF-β signaling is SMAD2/3 phosphorylation [8]. HSCs are also known to play an important role in hepatic regeneration as they express growth factors such as hepatocyte growth factor (HGF). HGF is a potent mitogen for hepatocytes and has been shown to induce a wide variety of other physiological activities such as anti-apoptosis, morphogenesis and angiogenesis [9,10]. When mature hepatocytes are extensively damaged or hampered in their replication, the liver progenitor cell (LPC) compartment is activated [11]. LPCs are bipotential and can differentiate both into cholangiocytes and hepatocytes [1,12,13].

Chronic liver disease is a worldwide health problem, which in later stages leads to ineffective regeneration, fibrosis, cirrhosis and tumour formation, and can only be solved by organ transplantation. New therapeutic options like antifibrotic strategies, or growth factor-mediated interventions are being developed in rodent model studies [2,14]. The step from principle to practice, however, remains large. The availability of a suitable large animal model with a comparable pathophysiology of chronic liver disease to man is lacking. The availability of such a model would greatly enhance the understanding of possibilities and drawbacks of new interventions in a clinical setting. Furthermore it could be tailored to develop effective and safe protocols before their first application in the clinic [14]. Recently, canine fibrotic liver diseases have been demonstrated to be highly comparable to their human counterparts in both pathophysiological and molecular mechanisms [13,15]. Comparable pathophysiology itself is not sufficient to become a model animal. A defined aetiology allows for more standardized experimental conditions. A potential dog model with a well-defined genetic aetiology is the Bedlington terrier (BT) which lacks the COMMD1 protein. The absence of COMMD1 protein in BT, due to a

deletion of exon-2 of the *COMMD1* gene, results in a progressive accumulation of copper in hepatocytes leading to chronic hepatitis and cirrhosis [16,17]. This canine disease was discussed as a potential model for human Wilson's disease (WD), an autosomal recessive inherited copper storage disorder due to a reduced or absent expression of the *ATP7B* gene [18-25]. Biochemical measurements focussing on the interaction between COMMD1 proteins and ATP7B proteins, explained similarity between the canine and human pathology [26,27]. Because of their size and their life-span, the availability of molecular tools, the possibility and easiness to take multiple and sequential liver biopsies, combined with a known genetic aetiology these dogs are potentially well suited for evaluation of new treatment protocols aimed at enhanced liver regeneration and reduction of fibrosis/cirrhosis. In this respect, it is highly advantageous when the pathogenesis of chronic liver diseases of model animal and human patients are much alike. Therefore we provide a detailed longitudinal analysis of regenerative and fibrotic pathways of dogs which develop chronic hepatitis caused by an inborn genetic defect.

Materials and methods

Animals

Five COMMD1 deficient dogs (two males, three females) were used for longitudinal follow-up. COMMD1 deficiency status was confirmed as described previously [28]. Dogs were examined every six months up to the age of 42 months (seven examination points). At each occasion the clinical symptoms were scored, physical examination was performed, and five liver biopsies were taken. Dogs were housed individually and received normal commercial dog food, not restricted for copper, once a day and water ad libitum. The procedures were approved by Utrecht University's Ethical Committee, as required under Dutch legislation.

Sampling and histopathology

Liver biopsy samples (five per dog per time-point) were obtained using a 14G Menghini needle [29]. Two biopsies were formalin-fixed, and 4μm thick paraffin sections were stained with haematoxylin and eosin (H&E), rubeanic acid (RA) for copper quantification, reticulin according to Gordon and Sweet, and Sirius red. Fibrosis scoring was performed by one board-certified veterinary pathologist (TvdI) according to a modified Scheuer classification (0 = normal, 1 = local mild centrolobular fibrosis, 2 = multifocal mild to moderate centrolobular fibrosis, 3 = moderate fibrosis with centro-central bridging or nodular transformation). Hepatitis was scored as 0 (absent), 1 (slight), 2 (mild), 3

(moderate) and 4 (marked) [30]. Copper accumulation (RA) was evaluated semi-quantitatively using a scale from 0 to 5 as previously described [31], and localization within the liver lobule was assessed. Three biopsies were fixed in RNAlater (Ambion, Austin, TX, USA) for a maximum of 24 hours and stored at -70 °C.

Immunohistochemistry

Alpha-smooth muscle actin (α-SMA), activated caspase-3 (Casp3), proliferation marker Ki67, Keratin 7 (K7), and Keratin 19 (K19) were stained as described elsewhere with slight modifications [5,32]. The antibodies used for IHC are described in Table 1. Three µm sections were mounted on poly-L lysine coated slides and stored for a maximum of 48 hours at room temperature (RT). Sections were deparaffinised followed by enzymatic (K7, K19) or heat-induced (Ki67) antigen retrieval (AR). Enzymatic AR was performed with proteinase K (DAKO, Glostrup, Danmark) for 40 minutes (K7) or 15 minutes (K19). Heat induced AR for Ki67 was achieved in 10 mM Citric acid buffer (pH 6) for 15 minutes in a microwave. Casp3 and α-SMA staining needed no AR. Endogenous peroxidase and unspecific binding were blocked in 0.3% H_2O_2 and 10% non-immune goat serum for 30 min, respectively. After application of the primary antibody over night (Casp3 and K7) or 1h at RT (α-SMA, Ki67, and K19), slides were washed in PBS and incubated with the appropriate Envision (DAKO) HRP-labeled polymer conjugated to the secondary antibody (anti-mouse or -rabbit) for 45 minutes at RT. After washing staining was detected using 3-3'-diaminobenzidine and nuclei were counterstained with 10% Mayer's haematoxylin.

α-SMA grading was performed as follows: 0: no staining of HSCs; 1: a few positive HSCs; 2: diffuse staining of HSCs; 3: mild increased staining of HSCs; 4: marked increased staining of HSCs. Results were compared with normal aged matched controls. The quantity of liver tissues from two needle biopsies were equal at all time points and in all animals, therefore both Ki67 and Casp3 were expressed as the number of positive hepatocytes per slide. K19 and K7 were graded as the number of positive cells in the limiting plate per portal area, either solitary or as small groups.

Table 1 *- Used antibodies in immunohistochemical experiments.*

Name	Antibody	Dilution	Incubation	Positive control	Supplier
α-SMA	mouse monoclonal	1:200 in PBS	60 minutes at RT	Hepatic artery smooth muscle	BioGenex, San Ramon, CA, USA
Caspase-3	rabbit polyclonal	1:200 in PBS + 1% BSA	O/N at 4°C	Canine acute hepatitis	R&D Systems, Minneapolis, MN, USA
Ki67	rabbit monoclonal	1:50 in TBS	30 minutes at RT	Canine duodenum	LabVision, Fremont, CA, USA
K19	mouse monoclonal	1:100 in TBS	60 minutes at RT	Bile ducts	Novacostra, Newcastle upon Tyne, UK
K7	mouse monoclonal	1:25 in TBS + 1% BSA	O/N at 4°C	Bile ducts	Dako, Glostrup, Denmark

RT: room temperature; O/N: overnight

RNA isolation and quantitative RT-PCR (q-PCR)

RNA was isolated from RNAlater fixed biopsies, using Qiagen RNeasy Mini Kit (Qiagen, Leusden, the Netherlands). RNA quality was analysed with the Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA). Q-PCR was performed on TFG-β1, TGF-β receptor type I (TGF-βR1), TGF-β receptor type II (TGF-βR2), hepatocyte growth factor (HGF), and c-MET protooncogene (c-MET). Q-PCR was performed using primers as described in Table 2 and was based on the high affinity double-stranded (ds) DNA-binding dye SYBR green I (iQSYBR Green Supermix, BioRad, Veenendaal, the Netherlands) [15]. Normalisation was performed using four reference-genes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyl transferase (HPRT), ribosomal protein S5 (RPS5) and S19 (RPS19)) [33]. Q-PCR results were related to the expression of six clinically and histologically determined healthy control dogs (one to three years of age). Minus RT and water controls remained negative.

Table 2 *- Nucleotide Sequences of dog specific primers for Quantitative Real-Time PCR*

Gene	Primer sequence (5'→ 3')	Annealing temperature (^{o}C)	Product size (bp)	Accession number
TGF-β1	Forward CCAGGATCTGGGCTGGAAGTGGA	66	113	L34956
	Reverse CCAGGACCTTGCTGTACTGCGTGT			
TGF-βR1	Forward AGTCACCGAGACCACAGACAAAGT	59	101	AY455799
	Reverse TGAAGATGGTGCACAAACAAATGG			
TGF-βR2	Forward GACCTGCTGCCTGTGTGACTTTG	61	116	AY455800
	Reverse GGACTTCGGGAGCCATGTATCTTG			
HGF	Forward AAAGGAGATGAGAAACGCAAACAG	58	92	BD105535
	Reverse GGCCTAGCAAGCTTCAGTAATACC			
c-MET	Forward TGTGCTGTGAAATCCCTGAATAGAAAT	59	112	AB118945
	Reverse CCAAGAGTGAGAGTACGTTTGGATGAC			
HPRT	Forward AGCTTGCTGGTGAAAAGGAC	56	100	L77488/9
	Reverse TTATAGTCAAGGGCATATCC			
GAPDH	Forward TGTCCCCACCCCCAATGTATC	58	100	AB038240
	Reverse CTCCGATGCCTGCTTCACTACCTT			
RPS5	Forward TCACTGGTGAG/AACCCCCT	62.5	141	XM 533568
	Reverse CCTGATTCACACGGCGTAG			
RPS19	Forward CCTTCCTCAAAAAGTCTGGG	61	95	XM 533657
	Reverse GTTCTCATCGTAGGGAGCAAG			

Western Blot analysis

Liver biopsies were lysed in RIPA buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1mM sodium orthovanadate, 1 µg/ml aprotinin, and 1mM PMSF. Extracted protein was quantified using a Lowry based assay (DC Protein Assay, Bio-Rad, Veenendaal, the Netherlands). To detect HGF, phospho-c-MET, (phospho-)STAT3 and (phospho-)Smad2/3 protein levels, 7.5 µg protein was

denatured for 3 minutes at 95°C, was run on a Criterion Tris-HCl polyacrylamide gel (Bio-Rad), transferred to Hybond-C nitrocellulose membranes (Amersham Biosciences, Europe, Roosendaal, the Netherlands) and blocked with blocking reagent from the ECL Advance Western Blotting Detection Kit (Amersham, Little Chalfont, UK) or with non-fat dry milk (HGF). The blots were incubated overnight at 4°C with the appropriate primary antibodies (Table 3).

Table 3 *- Used antibodies in Western blot experiments.*

	Primary antibody	Dilution	Incubation	Product size (kDa)	Supplier
phospho-Smad2 (Ser465/467)	Rabbit polyclonal	1:500	O/N at 4°C	52.5	Cell Signaling Technology, Beverly, MA, USA
Smad 2/3	Mouse monoclonal	1:500	O/N at 4°C	52.5	BD Transduction Laboratories, Franklin Lakes, NJ, USA
HGF	Rabbit polyclonal	1:1000	O/N at 4°C	83	Abcam, Cambridge, UK
phospho-c-MET (Y1230 + Y1234 + Y1235)	Rabbit polyclonal	1:750	O/N at 4°C	169	Abcam Cambridge, UK
phospho-STAT3 (Ser727)	Rabbit polyclonal	1:1,000	O/N at 4°C	86	Cell Signaling Technology, Beverly, MA, USA
STAT3	Mouse monoclonal	1:2,500	O/N at 4°C	86	BD Transduction Laboratories, Franklin Lakes, NJ, USA
beta-actin (ACTB)	Mouse monoclonal	1:2,000	O/N at 4°C	42	Thermo Fisher Scientific, Fremont, CA, USA

O/N: overnight

After this procedure STAT3, Smad2/3, and beta-actin blots were incubated with HRP conjugated anti-mouse antibodies (R&D Systems Europe, Abingdon, Oxfordshire, UK) dilution 1:20,000. HGF, phospho-STAT3, phospho-Smad2, and phospho-c-Met blots were incubated with HRP conjugated anti-rabbit antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) dilution 1:20,000. All secondary antibodies were incubated for 1 hour at RT. The ECL Advanced Western Blotting Detection Kit (Amersham) was used to achieve luminescence which was measured with a ChemiDoc XRS Imager (BioRad) and analyzed with provided software (Quantity-One 4.6.5).

Statistical analysis

Statistical analysis was performed in R (version 2.11.1) [34]. Quantitative PCR data were analyzed using linear mixed-effect modeling with the R package "nlme". The outcome variables were transformed by taking their natural logarithm in order to fulfill the criteria of normality and constant variance. Restricted maximum likelihood method was used to estimate the best fitting model. For all gene products the random intercept model was the best fitting model based on Akaike's information criterium. Maximum likelihood was used to estimate the fixed effect of the time in months (included in the model as a factor). The reference level of gene expression was set to be the expression at 6 months of age, and for every time-point differences from this reference were determined. A Bonferroni corrected p-value of 0.0083 (0.05 divided by 6 comparisons) was used to determine significance. True estimates and 95% confidence intervals were obtained by computing the exponents of the results from the mixed model fit on the ln transformed data.

Overall differences in immunohistochemical data at the 7 time points were determined by the Friedman test. When significant, the Wilcoxon signed rank test with continuity correction was performed as a post hoc test for comparisons of all time points with the reference time point (6 months). A Bonferroni corrected p-value of 0.0083 (0.05 divided by 6 comparisons) was used to determine significance.

Results

Animals

During the entire follow-up period COMMD1 deficient dogs developed normally and showed no abnormalities at physical examination during the whole time of the study.

Histopathological findings

At six months of age all five dogs showed a moderate centrolobular accumulation of

copper in hepatocytes; however no evidence of copper laden Kupffer cells or macrophages (derived from the destruction of copper laden hepatocytes) or other evidence of hepatitis was observed at this stage (Fig. 1). At 12 months of age all animals had extensive copper accumulation almost diffusely throughout the lobules and in two out of five animals a mild hepatitis was present. At 18 months all dogs showed a slight to mild hepatitis. Although some variation was seen between individual animals the activity of hepatitis became moderate to high at older age.

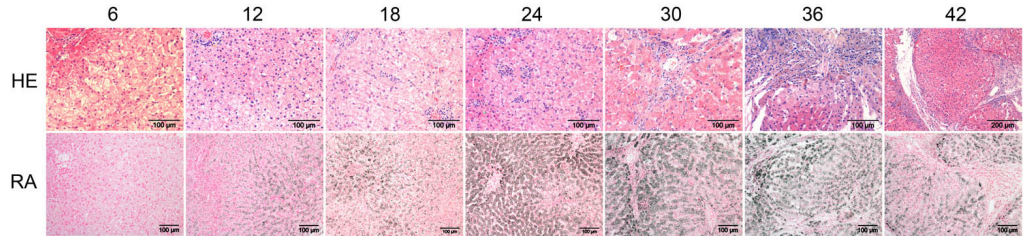


Fig. 1 *- Histological description.*
COMMD1 deficient dog livers were stained with H&E and RA to assess inflammation and copper accumulation, respectively. Representative pictures of a COMMD1 deficient dog over a period of 42 months are shown. Numbers indicate age in months.

Fibrogenesis

α-SMA showed an increasing presence similarly in the COMMD1 deficient dogs and normal age matched controls between 6 (grade 0-1) and 18 months (grade 2). Abnormal increased positivity for α-SMA was seen in all COMMD1 deficient dogs in the centrolobular areas starting from 18 months of age (Fig. 2 & Table 4).

The reticulin stain showed progressive centrolobular fibrosis starting in one of the five dogs at 24 months of age (Fig. 2 & Table 4) and present in all COMMD1 deficient dogs at 42 months of age, finally resulting in centro-central bridging fibrosis and cirrhosis in three of the five animals (at 42 months). Sirius red staining was similar to reticulin staining (data not shown).

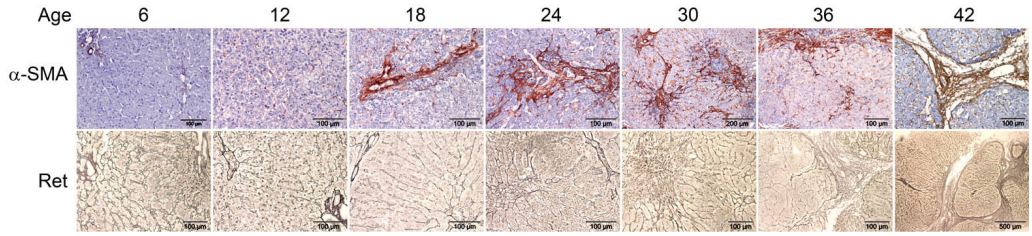


Fig. 2 *- Fibrosis. Histological description.*
COMMD1 deficient dog livers were stained with IHC for α-SMA and reticulin for collagen type III to assess fibrosis. Representative pictures of a COMMD1 deficient dog over a period of 42 months are shown. Numbers indicate age in months.

Apoptosis and regeneration

Having established that the COMMD1 dogs develop severe fibrosis with increasing age the fate of hepatocytes was investigated. Active caspase-3 remained undetectable up to 24 months and showed the first indication of activation in the hepatocytes from 30 months of age with a progressive 2-fold increase of the median at 42 months (Table 5).

A 2-fold increased median of the number of Ki67 positive hepatocytes was seen at 36 and 42 months (Table 5), particularly in the three of the five dogs with centro-central bridging fibrosis and cirrhosis, and in the areas with the least copper storage. To exclude regeneration from liver progenitor cells, K7 (not shown) and K19 (Table 5) immunohistochemical stainings were performed. Separate K7 and K19 stainings were highly comparable. An increase of LPCs was seen in the COMMD1 deficient dogs from 30 months onwards (median doubled at 30 months of age); there was no evidence of transition of progenitor cells into intermediate cells. Altogether, hepatocytic regeneration is derived solely from the hepatocyte compartment.

Table 4 *- Fibrosis.*
Fibrosis scoring: α-SMA (hepatic stellate cell activation) and reticulin grading (collagen type III) in five COMMD1 deficient dogs in a time-dependent copper-induced hepatitis. Data are presented as median (range). Age in months.
α-SMA grading; 0: no staining of HSCs; 1: a few positive HSCs; 2: diffuse staining of HSCs; 3: mild increased staining of HSCs; 4: marked increased staining of HSC and compared with age matches normal controls.
Reticulin grading was performed according to a modified Scheuer classification[30]; grade 0: normal, grade 1: local mild centrolobular increase, grade 2: multifocal mild to moderate centrolobular increase, grade 3: moderate increase with centro-central bridging or nodular transformation.
Overall differences in immunohistochemical data at the 7 time points were determined by the Friedman test (p-value of 0.0009 and 0.028 for α-SMA and reticulin, respectively). When significant ($p < 0.05$), the Wilcoxon signed rank test with continuity correction was performed as a post hoc test for comparisons of all time points with the reference time point (6 months). A Bonferroni corrected p-value of 0.0083 (0.05 divided by 6 comparisons) was considered significant. No significant differences on Bonferroni level were detected.

Age	6	12	18	24	30	36	42
α-SMA grading (0-4)	1 (-)	2 (1-2)	2 (2-2.5)	3 (2-4)	3 (3-4)	4 (2-4)	3 (2-4)
p-value	-	0.15	0.048	0.057	0.053	0.054	0.057
Reticulin grading (0-3)	0 (-)	1 (0-1)	0 (0-1)	1 (0-2.5)	0 (0-2.5)	2.5 (0-3)	2 (1-3)
p-value	-	0.15	1	0.17	0.37	0.17	0.058

Table 5 *- Apoptosis and regeneration.*
Number of (active) caspase-3 (Casp3) positive hepatocytes per biopsy (quantity of liver tissues (manual count) from two needle biopsies were equal at all time points and in all animals), number of Ki67 positive hepatocytes per biopsy (manual count), and number of K19 positive cells in the limiting plate per portal area in five COMMD1 deficient dogs in a time-dependent copper-induced hepatitis. Values are expressed as median (range).
Overall differences in immunohistochemical data at the 7 time points were determined by the Friedman test (p-value of 0.0074, 0.24, and 0.0037 for Casp3, Ki67, and K19, respectively). When significant ($p < 0.05$), the Wilcoxon signed rank test with continuity correction was performed as a post hoc test for comparisons of all time points with the reference time point (6 months). A Bonferroni corrected p-value of 0.0083 (0.05 divided by 6 comparisons) was considered significant. No significant differences on Bonferroni level were detected.

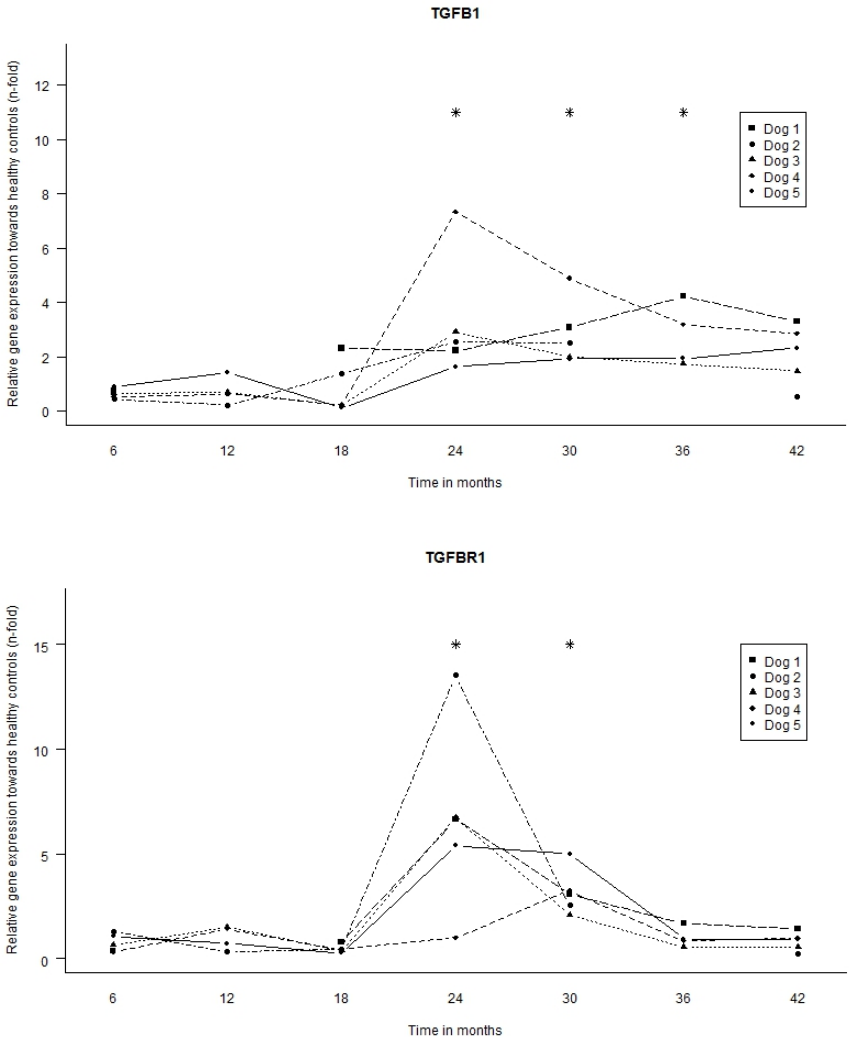
Age	6	12	18	24	30	36	42
Casp3	0 (-)	0 (-)	0 (0-1)	0 (-)	3 (0-7)	0 (0-17)	2 (0-22)
p-value	-	N/A	0.35	N/A	0.098	0.37	0.10
Ki67	6 (4-20)	5.5 (3-8)	8 (5-14)	3.5 (2-9)	5 (1-7)	16 (1-31)	11 (1-32)
K19	1.9 (1.2-3.2)	2.5 (1-3.3)	3.2 (2.7-3.4)	1.8 (1.5-3.3)	3.7 (2.5-5.3)	3.6 (2.8-7.4)	5.2 (4-9.6)
p-value	-	0.10	0.063	0.58	0.058	0.063	0.063

N/A: not available

Gene-expression profiling
Gene-expression of TGF-β1 and both its receptors increased significantly starting at 24 months. The mRNA levels of receptor TGF-βR1 and TGF-βR2 decreased after 30 months, whereas. TGF-β1 mRNA expression decreased after 36 months (Fig. 3A). The mRNA expression of HGF and its receptor c-MET showed a sharp increase (15 and 11-fold, respectively) occurring at 24 months of age in all dogs (Fig. 4A). This peak decreased gradually over time and returned to starting levels at 42 months of age.

Western blot analysis

For downstream signalling of the fibrosis and regeneration pathway, western blots were performed at the time points 12, 30, and 42 months of age. Smad2/3 was detectable at all time-points. Phosphorylated Smad2 was present in the COMMD1 deficient dogs at all time-points with no apparent differences (Fig. 3B). HGF was present at all measured time-points with individual differences. Phosphorylated c-MET was age-dependent increased. An age-dependent decrease in levels of total STAT3 was seen. Phosphorylated STAT3 was age-dependently increased in COMMD1 deficient dogs (Fig. 4B).



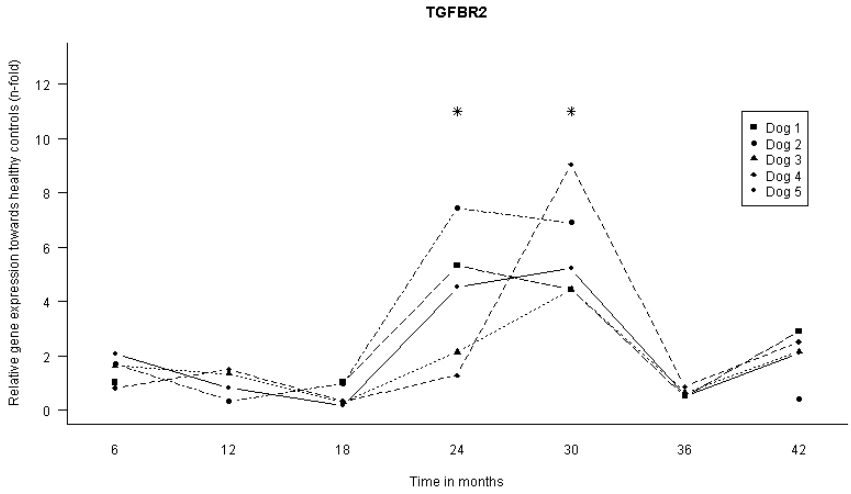


Fig. 3A *- TGF-β1 pathway.*
*Gene-expression profiling. Interaction plots of Q-PCR data of important mediators of fibrogenesis in a time-dependent copper-induced hepatitis in five COMMD1 deficient dogs. Q-PCR results were normalized against the expression of six control dogs (one to three years of age). Linear mixed-effect modeling was used; * indicates significant difference (p-value smaller than Bonferroni corrected p-value of 0.0083).*

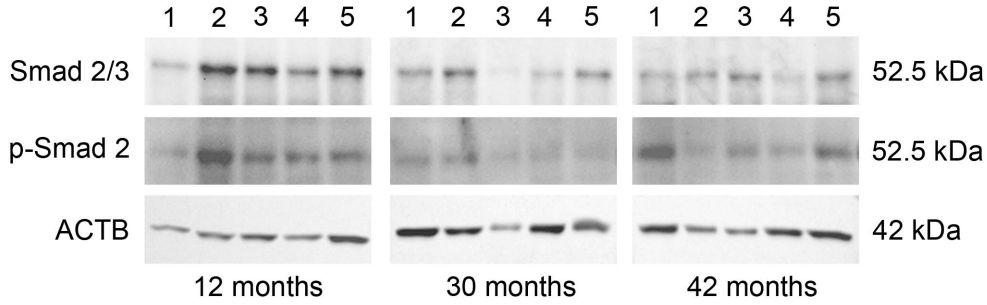


Fig. 3B *- TGF-β1 pathway.*
Western blot analysis on the activation of TGF-beta signalling (total Smad2 and phosphorylated Smad2 (Ser727)) of five COMMD1 deficient dogs at 12, 30, and 42 months. β-actin (ACTB) served as loading control.

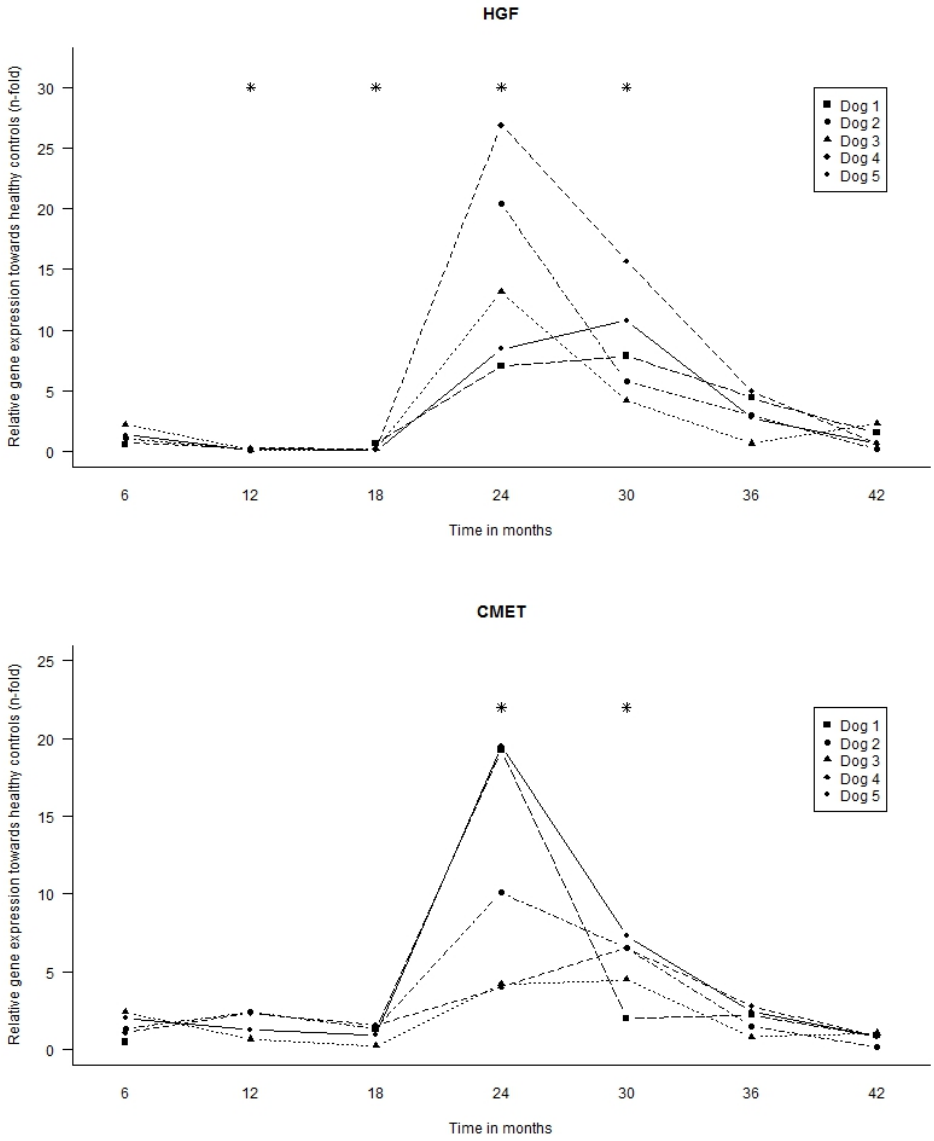


Fig. 4A *- HGF pathway.*
*Gene-expression profiling. Interaction plots of Q-PCR data of important mediators of regeneration in a time-dependent copper-induced hepatitis in five COMMD1 deficient dogs. Q-PCR results were normalized against the expression of six control dogs (one to three years of age). Linear mixed-effect modeling was used; * indicates significant difference (p-value smaller than Bonferroni corrected p-value of 0.0083).*

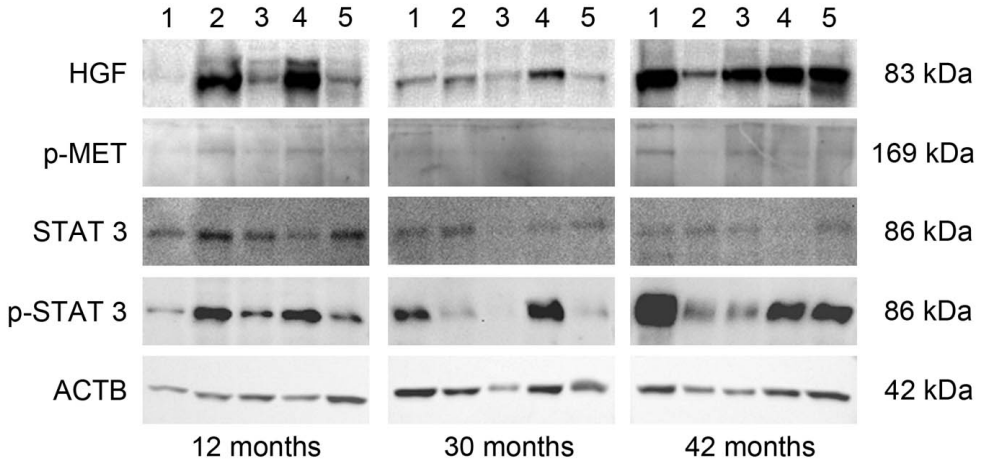


Fig. 4B *- HGF pathway.*
Western blot analysis on the activation HGF signalling (HGF, phosphorylated c-Met, (phosphorylated) STAT3) of five COMMD1 deficient dogs at 12, 30, and 42 months. β-actin (ACTB) served as loading control.

Discussion

In this study we evaluated COMMD1 deficient dogs as a possible large animal model for chronic hepatitis. Five dogs were part of a longitudinal study to assess inflammation, copper accumulation, fibrosis, regenerative pathways, and molecular pathways over a period of 42 months. Based on the similarities in the pathogenesis of hepatitis we propose the COMMD1 dog as a reproducible large animal model for chronic hepatitis and cirrhosis in man.

During the longitudinal follow up of five COMMD1 deficient dogs moderate chronic hepatitis was evident at 30 months of age, in some cases signs of hepatitis were detectable as early as 12 months. Copper had accumulated diffusely throughout the lobules at 12 months of age. Activation of HSCs based on α-SMA staining was apparent in centrolobular regions from 18 months of age and preceded fibrosis. HSC activation was accompanied by production of extracellular matrix indicated by the reticulin staining (collagen III), and slowly progressed further to fibrosis and cirrhosis.

Apoptosis was observed in the biopsies from 18 months of age and increased in time. Hepatocyte apoptosis has been well documented in HCV and HBV-associated chronic

liver diseases [35]. Also in COMMD1 deficient dogs apoptosis seems a component of the hepatocytic injury in this case associated with the copper storage in the hepatocytes. Free copper is highly toxic due to its ability to generate hydroxyl radicals and high hepatic levels of copper induce oxidative stress [36] and it has been demonstrated that hepatocellular apoptosis is triggered by copper-induced cell damage [37,38]. Signs of regeneration appeared when the inflammatory process had progressed. This was mainly associated with hepatocytic regeneration shown by Ki67 immunostaining. LPC activation, reflected by K19 immunostaining, was also present. In acute hepatitis LPCs differentiate through intermediate hepatocytes into hepatocytes within one week of the insult [39]. The fact that no intermediate hepatocytes were seen near the activated LPCs in the described dogs indicates a minor contribution of these cells to liver regeneration in this stage and model of hepatitis. The very slow onset of disease and regenerative capacity of hepatocytes may explain that the endogenous LPCs do not contribute to the regeneration process in this model. One important multifunctional cytokine during liver regeneration is HGF [9,10]. In this study, mRNA-expressions of HGF and its receptor c-MET were increased at 24 months of age, thereafter they returned to baseline levels. This HGF peak coincided with hepatic stellate cell activation (α-SMA), and the expression of TGF-β1 and its receptors. Although HGF and MET expression were temporarily present, HGF, c-MET activation, and the down-stream time-dependent increase in phospho-STAT3 indicated a continuous activation of the regenerative pathway. High HGF protein levels at 42 months are presumed to be from an extra hepatic source.

mRNA levels of TGF-β1 signalling components increased transiently from 24 months. Smad2/3 phosphorylation was present at all time-points and coincides with an increased presence of fibrosis as indicated by the reticulin staining (collagen type III).

Until now Bedlington terriers deficient for COMMD1 have been the best known dog model for hepatic copper toxicosis. Copper storage in COMMD1 deficient dogs causes chronic hepatitis, fibrosis and cirrhosis causing hepatic failure, portal hypertension, ascites, portosystemic collateral circulation, and hepatic encephalopathy [40]. Therewith all features common to not only copper-associated, but all forms of chronic hepatitis and cirrhosis are displayed. This reproducible autosomal recessive genetic disease may thus serve as a very appropriate large animal model for chronic hepatitis and cirrhosis in man. Our study adds to the understanding of the sequence of pathophysiological events occurring in copper associated hepatitis in COMMD1 deficient dogs and agrees with a semi-longitudinal study performed in *ATP7B*$^{-/-}$ mice [41]. However, our large animal model proves at least additive because of the ability to take multiple biopsies from the same individual, to obtain more material and study multiple parameters. No human copper storage disease thus far has been associated with a defect in COMMD1 [42,43]. Like other

model animals, the causative agent is not similar to the usual causative agents in human chronic hepatitis. These features make the COMMD1 deficient dogs model animals and not exact copies of human chronic hepatitis.

In conclusion, this longitudinal follow-up of COMMD1 deficient dogs with a copper induced chronic hepatitis demonstrates hepatocytic alteration, fibrosis and regeneration in a time dependent manner. This is the first well defined large animal model of chronic hepatitis. We anticipate that it may provide an important instrument for the evaluation of future regenerative and antifibrotic therapies in a preclinical model and helps to close the gap between commonly used rodent models and human pathology.

Acknowlegdements

We would like to thank of J.A. Drie – van Putten, R. Kisjes and H.G.H. van Engelen for technical assistance. H. Vernooij (Centre for Biostatistics, Utrecht University) assisted with statistical analysis.

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Chapter

8

A longitudinal study on copper-induced chronic hepatitis and interference with penicillamine in COMMD1 deficient dogs

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Manuscript in preparation

Abstract

Background/Aims: Free copper is highly toxic due to its ability to generate oxidative stress. Dogs with a deletion of exon-2 of the *COMMD1* gene develop copper-induced chronic hepatitis. This longitudinal study was undertaken to investigate the effect of the absence of COMMD1 on the hepatic copper regulation and oxidative stress and the effect of prolonged penicillamine treatment on these parameters.
Methods: From five COMMD1 deficient dogs, each half year liver biopsies were taken over a period of five years. Treatment with penicillamine was started at 43 months till the end of the study at 60 months. Biopsies were used for quantitative copper analysis and histology after staining with haematoxylin & eosin and rubeanic acid (semi-quantitative copper analysis). Gene-expression analysis was performed on genes involved in copper metabolism (COX17, CCS, ATOX1, ATP7A, ATP7B, MT1A, CP) and oxidative stress (SOD1, catalase, GPX1). In addition, immunohistochemistry (IHC) for ATP7B and COMMD1 was performed.
Results: Copper accumulation reached a maximum semi-quantitative score between 12 and 18 months which was associated with the start of hepatitis. Serum ALT increases from 24 months of age. After starting treatment with penicillamine, ALT decreased but remained above the upper reference limit. Semi-quantitative and quantitative copper scores decreased slowly during treatment but still remained increased. Gene expression of the copper chaperones CCS, COX17, and ATOX1 were, although not significantly, elevated until 24 months of age. Expression of ATP7A and ATP7B increased after 24 months of age. Both were reduced to normal at 36 and 30 months of age, respectively. Superoxide dismutase expression tended to be increased till 24 months of age (significant at 12 months) and decreased thereafter, without being altered during treatment with penicillamine. Catalase gene expression was increased at 24 months of age, coinciding with a severe increase of microscopically visible hepatitis, remained significantly increased during the rest of the follow-up, and remained, although not significant, five-fold increased during treatment with penicillamine. IHC in control dogs showed granular, both periportal and central acinar hepatocytic staining for COMMD1 and a canalicular distribution for ATP7B.

Conclusions: In the sequence of events, copper accumulation induced progressive hepatitis followed by a transient increase in gene products associated with intracellular copper trafficking and temporal activation of anti-oxidative stress mechanisms. Although penicillamine reduced intracellular copper levels and improved clinical parameters, it was of little influence on the expression of the gene products analysed.

Introduction

The trace element copper is actively taken up by cells via the copper transporter CTR1, and subsequently integrated in enzymes involved in several vital biologic processes [1-4]. Free copper is toxic due to its ability to generate reactive oxygen species, including the highly reactive hydroxyl radicals [5]. Consequently free intracellular copper levels are very low due to the presence of copper chaperones, sequestering proteins, and efflux pumps. ATOX1 delivers copper to copper-dependent ATPases, CCS (copper chaperone for SOD) distributes copper to Cu/Zn superoxide dismutase (SOD1), and COX17 delivers copper to cytochrome c oxidase in the mitochondria. Furthermore, free intracellular copper is sequestered by metallothioneins (MT) and MT1A mRNA and protein levels are rapidly increased when intracellular free copper levels rise [6]. Copper efflux occurs via the copper ATPase pumps encoded by the Wilson's disease (ATPase, Cu(2+)-transporting, beta polypeptide (ATP7B)) and Menkes disease genes (ATPase, Cu(2+)-transporting, alpha polypeptide (ATP7A)). Both ATPases exhibit copper-induced trafficking and redistribution properties in response to changes in copper abundance [7]. ATP7B is involved in the transfer of copper from the Golgi network to the apical membrane [8,9]. ATP7B deficiency results in a loss of biliary excretion of copper as well as a lack of copper incorporation into secreted proteins produced in the liver, with ceruloplasmin (CP) being the most notable example as it forms around 90% of the serum copper [10].

Positional cloning led to the discovery of a new gene product involved in copper handling, *copper metabolism (Murr1) domain containing 1 (COMMD1)*, which is ubiquitously expressed in all tissues [11,12]. Dogs with a deletion in exon-2 of *COMMD1* develop copper-induced chronic hepatitis. *In vitro* studies revealed increased copper accumulation in several cell types after *COMMD1* gene silencing [13,14]. Although its mechanism of action remains enigmatic, a functional relationship has been demonstrated between COMMD1 and ATP7B [15]. In addition, COMMD1 activity is associated with NFκB breakdown [12], and more recently with inhibition of metastasis [16]. In copper metabolism, COMMD1 protein is suggested to function as a traffic agent in escorting target proteins to the proteasome for further breakdown or to endosomes for incorporation in the apical membrane [17-22].

Several model animals have been proposed to investigate the human copper storage disorder Wilson's disease (WD), such as toxic milk mice, Long-Evans Cinnamon rats, and ATP7B knockout mice [23-26]. The effects of copper accumulation on the liver are in most cases observed when pathology has fully developed. Moreover, murine liver size clearly hampers sequential biopsies. Therefore, longitudinal studies following individual animals

animals in time until chronic hepatitis has developed and the effect of interference in copper uptake/metabolism at that late stage are lacking.

At the department of Clinical Sciences of Companion Animals of Utrecht University a dog population with the *COMMD1* mutation [27] has been created for longitudinal follow-up studies on the development of copper-associated chronic hepatitis. In contrast to *COMMD1*$^{-/-}$ mice, that die during gestation (possibly due to inferior placental development) [28] these dogs are born healthy but develop progressive chronic hepatitis later in life. Copper-associated hepatitis can be treated with dietary management, copper chelating agents, or zinc supplementation. In dogs good experience has been gained for decades with the use of penicillamine. With penicillamine, the copper overload in the affected liver is slowly reduced and controlled at a normal level within 6 to 12 months [29]. These features make the COMMD1 deficient dogs excellent animal models for a longitudinal study on the effects of COMMD1 deletion on the hepatic copper regulation. In the present longitudinal study we give detailed analysis of the time-course of the different intracellular players in copper metabolism related to development of hepatitis, and the effects of penicillamine treatment in COMMD1 deficient dogs are presented.

Materials and methods

Animals

All procedures were approved by Utrecht University's Ethical Committee, as required under Dutch legislation. Five COMMD1 deficient dogs (two males, three females) were used for longitudinal follow-up. COMMD1 deficiency status was confirmed as described previously [27]. The dogs were examined every six months up to the age of 60 months (10 examination points). At each occasion the clinical symptoms were scored, physical and blood examination was performed, and five liver biopsies were taken per animal at each time point. At 43 months of age penicillamine treatment (20 mg/kg/day) was started. The dogs were housed individually, fed once a day a normal commercial dog food not restricted for copper (Noblesse, Purina), and had free access to water.

Sampling and histopathology

The liver biopsy samples were obtained using a 14G Menghini needle under local anaesthesia [30]. Biopsies were stored differently to acquire optimal material for various analyses [31]. Two biopsies were fixed in neutral buffered formalin for one hour and then placed in 70% ethanol for 24 hours and embedded in paraffin. Slides were cut at 4 µm and stained with haematoxylin and eosin (HE), and rubeanic acid (RA) staining for copper. The

The presence and activity of hepatitis was scored as 0 (absent), 1 (slight), 2 (mild), 3 (moderate), and 4 (marked). The presence of copper was evaluated semi-quantitatively using a scale from 0 to 5 as previously described, as well as with respect to its localization in the liver lobules [32]. All slides were examined by one board-certified veterinary pathologist (TvdI). One biopsy was snap frozen and the other two biopsies were placed for 24 hours in RNA later (Ambion, Austin, TX) and all three were subsequently stored at -70 °C until assayed.

Copper assessment

The semi-quantitative assessment of the liver copper content was performed as described by Teske et al. [33]. Briefly, the RA stained slides were scored by one board-certified veterinary pathologist from 0 (no copper stained) till 5 (maximal copper stained). Besides the amount of copper, also the sublobular localisation (e.g. centrolobular) and cells (hepatocytes or macrophages) of the copper storage were determined. The quantitative copper analysis was performed using instrumental neutron activation analysis via the determination of ^{64}Cu [34]. In healthy dogs copper concentrations are ≤ 400 ppm [35].

Serum enzyme activity and fasting plasma bile acid concentration

Serum alanine aminotransferase (ALT) and fasting plasma bile acids were determined using a DXC-600 Beckman (Beckman Coulter, Woerden, the Netherlands). Reference range for ALT and bile acids is <54 U/l and <10 µmol/l, respectively.

RNA isolation and quantitative RT-PCR (q-PCR)

RNA was isolated from RNAlater fixed biopsies, using Qiagen RNeasy Mini Kit (Qiagen, Leusden, the Netherlands) according to the manufacturer's instructions. RNA quality was analysed with the Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA, USA). Q-PCR was performed on a total of seven gene products involved in copper homeostasis (ATPase, Cu(2+)-transporting, alpha polypeptide (ATP7A), ATPase, Cu(2+)-transporting, beta polypeptide (ATP7B), Ceruloplasmin (CP), Metallothionein 1A (MT1A), ATOX1 antioxidant protein 1 homolog (ATOX1), COX17 cytochrome c oxidase assembly homolog (COX17), Copper chaperone for superoxide dismutase (CCS)) and three gene products involved in oxidative stress (superoxide dismutase (SOD1), Catalase (CAT), and Glutathione peroxidase 1 (GPX1)). Q-PCR was performed using primers as described in Table 1 and was based on the high affinity double-stranded (ds) DNA-binding dye SYBR green I (iQSYBR Green Supermix, BioRad, Veenendaal, the Netherlands) [36]. Normalisation was performed using four reference-genes (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyl transferase (HPRT), ribosomal

protein S5 (RPS5) and S19 (RPS19)) [37]. Q-PCR results were related to the expression of six control dogs (one to three years of age). Minus-RT and no-template controls were negative indicating no contaminations.

Table 1 *- Nucleotide Sequences of Dog-Specific Primers for Quantitative Real-Time PCR.*

Gene	Primer sequence (5'→3')	Annealing temperature (°C)	Product size (bp)	Accession number
Copper related genes				
COX17	Forward ATC ATT GAG AAA GGA GAG GAG CAC	60	127	AY603041
	Reverse TTC ATT CTT CAA GGA TTA TTC ATT TAC			
CCS	Forward TGT GGC ATC ATC GCA CGC TCT G	64	96	AY572228
	Reverse GGG CCG GCC TCG CTC CTC			
ATOX1	Forward ACG CGG TCA GTC GGG TGC TC	67	137	AF179715
	Reverse AAC GGC CTT TCC TGT TTT CTC CAG			
ATP7A	Forward CTACTGTCTGATAAACGGTCCCTAAA	50	99	AY603040
	Reverse TGT GGT GTC ATC ATC TTC CCT GTA			
ATP7B	Forward GGT GGC CAT CGA CGG TGT GC	56	136	AY603039
	Reverse CGT CTT GCG GTT GTC TCC TGT GAT			
MT1a	Forward AGC TGC TGT GCC TGA TGT G	64	130	D84397
	Reverse TAT ACA AAC GGG AAT GTA GAA AAC			
CP	Forward AAT TCT CCC TTC TGT TTT TGG TT	62	97	AY572227
	Reverse TTG TTT ACT TTC TCA GGG TGG TTA			
Oxidative stress related genes				
Catalase	Forward TGA GCC CAG CCC TGA CAA AAT G	62	119	AB0112918
	Reverse CTC GAG CCC GGA AAG GAC AGT T			
SOD1	Forward TGG TGG TCC ACG AGA AAC GAG ATG	64	99	AF346417
	Reverse CAA TGA CAC CAC AAG CCA AAC GAC T			
GPX1	Forward GCA ACC AGT TCG GGC ATC AG	62	123	AY572225
	Reverse CGT TCA CCT CGC ACT TCT CAA AA			
	Reverse TGA AAG GAG CAT GTT CTG AAG TAG CAC T			
Endogenous reference genes				
HPRT	Forward AGCTTGCTGGTGAAAAGGAC	56	100	L77488/9
	Reverse TTATAGTCAAGGGCATATCC			
GAPDH	Forward TGTCCCCACCCCCAATGTATC	58	100	AB038240
	Reverse CTCCGATGCCTGCTTCACTACCTT			
RPS5	Forward TCACTGGTGAG/AACCCCCT	62.5	141	XM533568
	Reverse CCTGATTCACACGGCGTAG			
RPS19	Forward CCTTCCTCAAAAAGTCTGGG	61	95	XM533657
	Reverse GTTCTCATCGTAGGGAGCAAG			

Immunohistochemistry

Immunohistochemical characterisation of COMMD1 and ATP7B was performed on healthy and diseased canine liver tissue. Three livers of COMMD1 deficient dogs with an age of respectively six weeks, 42 months, and 72 months were included for ATP7B staining. Diseased tissue included chronic hepatitis samples with or without copper toxicosis. For ATP7B 4 µm frozen sections were mounted on poly-L lysine coated slides and fixed 5 minutes in ice-cold acetone for 5 minutes. For COMMD1, 4 µm sections were mounted on poly-L lysine coated slides and deparaffinised. Antigen retrieval was performed for 45 minutes in citrate buffer (pH 6) at 95^{o}C. Endogenous peroxidase activity of both antibodies was quenched by incubating the specimen for 5 minutes with DAKO Peroxidase Block (Dako, Glostrup, Denmark). Slides were blocked for 60 minutes in 10 percent goat serum and incubated overnight at 4^{o}C with rabbit anti-ATP7B (Sigma) diluted 1:5 in PBS or rabbit anti-COMMD1 (Sigma) diluted 1:300 in PBS. After washing slides were incubated for 45 minutes with the DAKO EnVision™ System for primary antibodies produced in rabbit. Staining of the slides was obtained with 3,3'-diaminobenzidine tetrahydrochloride (DAB), counterstained with hematoxylin, dehydrated and mounted.

Statistical analysis

Statistical analysis was performed in R (version 2.11.1) [38]. Quantitative PCR data were analyzed using linear mixed-effect modeling with the R package "nlme". The outcome variables were transformed by taking their natural logarithm in order to fulfill the criteria of normality and constant variance. Restricted maximum likelihood method was used to estimate the best fitting model. For all gene products the random intercept model was the best fitting model and for ALT the random slope model was the best fitting model based on Akaike's information criterium. Maximum likelihood was used to estimate the fixed effect of the time in months (included in the model as a factor). The reference level of gene expression was set to be the expression at 6 months of age, and for every time-point differences from this reference were determined for the 5 dogs that were followed until 42 months of age. A Bonferroni corrected p-value of 0.0083 (0.05 divided by 6 comparisons) was used to determine significance. For ALT, the reference level was set to be at 12 months of age and for every time point differences from the level at 12 months were determined for the 5 dogs that were followed until 42 months of age. A Bonferroni corrected p-value of 0.01 (0.05 divided by 5 comparisons) was used to determine significance. True estimates and 95% confidence intervals were obtained by computing the exponents of the results from the mixed model fit on the ln transformed data.

Overall differences in hepatitis score and copper score were determined by the

Friedman test. When significant, the Wilcoxon signed rank test with continuity correction was performed as a post hoc test for comparisons of all time points with the reference time point (6 months). A Bonferroni corrected p-value of 0.0083 (0.05 divided by 6 comparisons) was used to determine significance. Overall differences in gene expression per gene product and hepatitis and copper grading for the 3 dogs treated with penicillamine and followed until 60 months of age were determined for time points 42, 48, 54 and 60 months by the Friedman test. When significant, Wilcoxon signed rank test with continuity correction was performed as a post hoc test for comparisons of time points 48, 54 and 60 with the reference time point (42 months). A Bonferroni corrected p-value of 0.02 (0.05 divided by 3 comparisons) was used to determine significance.

Results

Animals

During the entire follow-up period dogs showed no abnormalities at physical examination. At 43 months of age, before penicillamine treatment, two dogs (both dogs had severe copper induced chronic hepatitis) died suddenly, one with a hemolytic crisis, the other without any clear cause.

Histopathological findings and copper assessment

HE stained histological slides showed in two out of five dogs that mild hepatitis was present at 12 months of age. At 18 months all five dogs showed a slight to mild hepatitis. Although some variation was seen between individual animals, hepatitis in all five dogs became moderate to marked in the later stages of the disease (Fig. 1a and Table 2(A)). After starting treatment with penicillamine, the activity of hepatitis did not decrease but progression was stopped (Fig. 1b and Table 2(B)).

Based on RA staining at 6 months of age all animals had a moderate centrolobular copper accumulation (grade 2) and at 12 months of age all animals had extensive copper accumulation (grade 4-5) almost diffusely throughout the lobules (Fig. 1a and Table 2(A)). After starting penicillamine treatment the semi-quantitative copper scores decreased moderately within 18 months from grade 5 till 3.5 (Fig. 1b and Table 2(B)). Follow-up of quantitative copper measurement revealed 3620 ppm (median) (range 1450-7200), 11500 ppm (8500-12400), and 8900 ppm (5620-12500) at 18, 36, and 42 months of age, respectively. Penicillamine treatment reduced quantitative copper levels to 6080 ppm (4960-6610), 3270 ppm (2670-6210), and 5510 ppm (4710-6200) at 48, 54, and 60 months of age, respectively.

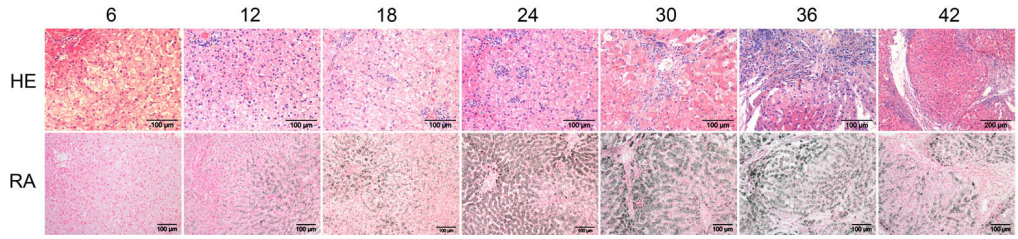


Fig. 1a *- Histological description.*
COMMD1 deficient dog livers were stained with H&E and RA to assess inflammation and copper accumulation, respectively. Representative pictures of COMMD1 deficient dogs over a period of 42 months are shown. Numbers indicate age in months.

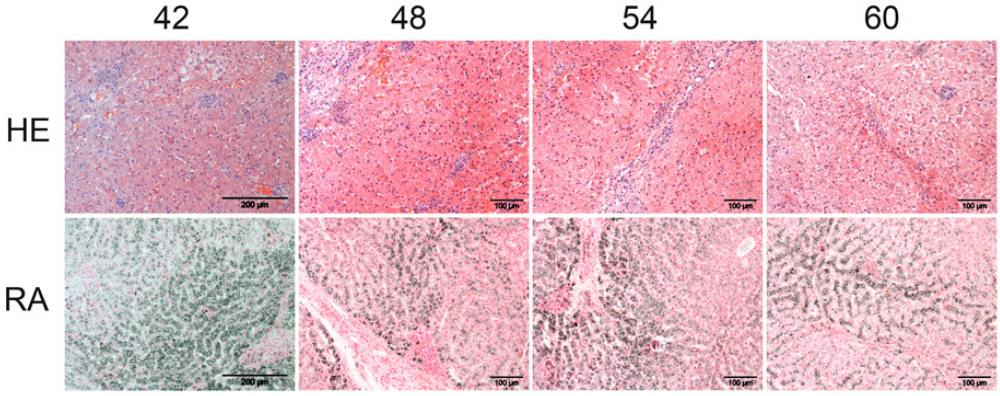


Fig. 1b *- Histological description.*
COMMD1 deficient dog livers were stained with H&E and RA to assess inflammation and copper accumulation, respectively, after starting treatment with penicillamine at 43 months of age (48, 54, and 60 months of age). Representative pictures of COMMD1 deficient dogs are shown. Numbers indicate age in months.

Table 2A and B *- Hepatitis and copper grading during aging in COMMD1 deficient dogs in a time-dependent copper-induced hepatitis before (A, n=5) and after (B, n=3) starting treatment with penicillamine at 43 months of age. Data are presented as median (range). Age in months. The presence and activity of hepatitis was graded as 0 (absent), 1 (slight), 2 (mild), 3 (moderate), and 4 (marked). The presence of copper was evaluated semi-quantitatively using a scale from 0 to 5; 0 – no copper; 1 – solitary liver cells and/or reticulohistiocytic (RHS) cells containing some copper positive granules; 2 – small groups of liver cells and/or RHS cells containing small to moderate amounts of copper positive granules; 3 – larger groups or areas of liver cells and/or RHS cells containing moderate amounts of copper positive granules; 4 – large areas of liver cells and/or RHS cells with many copper positive granules; 5 – diffuse presence of liver cells and/or RHS cells with many copper positive granules.*

(A)

Age	6	12	18	24	30	36	42
Hepatitis grading (0-4)	0 (-)	0 (0-2)	2 (2-2)	1 (1-3)	3.5 (2-4)	3 (2-4)	3 (2.5-3)
Bonferroni p-value	-	0.37	0.089	0.055	0.058	0.055	0.053
Copper grading (0-5)	2 (-)	4.5 (-)	5 (4.5-5)	5 (-)	5 (-)	5 (4.5-5)	5 (-)
Boferroni p-value	-	0.037	0.053	0.037	0.037	0.048	0.037

Overall differences in differences in hepatitis and copper grading data at the 7 time points were determined by the Friedman test (p-value of 0.00071 and 0.00022 for hepatitis and copper grading, respectively). When significant ($p < 0.05$), the Wilcoxon signed rank test with continuity correction was performed as a post hoc test for comparisons of all time points with the reference time point (6 months). A Bonferroni corrected p-value of 0.0083 (0.05 divided by 6 comparisons) was considered significant. No significant differences on Bonferroni level were detected.

(B)

Age	42	48	54	60
Hepatitis grading (0-4)	2.5 (2.5-3)	3 (-)	2.5 (2-3)	2.5 (2-3)
Copper grading (0-5)	5 (-)	4.5 (4.5-5)	4 (4-4.5)	3.5 (3.5-4)
Bonferroni p-value	-	0.35	0.17	0.17

Overall differences in hepatitis and copper grading for the 3 dogs treated with penicillamine and followed until 60 months of age were determined for time points 42, 48, 54 and 60 months by the Friedman test (p-value of 0.318 and 0.0322 for hepatitis and copper grading, respectively). When significant, Wilcoxon signed rank test with continuity correction was performed as a post hoc test for comparisons of time points 48, 54 and 60 with the reference time point (42 months). A Bonferroni corrected p-value of 0.02 (0.05 divided by 3 comparisons) was considered significant. No significant differences on Bonferroni level were detected.

Serum ALT and fasting plasma bile acids

Serum ALT started to increase from 24 months of age (2-3-fold). After starting treatment with penicillamine ALT decreased but remained above the upper reference limit. During the 60 months follow-up period, fasting plasma bile acids remained within reference limits (Table 3).

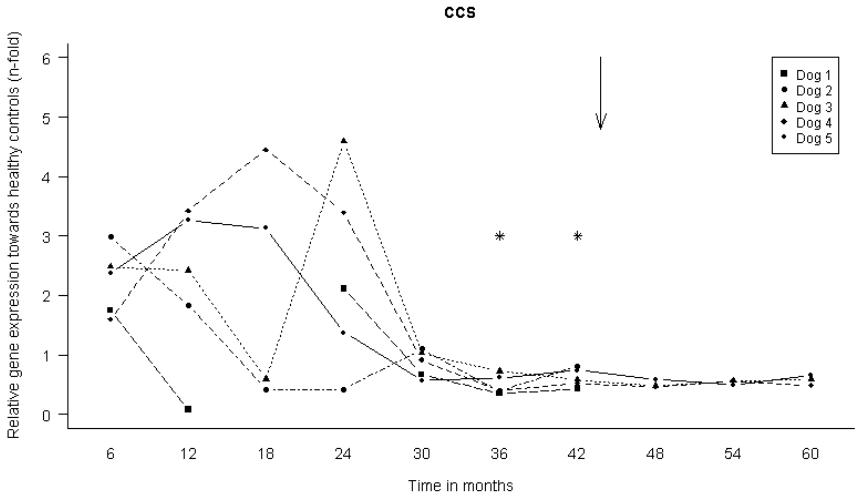
Table 3 *- Serum activity of ALT (ref value < 54 U/l) and concentration of bile acids (ref value < 10 µmol/l) during aging in COMMD1 deficient dogs before (n=5) and after (n=3) treatment with penicillamine (starting at 43 months of age). Values are expressed as median (range).*
** indicates a significant increased expression compared with 12 months of age (Bonferroni corrected p-value of < 0.01).*

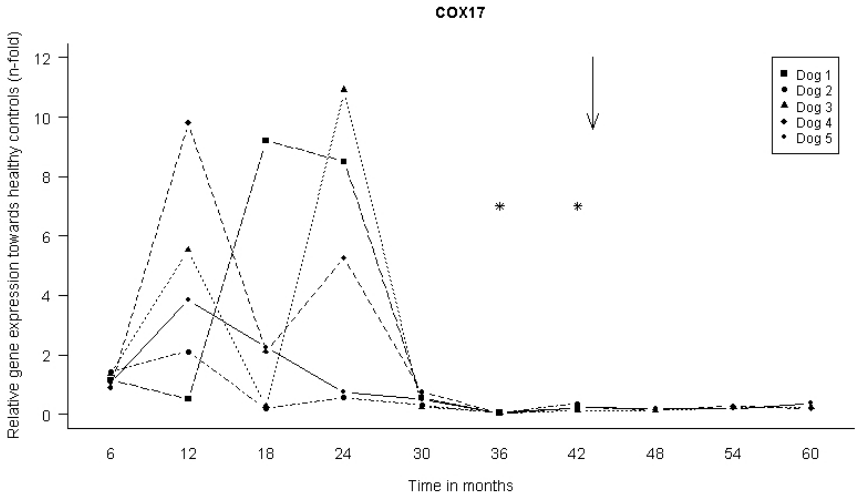
Age (months)	ALT	Bile acids
12	44 (34-75)	7 (3-11)
18	45 (35-60)	1 (1-2)
24	66 (43-257)	7 (6-12)
30	83 (62-153) *	4 (1-8)
36	180 (69-225) *	2 (0-3)
42	112 (55-201) *	2 (1-3)
48	139 (58-157)	2 (1-2)
54	72 (56-116)	2 (1-3)
60	64 (60-79)	3 (0-8)

Q-PCR of copper-related gene products
Gene-expression of the intracellular copper chaperones (CCS, COX17, ATOX1) were, although not significant, elevated until 24 months of age and thereafter dropped to about normal levels. During penicillamine treatment expression levels of all 3 copper chaperones remained at these normal expression levels (Fig 2a).

The expression of MT1A was increased over ten-fold at 12 months and then rapidly decreased to normal expression levels at 36 months. During penicillamine treatment MT1a expression levels remained within normal limits (Fig. 2b). CP expression levels varied but were increased at 12 and 24 months and thereafter returned to normal levels and remained unchanged during penicillamine treatment (Fig 2b).

For the excretory regulators, the expression of ATP7A was significantly increased at 24 and 30 months of age, the expression of ATP7B was significantly increased at 30 months of age. The over ten-fold increased ATP7A expression was reduced to normal expression levels at 36 months of age and remained at this level, also during penicillamine treatment. The over five-fold increased ATP7B expression was reduced from 36 months of age, but tended to be increased, also during penicillamine treatment (Fig 2c).


CCS
Relative gene expression towards healthy controls (n-fold)
0
1
2
3
4
5
6
Dog 1
Dog 2
Dog 3
Dog 4
Dog 5
*
*
6
12
18
24
30
36
42
48
54
60
Time in months


COX17
Relative gene expression towards healthy controls (n-fold)
0
2
4
6
8
10
12
Dog 1
Dog 2
Dog 3
Dog 4
Dog 5
*
*
6
12
18
24
30
36
42
48
54
60
Time in months

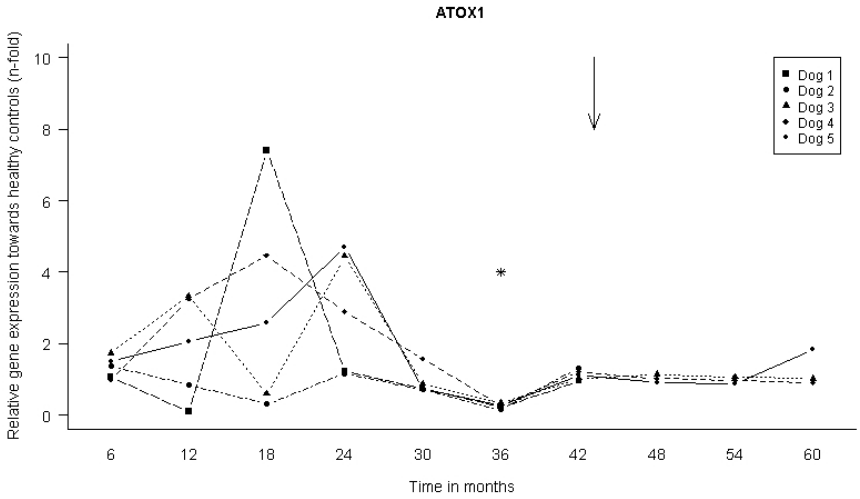


Fig. 2a *- Gene-expression profiling. Interaction plots of Q-PCR data of important mediators of copper homeostasis (the copper chaperones) in a time-dependent copper-induced hepatitis in COMMD1 deficient dogs (n=5) and in COMMD1 deficient dogs in a time-dependent copper-induced hepatitis after starting treatment with penicillamine at 43 months of age (n=3, indicated by the arrow). Age in months. Q-PCR results were normalized against the expression of six control dogs (one to three years of age). Linear mixed-effect modeling was used;* ******* *indicates significant difference (p-value smaller than Bonferroni corrected p-value of 0.0083).*

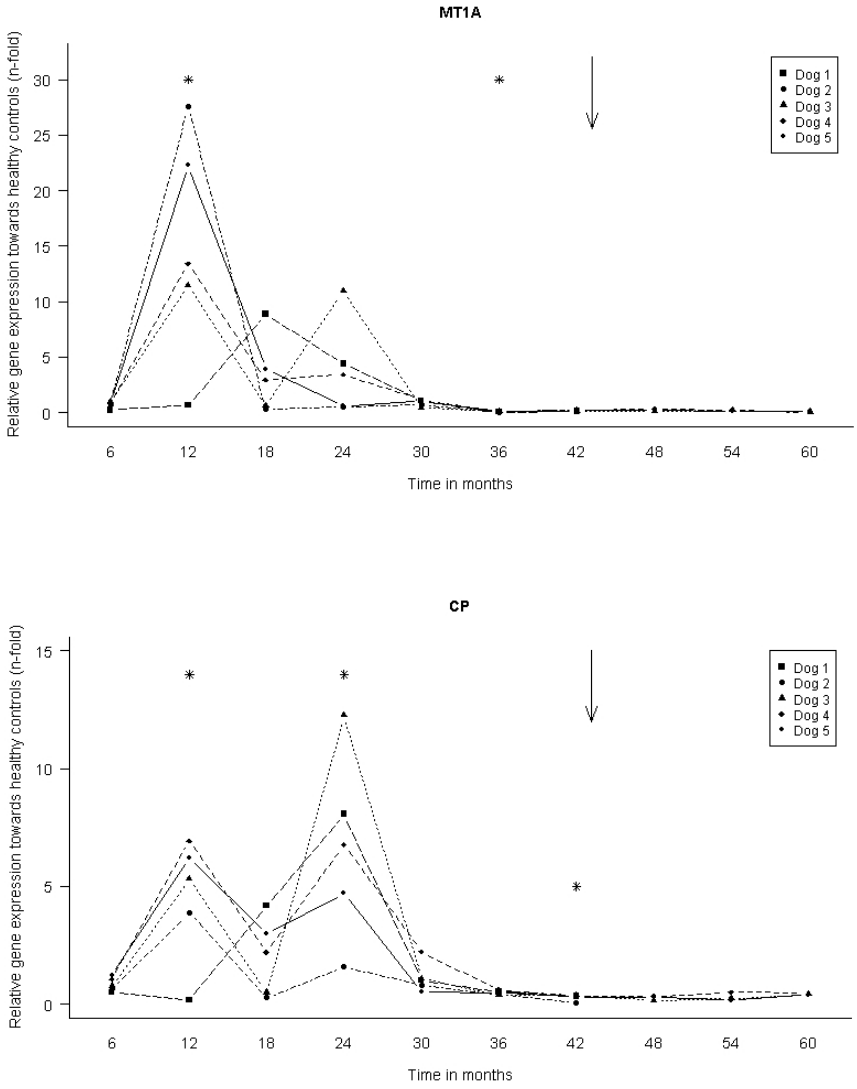


Fig. 2b *- Gene-expression profiling. Interaction plots of Q-PCR data of important mediators of copper homeostasis (copper storage MT1A and excretion CP) in a time-dependent copper-induced hepatitis in COMMD1 deficient dogs (n=5) and in COMMD1 deficient dogs in a time-dependent copper-induced hepatitis after starting treatment with penicillamine at 43 months of age (n=3), indicated by the arrow. Age in months. Q-PCR results were normalized against the expression of six control dogs (one to three years of age). Linear mixed-effect modeling was used; * indicates significant difference (p-value smaller than Bonferroni corrected p-value of 0.0083).*

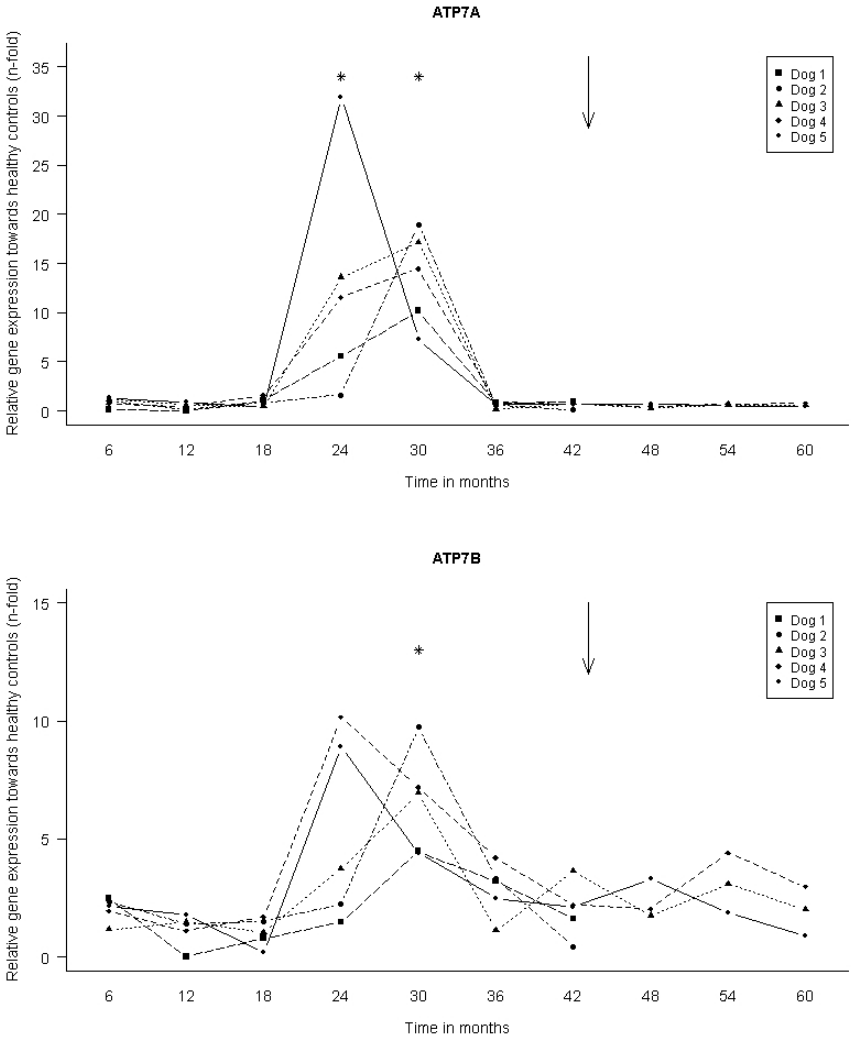
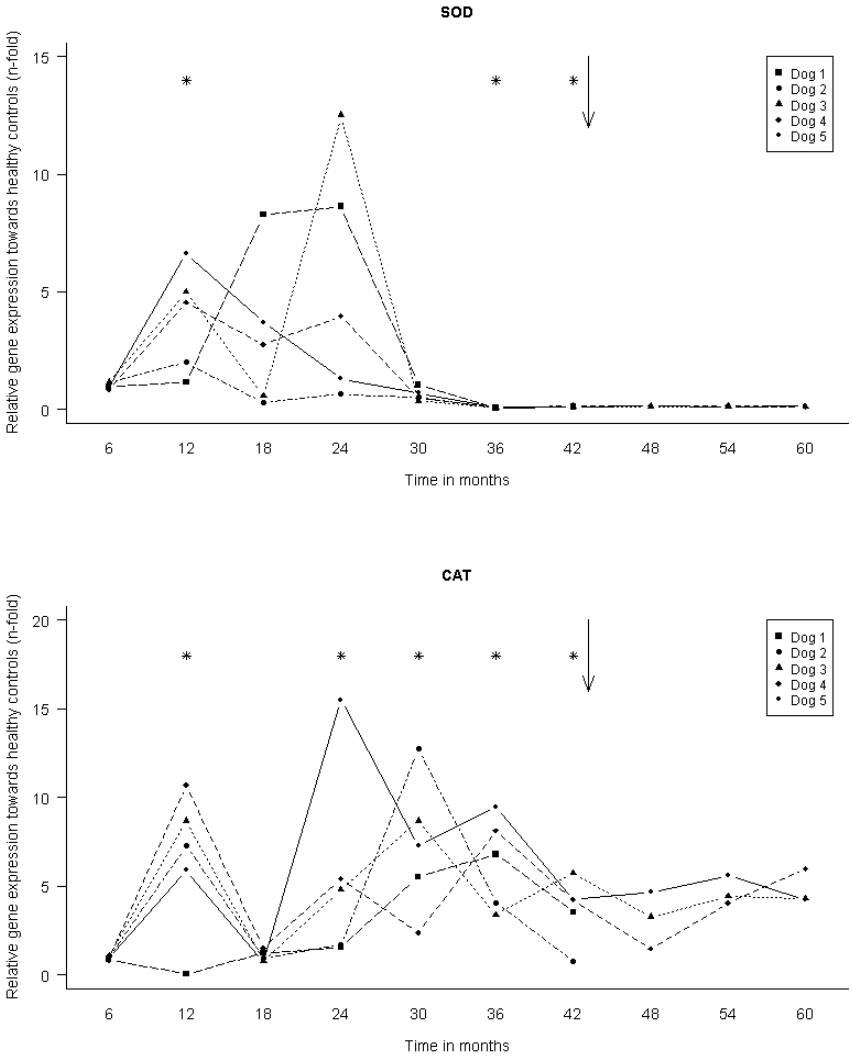


Fig. 2c *- Gene-expression profiling. Interaction plots of Q-PCR data of important mediators of copper homeostasis (copper excretion (ATP7A&B)) in a time-dependent copper-induced hepatitis in COMMD1 deficient dogs (n=5) and in COMMD1 deficient dogs in a time-dependent copper-induced hepatitis after starting treatment with penicillamine at 43 months of age (n=3), indicated by the arrow. Age in months. Q-PCR results were normalized against the expression of six control dogs (one to three years of age). Linear mixed-effect modeling was used; * indicates significant difference (p-value smaller than Bonferroni corrected p-value of 0.0083).*

Q-PCR of oxidative stress-related gene products

SOD mRNA levels tended to be increased (significant at 12 months of age) till 24 months of age, then returned to normal, and were unaltered upon penicillamine treatment. Catalase expression was significantly increased (around five-fold) between 12, and 24 until 42 months of age, and remained, although not significant, five-fold increased during penicillamine treatment. GPX1 expression was significantly over ten-fold increased only at 30 months of age (Fig. 3).



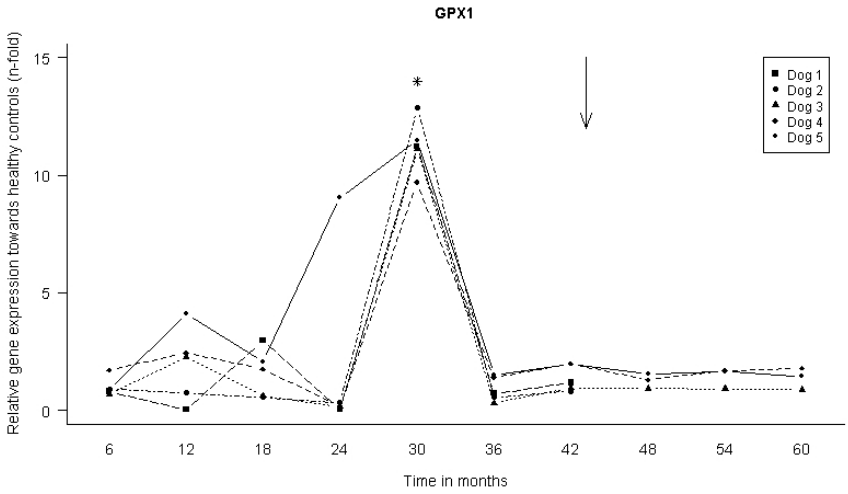


Fig. 3 *- Gene-expression profiling. Interaction plots of Q-PCR data of important mediators of oxidative stress in a time-dependent copper-induced hepatitis in COMMD1 deficient dogs (n=5) and in COMMD1 deficient dogs in a time-dependent copper-induced hepatitis after starting treatment with penicillamine at 43 months of age (n=3), indicated by the arrow. Age in months. Q-PCR results were normalized against the expression of six control dogs (one to three years of age). Linear mixed-effect modeling was used; * indicates significant difference (p-value smaller than Bonferroni corrected p-value of 0.0083).*

Immunohistochemistry

Immunohistochemistry (IHC) on COMMD1 in the histologically healthy liver of a control dog (COMMD1 positive) showed a diffuse granular cytoplasmic staining of hepatocytes in the centrolobular area, as well as in the first layer of hepatocytes around the portal area. IHC for ATP7B in the histologically healthy liver of the control dog (COMMD positive) showed a clear canalicular staining throughout the lobule. Centrolobularly a more pronounced granular staining was observed (Fig. 4). In the COMMD1 deficient dog livers, COMMD1 staining was absent.

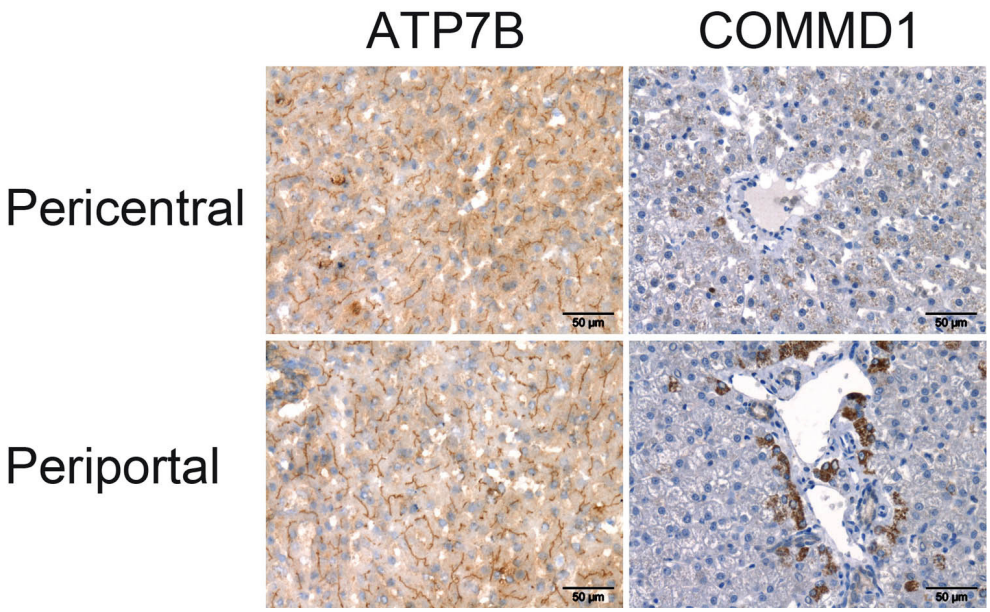


Fig. 4 *- IHC of COMMD1 and ATP7B in a histological normal liver of a COMMD1 positive dog. Size indicated by indicator bar.*

Discussion

The present paper describes a longitudinal study in COMMD1 deficient dogs characterized by a progressive copper accumulation in the liver and subsequent development of hepatitis. Furthermore, the effect of prolonged treatment with the copper chelator penicillamine on these parameters was investigated.

At 12 months of age there was already massive copper accumulation which reached a maximum score between 18 and 24 months. The first evidence of copper-associated hepatitis was seen at 12 months in two dogs and progressed over time to chronic hepatitis and cirrhosis from 30 months of age. Copper accumulation and histological hepatitis were obvious long before clinical symptoms started. Treatment with penicillamine, which was started at 43 months, demonstrated a slowly progressive decline in hepatic copper content and improvement of hepatitis activity histologically. This is in line with previous reports [29] in other dog breeds.

The increased plasma activity of ALT, a sensitive indicator for hepatocellular damage, coincided with the occurrence of histologically mild to moderate hepatitis. The plasma

activity of ALT (2-3-fold increase) was lower than that reported by Huster et al. in *ATP7B*$^{-/-}$ knockout mice (10-fold increase) [26]. This may indicate a slower progression of cellular damage in the COMMD1 deficient dogs compared to the knockout mice. After starting anti-copper treatment, ALT plasma concentration slowly declined, but remained increased during the next 18 months consistent with the persistence of hepatitis.

The absence of COMMD1 in dogs caused various time-dependent effects on the copper related gene products. Taking into account the limitations of mRNA levels compared to protein levels still a logical pattern of sequential events arises. MT1A is an important protein for intracellular copper storage and MT1A mRNA expression in our dogs was significantly increased at 12 months of age, coinciding with the progressive and massive accumulation of copper. At this age pathological changes were absent, presumably since the MT-bound copper appeared to be sequestered in lysosomes [39,40] between 6 and 12 months. These findings are in accordance with the results of a study in *ATP7B*$^{-/-}$ mice by Huster et al [41]. Spee et al. demonstrated a reduced mRNA expression of MT in dogs with progressed copper-induced hepatitis [36,42], in accordance with the reduced mRNA expression of MT in our dogs when hepatitis was present. When the liver copper values reach a maximal level, expectedly MT1A protein levels are maximally up-regulated and saturated. Prolonged copper overload leads subsequently to hepatocellular damage and the onset of hepatitis. At that late stage the expression of the copper excretory regulators ATP7A and ATP7B and anti-oxidative stress mechanisms (catalase, GPX1) were significantly increased. Adult livers do normally not express ATP7A to any significant degree, but instead express the homologous ATP7B copper transporter that facilitates copper excretion into the bile [3,43]. We demonstrated a temporarily increased expression of ATP7A, possibly as a rescue mechanism as a consequence of a reduced functional ATP7B excretory pathway despite increased mRNA levels. Weiss et al. demonstrated that the COMMD1 protein is not involved in copper-mediated translocation but has a function in the pathway of copper excretion [22]. De Bie et al. [15] demonstrated a relation between ATP7B and COMMD1, indicating copper accumulation in COMMD1 deficiency is the result of a reduced function of ATP7B leading to reduced copper excretion. Recently, Burkhead et al. suggested that the COMMD1 protein is involved in the regulation of structurally unrelated membrane transporters [21].

Although the molecular processes underlying copper-induced cytotoxicity remain unclear, it is commonly agreed that excess of copper results in elevated ROS production in mammals [44,45]. In LEC rats with copper induced hepatitis as well as experimentally increased copper ingestion in normal Wistar rats, liver hydroxyl radical (*OH) production was increased. This suggests that accelerated generation of *OH catalyzed by free copper may play a role in the pathogenesis of acute hepatitis in LEC rats [46]. Elevated copper

levels cause relevant changes in intracellular structures of hepatocytes, particularly mitochondria [47,48], the main cellular source of *OH [46]. Higher increased lipid peroxidation levels were observed in post-mitochondrial supernatant (S-9) fraction of livers from LEC rats with hepatitis than in those from healthy Wistar rats [49]. In the COMMD1 deficient dogs the expression levels for the antioxidant catalase was significantly increased from 24 months onwards and remained high throughout the whole observation period, also during penicillamine treatment. When antioxidative systems are overwhelmed, the produced *OH may rapidly accelerate the disease process. Even after treatment with penicillamine for 18 months, when copper levels were reduced and the progression of hepatitis was stopped or even reduced, catalase expression remained, although not significant, five-fold increased indicating that oxidative stress was still potentially present despite clinical improvement. Our findings regarding the involvement of oxidative stress in COMMD1 deficient dogs are in accordance with the results of Huster et al. [41]. They found little evidence for oxidative stress response in the very early stages at the liver mRNA level (SOD and catalase) in *ATP7B*$^{-/-}$ mice, despite a 20-40-fold increase in its copper content, as we did regarding SOD, GPX and catalase expression. All together these results suggest that oxidative stress plays a minor role in the initial phase (until the age of 12 months) when it is kept under control. When the initial defense systems are overwhelmed, hepatitis progressively develops.

There are clearly similarities between WD in man, rodent models such as LEC rats, *ATP7B* $^{-/-}$ mice, and COMMD1 deficient dogs. In all cases there are age dependent increases in liver copper concentration due to an absent or reduced function of ATP7B and development of pathological changes associated with increased free cytoplasmic copper and production of oxygen radicals. By adding increased amounts of copper to the diet, these processes can be accelerated. A difference between COMMD1 deficient dogs, WD, and the rodent models is the fact that in dogs with copper-induced chronic hepatitis, the start of the accumulation of copper is always in the centrolobular area of the liver lobules. This histological copper storage precedes hepatocytic alteration, whereas in WD hepatocytic alteration is observed before the presence of histologically recognizable copper storage. The reason for this initial zonal difference is unclear, but based on the IHC stainings for ATP7B and COMMD1 one might speculate that in COMMD1 deficient dogs the trafficking of ATP7B is disrupted especially in the centrolobular area. Deficiency of COMMD1, a traffic agent for ATP7B [15,21], might play an important role in this initial zonal distribution. In normal healthy dog liver ATP7B stained positive at the canalicular membrane of hepatocytes diffusely throughout the lobules. This is in part comparable with the result of Schaefer et al. who demonstrated that in human liver ATP7B is predominantly present in trans-Golgi vesicles in the pericanalicular area, whereas small amounts were

amounts were localized to the canalicular membrane [8]. It has been demonstrated in polarized hepatoma cells that with increased copper levels ATP7B redistributed from the trans-Golgi network to the apical membrane [9,50]. Further research is necessary to unravel the process of the endosomal transport for ATP7B under the influence of COMMD1 to the apical membrane. Despite several differences, inherent to model animals, the molecular tools available for dogs studies, the ease to take multiple and sequential liver biopsies, the known genetic aetiology, and the dietary tools to affect intrahepatic copper levels, position the COMMD1 deficient dogs as additive model animals to study WD.

Acknowlegdements

We would like to thank of J.A. Drie – van Putten, R. Kisjes and H.G.H. van Engelen for technical assistance. H. Vernooij (Centre for Biostatistics, Utrecht University) assisted with statistical analysis.

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Chapter

9

General discussion

Pathways of regeneration and fibrosis in the liver can be studied *in vitro* in cell culture or tissue slices, and *in vivo* in zebrafish, mouse, or rat models. The availability of suitable large animal models for independent evaluation of proof of principle can bridge the giant step from the fundamental (fish or rodent) animal model studies to clinical application in human medicine. Recent studies have shown that pathways of fibrosis, regeneration, adult stem cell activation and tumour formation in the liver are very similar in dogs and humans [1-4]. The central aim of this thesis is to analyse hepatitis in dogs in general and specifically the in-depth description of the pathogenesis of hepatitis in COMMD1 deficient dogs with hepatic copper accumulation.

In the clinically-oriented **part 1** (Canine Hepatitis: the veterinary clinic) we evaluated the canine patient population with hepatitis referred to the Utrecht University Clinic for Companion Animals (UUCCA).

In **chapter 3** an overview has been presented for diagnosis and treatment of different forms of canine hepatitis. Although "state of the art" treatments are mentioned, additional treatments are really necessary since treatment is often symptomatic rather than cause-directed. Therefore more research into the causes and progression of idiopathic hepatitis is of the utmost importance. Finding the cause will permit an aetiology based treatment in order to prevent progression from acute to chronic hepatitis to cirrhosis [5,6].

In **chapter 4** we performed a retrospective study to evaluate which forms of hepatitis are diagnosed in the UUCCA referral population [6]. Chronic hepatitis (CH) appeared to be the most frequently encountered form of hepatitis in our population. We have evaluated the potential role of copper accumulation as a factor in aetiology for canine hepatitis. This revealed that approximately in 1/3 of all dogs with acute and chronic forms of primary hepatitis the liver copper level was pathologically increased. This observation was based on the current routine procedure to stain and histologically evaluate biopsies for semi-quantitative copper levels. Besides copper, no other possible aetiologies were discovered and therefore the majority of the cases still remained idiopathic. These canine patients with idiopathic acute or chronic hepatitis may have different aetiologies (e.g. infectious, autoimmune). One of the most interesting candidates are viruses. Decades ago Jarret et al. proposed a viral cause [7,8]. More recent data indicated that indeed liver homogenates from dogs with chronic hepatitis administered to healthy ferrets induced a slight hepatitis in some ferrets [9]. Unfortunately, economic restrictions limited this study to only nine weeks after exposure. Recently a potential hepatitis virus was found in Welsh Springer spaniels [10]. It seems, however, unlikely that a virus will only be able to produce hepatitis

in one single breed. A breed over-representation could be due to the effect of sexual transfer by a popular breeding sire affected with a transmittable virus. In close cooperation with the AMC-Virus discovery group we have used the VIDISCA-454 next generation sequencing strategy to investigate the presence of viruses in a sequence independent manner. This work, which was initiated early 2010 by the Honours Programme student M. van der Heijden resulted in a number of potential sequences which could be associated with viral aetiologies for canine idiopathic hepatitis. These combined preliminary data may indicate that like in man, hepatitis in dogs will become a predominant viral rather than an idiopathic disease.

In **chapter 5** we evaluated the therapeutic effects of prednisone for dogs with idiopathic chronic hepatitis (CH(i)). For CH(i) there is little consensus about the most effective treatment. The milestone study on which prednisone treatment is based was published in 1988 [11]. However, experts in the field have hesitation about the therapeutic value of prednisone treatment. A drug with prominent side effects should not be prescribed without having proven effect. Moreover, in light of the discussion about possible viral etiologies, immunosuppression could even be contra-indicated. The general impression amongst experts in the field is that corticosteroid therapy improves survival and/or reduces hepatic inflammation of dogs with CH(i) and that withholding prednisone therapy is unethical. Therefore a randomized, placebo controlled, double-blinded, prospective study evaluating the therapeutic effect of prednisone against a placebo is not possible. In order to find more objective evidence we performed a retrospective study in our University Hospital population of dogs with CH(i), based on the WSAVA-diagnostic criteria [12]. Acknowledging the limitations of a retrospective study, at this moment this is the best available material to support this discussion on whether or not prednisone can be avoided as the preferred treatment. This is essential for a rational decision on the design of a future prospective multicenter study to find the best treatment for idiopathic canine hepatitis.
Based on these findings we concluded that prednisone treatment had beneficial effects in some dogs with CH(i), which was in agreement with the conclusions of Strombeck et al. [11]. An important difference between our study and the study of Strombeck et al. [11] is the fact that the latter one did not make any difference between idiopathic and copper-associated chronic hepatitis, leading to a more heterogeneous population and a different clinical outcome. Important prognostic factors for the survival of dogs with hepatitis (such as hepatic inflammation, fibrosis, and coagulation parameters) were on average beneficially altered after treatment with prednisone. Dogs that had not developed hepatic cirrhosis had a long survival time, and prednisone reduced the hazard of hepatitis-related death in the entire study group. Our overall conclusion was that the main effect of

prednisone medication is a better survival. In addition we demonstrated that prednisone treatment had a favourable and almost immediate effect on coagulopathy associated with canine CH(i). Based on these findings studies to find the underlying mechanisms for the corticosteroid-induced quick recovery of hepatitis-associated coagualopathy will be conducted. These may be of broader interest than for veterinary medicine alone.

In a model-animal centered **part 2** (Canine Hepatitis: from clinic to translational model) we have evaluated dogs with COMMD1 deficiency resulting in a progressive copper induced hepatitis as a possible model for translational medicine. Large animal models allow the serial sampling of tissue in the volume required for detailed studies of cellular and molecular pathogenesis. In 2002 van Sluijs et al. discovered a clear association between a mutation in the *COMMD1* gene and copper-induced hepatitis in Bedlington terriers [13]. Two papers showed that siRNA mediated COMMD1 silencing resulted in increased copper accumulation in human embryonic kidney cells and in a canine hepatic cell line [14,15].

In **chapter 6** we have published a diagnostic DNA test for COMMD1 carriers and diseased animals based on the quantitative PCR of exon-3 and exon-2 of the *COMMD1* gene [16]. Although this unusual use of quantitative PCR was initially developed for the analysis of large (>5kb) deletions, it is applicable with minor modifications for other large deletions for which no regular PCR test can be developed. Our work has been replicated two years later [17]. Shortly after our publication [16] another paper was published by Forman et al. [18] about testing COMMD carriers and homozygous diseased dogs based on a regular PCR method showing different amplicons for dogs with the mutation compared with healthy dogs. This DNA based test is now the first choice method: it does not require expensive quantitative PCR equipment and it can easily be performed on genomic DNA.

In **chapter 7** we performed a time-dependent characterization of fibrogenesis and regeneration in dogs deficient for COMMD1. The longitudinal follow-up of COMMD1 deficient dogs with a copper-induced chronic hepatitis demonstrated an early onset of fibrosis, starting with hepatic stellate cell (HSC) activation. Hepatic regeneration occurs through hepatocyte replication even when the process of fibrogenesis has already severely progressed. Liver progenitor cell (LPC) proliferation is present, as indicated by the increased K19 staining of non-biliary cells, but they do not seem to contribute to hepatocytic regeneration as differentiation to adult hepatocytes was not observed. It is interesting to speculate why the progenitor compartment starts to proliferate but does not

differentiate. LPC activation and differentiation could be driven by different signals acting onto the niche as result of the stage (acute or chronic). Spee et al. demonstrated in acute and chronic human liver disease that activation of the Wnt pathway is involved in LPC proliferation [19]. This finding in human liver disease warrants further research into the role of the Wnt/β-catenin pathway in COMMD1 deficient dogs.

Although not published yet, we obtained interesting data supporting a possible role of hypoxia inducible factor-1 (HIF-1) in the process of fibrogenesis. Evidence had been found that HIF-1 plays an important role in the initiation of fibrogenesis in kidneys and liver [20-24]. Sasabe et al. demonstrated that intracellular reactive oxygen species (ROS) up-regulate HIF-1α expression by inhibiting degradation, and enhancing the transcription and translation of HIF-1α [25]. Recently, van de Sluis et al. demonstrated that in knockout mice deficiency of COMMD1 is associated with fetal death around day 9 (due to inadequate placenta development) and an increased HIF-1α activity [26]. Furthermore, over-expression of COMMD1 enhanced the protein degradation of HIF-1α in an ubiquitin-independent manner [27]. We performed a qPCR for HIF-1α during our longitudinal follow-up and found the expression of HIF-1α to be up-regulated starting at 12 months of age, before HSC activation and the process of fibrosis. This HIF-1α up-regulation (in dogs at mRNA level) corresponds with the data obtained in *COMMD1*$^{-/-}$ mice [26]. These preliminary results in the COMMD1 deficient dogs suggest an initiating role for HIF in the development of fibrosis. For interpretation of these findings, measurements at the protein level will be of utmost importance. Therefore, work is in progress on an IHC for HIF-1α in our COMMD1 deficient dogs.

In **chapter 8** we have evaluated the process of copper metabolism and oxidative stress in the absence of the COMMD1 protein, before and after intervention with penicillamine, a copper chelating agent. The COMMD1 mutation affects the function of ATP7B. Mutations in this ATP7B gene cause different forms of Wilson's disease (WD) in man. Although the affected gene for WD is known, and progress has been made into understanding the molecular events leading to WD [28] further molecular characterization is feasible and necessary and this canine model can be helpful in unraveling the underlying processes.

It is commonly agreed that chronic copper exposure results in elevated ROS production in mammals [29,30]. In our longitudinal study, genes involved in the regulation of oxidative stress (SOD, catalase, GPX) are initially not up-regulated, whereas copper at the age of one year has been stored in high amounts. In this phase, the exposure of cellular organelles

to free cytoplasmic copper is likely minimal, due to the fact that copper is bound to metallothionein (MT) and sequestered in the lysosomal compartment [31]. Although hard to analyze in stored biopsies, a non-conclusive decrease in GSH/GSSG ratio occurred gradually during ageing. In this respect these data are in accordance with the results of a study performed in ATP7B knockout mice [32]. Ultimately, when the storage capacity is overwhelmed, the oxidative defense systems are activated as indicated by an up-regulation especially of catalase in our dogs from two years of age onwards, which persists also during treatment with a copper binding agent. At this age of two years, hepatitis is histologically clearly visible. At the same time the gene transcription of expression of the copper efflux pumps ATP7B and ATP7A is up-regulated, also indicating that the intracellular storage compartment (MT) is overwhelmed. Despite these defense mechanisms, the pro-oxidants prevail and hepatitis progressively evolves.

To unravel the function of the COMMD1 protein further research is necessary. The findings in this thesis point to, as suggested by other studies, a trafficking agent involved in the regulation of other proteins [33,34]. The reason why COMMD1 deficient dogs do accumulate copper is still not explained. Our research reveals an important role for ATP7B for the aberrant metabolic handling of hepatic copper in COMMD1 deficiency, as was suggested by others [33,34]. Our findings (gene expression and protein (IHC) level of ATP7B and COMMD1) underline the importance of COMMD1 as a transport regulator of ATP7B and its role in copper homeostasis. Together with Dr. van IJzendoorn (UMCG) we will perform studies on ATP7B trafficking in wild type and COMMD1 deficient dog livers [35-37].

COMMD1 deficient dogs demonstrate clear similarities with WD in man. We think this canine disease is an excellent large animal model for chronic hepatitis in man, and specifically for WD. In the dogs it is ATP7B which fails to function under the influence of the absence of the COMMD1 protein. Therefore in both species copper accumulation in the liver is caused by deficient function of ATP7B, resulting in chronic progressive hepatitis and cirrhosis. A general species related difference between dogs and man is that dogs have higher liver copper concentrations, also in health. Apparently the combined effect of pathways regulating copper homeostasis causes a higher plateau in dogs. The hepatic copper concentrations in patients with WD are accordingly lower than in dogs with copper-induced hepatitis (on average 150 ppm in WD vs 1500->8000 ppm in dogs), although in both species this leads to progressive hepatitis and finally cirrhosis. This species difference makes it much easier to monitor effects of interventions in dogs than in man. By adding increased amounts of copper to the diet, the disease may also be rapidly

inducible in COMMD1 deficient dogs so that evaluation of interventions may be performed before the age of 1.5 years. Besides clear similarities also differences exist between dog and man. In contrast to human WD neurological defects have not been observed and ceruloplasmin concentrations are normal in dogs without COMMD1 [38]. Dogs with copper-induced hepatitis start to accumulate copper in the centrolobular zone of the liver lobules, whereas a zonal distribution is not distinct in humans.

All together, we conclude that for pathomechanistic studies into aberrant copper metabolism for WD COMMD1 deficient dogs are a suitable large animal model in addition to the ATP7B deficient or knockout rodent animals [32,39-43]. In addition as a model for WD, the COMMD1 deficient dog is a very valuable tool to unravel copper transport and oxidative stress involved mechanisms in general, and to evaluate the effects of new medical copper and oxidative stress interference studies. A model is not necessarily an exact copy of the disease of interest. The known genetic aetiology and the inducibility with dietary modulations make these dogs attractive to evaluate new clinical approaches to reduce fibrosis and improve regeneration of the liver.

Conclusions

We have established a dog model with excellent properties for the evaluation of anti-fibrotic and anti-oxidant therapies and liver cell transplantation experiments. Different pathways (fibrosis, regeneration, copper metabolism and oxidative stress) have been characterised. These dogs combined with state-of-art clinical treatment options, close collaboration with owners and breeding organisations, detailed histological knowledge and almost unlimited molecular tools allow to investigate the feasibility of hepatocyte, or (adult) stem cell- and/or gene-therapy to cure chronic hepatitis in man (‘s best friend) and to evaluate new therapeutic strategies for WD.

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Chapter

10

Samenvatting (Summary in Dutch)

Achtergrond

De lever is één van de grootste organen van ons lichaam en speelt een zeer belangrijke rol in de stofwisseling en het handhaven van de homeostase. Wanneer de lever beschadigd raakt, acuut of chronisch, worden er processen geactiveerd om deze schade te herstellen. Wanneer dit niet of onvoldoende lukt en de schade, ongeacht de oorzaak, aan blijft houden wordt er progressief bindweefsel gevormd wat uiteindelijk zal resulteren in levercirrose. Levercirrose is wereldwijd een veel voorkomend probleem bij mens en dier. Vroegtijdige diagnose van een (chronisch) leverprobleem kan met een juiste behandeling mogelijk erger voorkomen. Als er éénmaal sprake is van cirrose met ernstige functionele problemen is dit niet anders te behandelen dan met een levertransplantatie. Er zijn echter onvoldoende donorlevers om aan de vraag voor transplantatie te kunnen voldoen, ondanks verschillende initiatieven om het aantal orgaandonoren te verhogen. Derhalve zijn alternatieven voor het transplanteren van een hele lever zeer gewenst. Deze alternatieven kunnen bestaan uit transplantatie van delen van levers of levercellen, of anti-fibrose en regeneratie stimulerende therapieën. Veel onderzoek naar dit soort alternatieven heeft de afgelopen decennia plaatsgevonden in knaagdiermodellen. De stap rechtstreeks van deze kleine diermodellen naar de humane patiënt is groot en daarom is het wenselijk om een groot diermodel te realiseren wat grote overeenkomsten vertoont met de pathologie en pathofysiologie van leverziekten zoals die bij de mens voorkomen. De hond is een goed voorbeeld van een dergelijk diersoort. Belangrijke randvoorwaarden van een dergelijk diermodel zijn dat het goed controleerbaar en reproduceerbaar is. De Bedlington terrier (BT), waarbij een koper-geïnduceerde hepatitis voorkomt door een gendefect, lijkt aan deze randvoorwaarden te voldoen. BTs stapelen koper in hun lever als gevolg van een mutatie in het *COMMD1* gen. Deze mutatie is in 2002 ontdekt. Wij hebben BTs met deze mutatie gekruist met gezonde Beagles en nakomelingen hiervan weer onderling gekruist. Op deze manier hebben wij een kolonie honden gecreëerd die COMMD1 deficiënt zijn. Het deficiënt zijn van COMMD1 leidt tot een dysfunctie van ATP7B, een eiwit in de lever dat een zeer belangrijke rol speelt bij de uitscheiding van koper naar de gal. Een dysfunctie van ATP7B resulteert in koperstapeling in de lever en leidt tot een ziektebeeld wat bij mensen de ziekte van Wilson wordt genoemd. Aangezien de dysfunctie van ATP7B de oorzaak is voor de koperstapeling, is de pathogenese van mensen met de ziekte van Wilson en COMMD1 deficiënte honden vergelijkbaar. Indien de ziekte van Wilson niet behandeld wordt, ontwikkelen deze patiënten uiteindelijk levercirrose en in een aantal gevallen zal dan een levertransplantatie nodig zijn. Echter, een tijdige behandeling met een laag koper dieet en koperbindende medicijnen kan het ziektebeeld onder controle krijgen.

Indeling

Dit proefschrift is opgedeeld in 2 delen. Deel één (hoofdstuk 3 t/m 5) gaat over het voorkomen van hepatitis bij de hond in de veterinair klinische *setting*. Deel twee (hoofdstuk 6 t/m 8) gaat over de hond met een deficiëntie van COMMD1 als modeldier voor chronische hepatitis bij de mens en in het bijzonder de koper-geïnduceerde hepatitis.

In **hoofdstuk 3** wordt beschreven welke vormen van hepatitis er bij de hond bekend zijn. Ook wordt hier beschreven hoe de diagnose van elke van deze vormen gesteld kan worden en wat de meest recente behandeling is.

Hoofdstuk 4 is een retrospectieve analyse van een groep honden met een vorm van primaire hepatitis die verwezen zijn naar de kliniek voor gezelschapsdieren van de faculteit diergeneeskunde van de Universiteit Utrecht. Belangrijke conclusies die getrokken konden worden waren dat chronische hepatitis de meest voorkomende vorm van hepatitis is in deze verwijspopulatie en dat koperstapeling in de lever voor 1/3^{e} deel van de gevallen van chronische hepatitis de oorzaak was, ongeacht het ras. Op basis van deze conclusie wordt nu het advies gegeven om bij het insturen van een leverbiopt van een hond verdacht van hepatitis naast een routine HE kleuring ook een koperkleuring aan te vragen, omdat als een verhoogde koperconcentratie in de lever de oorzaak is, hier een andere behandeling (penicillamine, dieetmaatregelen) voor wordt ingesteld dan als de oorzaak onbekend is (prednison). Ook werd aangetoond dat acute hepatitis in een aantal gevallen (ongeveer 20%) overgaat in chronische hepatitis. Daarom wordt eigenaren geadviseerd om na 6 weken een controle leverbiopt te laten nemen om vast te stellen of de acute hepatitis genezen is of over gegaan is in chronische hepatitis.

In **hoofdstuk 5** behandelt een retrospectieve evaluatie van het therapeutisch effect van prednison als medicijn voor de behandeling van chronische hepatitis met onbegrepen oorzaak (idiopathisch) (CH(i)). De enige onderbouwing tot nu toe voor het gebruik van prednison als medicijn voor CH(i) is een retrospectieve studie uit 1988 uitgevoerd bij 151 honden. Uit deze studie kwam als belangrijkste conclusie naar voren dat prednison een levensverlengend effect heeft bij honden met CH(i). Echter, in de samenstelling van deze groep van 151 honden was geen onderscheid gemaakt tussen idiopathische en koper-geinduceerde hepatitis. Daarnaast komen er steeds meer aanwijzingen dat een deel van de CH(i) mogelijk een virale oorzaak heeft. Indien dit waar is, is in deze gevallen immuunsuppressie door prednison niet wenselijk en zelfs gecontra-indiceerd. Deze

aspecten zijn een reden geweest waarom wij een retrospectieve analyse van 36 honden met CH(i) die met prednison zijn behandeld in onze kliniek voor gezelschapsdieren hebben uitgevoerd. Uit deze evaluatie kwam naar voren dat levercirrose een sterke negatieve prognostische factor is, dat prednison de progressie van fibrosering lijkt af te remmen en dat prednison een positief effect op de overleving heeft bij patiënten met CH(i). Ook is via dit onderzoek duidelijk aangetoond dat het geven van prednison aan honden met een stollingsprobleem als gevolg van CH(i) na één week een sterke verbetering van de bloedstolling geeft. Het mechanisme van dit positieve effect op de bloedstolling bij patiënten met hepatitis is nog niet opgehelderd en zeer zeker reden voor verder onderzoek. Op basis van deze gegevens lijkt het dus onverstandig om honden met CH(i) niet te behandelen met prednison. In de nabije toekomst zullen de therapeutische effecten van nieuwe medicijnen in een gerandomiseerde dubbelblinde placebo gecontroleerde studie in aanvulling op prednison getest moeten worden. Daarnaast blijft het belangrijk dat de oorzaken voor chronische hepatitis gevonden gaan worden. Alleen als de oorzaak bekend is, kan er echt een gerichte therapie gegeven worden.

Hoofdstuk 6 beschrijft de ontwikkeling van een diagnostische test om dieren met een mutatie in het *COMMD1* gen op te sporen. Voordat de door ons beschreven test ontwikkeld werd, was voor de diagnostiek RNA nodig, een kwetsbaar materiaal dat direct na afname van de patiënt geïsoleerd dient te worden. Praktischer zou het zijn als DNA, wat eenvoudig te isoleren is uit een bloedmonster dat per post te versturen is, gebruikt kan worden. Het principe van onze test is gebaseerd op de kwantitatieve PCR techniek (qPCR). Deze techniek wordt normaliter gebruikt om geïsoleerde RNA hoeveelheden te kwantificeren om zo een beeld van de expressie van een gen van interesse te krijgen. Wij hebben deze qPCR techniek gebruikt voor het *COMMD1* gen op DNA niveau in plaats van RNA niveau. Het COMMD1 gen bestaat uit 3 exonen. Bij het gemuteerde gen ontbreekt exon-2. Door op DNA niveau exon-2 in een qPCR reactie te bepalen ten opzichte van een bepaalde altijd aanwezige referentie (door ons is hiervoor exon-3 van het *COMMD1* gen gekozen (en een veel gebruikt controle gen GAPDH gaf vergelijkbare resultaten)), kan dan worden vastgesteld of we te maken hebben met een homozygoot gezond dier (een ratio van 1), een drager (een ratio van 0.5), of een lijder (ratio van 0). Uit onze onderzoek is gebleken dat deze benadering van de mutatie op DNA niveau met behulp van de qPCR inderdaad werkt. Een half jaar na onze publicatie verscheen er een publicatie met een nog praktischer test. Deze test is gebaseerd op een PCR reactie op DNA niveau. Deze onderzoeksgroep heeft namelijk het gehele *COMMD1* gen gesequenced en kon daarom ook vaststellen welke primersequenties nodig waren om de deletie op te sporen zonder gebruik te hoeven maken van de duurdere qPCR techniek. Doordat de regio

van het COMMD1 gen veel repeterende DNA sequenties bevat, was het vaststellen van de DNA volgorde tot dan toe niet volledig gelukt.

Hoofdstuk 7 beschrijft een longitudinale follow-up studie met betrekking tot de ontwikkeling van fibrose en regeneratie in vijf COMMD1 deficiënte honden. Als gevolg van het ontbreken van COMMD1 stapelen deze honden koper in hun lever wat uiteindelijk aanleiding geeft tot de ontwikkeling van een koper-geïnduceerde hepatitis. Deze studie heeft aangetoond dat koper op 1 jaar leeftijd al maximaal gestapeld is, maar dat hepatitis dan nog afwezig is. Het eerste wat op valt is de activatie van de "hepatic stellate cells" (HSCs, een celtype in de lever dat een centrale rol speelt in de bindweefselvorming) vanaf 18 maanden leeftijd, waarbij er dan sprake is van een zeer geringe hepatitis. De HSC activatie wordt in de tijd gevolgd door een progressieve bindweefselvorming leidend tot levercirrose bij 3 van de 5 honden op 42 maanden leeftijd. Verrassend genoeg loopt min of meer gelijktijdig met de bindweefselvorming een regeneratief proces, dat blijkbaar ontoereikend is. Deze regeneratie komt geheel voor rekening van de volwassen levercellen. Er is ook sprake van lever progenitor cel (LPC) activatie, maar deze cellen ontwikkelen zich echter niet verder tot volwassen levercellen. Ondanks het feit dat gedurende het ouder worden er zich een steeds heftiger wordende hepatitis met cirrose ontwikkelt, lijken de dieren zelf daarvan weinig of geen last van te hebben: ze vallen niet af, er is geen sprake van braken en/of diarree, ze hebben geen verminderde eetlust en ze blijven actief. De door ons gekarakteriseerde processen tijdens de ontwikkeling van chronische hepatitis in de COMMD1 deficiënte honden vertonen zeer grote overeenkomsten met deze processen zoals die bij de mens met chronische hepatitis voorkomen. Dit maakt deze honden, met een bekende genetische afwijking, zeer geschikt als diermodel voor het bestuderen van toekomstige anti-fibrose of groeistimulerende behandelingen of het uitvoeren van celtransplantaties gericht op het uiteindelijk behandelen van humane leverpatiënten.

In **hoofdstuk 8** zijn het kopermetabolisme en de rol van oxidatieve stress onderzocht bij het ontstaan van chronische hepatitis als gevolg van koperstapeling. Ook hebben we gekeken naar de effecten van penicillamine, een koperbindende stof, op het kopermetabolisme en oxidatieve stress. (Chronische) blootstelling aan een verhoogde hoeveelheid koper in de lever leidt tot de vorming van radicalen. Radicalen zijn schadelijk en kunnen onderdelen van cellen kapot maken. Belangrijke onderdelen die schade kunnen ondervinden zijn membranen en het DNA in de cel. Gelukkig zijn er beschermingsmechanismen in de vorm van anti-oxidanten en enzymen die radicalen kunnen omvormen in minder schadelijke stoffen. In onze longitudinale follow-up studie

hebben we laten zien dat koper in het eerste levensjaar tot zeer hoge waarden gestapeld kan worden, maar dat de anti-oxidatieve beschermingsmechanismen nog niet actief zijn, evenals het feit dat er vrijwel nog geen sprake is van zichtbare hepatitis. De verklaring hiervoor is dat bij honden koper in eerste instantie in grote hoeveelheden veilig gebonden kan worden door metallothioneïne en daardoor geen schade veroorzaakt. Maar na ongeveer twee jaar lijkt de buffercapaciteit te kort te schieten, worden de anti-oxidant systemen geactiveerd en ontwikkelt er zich tevens hepatitis. Op dit moment zien we ook dat er een verhoogde genexpressie ontstaat van zowel ATP7A als ATP7B, eiwitten die koper de cel uit moeten pompen. Ondanks dat er sprake lijkt te zijn van een verhoogde activiteit om koper de cel uit te werken, neemt de hepatitis verder toe. Op een leeftijd van 42 maanden zijn we gestart met de behandeling met penicillamine en hebben deze behandeling gedurende 18 maanden vervolgd. In deze maanden daalt het kopergehalte geleidelijk, is er geen verdere progressie van de hepatitis en blijven de genexpressie niveaus voor zowel katalase (een anti-oxidatieve enzym) als ATP7B verhoogd tot expressie komen.

Het is duidelijk geworden dat COMMD1 deficiënte honden een geschikt diermodel zijn in aanvulling op de kleinere knaagdiermodellen voor de ziekte van Wilson bij mensen. In beide gevallen betreft het een deficiënte functie van ATP7B wat resulteert in koperstapeling met chronische hepatitis als gevolg. Honden stapelen wel veel meer koper dan patiënten met de ziekte van Wilson alvorens er hepatitis ontstaat. Dit maakt deze honden nog geschikter voor het evalueren van interventies in het koperstapelingsproces aangezien de afwijkingen duidelijker zijn. Ook kunnen we deze honden meer koper via het dieet aanbieden zodat de processen versneld kunnen worden.

Conclusie

In een verwijspopulatie is chronische hepatitis bij de hond de meest voorkomende vorm van hepatitis en in ongeveer 30% van de gevallen is deze vorm van hepatitis geassocieerd met een verhoogd kopergehalte in de lever. Een behandeling met prednison heeft een levensverlengend effect bij honden met chronische hepatitis die niet door een verhoogd kopergehalte wordt veroorzaakt (CH(i)). Ook heeft prednison een zeer positief effect op de bloedstolling indien deze afwijkend is bij honden met CH(i).
Onze methode om de qPCR techniek te gebruiken op DNA om te screenen voor aan/afwezigheid van grote deleties (in ons geval COMMD1, maar is algemeen toepasbaar)

is door de snelle vooruitgang van sequencing methodes een paar jaar na ontwikkeling al ingehaald.

We hebben gedetailleerd de ontwikkeling van koper-geïnduceerde chronische hepatitis in een hondenmodel beschreven. Verschillende processen zoals het ontstaan van fibrose, het herstelvermogen, koper metabolisme en het ontstaan van oxidatieve stress zijn uitvoerig onderzocht. De ontwikkeling van chronische hepatitis bij de hond is zeer vergelijkbaar met chronische hepatitis bij de mens. We hebben laten zien dat onze COMMD1 deficiënte honden grote overeenkomsten vertonen met mensen met de ziekte van Wilson. Ons model heeft dan ook zeer geschikte eigenschappen om anti-fibrose, groeistimulerende of anti-oxidatieve therapieën, levercel transplantaties en nieuwe therapeutische strategieën voor de behandeling van koperstapeling bij patiënten met de ziekte van Wilson te evalueren. Met dit model slaan we een brug tussen onderzoeken uitgevoerd in kleine diermodellen zoals ratten en muizen en de uiteindelijke verantwoorde toepassing van deze onderzoeksgegevens bij de mens.

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List of publications and conference proceedings

Curriculum Vitae

The author of this thesis was born on the 14th of October 1973 in Leiden, the Netherlands. As secondary schools he attended the Christelijke Scholengemeenschap in Emmen from 1985 to 1987 and the Buys Ballot College in Goes from 1987 to 1991 and then went on to study veterinary medicine at the Faculty of Veterinary Medicine of the University of Utrecht. From September 1995 to August 1996 he followed a research master at the Department of Companion Animals of the same faculty (excellent trace), which was finished with a Master of Veterinary Research degree. From October 1996 to May 1999 he followed the post doctoral phase and graduated as a veterinarian May 1999. In June 1999 he started with an **internship** of Small Animal Internal Medicine at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, University of Utrecht, which was followed by a **residency** of Small Animal Internal Medicine in September 2000, sponsored by the **Hill's** animal pet food company. This residency was finished in August 2003. In 2003 he started his Ph.D. research project on "Canine hepatitis and the pathomechanisms of copper-induced hepatitis in COMMD1 deficient dogs" in the same department. The results are described in this thesis. Besides his research projects he is and further will be raised within the field of companion animal hepatology and since September 2009 he is a staff member at the same department. The author likes, besides working within the Veterinary Science, long-distance running, Salvador Dali, Spanish food and red wine.

De auteur van dit proefschrift werd op 14 oktober 1973 geboren in Leiden, Nederland. Zijn middelbare school opleiding volgde hij in Emmen op de Christelijke Scholengemeenschap van 1985 tot 1987 en in Goes op het Buys Ballot College van 1987 tot 1991, waarna hij diergeneeskunde ging studeren aan de Universiteit Utrecht. In september 1995 startte hij zijn Excellent Tracé bij het departement geneeskunde van gezelschapsdieren. Vervolgens heeft hij zijn co-schappen doorlopen, om in mei 1999 af te studeren als dierenarts. In juni 1999 begon hij als roulant bij het departement geneeskunde van gezelschapsdieren van de veterinaire faculteit te Utrecht, waar hij in september 2000 werd aangenomen als specialist in opleiding op het gebied van de Interne geneeskunde van Gezelschapsdieren wat werd afgesloten in augustus 2003. Zijn opleiding werd gesponsord door **Hill's** animal pet food company. In 2003 is hij begonnen met zijn promotieonderzoek, met als onderwerp "Hepatitis bij de hond en pathomechanismen van koper-geïnduceerde hepatitis in COMMD1 deficiënte honden" bij het departement geneeskunde van gezelschapsdieren. De resultaten zijn beschreven in dit proefschrift. Naast zijn onderzoeksprojecten is hij zich verder gaan verdiepen in het gebied van de hepatologie van gezelschapsdieren en sinds september 2009 is hij staflid bij hetzelfde departement. Behalve van zijn werk in de diergeneeskunde houdt de auteur van hardlopen, Salvador Dali, de Spaanse keuken en van rode wijn.

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Acknowledgements (Dankwoord)

Er zijn genoeg inzichten verschaft om te promoveren. Het is het resultaat van op gezette tijden hard werken. Veel is er veranderd in mijn leven sinds de start van deze promotie. Er is veel geleerd, toekomstbeelden zijn bijgesteld, een groter relativeringsvermogen is ontwikkeld en bovenal is er intenser het leven geleefd sinds de bewustwording van de eindigheid ervan.

Veel koffie is er gedronken, rond de tafel, op de werkvloer, achter de computer. Ik heb dat drinken zeker niet alleen gedaan, maar samen met velen. Deze velen zou ik graag willen bedanken voor alle hulp, op wat voor manier dan ook.

Prof. dr. Jan Rothuizen, mijn promotor
Beste Jan, bedankt dat je samen met mij een onderzoeksvoorstel wilde schrijven en mij hebt opgeleid tot een veterinair hepatoloog. Sinds de allereerste dag dat wij samenwerken heb jij mij heel, heel veel vrijheid gegeven. Aan een enkel woord hebben we genoeg. Ik ben je ook dankbaar voor alle letterlijk ongelimiteerde tijd die ik kreeg om thuis en in het ziekenhuis te zijn toen dat helaas zo nodig was.

Mijn co-promotoren dr. Louis Penning en dr. Bart Spee
Beste Louis, je geestdrift is enorm. Je bent altijd aanspreekbaar en denkt mee. Vele ideeën worden door jou in een rap tempo gegenereerd. Aanvankelijk had ik grote moeite het tempo van je denkbeelden te volgen. Totdat ik doorkreeg dat ik ze veelal even naast me neer moest leggen om er vervolgens dan weer een week later op terug te komen, waarop jij dan soms even niet meer precies wist hoe en wat. Voor mij zeer geruststellend en ik wil je vragen er niet mee te stoppen.

Beste Bart, jij kan overal ter wereld aan de slag en toch ben je bij ons gebleven. Wat ben ik daar blij om. Je biochemisch probleem oplossend vermogen is erg groot en lijkt zelfs oneindig te zijn. Briljant. Heel binnenkort word je vader, een nieuw moment in je leven, geniet ervan, samen met Brigitte. Dank voor al je hulp en ondersteuning!

Dr. Ted van den Ingh
Beste Ted, veterinair patholoog in het algemeen en leverpatholoog in het bijzonder. Wat een enorme expertise heb jij opgebouwd in de veterinaire pathologie. Ik heb hier een goed gebruik van kunnen maken. Snel en efficiënt. Jij hebt een zeer groot aandeel in de gegevens die in dit proefschrift zijn beschreven. Je was en bent altijd bereikbaar voor overleg, of je nu in Nederland of in Frankrijk bent.

De technische ondersteuning, Harry van Engelen, Inge van Duiven, Ries Pel, Martine de Ruyter Korver, Bas Brinkhof, Ank van Wees, Anette van Drie en Ronald Kisjes.

Beste Harry, technician. Ik ken je al heel lang, onze eerste samenwerking dateert van 1995, toen ik voor het eerst wat onderzoek mocht doen in de kliniek voor gezelschapsdieren. Wat ben jij van onschatbare waarde geweest bij het verzamelen van mijn onderzoeksmateriaal, een gouden praktische spil. Het fixeren van de honden, feilloos. Eigenlijk was jij het die de lever biopten nam, ik hield enkel de naald vast. Ook je belangstelling naar de voortgang van mijn onderzoek, mijzelf en mijn gezin heeft mij elke keer weer aangenaam verrast.

Beste Inge, pleegmoeder van mijn honden, Elles, Paula, Sylvia, Peter, Asterix en Viola. Fantastisch hoe jij je inzet voor het welbevinden van al onze kliniekhonden. Gelukkig was en ben je niet terughoudend om mij zo nu en dan details te geven hoe ik mijn onderzoeksaanvragen voor mijn hondjes kon optimaliseren om te voorkomen dat ik in het gevang wasknijpers moest gaan maken.

Beste Ries en Martine, bedankt voor het vast houden van mijn honden en alle hulp bij het verzamelen van de bloedmonsters.

Beste Bas en Ank, data genereren, aan één stuk door. Het zijn erg veel data geworden. Ank, ik hoop dat je nu volop geniet van je vrije tijd. En Bas, succes met je pas gestarte promotie.

Beste Anette, bedankt voor het snijden en kleuren van de vele coupes. Altijd op tijd klaar, ook in een periode dat je het zelf moeilijk had. Maar het is helemaal goed gekomen.

Beste Ronald, begonnen als technische ondersteuning voor de immunohistochemische kleuringen en inmiddels coördinator van deze technische ondersteuning. Altijd kritisch en terecht niet snel tevreden. Goed om mee te sparren en te ontdekken dat “gewoon even een kleuring doen” nooit gewoon is, ik heb er veel van geleerd.

De medewerkers van het UVDL, in willekeurige volgorde: Steve Rijnbout, Gaby Purimahuwa, Tjeerd Altena, Femke Altena van Drunen, Marije van der Schaar, Martin van Leeuwen, Marina van Houten, Hans Koeslag, Lide Bijlsma, Ellen Spithoven, Lida Overbeek, Martine Dantuma, Patrick van Hoorn, Marianne Endert van Dobben de Bruyn, Meidert Hogendoorn, Jos Vossen, Erna Meijer, en Lenie Boswijk-Appelwijk: bedankt voor het verwerken en uitvoeren van de vele door mij aangevraagde bloedonderzoeken. En Tjeerd, ik heb het gewaardeerd dat je mij speciaal op mijn vakantie belde om te vertellen dat je met Femke ging trouwen. Nu zijn jullie inmiddels 3 kinderen verder.

De “liver research” groep, in willekeurige volgorde en door de jaren heen: Brigitte Arends, Baukje Schotanus, Sacha Boomkens, Jooske IJzer, Conny Viebahn, Joost Poldervaart,

Heleen ter Borg, Giora van Straten, Frank van Steenbeek, Gaby Hoffmann, Hille Fieten, Hedwig Kruitwagen: bedankt voor alle reflectie over levergerelateerde en niet levergerelateerde zaken.

Voor alle hulp bij het ontwerp en de opmaak van het tastbare resultaat wat u nu in handen heeft wil ik de afdeling Multimedia van de Faculteit Diergeneeskunde, in het bijzonder Harry Otter (lay-out), Joop Fama (histologie en Western blot figuren), Anjolieke Dertien (de omslag), Lisanne van der Voort (fotografie omslag) hartelijk bedanken. Soepel en toegankelijk.

Mijn paranimfen, Quirine Stassen en Krista Visser ‘t Hooft.
Quirine, lieve Quirine, in juni 1999 heb ik je voor het eerst ontmoet, we startte tegelijk als roulant, samen op één kamer. Vanaf die dag hebben wij hetzelfde “werkparkoers” gevolgd en zijn we, ondanks alle doorgemaakte verhuizingen, altijd kamergenoten gebleven. Ik hoop dat dit de rest van onze werklevens ook zo zal blijven, wij hoeven elkaar niet uit te leggen waarom de combinatie van kliniek, onderwijs en onderzoek kan knellen. We lachen wat, we huilen wat en we gaan weer naar huis.

Krista, lieve Kris, in 2002 heb ik je leren kennen als een collega, steeds beter en je bent een ontzettend goede vriendin geworden. Wat kunnen wij samen lachen, je maakt me altijd vrolijk! Ik hoop dat je over niet al te lange tijd weer bij ons in de buurt komt wonen en werken.

Mijn familie.
Mijn lieve ouders, uitkijkend naar mijn promotie, bedankt voor alles wat jullie mij hebben meegegeven om te komen waar ik nu ben aangekomen. Lieve Dinand, mijn broertje, ook dierenarts en lieve Irene, mijn zusje, neuroloog, bedankt voor het willen aanhoren hoe ongelofelijk druk ik het wel niet heb gehad.

Lieve Rob en Lis, mijn schoonouders, en mevrouw Jacobson, oma, voor alle intellectuele interesse in mijn promotieonderzoek, het meeleven en de praktische ondersteuning.

Lieve Liesbeth, allerliefste Liesje. Sinds ik jou ben tegengekomen is er heel veel veranderd in mijn leven. Wat een steun en tijd heb ik van jou gekregen om te kunnen doen wat ik heb gedaan. Nu is het tijd voor jou om te gaan doen wat jij wilt doen. Ik hoop dat we heel erg lang samen blijven, samen met Tobias, Hanna en Eva. Wat heerlijk is het met jullie samen.

Vanaf nu ga ik samen met veel van de hierboven genoemde personen verder werken aan het ontwikkelen van alternatieven voor "*whole liver transplantation*". Het doet mij goed om dit te kunnen en mogen doen!

Amersfoort, januari 2011
Robert

Abbreviations

AH	acute hepatitis
ALT	alanine aminotransferase
AP	alkaline phosphatase
ATOX1	anti-oxidant protein 1
ATP7A	ATPase Cu(2+)-transporting alpha polypeptide
ATP7B	ATPase Cu(2+)-transporting beta polypeptide
CACH	copper associated chronic hepatitis
CAT	catalase
CAV-1	canine adenovirus-1
CCS	copper chaperone for superoxide dismutase
CH(i)	idiopathic chronic hepatitis
CIRR	cirrhosis
CMET	c-Met proto-oncogene product
COMMD1	copper metabolism MURR1 domain-containing protein 1
COX17	cytochrome c oxidase assembly protein
CP	ceruloplasmin
CT	copper toxicosis
CTR	copper transporter
ECM	extracellular matrix
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPX1	glutathione peroxidase 1
GR	glutathione reductase
GSH	reduced glutathione
GSS	glutathione synthetase
GSSG	oxidized glutathione
HGF	hepatocyte growth factor
HIF-1α	hypoxia inducible factor-1α
HPRT	hypoxanthine phosphoribosyl transferase
HSC	hepatic stellate cell
IL-6	interleukin-6
K	cytokeratin
LDH	lobular dissecting hepatitis
LEC	Long Evans Cinnamon

LPC	liver progenitor cell
MMP	matrix metalloproteinase
mRNA	messenger RNA (ribonucleic acid)
MT1A	metallothionein 1A
OLT	orthotopic liver transplantation
PCR	polymerase chain reaction
UPA	urokinase plasminogen activator
Q-PCR	quantitative real-time polymerase chain reaction
ROS	reactive oxygen species
RPS5	ribosomal protein S5
siRNA	small infering RNA
SOD1	Cu/Zn superoxide dismutase
STAT	signal transducer and activator of transcription
TGF-alpha	transforming growth factor alpha
TGF-β1	transforming growth factor β1
TGF-βR	transforming growth factor β1 receptor
TIMP	tissue inhibitor of metalloproteinase
WD	Wilson's disease
XIAP	X-linked inhibitor of apoptosis