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# DOBERMAN HEPATITIS comparison of subclinical and clinical stages and evaluation of etiopathogenesis of the disease

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# ACADEMIC DISSERTATION

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Yliopistopaino Helsinki 2004 To my children Elias and Maria



# CONTENTS

1.	ABSTRACT	8
2.	LIST OF ORIGINAL PUBLICATIONS	11
3.	ABBREVIATIONS	12
4.	INTRODUCTION	13
5.	REVIEW OF THE LITERATURE	16
	5.1. Definition of chronic hepatitis	16
	5.2. Laboratory tests to diagnose chronic hepatitis	16
	5.2.1. Alanine aminotransferase (ALT)	16
	5.2.2. Alkaline phosphatase (AP)	17
	5.2.3. Total bilirubin	17
	5.2.4. ALT, AP, and total bilirubin in Doberman hepatitis (DH)	18
	5.3. Histological findings in DH	19
	5.3.1. Interpretation of the histological findings in liver samples	19
	5.3.2. Histopathology of DH	20
	5.4. Classification of chronic hepatitis in man based on the etiology	22
	5.4.1. Infection	22
	5.4.2. Metabolic disorders	23
	5.4.3. Drugs and toxins	24
	5.4.4. Autoimmunity	25
	5.5. Possible etiological factors of DH	
	5.5.1. Infection	
	5.5.2. Metabolic disorders	27
	5.5.3. Drugs and toxins	29
	5.5.4. Autoimmunity	29
	5.6. Major histocompatibility complex (MHC) class II expression	
	5.6.1. MHC class II in diseases	
6.	AIMS OF THE STUDY	
7.	MATERIALS AND METHODS	
	7.1. Study material	
	7.2. Diagnostic methods	
	7.2.1. Biochemistry	

	7.2.2. Histology	
	7.2.3. Immunohistochemistry	
	7.2.4. Quantitative analysis of liver copper content	
	7.3. Interpretation of histological findings in liver samples	
	7.3.1. Classic lobule model	
	7.3.2. Scoring and grading of histological lesions	
	7.3.3. Cholestasis	40
	7.3.4. Copper	40
	7.3.5. Criteria for MHC class II expression	41
	7.4. Treatment	
	7.5. Statistical analyses	
8.	RESULTS	44
	8.1. Criteria for subclinical DH	44
	8.2. Biochemistry	44
	8.2.1. Serum parameters in the subclinical stage of DH	44
	8.2.2. Serum parameters in the clinical stage of DH	45
	8.2.3. Serum parameters of subclinical versus clinical stages of DH	47
	8.3. Evaluation of liver samples	47
	8.3.1. Biopsy samples in DH	47
	8.3.2. Autopsy samples in DH	
	8.3.3. Biopsy versus autopsy samples in DH	
	8.3.4. Liver samples of control Dobermans	
	8.3.5. Relationship of mononuclear inflammation to liver copper gran	ales 53
	8.3.6. Relationship of mononuclear inflammation to MHC class II ex	pression
	in hepatocytes	
	8.3.7. Comparison of rubeinic acid and rhodanine stains for copper det	ection 55
	8.3.8. Evaluation of hepatic copper concentrations	
	8.4. Effect of corticosteroid treatment in DH	
9.	DISCUSSION	
	9.1. Serum parameters	
	9.2. Diagnosis of subclinical DH	60
	9.3. Role of copper in DH	
	9.4. MHC class II expression in hepatocytes and etiology of DH	
	9.5. MHC class II expression in hepatocytes and pathogenesis of DH	69

10.	CONCLUSIONS	72
11.	ACKNOWLEDGMENTS	77
12.	REFERENCES	80

# 1. ABSTRACT

Doberman hepatitis (DH) is a chronic liver disease which is usually diagnosed when the dog has clinical signs of liver failure. At this stage of the disease, the prognosis is poor. Detection of affected individuals in the asymptomatic subclinical stage would likely improve survival time. To date, diagnosis of subclinical DH has been difficult since criteria for this stage are lacking and the etiopathogenesis of DH is unknown.

The aim of this work was manifold. First, the serum parameters most effective for screening subclinical DH and indicating progression from the subclinical stage to the clinical stage were determined. Second, the histopathological lesions in the subclinical stage of DH were described. Third, the role of increased hepatic copper (Cu) in DH was studied. And, fourth, the etiology and the pathogenesis of DH were evaluated based on the observation that hepatocytes in DH express major histocompatibility complex (MHC) class II molecules.

Serum concentrations of both alanine aminotransferase (ALT) and alkaline phosphatase (AP) are routinely used to detect liver failure in dogs. Based on the findings from the first phase of this study, ALT was determined to be the more reliable liver enzyme in detecting subclinical DH. During the early stage of the disease elevated concentrations of ALT can be seen, followed by an increase in AP. Serum concentrations of ALT and AP were shown to be abnormally high for several months or even years before onset of clinical signs of DH. Fluctuation of these parameters was common during the subclinical stage, while total bilirubin concentration in serum remained normal. An increase in total bilirubin was therefore deemed to be the best serum parameter for predicting the disease's progression to the clinical stage.

In the second phase of the study, the most characteristic histological findings during the subclinical stage were parenchymal and portal inflammation. The inflammatory cells were mostly lymphocytes and macrophages, with neutrophils and plasma cells being few in number. Periportal inflammation was minimal. Bridging necrosis was also seldom observed. A scoring system was used to determine how histological lesions in biopsy differed from those in autopsy liver samples of the same dog. With this system,

DH was shown to be progressive in nature. When clinical signs appear, the histological findings resemble those typical of chronic hepatitis with piecemeal and bridging necrosis, fibrosis, and cirrhosis.

During the third phase of this study elevated hepatic Cu content was recorded both in the subclinical and clinical stages of DH. In the subclinical stage, the location of Cu was first observed in hepatocytes in the parenchymal area. Periportal areas were mainly intact. In the clinical stage, the Cu was mostly located adjacent to piece-meal and bridging necrosis areas. No intrahepatic cholestasis was evident, bile plugs were observed very rarely, and serum total bilirubin values were within the normal range during the subclinical stage of DH. Increased hepatic Cu content was also demonstrated to be associated with inflammation of the tissue. Based on these findings, Cu accumulation in hepatocytes was speculated to be caused by mononuclear inflammation, which in turn disturbs the secretion of Cu from hepatocytes to the bile canaliculus.

From the combined findings of the first three study phases, the diagnosis of subclinical DH was determined to be based on the following four criteria: 1) the dog is clinically asymptomatic; 2) the dog has an elevated serum ALT concentration that is at least three times above the normal upper value in two consecutive blood samples; 3) mononuclear inflammation is present in the liver; 4) increased hepatic Cu content is detected by a special stain.

Since normal hepatocytes do not express MHC class II molecules, their expression in hepatocytes in DH indicates that hepatocytes are the target cells of this disease. The etiological background of DH was analyzed in the fourth phase of this study based on findings of abnormal MHC class II expression in hepatocytes during different stages of DH. MHC class II molecules were expressed in hepatocytes in 87% of biopsies taken during the subclinical stage of the disease and in 100% of autopsy cases. This finding is a strong indicator of the autoimmune background of DH.

A significant correlation between mononuclear inflammation and MHC class II expression in hepatocytes was also noted. This finding suggests that cytokines produced

by mononuclear inflammatory cells could induce abnormal MHC class II expression in hepatocytes.

Staining patterns of MHC class II expression in hepatocytes vary according to the stage of the disease. In the subclinical stage, expression was observed to be mostly cytoplasmic, whereas in the clinical stage molecules were detected mainly on the cell membrane. These findings indicate that while the MHC class II molecule and antigen are in the cytoplasm, the hepatocyte is protected from the immune system. However, as soon as the MHC class II:autoantigen complex reaches the cell surface, the immune system will recognize the autoantigen and be activated.

Thus in DH , the MHC class II molecule apparently presents an autoantigen to the CD4+ T-cell, which in turn becomes activated, initiating the immune attack against hepatocytes. The T-cells are therefore autoreactive and DH is a T-cell-mediated disease.

# 2. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals (I-IV):

- I Speeti M, Ihantola M, Westermarck E. Subclinical versus clinical hepatitis in the dobermann: evaluation of changes in blood parameters. Journal of Small Animal Practice 1996; 37: 465-470.
- II Speeti M, Eriksson J, Saari S, Westermarck E. Lesions of Subclinical Doberman Hepatitis. Veterinary Pathology 1998; 35: 361-369.
- III Speeti M, Eriksson J, Westermarck E. Some new aspects of the role of copper in doberman hepatitis. European Journal of Veterinary Pathology 1999; 5: 51-56.
- IV Speeti M, Ståhls A, Meri S, Westermarck E. Upregulation of major histocompatibility complex class II antigens in hepatocytes in Doberman hepatitis. Veterinary Immunology and Immunopathology 2003; 96: 1-12.

# 3. ABBREVIATIONS

ALT	alanine aminotransferase	HNO <sub>3</sub>	nitric acid	
ANA	antinuclear antigen	HRP	horseradish peroxidase	
AP	alkaline phosphatase	LKM	liver kidney microsome	
APC	antigen-presenting cell	LMA	liver membrane antibody	
BSA	bovine serum albumin	MHC	major histocompatibility	
САН	chronic active hepatitis		complex	
CAV-1	canine adenovirus-1	mmol/L	millimole/liter	
Cu	copper	PBC	primary biliary cirrhosis	
DH	Doberman hepatitis	SD	standard deviation	
DNA	deoxyribonucleic acid	SMA	smooth muscle antigen	
dw	dry weight	TBS	Tris Buffer Saline	
ER	endoplasmatic reticulum	U/1	Unit/liter	
HAI	histology activity index	VG	van Gieson	
HBV	hepatitis B virus	α-1-A	alfa-1- antitypsin	
HCV	hepatitis C virus	CD	cluster of differentation	
HE	hematoxylin and eosin	PAS	peroxid acid stiff	

# 4. INTRODUCTION

Chronic hepatitis in dogs refers to a chronic inflammatory process that destroys the liver parenchyma, resulting in liver failure (Strombeck and Gribble 1978, Sarli 1992). In dogs, chronic hepatitis is among reactive hepatopathies the most commonly diagnosed liver disorders (Twedt 1998b). Certain breeds, such as Bedlington Terriers, West Highland White Terriers, Cocker Spaniels, Labrador Retrievers, and Doberman Pinschers are more prone to chronic hepatitis than other breeds (Twedt et al. 1979, Doige and Lester 1981, Sevelius 1995, Thornburg et al.1996, Fuentealba et al. 1997).

In the literature, different names can be found for chronic hepatitis in Dobermans. "Chronic active hepatitis", formerly the predominant term, was adapted from the terminology for human hepatitis (Meyer et al. 1980, Fiorito 1985, van den Ingh et al. 1988, Searle 1991). The initial classification of chronic hepatitis in human medicine was based on histology since the etiology of the disease was largely unknown. A distinction was made between a milder form of the disease, i.e. chronic persistent hepatitis, and variants with more severe disease activity, i.e. chronic active hepatitis (De Groote et al. 1968). Because chronic hepatitis in Dobermans histologically resembles human chronic active hepatitis, the name was also used for Dobermans.

In human medicine today, the classification of chronic hepatitis is based on different etiological factors (Ludwig 1993). The terms ''chronic persistent hepatitis'' and ''chronic active hepatitis'' have thus been replaced with ''chronic hepatitis''. Likewise, for Dobermans, the term ''chronic active hepatitis'' has been replaced with ''chronic hepatitis'' (Zawie 1986, Center 1996b). Other commonly used names for hepatitis in Dobermans are ''copper-associated hepatitis'' and ''Doberman hepatitis'' (DH) (Hardy 1986, King 1990). The emphasis in copper-associated hepatitis is on the typical Cu accumulations in the liver, while the term DH is used to stress that the disease in Dobermans differs from chronic hepatitis in other breeds.

Several features are typical of DH. One of these is the aggressive nature of the disease. After the clinical signs of liver failure become obvious, the dog's condition will deteriorate rapidlyvery fast (Meyer et al. 1980, Fiorito 1985, Rothuizen and Meyer 2000). Unfortunately, these clinical signs are usually not detectable until the liver has already been severely damaged. Another typical feature of DH is its poor response to treatment. The most commonly used medication is corticosteroids, with an immunosuppressive dosage to reduce the inflammatory process in the liver. However, corticosteroids, when started at the onset of clinical signs, only temporarily improve the dog's condition (Crawford et al. 1985, van den Ingh et al. 1988, Strombeck et al. 1988). This is in contrast to the treatment of chronic hepatitis in other breeds, where results are more favorable (Rothuizen 1997). A third typical feature of DH is that most of the dogs affected are female. The reason for this is unknown.

Clinical signs usually appear at the age of 3-6 years. The youngest Doberman reported to suffer from DH was 1.5 years, and the oldest 11 years (Crawford et al. 1985, Fuenealbe et al. 1997). Clinical symptoms are not pathognomonic of DH. The most common signs are polydipsia and polyuria, anorexia, weight loss, and lethargy. Other signs include vomiting and diarrhea (Crawford et al. 1985, van den Ingh et al. 1988, Cornelius 1989a).

Although the exact pathophysiological connection between chronic hepatitis and gastrointestinal problems has not been elucidated, chronic hepatitis is known to cause gastrointestinal symptoms (Kamath et al 2000). This is particularly evident when the patient is on corticosteroid treatment (Center 1996a). Icterus in DH manifests with discoloration of the sclera, mucous membranes, and urine, and in the advanced stages, also of the skin. Since the liver has a central role in blood coagulation, blood may be present in the stool, and bleeding can be associated with obtaining liver samples or with other surgical procedures in the advanced stages of DH. In the later stages of the disease, ascitic fluid accumulates in the abdomen, and hepatic encephalopathy with altered mental status, including stupor, may be observed (Doige and Lester 1981, Rothuizen 1997).

The etiology and pathogenesis of DH remains unknown, and studies on histological lesions in the subclinical stage of the disease are scarce. Most published cases were diagnosed when inflammation of the liver had already caused obvious damage to the parenchymal cells. Identifying affected individuals as early as possible is paramount. In the early stages of the disease, tissue destruction has just begun and the activated

immune system has not yet altered the original cell composition of the inflammatory cells.

While Dobermans are known to be particularly susceptible to chronic hepatitis, the prevalence of the disease in this breed has not been well established. In one study, the occurrence of chronic hepatitis in Dobermans (2%) was considerably higher than in other breeds (0.12%) (Johnson et al. 1982). However, a survey of 106 healthy Dobermans (age 3 years) evaluating subclinical cases of DH reported a prevalence of 21% (Mandigers et al. 2000). These high prevalences of chronic hepatitis in Dobermans suggest a genetic pre-disposition (Johnson et al. 1982, Thornburg et al. 1984, Hardy 1985).

# 5. REVIEW OF THE LITERATURE

# 5.1. Definition of chronic hepatitis

In humans, an accurate diagnosis of chronic hepatitis requires a period of at least six months for both clinical and laboratory examination to indicate inflammation and death of liver cells (Hassi 1986, Desmet et al. 1994). Histologically, chronic hepatitis causes ongoing injury to liver cells, which is characterized by mononuclear inflammation in the portal and lobular areas with differing degrees of piecemeal necrosis that disrupts the limiting plate surrounding the portal area. Parenchymal inflammation varies. Bridging necrosis arises from portal to portal, portal to central, or central to central areas. For dogs, chronicity is generally defined as persistently abnormal liver tests and specimens over 4-6 months (Sarli 1992).

# 5.2. Laboratory tests to diagnose chronic hepatitis

Signs of chronic hepatitis are not pathognomonic of liver dysfunction. Therefore, biochemical tests are used to establish the presence of liver tissue damage. Several laboratory tests are available, but measurement of serum liver enzymes is the most common indicator of hepatocellular damage. Serum liver enzyme tests, based on measurement of cellular contents that have been released into the circulation, can be divided into those reflecting hepatocellular injury and those indicating increased enzyme production (Meyer et al. 1992).

## 5.2.1. Alanine aminotransferase (ALT)

Alanine aminotransferase (ALT) is considered to be the most specific liver enzyme in dogs. In addition to its physiological functions as an enzyme, ALT is useful for diagnosing disorders of the liver. It is a soluble cytoplasmic enzyme that is easily released through the cell membrane even when hepatocytes appear microscopically intact (Bush 1991, Center 1997). ALT can also be found in the heart, kidneys, and muscles. However, its concentrations in hepatocytes are several times higher than in other tissues (Center 1996c). Increased serum concentrations of ALT are therefore a good indicator that hepatocytes are somehow affected. The serum half-life of ALT in

dogs is approximately 48 hours (Richter 1996). However, increased production and release by viable and regenerating hepatocytes may contribute to changes in the serum ALT concentration. Elevated ALT values will not return to the normal range in just a few days. Moreover, elevated ALT values do not discriminate between the primary and secondary reasons for altered hepatocytes (Twedt 1998b).

#### 5.2.2. Alkaline phosphatase (AP)

Alkaline phosphatase (AP) is commonly used, usually in combination with ALT, to detect liver diseases in dogs. AP is a membrane-bound enzyme that is primarily located on the bile canalicular membrane of hepatocytes and the luminal surface of biliary epithelial cells (Leveille-Webster 2000). Elevated AP levels are the result of increased synthesis of the enzyme and are not due to the leakage of the enzyme from the cells, as described for ALT (Bush 1991, Meyer 1996). Several isoenzymes of AP can be found in the liver, kidney, intestine, bone, and placenta. The half-lives of isoenzymes for the kidney, intestine, and placenta are less than 10 minutes, and therefore, they are not clinically significant (Sevelius and Andersson 1995). In dogs, AP levels will increase after induction by glucocorticoids and anticonvulsants (Bush 1991, Cornelius 1997). In an adult dog's serum, AP originates from the liver, is corticosteroid- or epileptic drug-induced, or is the result of a pathologic bone condition. In growing dogs, AP is associated with increased osteoblastic activity (Leveille-Webster 2000).

## 5.2.3. Total bilirubin

Serum bilirubin concentration is used to detect liver failure in addition to liver enzymes. Bilirubin is an end-product of heme metabolism. Approximately 80% of the bilirubin produced by a normal dog is derived from the removal of aged erythrocytes from the circulation by macrophages. Degradation of heme from other sources, including myoglobin, cytochromes, peroxidase, and catalase, accounts for the remaining bilirubin production (Bush 1991, Leveille-Webster 2000). In the plasma, bilirubin is in an unconjugated form and is carried to hepatocytes bound to albumin. Unconjugated bilirubin is water-insoluble and therefore must be conjugated to glucuronic acid in hepatocytes before being excreted into bile canaliculi. Conjugated bilirubin is poorly absorbed from the small intestine. In the large intestine, it is changed to urobilinogen by enzymes and is mainly secreted in the feces. Only a small portion is reabsorbed to the portal blood reaching the liver and another small portion to the systemic circulation to be excreted by the kidneys (Bush 1991, Leveille-Webster 2000). The measurement of total bilirubin includes both unconjugated and conjugated forms. The balance between bilirubin production from the hemoglobin and excretion from the bile duct is meintained under normal conditions. Hyperbilirubinemia then jaundice occur when this balance is upset due to increased breakdown of erythrocytes, impaired uptake of bilirubin to hepatocytes, or impaired release of bilirubin from hepatocytes to the canaliculus (Richter 1996, Kaneko et al. 1997). An increase in total bilirubin results in a yellow color in plasma when it is greater than 1 mg/dL (or 17.1 umol/L) and in other tissues at concentrations exceeding 2 -3 mg/dL (or 34.2 - 51.3 umol/L) (Meyer et al. 1992).

#### 5.2.4. ALT, AP, and total bilirubin in Doberman hepatitis (DH)

ALT, AP, and total bilirubin serum concentrations were gathered from different research publications of DH. Only those reports were included where precise numerical values were available and the reference values of the laboratory were mentioned. Several articles fullfilled these criterions (Meyer et al. 1980, Doige and Lester 1981, Johnson et al. 1982, Thornburg et al 1983, Thornburg et al. 1984, Crawford et al. 1985, van den Ingh et al. 1988, Thornburg 1988, Cornelius 1989, Röcken et al. 1991, Fuentealba et al. 1997), providing a total of 91 blood samples from 61 Dobermans with DH.

ALT values had been measured in all blood samples. Seventeen ALT values from eight dogs with subclinical hepatitis were reported in five different articles (Johnson et al. 1982, Crawford et al. 1985, Thornburg 1988, Röcken et al. 1991, Fuentealba et al. 1997); the serum ALT values were all abnormal, being 1.7-24.6 times higher than the normal upper value. In the remaining 74 blood samples taken from Dobermans with clinical signs of liver failure, the ALT value was abnormal in 71 samples, ranging from 1.6- to 58-fold the normal upper value. In the clinical stage of disease, ALT values of three of 74 blood samples (0.04 %), were within the normal range.

AP values had been determined for 88 blood samples from 60 Dobermans with chronic hepatitis. Seventeen samples, as reported in five different articles, had been taken in the subclinical stage from eight Dobermans (Johnson et al. 1982, Crawford et al. 1985, Thornburg 1988, Röcken et al. 1991, Fuentealba et al. 1997); AP values ranged from

0.7- to 19.4-fold the normal upper value, being normal in one sample. In the clinical stage, the AP value measured from 71 blood samples was 0.7-75 times higher than the normal upper value.

Total bilirubin value had been analyzed for 62 blood samples from 49 Dobermans with chronic hepatitis. In four studies, seven samples were measured from five Dobermans in the subclinical stage of the disease. The total bilirubin values were all in the normal range (Johnson et al. 1982, Crawford et al. 1985, Thornburg 1988, Fuentealba et al. 1997). The remaining 55 blood samples were taken when dogs had clinical signs of liver failure. In this stage, total bilirubin was 0.3- to 51-fold the normal upper value, except for five samples in which it remained normal.

# 5.3. Histological findings in DH

## 5.3.1. Interpretation of the histological findings in liver samples

In veterinary medicine, the two accepted models for histological evaluation of the liver are the classic lobule model and the acinar unit model (Meyer 1994). Based on these models, the location of different histological lesions can be recorded.

In the classic lobule model, the lobule is the structural unit of the liver and it is rather easy to observe. A lobule is roughly hexagonal in shape and is composed of stacks of anastomosing plates of hepatic cells separated by the anastomosing system of sinusoids that perfuse the cells with the mixed portal and arterial blood. The central vein, into which the sinusoids drain, is located in the center of the lobule. The portal areas are at the angles of the hexagon (Maclachlan and Cullen 1995).

In the acinar unit model, the acinus is a unit that is of more relevance to hepatic function because it is oriented around the afferent vascular system. However, the acinus is difficult to visualize because the zone has no sharp boundaries. The acinus consists of a roughly ellipsoidal mass of hepatocytes aligned around the hepatic arterioles and portal venules as they anastomose into sinusoids. The axis of the acinus is defined by the terminal branches of the portal triad that lie along the border between two classic lobules. The hepatocytes in the acinus are divided into zones corresponding to the distance from the arterial blood supply (Maclachlan and Cullen 1995). Hepatocytes closest to the arterioles (zone 1) are oxygenated best, while those farthest from the arterioles (zone 3) have the poorest supply of oxygen.

In human medicine, liver specimens undergoing analysis for chronic hepatitis are also evaluated with the models described above. In addition, liver samples are analyzed for the severity of the necroinflammatory process (grade) and the extent of fibrosis (stage) (Hubscher 1998, Jevon 2001). While several systems are available, the Knodell histology activity index (HAI) was the first system developed and is today widely regarded as the golden standard (Knodell et al. 1981). In this system, four individual components (periportal necrosis +/- bridging necrosis, intralobular degeneration and focal necrosis, portal inflammation, and fibrosis) are analyzed. The degree of lesions in each component is scored, and the numerical value of the components is summed to produce an overall activity index (the HAI). Other grading and stating systems have subsequently been developed (Scheuer 1991, Ishak et al. 1995, Brunt 2000, Jevon 2001). These systems provide a systematic means of comparing pre- and post-therapy biopsies and can therefore be used to evaluate disease progression and response to therapeutic intervention. With these systems, the severity of inflammation and the degree of fibrosis in liver tissue can be objectively described (Desmet et al. 1994, Jevon 2001).

## 5.3.2. Histopathology of DH

Histological findings in chronic hepatitis have been presented in numerous reports on DH (Meyer et al. 1980, Doige and Lester 1981, Johnson et al. 1982, Thornburgh et al. 1984, Crawford et al. 1985, Fiorito 1985, van den Ingh et al 1988, Thornburg 1988, Cornelius 1989, Fuenteable et al. 1997, Thornburg 1998). These are mainly cases with advanced liver destruction that also show clinical signs of liver failure. In the majority of these, the cellular infiltration consisted of lymphocytes and pigment-filled macrophages. Neutrophils and plasma cells were also found but were few in number. In the parenchymal area, especially in severe cases, the parenchyma was clearly distorted. The inflammation varied from mild to severe. Inflammatory cell infiltration was virtually always recorded in the portal areas. In some cases, the limiting plates were intact, but usually piecemeal necrosis characterized by the erosion of the limiting plate was found. The portal areas were often expanded by a loose fibroplasia. Collagen and

reticular fibers extended into the lobulus. These fibers distorted the parenchyma, producing pseudolobulus of various sizes. The inflammatory cells were associated with connective tissue. Bile duct hyperplasia was commonly seen. Cholestasis was noted as bile plugs in canaliculus and depris filled-macrophages.

The findings in one research article differed from other published observations (Thornburgh 1998). In this study, the first histological findings were observed in zone 3, around central veins and adjacent hepatocytes. An inflammation composed of lymphocytes, plasma cells, and macrophages in the walls of hepatic veins and loss of surrounding hepatocytes was seen. All cases featured acidophilic cells in zone 3, with the number of these cells increasing as the disease became more severe. For advanced cases of liver failure, inflammation in zone 3 and in the portal area was obvious, and scar tissue radiated from central veins towards the adjacent acinus. Cholestasis was observed in two of the 36 liver samples. No granules were detected in macrophages by special staining.

Immunohistochemistry techniques have been used in two studies (Thornburgh 1998, Boisclair et al. 2001). In both of these studies, stainings were performed using paraffinembedded tissue in order to analyze the composition of lymphocytes. In the first study (Thornburgh 1998), T-cells were demonstrated using rabbit-origin polyclonal human CD3 antibody. B-cells and plasma cells were visualized by polyclonal rabbit-origin IgG antibody. The antibody for B-cells and plasma cells recognizes the Fc fragment of canine IgG. CD3+ T-lymphocytes were observed in portal tracts and walls of the hepatic veins, but the number of T-cells was few. Anti-IgG antibody reacted only with plasma cells. These cells were also few in number and were seen around hepatic veins and portal tracks.

In the second study (Boisclair et al 2001), lymphocyte compositions were evaluated using antibodies against CD3, which detected T-cells, and antibodies against alpha and kappa light chains, which detected B-cells. In the portal area, most of the lymphocytes were CD3+ lymphocytes. Also in the centolobular area CD3+ lymphocytes were the most abundant. Intralobular kappa and lamda chain-positive cells were not evaluated because of difficulties in differentiating between intrasinusoidal and intralobular cells. In most cases, more kappa chain-positive cells than lambda chain-positive cells were

present. A significant correlation was found between necrosis and the number of CD3+ lymphocytes and lambda and kappa chain-positive cells in the portal and lobular areas.

# 5.4. Classification of chronic hepatitis in man based on the etiology

The response of the liver to injury caused by a variety of etiologic agents results in similar microscopic patterns, making it difficult to distinguish specific disease entities. However, after the diagnosis of chronic hepatitis has been made, a causative agent should be sought to ensure effective treatment.

In human medicine, the etiology of chronic hepatitis is divided into infectious agents, metabolic disorders, toxins and drugs, and autoimmune diseases. These divisions are based on the correlations between clinical history, serological studies, and biopsy findings. Sometimes the etiology of chronic hepatitis cannot be determined because of the lack of serological markers for virus or autoimmune hepatitis and the absence of information about any drug. In these cases, the diagnosis made is idiopathic chronic hepatitis (Desmet et al. 1994, Jevon 2001).

#### 5.4.1. Infection

In humans, viruses, including hepatitis B and C (HBV, HCV) are the main etiological factor causing chronic hepatitis (Walsh and Alexander 2001). **HBV** is a DNA virus in which the major modes of transmission are prenatal, sexual, via blood products, and via contaminated needles in intravenous drug users (Lau et al. 1997, Walsh and Alexander 2001). Of adults, 90% infected with HBV recover completely because of an effective immune system. A child whose immune system is not fully developed cannot eliminate the virus but instead will be chronically infected (Lau et al. 1997, Huang and Lok 2003). The virus replicates within infected hepatocytes, causing the liver cell to make copies of HBV DNA. After HBV replication, hepatocytes secrete hepatitis B antigens and release intact virions which contain HBV DNA into the bloodstream. These viral copies can infect other liver cells, thus replicating effectively (Walsh and Alexander 2001). The cells of the immune system recognize HBV-infected cells and induce them to undergo apoptosis. In addition to the killing of infected hepatocytes, activated T-cells secrete interferon-gamma (IFN-g), which interferes with the life cycle of the pathogen.

In chronic hepatitis B, the immune system is not capable of terminating the infection but progressively destroys some of the infected hepatocytes, leading to cirrhosis (Lau et al. 1997). The diagnosis of HBV infection is based on detection of HBV antigen, antibodies against HBV, or HBV DNA in the patient's serum (Walsh and Alexander 2001).

At present, **HCV** infection is the leading cause of chronic liver disease worldwide. (Verbaan et al. 1998, Busch 2001). HCV is a small RNA virus that is transmitted primarily through contaminated blood and less effectively by human body secretions. Vertical transmission can also occur (Busch 2001, Walsh and Alexander 2001). The characteristic feature of HCV infection is its high rate of viral persistence and its ability to progress silently to chronic liver disease. In contrast to HBV, where the virus is eliminated in up to 80% of patients, HCV infection almost invariably progresses to chronic hepatitis (Busch 2001). The mechanisms of persistence, replication, and cellular injury by HCV are not, however, well characterized (Marrogi et al. 1995, Wejstål 1995). Persistence appears to arise from the ability of the virus to undergo a high rate of mutation, resulting in a series of immunologically distinct variants that allow the virus to escape recognition by the immune system (Pouti et al. 1997). Chronic hepatitis C is diagnosed when serum transaminases are persistently elevated in the presence of HCV antibodies.

#### 5.4.2. Metabolic disorders

Inherited metabolic disorders can also manifest as chronic hepatitis in humans. The most common of these conditions are Wilson's disease and alpha-1 antitrypsin ( $\alpha$ -1-A) deficiency.

**Wilson's disease** is an autosomally recessive inherited disease of copper metabolism. The mutation results in dysfunction in the gene (APT7B) encoding copper (Cu) transporter ATPase (Schilsky 2002, Subramanian et al. 2002). APTase transports Cu from one side of the hepatocytic membrane to the other. A defect in this protein leads to impaired biliary excretion of Cu and its retention by hepatocytes. When the hepatic storage capacity of Cu is exceeded, parenchymal inflammation followed by cell death ensues, with Cu release into the plasma, causing hemolysis. Copper also accumulates in the brain, cornea, and kidneys (Brewer 2000). The diagnosis of Wilson's disease is

made when decreased serum ceruloplasmin, increased urinary copper content, and elevated hepatic copper concentration are observed. Kayser-Fleischer rings, i.e. rusty brown rings around the cornea of the eye, are also diagnostic of Wilson's disease (Loudianos et al. 2000, Schilsky 2002).

 $\alpha$ -1-A is the predominant genetic cause of liver disease in children and the most common genetic disorder for which children undergo liver transplantation (Perlmutter 1996). The  $\alpha$ -1-A molecule is a serum glycoprotein that is predominantly expressed by hepatocytes but is also synthesized by a number of extrahepatic cell types and tissues. It is an acute phase protein and its concentration in plasma increases during host response to tissue injury or inflammation. Its major physiological function is inhibition of the destructive neutrophilic protease elastase (Hussain et al. 1991, Lomas 1996). Several phenotypes of  $\alpha$ -1-A protein exist. However, from a pathological perspective, only one type, PIZZ, is important.. In this phenotype, both gene alleles are mutated. This mutated  $\alpha$ -1-A molecule is responsible for an alteration in the folding of the  $\alpha$ -1-A protein, resulting in its retention in the endoplasmic reticulum (ER) of hepatocytes (Qu et al. 1997). The hepatocytic accumulation causes the serum  $\alpha$ -1-A concentration to decrease below physiological level. The consequence of this in most affected individuals is progressive lung disease since an insufficient level of  $\alpha$ -1-A cannot prevent the neutrophilic enzyme from destroying the collagen of the lung (Crystal 1990). In contrast to the pathogenesis of lung in  $\alpha$ -1-A deficiency, no evidence exists that liver injury is due to a deficiency in elastase inhibitory capacity. Most evidence favors the concept that the accumulation of  $\alpha$ -1-A in the ER is directly related to liver cell injury (Lomas et al. 1992, Perlmutter 1996, Qu et al. 1997). Affected individuals may be diagnosed based on detection of periodic acid-Schiff (PAS)-positive, diastase-resistant globules in the hepatocytic ER. However, in cases where these globules are absent, identification of the  $\alpha$ -1-A phenotype is important in diagnosis of the disease (Crystal 1990).

## 5.4.3. Drugs and toxins

The liver is the major site of drug metabolism and is therefore a common target of adverse drug reactions (Beaune and Lecoeur 1997). Most drug-induced chronic hepatitis is idiosyncratic in nature (Pirmohamed et al. 1996, Uetrecht 1997, Castel 1998). An idiosyncratic reaction often has serious consequences, and it is impossible to predict

who will develop such a reaction. Idiosyncratic reactions are also dose-independent such that most individuals will have no adverse response to the drug at any dose while a few may have an exaggerated response to a very low dosage. Under normal conditions, most drugs undergo some degree of bioactivation without causing any harm. This is due to the metabolites being effectively detoxified. However, harmful chemically reactive metabolites are formed when inadequate detoxification occurs. This may be the result of a genetically determined deficiency of enzymes involved in detoxification or a disturbance caused by environmental factors such as infections, diet, or concomitant drug intake (Pirmohamed et al. 1996). The mechanisms involved in the pathogenesis of idiosyncratic drug reactions remain obscure. Harmful metabolites are speculated to cause a toxic insult on the cell either directly or indirectly by acting as a hapten and initiating an immune-mediated reaction (Castel 1998). No specific markers or tests for drug-induced liver injury are available. Therefore, the diagnosis is usually based on pharmacological history, the relationship between drug intake and the onset of clinical signs, and the exclusion of other etiological factors. In humans, a clinical diagnostic scale has been developed for evaluating drug-induced liver injuries (Vasco and Rui 1997, Aithal et al. 2000).

#### 5.4.4. Autoimmunity

Autoimmune hepatitis is a disorder of unknown etiology in which progressive destruction of the hepatic parenchyma occurs. Females seem to be more susceptible, and often there is a family history of other disorders thought to have an autoimmune etiology (Al-Khalidi and Czaja 2001). A typical feature of autoimmune chronic hepatitis is a good response to corticosteroid therapy (Czaja 2002). Althouhg the pathophysiological mechanisms are not yet fully understood, evidence suggests that autoimmune hepatitis develops after genetically susceptible individuals have been exposed to an initiating factor, such as a hepatotropic viral infection or an idiosyncratic reaction to a drug or other hepatotoxin (Czaja 2002). The immune reaction may be related to defects in the immunological control of autoreactivity, with consequent loss of self-tolerance to liver auto-antigens (van den Berg 1998). As there are no morphological features that are pathognomonic of the condition to distinguish autoimmune chronic hepatitis from other forms of hepatitis, the diagnosis is based on laboratory results, after ruling out other causes of chronic hepatitis. Serologically, the disease is characterized by hypergammaglobulinemia with elevated levels of IgG and

high titers of a wide range of circulating auto-antibodies. The most common autoantibodies are ANA, SMA, and LKM-1 (Luxon 2003). In addition, no serological signs of viral infections are present. Other findings include elevated aminotransferase levels, normal serum  $\alpha$ -1-A and ceruloplasmin concentrations, normal liver Cu content, and no recent use of known hepatotoxic drugs (van den Berg 1998, Al-Khalidi and Czaja 2001).

# 5.5. Possible etiological factors of DH

The etiology of DH is unknown partly due to the lack of specific serological tests for humans. Disease categorization is therefore not based on etiological factors, and DH is classified together with copper-associated hepatitis, breed-associated hepatitis, or hepatitis with familial predisposition (Johnson 1982, Hardy 1986, Dill-Mackey 1995, Sterczer et al. 2001).

## 5.5.1. Infection

Infection as a cause of DH has been proposed, although the specific agent remains to be determined (Rothuizen 1997). To date, **canine adenovirus 1 (CAV-1)** is the best-known viral cause of chronic hepatitis in dogs (Chouinard et al. 1998). This highly contagious virus is transmitted by contact with infected animals and contaminated fomites (Sherding 2000). Since an effective vaccination is available, the incidence of adenovirus-caused hepatitis is under control. Definitive diagnosis is based on increased antibody titers to CAV-1 and viral isolation from affected tissues (Sherding 2000).

In addition to CAV-1-induced chronic hepatitis, **asidophilic cell hepatitis** is a distinct form of chronic hepatitis caused by a transmissible agent which is most probably a virus (Jarrett et al. 1987). This hepatitis could be transmitted to a healthy dog via the infected serum and liver homogenate of a euthanized dog. Asidophilic cell hepatitis is histologically characterized by acidophilic cells and only a small number of mononuclear cells.

Leptospira is a potential agent of chronic hepatitis in Dobermans. This bacteria is spiral-haped and has several serotypes. Leptospira interrogans serovars

icterohemorhagiae and canicola previously were the most common leptospiras causing liver failure (Nielsen et al. 1991, Harkin and Gastrell 1996). However, other serovars, such as grippotyphosa, pomona, bratisvala, and australis, have replaced icterohemorhagiae and canicola partly because a vaccination against L. icterohemorhagigae and L. canicola is available decreasing their role as causative agents of liver failure (Bishop et al. 1979, Nielson et al. 1991, Brown 1996, Harkin and Gastrell 1996, Adamus et al. 1979). Animals become infected through contact with contaminated urine, water, or soil. The bacteria enter the body through the skin or mucous membranes. Our current understanding of the pathogenesis of leptospirosis is incomplete. During tissue invasion, there seems to be direct leptospiral cytotoxic effect on endothelial and hepatocytic membranes (Alves et al. 1992). The diagnosis depends on detecting the leptospira in clinical specimens and/or demonstrating an increase in antibody titers to one or more leptospiral serovars (Harkin and Gastrell 1996).

### 5.5.2. Metabolic disorders

Liver Cu content in a healthy dog does not exceed above 400  $\mu$ g/mg dw (dry weight) (Rolfe and Twedt 1995, Twedt 1997). At this concentration, Cu is not histologically detected (Thornburg et al. 1985). Hepatitis is not however, apparent until Cu concentration exceeds 2000  $\mu$ g/mg (Thornburg et al. 1990, Rolfe 2000).

In Dobermans with chronic hepatitis, increased hepatic **Cu** levels have been widely observed (Doige and Lester 1981, Johnson et al. 1982, Thornburg et al. 1984, Crawford et al. 1985, Hardy 1986, van den Ingh et al. 1988, Cornelius 1989a). However, the role of Cu in the disease is contradictory. The most common theory is that increased Cu concentration is secondary to cholestasis (Johnson et al. 1982, van den Ingh et al. 1988, Cornelius 1989a). This suggestion is based on histological evidence of biliary stasis, characterized by the accumulation of biliary pigments in hepatocytes and plugs in ducts. Dogs almost invariable have concomitant hyperbilirubinemia. Histologically, Cu is located in the periportal area or in the lobular periphery, providing evidence of the cholestatic nature of Cu accumulation (Meyer et al. 1980, Doige and Lester 1981, Johnson et al. 1982, Crawford et al. 1985, van den Ingh et al. 1988, Cornelius 1989a).

Based on a study of two Dobermans with subacute hepatitis, Dobermans, similar to Bedlington Terriers, were proposed to be affected with a hereditary defect in Cu metabolism (Thornburgh et al. 1984). While livers in this study histologically normal, abundant Cu-positive granules were present in the centrolobular hepatocytes.

Other studies suggest that Cu is neither the cause nor the effect of chronic hepatitis in Dobermans but is incidental, also suggesting that hepatic Cu concentration can normally be above 400  $\mu$ g/mg dw without predisposing to chronic liver disease (Thornburgh et al. 1990, Thornburgh 1998). No evidence has been focused that Dobermans, or other breeds, accumulate Cu secondary to chronic cholestasis. This is unlike in human patients with chronic biliary disease, such as primary biliary cirrhosis, in which it usually takes years for Cu to accumulate in hepatocytes. In this study (Thornburgh 1998), comprising 35 Dobermans with chronic hepatitis, 30 dogs had elevated hepatic copper concentrations and five dogs had normal levels. In all 30 samples in which increased Cu was present in rhodanine staining, the Cu-laden hepatocytes were restricted to zone 3 of the acinus, adjacent to the hepatic vein branch, or adjacent to zone 3 scar tissue.

In the Netherlands, 3-year-old Dobermans were evaluated for subclinical hepatitis (Mandigers et al. 2000). Liver samples were analyzed for inflammation as well as increased Cu content. Results indicated that most Dobermans with subclinical hepatitis also had elevated liver Cu content.

 $\alpha$ -1-A was evaluated in 13 dogs with chronic hepatitis, including three Dobermans (Sevelius et al. 1994). The  $\alpha$ -1-A phenotype was divided into the following three categories: fast (F), intermediate (I), and slow (S). These appeared in either a homozygous (FF, II, SS) or a heterozygous (FI, FS, IS) form. In dogs considered to have true  $\alpha$ -1-A accumulation, the phenotype was either II or FI. Immunohistochemical examination revealed three staining patterns, and globular staining indicated the true accumulation in hepatocytes. Granular and diffuse staining are not regarded as an aggregation of  $\alpha$ -1-A but as a secondary phenomena to inflammation. In all three Dobermans,  $\alpha$ -1-A presented as diffuse staining in the periportal and centrolobular areas. The phenotype was homozygous fast (FF). The serum concentration of  $\alpha$ -1-A in these three dogs was elevated compared twith control dogs.

#### 5.5.3. Drugs and toxins

Drugs can also cause chronic hepatitis in Dobermans. Anticonvulsants, primidone, phenytoin, and phenobarbital have been shown to have hepatotoxic effects, especially in long-term treatment of dogs (Bunch et al. 1982). Diethylcarbamazine with oxibendadazole, drugs used for heartworm prophylaxis, are also known to cause hepatitis in Dobermans (Vaden et al. 1988).

#### 5.5.4. Autoimmunity

Cell destruction in DH may be caused by a malfunctioning autoimmune system. Evidence for this hypothesis is, however, lacking (Rothuizen.et al. 1998). The autoimmune background in DH has been evaluated mainly by measuring autoantibodies in the serum of affected dogs (Crawford et al. 1985, Cornelius 1989a, Andersson et al. 1992, Sevelius et al. 1993, Weiss et al. 1995). Although the pathogenic role of autoantibodies in autoimmune disorders is unclear, they serve as markers for autoimmune diseases and are widely used as a diagnostic tool. In humans, antinuclear antibodies (ANA) are detected in cases of autoimmune hepatitis type I (Luxon 2003). Altogether, 15 different Dobermans with chronic hepatitis were analyzed antibodies for ANA (Crawford et al. 1985, Cornelius 1989a, Sevelius et al. 1993). In 4 of 15 cases (27%), elevated antibody titers against nuclear antigens were present. In humans, antibodies against smooth muscle antigens (SMA) are also found in the majority of individuals with autoimmune hepatitis. Antibodies against mitochondrial antigens are found in 98% of cases of primary biliary cirrhosis (Kaplan 1993, Galperin and Gershwin 1996). Sevelius et al. (1993) evaluated the serum samples of Dobermans with chronic hepatitis for antibodies against SMA and mitochondrial antigens. Neither of these autoantibodies was found. Antibodies against liver-specific antigens were evaluated in two studies. Sevelius et al. (1993) measured liver membrane autoantibodies (LMA) in the serum of three Dobermans with chronic hepatitis but found no antibodies. In a second study, antibodies against liver membrane protein (LMP) were evaluated in the serum of four Dobermans with chronic hepatitis. In two of these four Dobermans, the anti-LMP titer was four times higher than in controls. The other two, with anti-LMP titers equal to those in controls were receiving corticosteroid treatment (Weiss et al. 1995).

# 5.6. Major histocompatibility complex (MHC) class II expression

MHC molecules are special inherited proteins expressed on the surface of an animal's cells. As tissue histocompatibility proteins, they present antigenic peptides to T-lymphocytes. They are composed of two polypeptides, an alpha and a beta subunit, which form a groove where the antigen is located (Rask et al. 1991, Day 1999). MHC class I antigens are more ubiquitously distributed in all cells than MHC class II antigens, which are mainly located in so-called professional antigen-presenting cells (APCs) (Mellins 1992, Tizard 1996). MHC class II molecules have two major functions. In the thymus, they are expressed on thymocytes, where they present different autoantigens to developing T-cells. In positive selection, only T-cells that can react with MHC class II molecules survive. Afterwards, in negative selection, those T-cells reacting with auto-antigens presented by MHC class II molecules are destroyed. Through positive and negative selection, over 90% of bone-derived T-cells are prevented from entering the bloodstream (Janeway et al. 1999a).

The second function of MHC class II is to present processed antigens to CD4+ T-cells (Janeway et al. 1999b). Outside the thymus, MHC class II molecules are expressed exclusively on very few cell surfaces. These cells consist of macrophages, dendritic cells, and B cells, and are collectively refered to as APCs (Mellins 1992, Tizard 1996). MHC proteins are synthesized and integrated with an antigen in the cytoplasm of an APC. The stable MHC antigen complex is then transported to the cell membrane and presented to T-cell receptors (TCRs). Helper T-cells have a molecule called CD4, which binds to MHC II. Moreover, an antigen located in the groove of MHC class II on the cell surface of APCs is bound by highly specific TCRs, also expressed on the surface of helper T-cells (Janeway et al. 1999b). These interactions, with the help of costimulatory factors, are necessary for the activation of helper T-cells. After activation, helper T-cells initiate the release of cytokines that regulate other processes associated with immune responses (Day 1999).

### 5.6.1. MHC class II in diseases

Under normal conditions, the body has a tolerance to its own antigens. This tolerance is mainly due to the deletion of autoreactive T-cells in the thymus before their entry into

the bloodstream. This ensures that T-cells, which react with the body's own antigens, are destroyed (Janeway et al. 1999a). However, this mechanism is not perfect, and as a result, all living being have autoreactive cells in their bodies. Autoimmunity is a phenomenon where the body's own immune system has failed to arrest these autoreactive cells. Alternatively, autoantigens which the immune system has not encountered before may become exposed. Autoimmune response develops because there is no tolerance to these antigens (Janeway et al. 1999c).

A genetic association exists between certain autoimmune diseases and the expression of specific MHC class II molecules (Nepon 1991, Rask et al. 1991, McDevitt 1998). This association is hypotherized to arise from an altered thymic selection. Autoreactive T-cells may escape selection in the thymus as a consequence of the poor peptide-binding properties of disease-associated MHC class II molecules (Ridgway and Fathman 1998). Among other possibilities, disease-predisposing MHC class II molecules may preferentially bind and present to pathogenic peptides.

In addition to the genetic association between certain MHC class II molecules and autoimmune diseases, abnormal MHC class II expression in non APCs is also a common finding in several autoimmune diseases (Selby et al. 1983, Ballardini et al. 1984, Lindahl et al. 1985, Altomonte et al. 1999). It is hypothesized that aberrant MHC class II on non immune cells allows cells to become antigen-presenting cells, to present antigens to T-cells leading to T-cell activation, and the development of autoimmune disease or local immune damage to tissues.

# 6. AIMS OF THE STUDY

A framework was sought to facilitate diagnosis of subclinical Doberman hepatitis (DH) and evaluation of the etiopathogenesis of the disease. The thesis was divided into four parts with specific goals as follows:

**Study I :** - to determine which enzyme, ALT or AP, is the most practical and reliable serum parameter for screening Dobermans with subclinical hepatitis.

- to evaluate which serum parameters are the best indicators showing that the disease is progressing from the subclinical to clinical stage.

Study II: - to describe histopathological changes in the liver in subclinical DH.

- to compare histopathological changes between the subclinical and clinical stages of DH

- **Study III:** to clarify the role of copper in DH based on biochemical, histological, and histochemical findings. Emphasis was on subclinical DH, comparing the distribution of copper in the subclinical stage with that in the clinical stage.
- **Study IV:** to evaluate MHC class II expression in hepatocytes in DH from etiological and pathophysiological perspectives

- to evaluate the correlation of mononuclear inflammation with MHC class II expression in hepatocytes.

# 7. MATERIALS AND METHODS

Characteristics of the dogs in Studies I-IV are shown in Table 1.

# 7.1. Study material

Study I: Subclinical hepatitis was evaluated in a population of 626 (425 female, 201 male) randomly selected, clinically healthy Finnish Dobermans between 1987 and 1994. The age of these dogs ranged from 1.5 to 9.5 years. Blood samples were collected mostly at dog shows and breeding association meetings. In 55 dogs, ALT concentration was greater than three times the normal upper value. ALT value was rechecked and if it remained elevated, the owner was asked for permission to take a liver biopsy from their dog. Thus, a liver biopsy was performed for 23 dogs. Those Dobermans considered to have subclinical hepatitis based on histological findings of the liver were further studied through an ongoing evaluation of their clinical status and measurement of serum parameters, including ALT, AP, and total bilirubin. The serum biochemical data of dogs with subclinical hepatitis were then compared with serum parameters of 22 Dobermans with clinical signs of chronic hepatitis. These 22 Dobermans were composed of six dogs diagnosed in the subclinical stage but who had clinical signs of liver failure during follow-up and 16 dogs diagnosed while having clinical signs of DH. All the clinically affected dogs were euthanized, and the diagnosis of advanced DH was based on histological evaluation of postmortem liver samples.

**Study II:** Histological changes observed during liver biopsy of 20 Dobermans with subclinical hepatitis were recorded. During follow-up eight of these dogs were euthanized for reasons other than DH; as follows: aggression (3), postoperative bleeding (1), thrombocytopenia (1), lung tumor (1), renal failure (1), and gastric ulcer (1). For five of eight, an autopsy was performed. Since these dogs showed no clinical signs of liver failure, they were considered to be in the subclinical phase. Six dogs within the remaining population of 12 Dobermans with subclinical hepatitis showed typical signs of liver failure and were euthanized. An autopsy liver specimen was obtained from four of these six dogs. Histological findings of Dobermans with subclinical hepatitis were compared with histological observation of autopsy samples of clinically affected Dobermans.

**Study III:** Only these dogs with DH were included for whom Cu could be analyzed quantitatively and qualitatively. The role of Cu in DH was evaluated based on results obtained from biopsies of 17 Dobermans with subclinical hepatitis and from autopsies of 23 Dobermans euthanized because of the disease. The control group consisted of postmortem liver specimens obtained from 12 Dobermans euthanized for reasons other than DH. Reasons for euthanasia were aggression (1), dilatation and torsion of the stomach (1), adenocarcinoma of the duodenum (2), unknown reason (3), changed family situation (4), and car accident (1). Histologically, none of these dogs had signs of hepatitis in the liver.

**Study IV:** MHC class II expression in hepatocytes was studied in 18 biopsies of dogs in the subclinical stage of DH, in 6 autopsies of dogs suffering from DH and euthanized for reasons other than liver failure, in 14 autopsies of dogs suffering from DH and euthanized because of the end-stage of the disease, and in 6 control Dobermans. From 10 of the dogs with DH, both biopsy and autopsy samples were available for analysis of MHC class II expression changes during disease progression. The liver samples of dogs in this study were embedded in paraffin within one week in order to avoid changes in antigens in the formalin.

Study	Number of animals	Sex Age, yrs (mean)	Signs of liver failure	ALT value	Liver biopsy / autopsy	Copper in rubeinic acid stain	Immuno- histology
I	626 Dobermans <sup>1</sup>	F 425, M 201 1.5 - 9.5	No				
	21 Dobermans with sDH	F 17, M 4 2 - 7 (3.8)	No	Incr <sup>2</sup>	b	pos	
	22 Dobermans with cDH	F 13, M 9 2.5 - 10 (6)	Yes	Incr	а	pos	
II	18 Dobermans with sDH	F 15, M 3 2 - 7 (4.1)	No	Incr <sup>2</sup>	b	pos	
	5 Dobermans with sDH $^3$	F 4, M 1 4 - 7 (5.2)	No	Incr	а	pos	
	4 Dobermans with cDH <sup>4</sup>	F 4 2 - 9 (6.6)	Yes	Incr	а	pos	
	2 Dobermans with suspected sDH	F 1, M 1 4 - 6 (5)	No	Incr <sup>2</sup>	b <sup>1</sup>	neg	
111	17 Dobermans with sDH	F 13, M 4 2 - 7.9 (4.4)	No	Incr <sup>1</sup>	b	pos <sup>5</sup>	
	23 Dobermans with cDH	F 15, M 8 2.9 - 10 (5.8)	Yes	Incr	а	pos <sup>5</sup>	
	12 control Dobermans	F 8, M 4 2 - 13 (5.6)	No	ND	а	pos <sup>5</sup>	
IV	18 Dobermans with sDH	F 15, M 3 2.2 - 7.9	No	Incr <sup>1</sup>	b	pos	Yes <sup>6</sup>
	6 Dobermans with sDH $^3$	F 5, M 1 4 - 7 (5.6)	No	Incr	а	pos	Yes <sup>6</sup>
	14 Dobermans with cDH	F 10, M 4 3.5 - 10 (6.4)	Yes	Incr	а	pos	Yes <sup>6</sup>
	6 control Dobermans	F 3, M 3 3.5 - 12 (6.4)	No	ND	b <sup>1</sup>	neg	Yes <sup>6</sup>

## Table 1. Characteristics of animals in the different study groups.

ALT = alanine aminotransferase sDH = subclinical Doberman hepatitis; cDH = clinical Doberman hepatitis;

F = female; M = male; ND = not determined

a = autopsy; b = liver biopsy revealed mononuclear inflammation in parenchymal and portal areas;

b1 = liver biopsy; revealed no mononuclear inflammation in parenchymal and portal areas

<sup>1</sup> randomly selected clinically healthy Dobermans; <sup>2</sup> ALT values exceeding the normal maximum by at least three times in two concequtive blood samples; <sup>3</sup> diagnosed in the subclinical stage of DH but euthanized for reasons other than DH; <sup>4</sup> diagnosed in the subclinical stage of DH and euthanized because of DH;

<sup>5</sup> quantitative copper analysis of the liver was available; <sup>6</sup> Major histocompatibility complex (MHC) class II detection using anti MHC class II antibody

# 7.2. Diagnostic methods

#### 7.2.1. Biochemistry

Alanine aminotranspherase (ALT), alkaline phosphatase (AP), and total bilirubin Serum ALT and AP levels were analyzed according to the recommendations of the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology (1974). ALT and AP values were determined from patients both in the subclinical and in the clinical stages of DH. Serum bilirubin was determined by using the azo-bilirubin method (Michaelson 1961). Blood parameters (ALT, AP, total bilirubin) were compared between stages of DH to evaluate which serum parameters were best for indicating progression of the disease from the subclinical to the clinical stage. All samples were analyzed in the same laboratory.

## 7.2.2. Histology

Liver biopsies were obtained via laparatomy by ligation from the left medial lobe of the liver. The autopsy samples were taken immediately after euthanasia to minimize any postmortem changes.

Liver specimens were placed in 10% buffered formalin solution for fixation. After fixation, the samples were embedded in paraffin. All sections were stained with hematoxylin and eosin (HE) and rubeinic acid. Some of the samples were also stained with van Gieson (VG) for analysis of connective tissue, with Unna Pappenheim for evaluation of plasma cells (Study II), with Rhodanine for Cu examinations (Study III), and immunohistochemically with antibodies against MHC class II antigens (Study IV).

#### 7.2.3. Immunohistochemistry

All samples were fixed in 10% unbuffered formalin and embedded in paraffin. Liver samples were sectioned and mounted on poly-l-lysine-coated slides for immunohistological staining (DAKO EnVision<sup>TM</sup>+System, Peroxidase, HRP, Carpinteria, USA). The sections were first passed through graded xylene and alcohol solutions. The unmasking of antigens was performed by heating the sections in a microwave oven in 10 mM citrate buffer (pH 7.2) twice for seven minutes. Endogenous peroxidase was blocked by incubating the sections with 0.03% hydrogen peroxide for
five minutes. The slides were subsequently incubated with the primary anti-MHC class II antibody for 30 minutes (anti-human HLA-DR alpha chain MoAb at 1:25 dilution, clone TAL 1B5, DAKO). To demonstrate the presence of MHC class II antigens, a horseradish peroxidase (HRP)-labeled conjugated secondary antibody was used for 30 minutes. The reactions were developed by the addition of 3,3'-diaminobenzidine chromogen solution (DAB), and the sections were counter-stained with Mayer's hematoxylin. The primary antibodies were diluted in bovine serum albumin (BSA) diluted in Tris Buffered Saline (TBS). The sections were washed three times in TBS between each incubation stage, and all incubations were performed at room temperature. Canine tonsils served as positive controls and negative controls consisted of specimens in which the primary antibody was replaced with BSA/TBS.

#### 7.2.4. Quantitative analysis of liver copper content

For quantitative hepatic Cu assay, liver samples were stored at  $-20^{\circ}$ C, then dried at  $105^{\circ}$ C overnight, weighed, and digested in 0.5 ml of concentrated HNO3. The digests were diluted to 3 ml with water, and Cu was determined by flame atomic absorption spectrometry. A Perkin Elmer 2100 spectrometer was used according to the manufacturer's instructions.

# 7.3. Interpretation of histological findings in liver samples

#### 7.3.1. Classic lobule model

The liver sections were analyzed using the classic lobule model, where lobular area was defined as an area with a central vein in the midle and portal areas at the angles. The lobular area was studied as a different area in order to better localize findings in the liver section. The lobular area was divided into parenchymal, portal, and periportal regions where parenchyma refers to the lobular area excluding the portal and periportal areas. In Study II, the term lobular area was replaced with lobule. In that study, lobule had been defined as an area with a portal region, a central vein, and parenchyma was divided into periportal and centrolobular regions. Bridging necrosis refered to the necrotic areas between the two portal regions. In Study II, when evaluating the histological lesions in the subclinical and clinical stages of the disease, the bridging necrosis areas

were added to the piecemeal necrosis areas. In Studies III and IV, bridging necrosis areas were recorded separately for analysis.

### 7.3.2. Scoring and grading of histological lesions

Histological findings were numerically recorded to enable statistical analysis of lesions in specific locations in different liver samples and comparison of severity of lesions in samples of the same dog obtained at different time intervals. In Study II, the grading of portal inflammation, periportal+- bridging necrosis, and fibrosis were modified from the system used by Knodell et al (1981). In the Knodell system, periportal +/- bridging necrosis was weighted more heavily than other parameters because it appears to be more relevant in determining the severity of chronic hepatitis. The grading system used here for inflammation in the parenchyma, portal expansion, and bile duct proliferation was created by the author (Table 2). In Study III, the grading criteria for inflammation were based on Table 2, where the intensity of mononuclear inflammation in the centrolobular area was analyzed as inflammation in the parenchymal area. In Study IV, the grading criteria for inflammation was also based on Table 2, but in this study the scores were 0, 1, 2, and 3, as opposed to 0, 1, 3, and 4 as in Studies II and III. In Study III, the intensity of the inflammatory reaction in the bridging necrosis area was graded as follows: 0=none, 1=mild (sprinkling of inflammatory cells in <35% of the bridging necrosis area), 3=moderate (increased inflammatory cells in 35-65% of the bridging necrosis area), 4=marked (dense packing of inflammatory cells in >65% of the bridging necrosis area). In Study IV, grading of the intensity of inflammatory reaction in the bridging necrosis area was the same as in Study III, except the scores were 0, 1, 2, and 3.

Score	Parenchymal inflammation**	Portal inflammation	Periportal +/- bridging necrosis	Portal expansion	Bile-duct proliferation	Fibrosis
0	None	None	None <sup>a</sup>	None	None <sup>d</sup>	None
1	Mild (sprinkling of small groups and/or single inflammatory cells in parenchymal area)	Mild (sprinkling of inflam- matory cells in <35% of portal tracts)	Mild piecemeal necrosis (limiting plate ruptured in some portal areas)	Mild (<50% of portal tracts expanded. Bor- ders irregular and ex- hibiting fibrous spurs)	2-4 bile ducts / portal area, on average	Fibrous portal expansion
3	Moderate (inflammatory cells in sinusoids are intermediate)	Moderate (increased in- flammatory cells in 35-65% of portal tracts)	Moderate piecemeal ne- crosis (<50% of the circumference of most portal tracts involved)	Moderate (>50% of por- tal tracts expanded. Borders irregular and exhibiting fibrous spurs)	5-6 bile ducts/portal area, on average	Bridging fibrosis (portal- portal or portal-central linkage)
4	Marked (severe inflam- mation in parenchyma)	Marked (dense packing of inflammatory cells in >65% of portal tracts)	Marked piecemeal necro- sis (>50% of the circum- ference of most portal tracts involved)	Marked (portal tracts ex- panded severely and connective tissue trib- utaries from portal ar- eas throughout the liver)	>6 bile ducts/portal area, on average	Cirrhosis <sup>e</sup>
			Moderate piecemeal ne- crosis plus bridging necrosis <sup>b</sup>			
			Marked piecemeal necro- sis plus bridging necro- sis <sup>b</sup>			
			Multilobular necrosis <sup>c</sup>			
** Pare <sup>a</sup> Limiti <sup>b</sup> Bridg <sup>c</sup> Two c	enchyma is referred to as lob ng plate intact in every portal ing defined as > two bridges i or more contiguous lobules wi	ular area excluding portal and pe area. n one liver specimen; no distincti th panlobular necrosis	riportal areas. on made between portal-portal a	nd portal-central linkage.		

## Table 2. Grading of histopathological findings for hepatitis in Dobermans.

<sup>d</sup> < 2 bile ducts / portal area, on average.</li>
 <sup>e</sup> Loss of normal hepatic lobular architecture with fibrous septa separating and surrounding nodules.

#### 7.3.3. Cholestasis

In Study III, cholestasis was histologically evaluated using HE stain. Cholestasis stands out microscopically as greenish-brown bile thrombi between the hepatocytes in the canaliculus and was detected with oil immersion (1000 x) magnification. The number of bile plugs was graded as 0 (no plugs/liver section), 1+ (1-10 plugs/liver section), and 2+ (>10 plugs/liver section).

#### 7.3.4. Copper

Copper-positive granules were evaluated in both rubeinic acid and rhodanine stains to compare how effective these two stains are in detecting Cu granules. In addition, the numbers of Cu-positive granules with the rhodanine stain were classified to enable evaluation of the relationship between location of mononuclear inflammatory reaction and associated Cu accumulation. Rhodanine stain was used because the counterstain allows for the evaluation of the location of Cu-positive granules in liver samples. The number of Cu-positive granules with the rhodanine stain was classified as follows: 0=none, 1=solitary liver cells and/or macrophages containing some Cu-positive granules, 2=small groups of liver cells and/or macrophages containing small to moderate numbers of Cu-positive granules, 3=larger groups or areas of liver cells and/or macrophages containing moderate numbers of Cu-positive granules, 5=diffuse presence of liver cells and/or macrophages with many Cu-positive granules. This classification was based on the system described by van den Ingh et al. (1988).

#### 7.3.5. Criteria for MHC class II expression

MHC class II antigen expression was evaluated in the liver sections of Dobermans suffering from chronic hepatitis and of control Dobermans, with special attention being given to the hepatocytes' ability to produce the molecule. The expression of MHC class II in liver samples was studied using an anti-human MHC class II antibody. This antibody is commercially available, works in paraffin-embedded sections, and has in previous studies been shown to cross-react with canine MHC class II antigen (Day 1996, Tafti et al. 1996, German et al. 1998). To determine the relationship between inflammatory reaction and MHC class II expression in hepatocytes, expression was evaluated in the lobular areas. The number of hepatocytes expressing MHC class II in immunohistological staining was classified as shown in Table 3.

Score	Parenchymal area	Periportal area	Bridging necrosis area
0	None	None	None
1	Solitary hepatocyte expresses MHC class II	Solitary hepatocyte around portal area expresses MHC class II	Solitary hepatocyte adjacent to bridging necrosis areas expresses MHC class II
2	Small groups of hepatocytes express MHC class II	Small groups of hepatocytes around portal area express MHC class II	Thin band of hepatocytes adjacent to bridging necrosis areas expresses MHC class II
3	Moderate-sized groups of hepatocytes express MHC class II	Less than 50% of circum- ference of most portal tracts is involved	Obvious band of hepatocytes adjacent to bridging necrosis areas expresses MHC class II
4	Large areas of hepatocytes express MHC class II	More than 50% of circum- ference of most portal tracts is involved	Thick band of hepatocytes adjacent to bridging necrosis areas expresses MHC class II
5	Diffusely spread hepatocytes express MHC class II		

**Table 3.** Grading criteria for major histocompatibility complex (MHC) class II expression in hepatocytes in liver specimens of Dobermans with hepatitis.

## 7.4. Treatment

**In Study II**, after the subclinical DH diagnosis had been confirmed, treatment was begun for seven of these dogs for which both biopsy and postmortem samples were available. The treatment consisted of prednisolon 0.1-0.5 mg/kg/day with or without d-penicillamin 5 mg/kg/day. Prednisolon was given to five dogs for the entire follow-up period. D-penicillamin was stopped, for every dog, except one, months before euthanasia.

**In Study IV**, seven Dobermans euthanized for end-stage DH had received corticosteroids (0.1-0.5 mg/kg/day) for 4-5 months before liver autopsy specimens were obtained. MHC class II expression in hepatocytes of these dogs were compared with expression in seven Dobermans euthanized for of end-stage DH that had received no treatment.

## 7.5. Statistical analyses

**Study I:** The pair samples t-test and the Wilcoxon signed rank test were used to compare changes in blood parameters between the different stages during the subclinical period. The independent samples t-test and the Mann-Whitney U-test were used to evaluate differences between dogs with subclinical versus clinical DH.

**Study II:** Histopathologic changes in the biopsies and in the postmortem liver samples from the same dogs were compared. Postmortem samples were further divided into two groups according to cause of death; five dogs died for reasons other than liver failure, and four dogs were euthanized because of advanced liver failure. The Wilcoxon signed rank test was used to study changes in histological parameters from biopsy sample to postmortem sample. The Mann-Whitney U-test served to evaluate differences in histological parameters between the two groups of postmortem samples.

**Study III:** The difference in hepatic quantitative Cu concentrations between the DHpositive groups was compared by a two-sample t-test. The control group was compared with the DH groups by a two-sample t-test with unequal group variances. Differences in intensity of mononuclear inflammation and number of Cu-positive granules between centrolobular, periportal, and bridging necrosis areas were tested by Friedman two-way analyses of variance followed by a multiple comparison test. P-values less than 0.05 were considered significant.

**Study IV:** The Wilcoxon signed rank test was used when testing differences of MHC class II expression in hepatocytes between biopsy and autopsy samples, and the Mann-Whitney U-test when testing the effect of treatment on MHC class II expression in hepatocytes. Spearman rank correlation coefficients were used to test the association between MHC class II expression and inflammation within areas. Results are expressed as means (+/- SD). P-values less than 0.05 were considered significant.

# 8. **RESULTS**

## 8.1. Criteria for subclinical DH

Diagnosis of subclinical DH was based on four criteria. Dogs had to be clinically asymptomatic, with no signs of liver failure or any other disease at the time blood samples and the liver biopsy were taken. Dogs also had to have serum ALT values which were least three times the normal upper value in at least two consecutive samples. Histologically, an inflammation composed of mononuclear cells in the liver tissue must have present. And, finally, increased hepatic Cu content must here been observed by special stains.

## 8.2. Biochemistry

#### 8.2.1. Serum parameters in the subclinical stage of DH

A greater than threefold rise in serum ALT concentration was detected in 55 (8.8%) of the 626 randomly selected Dobermans. To investigate the reason for persistently increased ALT levels, a liver biopsy was obtained from 23 Dobermans. For 21 of these dogs, histological examination revealed hepatic changes compatible with the criteria set for DH.

These 21 dogs with subclinical DH were followed up by evaluation of their ALT, AP, and total bilirubin values. The follow-up period, from the first blood sample to the first blood sample with clinical signs, lasted from 3 to 48 (average 19) months. The blood sampling interval ranged from one to six months. Altogether 102 blood samples were collected during this period.

**ALT** values were recorded from all 102 blood samples. ALT values measured from these blood samples ranged from 87 to 2166 (normal value 15 to 85) U/l. The number of consecutive blood samples from the same dog in the subclinical period varied from 2 to 10 (average 4.9) samples. The mean ALT of individual dogs ranged from 256 to 1575 U/l. A marked variation was observed between consecutive samples, with ALT levels being very high in one sample but nearly normal in the next. However, in comparing

ALT in the first blood sample with that in the last in the subclinical stage, no significant (p>0.05) difference was present. In the follow-up, ALT did not return to the normal range in any of the dogs. Dogs that later developed clinical DH did not have higher ALT values in their first blood samples compared with dogs with subclinical DH that stayed asymptomatic.

**AP** values, measured in 92 of the 102 blood samples, ranged between 134 and 3165 (normal value 39 to 220) U/l. The consecutive AP value in the same dog ranged from 1 to 10 (average 4.4) blood samples. The mean AP of individual dogs ranged from 297 to 2119 U/l. The values for AP fluctuated during the follow-up, but as with ALT, in comparing the AP in the first blood sample with that in the last in the subclinical stage, no significant (p>0.05) change had occured. Three dogs had normal a AP value in the first blood sample. In the second and third blood samples, only one dog maintained a normal AP concentration. Thereafter, the AP of every dog increased.

**Total bilirubin** values were measured for 15 of 21 dogs with subclinical DH. Thus, bilirubin was analyzed in 45 of the 102 blood samples. The values were in the normal range in 42 of these samples, ranging from 2.1 to 29.8 (normal values, 0 to 14) mmol/l. This blood parameter was analyzed from one to seven (average 3) times per dog. Mean total bilirubin of individual dogs ranged from 6.1 to 14.4 mmol/l.

#### 8.2.2. Serum parameters in the clinical stage of DH

Serum biochemistry was measured from 41 blood samples of 22 different Dobermans with clinical signs of chronic hepatitis. These 22 dogs included six who were initially in the subclinical stage but who during the follow-up showed signs of liver failure and were subsequantly euthanized because of DH. Sexteen dogs were diagnosed with DH only after the appearance of clinical signs.

The follow-up period for the six dogs initially entering the investigation as subclinical patients but who later progressed to the clinical stage varied from 1 to 21 months. This period was measured from the first blood sample with clinical signs to the last sample before death. The survival time of these dogs, i.e.the time from the first blood sample with clinical signs until death ranged from 1.3 to 22 months. Altogether 16 blood samples were collected during this period from these six dogs.

The survival time i.e. the time from the first blood sample with clinical signs to death, of the 16 dogs diagnosed with DH only after clinical signs had appeared ranged from 2 to 284 days. Altogether 25 blood samples were collected from these dogs.

**ALT** values were recorded in all 41 blood samples during the clinical stage of DH. These values ranged from 279 to 2025 U/l. The number of consecutive samples taken from a single Doberman ranged from one to five (average 1.9). Mean ALT for individual dogs ranged from 279 to 1286 U/l. In 13 clinically affected dogs for whom more than one blood sample was collected, serum parameters for the first blood samples collected after onset of clinical signs were compared with those of the last samples before euthanasia. A decrease in ALT levels occured as the disease progressed, but the change was not significant (p>0.05). In none of the dogs was the ALT level in the last blood sample lower than three times the normal value. The lowest ALT value among these dogs in the clinical stage was 279 U/l, which is at least three times the normal upper value.

**AP** values, determined from 40 blood samples in the clinical stage, varied from 403 to 6776 U/l. The number of consecutive samples taken from a single Doberman ranged from one to five (average 1.8). Mean AP of individual dogs ranged from 501 to 5347 U/l. In 13 clinically affected dogs for whom which more than one blood sample was collected, serum parameters for the first blood samples collected after onset of clinical signs were compared with those of the last samples before euthanasia. AP levels increased as the disease progressed, but this change was not significant (p>0.05).

**Total bilirubin** levels were recorded for 18 of 22 Dobermans in the clinical stage of DH. Total bilirubin was analyzed from a total of 25 blood samples, with values ranging from 2.9 to 550.6 (normal value 0 to14) mmol/ 1. Bilirubin concentration was normal only in four out of these blood samples. The number of consecutive samples taken from a single Doberman ranged from one to four (average 1.4). Mean total bilirubin of individual dogs ranged from 9.4 to 550.6 U/l.

#### 8.2.3. Serum parameters of subclinical versus clinical stages of DH

The **ALT** values in the first blood samples of dogs in the subclinical stage of hepatitis were compared to ALT values measured in the first blood samples of dogs with clinical DH. No significant difference was observed (p>0.05). Comparison of all blood samples collected during the subclinical stage (n=102) with the corresponding samples collected during the clinical stage (n=41) yielded similar results (p>0.05).

The levels of **AP** were, however, significantly higher among clinically affected dogs compared with the first blood samples of the dog with subclinical stage with the corresponding samples collected in the clinical stage of the disease (p<0.001). When comparing all blood samples collected in the subclinical stage (n=92) with those collected in the clinical stage of the disease (n=40), AP concentrations were significantly (p<0.001) higher among dogs with clinical DH.

**Bilirubin** levels were significantly (p<0.001) higher among clinically affected dogs than among dogs with subclinical DH.

## 8.3. Evaluation of liver samples

#### 8.3.1. Biopsy samples in DH

Biopsy samples from dogs in the subclinical stage of DH were obtained via laparatomy. On macroscopical evaluation, the liver of all dogs with DH appeared normal in size, with a smooth and unremarkable surface.

The most significant changes in the subclinical stage of DH were diffuse inflammation with multifocal clusters of inflammatory cells in the parenchymal area, and portal inflammation (Fig 1). The degree of parenchymal inflammation in most of the dogs was graded as moderate to marked. Most of the inflammatory cells were lymphocytes. Neutrophils and macrophages were also present, but the number of plasma cells was low. The parenchyma was usually not distorted. Portal inflammation was also moderate to marked. In contrast to findings in the parenchymal and portal areas, lesions in the periportal areas were less severe. Periportal necrosis was generally mild to moderate, and the limiting plate surrounding the portal area was never markedly ruptured. Only

three dogs had bridging necrosis which appeared minimal. Portal expansion and fibrosis were not common findings, and when found, were usually mild. Bile duct proliferation was typically absent, being and recorded in only two of 18 biopsies. Intrasinusoidal bile plugs, as a sign of intrahepatic cholestasis, were evaluated at high magnification for 17 samples. Bile plugs were noted in only two samples.

The location of Cu-positive granules was evaluated in 17 dogs with subclinical DH using a rhodanine stain. Cu accumulation was observed in every biopsy sample. In the subclinical stage, the most numerous Cu-containing granules occured in the centrolobular area around the central vein. In only four of 17 liver samples was Cu observed in small groups of periportal hepatocytes. Bridging necrosis was demonstrated in five of the 17 samples, and Cu-positive granules were rarely seen in these areas.

MHC class II expression was observed in the hepatocytes in 15 of 18 biopsy samples (83%). In the parenchymal area, in 10 of 15 positive cases, expression was noted only around the central vein. In the periportal area, MHC class II expression in hepatocytes was seen in seven of 15 DH cases. Bridging necrosis was noted in five biopsies, and MHC class II expression in hepatocytes was present adjacent to these necrosis areas in three samples. In the biopsy samples, the most prominent mode of expression for MHC class II was cytoplasmic, which was characterized by granules in the cytoplasm of hepatocytes (Fig 2). Membranous expression, indicated as a rim around hepatocytes, was seldom observed at this stage of the disease.

In biopsy samples, MHC class II were also expressed by Kuppfer cells and lymphocytes. Only a few bile ducts in the portal areas expressed MHC class II.

#### 8.3.2. Autopsy samples in DH

In autopsy samples of Dobermans, from which the diagnosis of DH was made during the subclinical stage of the disease and an autopsy specimen was obtained when these dogs were eutahanized for reasons other than DH, inflammation in the parenchymal area was moderate in four dogs and minimal in one. Portal inflammation was moderate to marked. All dogs had at least moderate piecemeal necrosis. Bridging necrosis was detected in three samples, and in one of these, it was diagnosed as multilobular necrosis. Although portal expansion was moderate to marked, bile duct proliferation was evident in only two dogs. One dog had cirrhosis.

In those dogs for whom the diagnosis of DH was made in the subclinical stage and who were subsequently euthanized because of DH, the liver was clearly distorted, its surface covered with nodules. Histopathological findings were characterized by moderate to marked piecemeal and bridging necrosis. In one dog, multilobular necrosis was observed. Portal expansion was moderate to marked. In every sample, bile duct proliferation and fibrosis were also present. Although portal expansion was moderate to marked, in two dogs only small groups of cells or single inflammatory cells were randomly distributed in the parenchymal area. In two other dogs, multifocal parenchymal inflammation was moderate. All dogs had most of the inflammatory cells, which were composed of lymphocytes and macrophages, situated in the periportal and bridging necrosis areas (Fig 3). Bile plugs were observed in every autopsy sample obtained from these dogs (Study III). The number of plugs was 1+ in six samples and 2+ in 17 samples.

Cu was detected histologically in every autopsy sample of dogs with DH (II, III). In the clinical stage of DH, Cu was mainly located in the periportal and bridging necrosis areas (III).

In the autopsy samples of all 20 dogs diagnosed with DH, hepatocytes expressed MHC class II molecules. Although MHC class II expression was also observed in the parenchymal area, the most obvious expression was noted in the hepatocytes adjacent to the periportal and bridging necrosis areas. The prominant staining pattern was a membranous rim around hepatocytes (Fig 4). Cytoplasmic staining patterns for anti-MHC class II antigens were also seen.



**Figure 1**. Liver biopsy from a Doberman suffering from subclinical DH which shows scattered inflammatory cells in sinusoids and a cluster of cells in the parenchymal area.



**Figure 2**. MHC class II expression in a liver specimen from a Doberman with subclinical DH. A focal group of hepatocytes around the central vein stained positive for MHC class II antigens. The inset shows staining for MHC class II molecules in hepatocyte



**Figure 3.** Postmortem liver sample from a dog euthanized for DH. The inflammatory reaction is located in the bridging necrosis area.



**Figure 4**. Autopsy specimen from a Doberman euthanized for DH. No immunosuppressive medication was received before euthanasia. Hepatocytes staining positively for MHC class II antigens were adjacent to bridging necrosis areas with mononuclear cell infiltration. The inset reveals a rim around the hepatocytes, denoting membranous staining of MHC class II molecules.

#### 8.3.3. Biopsy versus autopsy samples in DH

In five of the dogs for whom diagnosis of DH was made in the subclinical stage but who were subsequently euthanized for reasons other than DH, the time between biopsy and autopsy samples ranged from 3.5 to 65 months. Four dogs diagnosed with DH in the subclinical stage subsequently developed clinical signs of liver failure 5 to 35.5 months after the biopsy and were euthanized 0.5 to 20.5 months later. In all nine of these dogs, the disease has progressed. Comparing histopathological changes in these dogs with both the biopsy and the postmortem sample, the most notable changes were expanded portal areas (p=0.008), increased periportal and bridging necrosis (p=0.008), increased fibrosis (p=0.016), and increased proliferation of bile ducts (p=0.063). Diffuse inflammation with clusters of inflammatory cells in the parenchyma tended to occur more often in biopsy than in postmortem samples, but this difference was not significant (p=0.250) (II).

When comparing Cu accumulation between biopsies obtained in the subclinical stage of DH and autopsies obtained after euthanasia because of DH, accumulation was higher in the periportal and bridging necrosis areas of autopsy samples (III). The values in biopsy and autopsy specimens in periportal areas were 0.5 and 1.8, respectively. The corresponding values in bridging necrosis areas were 0.8 and 2.4. By contrast, in parenchymal areas, Cu granules were fewer in autopsy than in biopsy samples. The values in biopsy and autopsy specimens in parenchymal areas were 2.6 and 1.4, respectively. Comparing staining patterns of Cu granules, patterns were "pathy-like" in biopsy samples and "network-like" in autopsy samples.

From 10 Dobermans, both biopsy and autopsy samples were available for evaluating changes in MHC class II expression during disease progression. In two dogs, hepatocytes showed no positive staining for MHC class II in biopsy specimens. However, in the autopsy samples of both dogs, hepatocytes did express MHC class II molecules. The intervals between biopsy and autopsy specimens for these two dogs were 3.5 and 57 months. In the remaining eight dogs, the amount of MHC class II expression in hepatocytes in the parenchymal area did not differ significantly between biopsy and autopsy samples. In the periportal area (p<0.05) and the region adjacent to bridging necrosis areas (p<0.05), by contrast, MHC class II expression increased significantly in hepatocytes during disease progression. In these dogs, biopsy and

autopsy samples were taken from 5.5 to 65 months apart. MHC class II expression in/on hepotocytes was greater when the interval between the biopsy and autopsy was larger. However, the correlation ( $r_s=0.49$ ) was not statistically significant (p>0.15).

#### 8.3.4. Liver samples of control Dobermans

In the biopsy of two Dobermans with elevated ALT values in consecutive blood samples, neither inflammation nor copper accumulation was observed in liver tissues. Histological findings instead revealed vacuolization of liver cells (II). Studies III and IV also revealed no inflammation in liver samples of control Dobermans.

In two Dobermans, euthanized because of adenocarcinoma in the beginning of duodenum or dilatation and torsion of the stomach, some Cu granules were present in a few hepatocytes (III).

In the control Dobermans, no MHC class II molecules were detected in hepatocytes or endothelial cells of bile ducts. However, Kupffer cells and the few existing mononuclear leukocytes in the sinusoids did express MHC class II molecules.

#### 8.3.5. Relationship of mononuclear inflammation to liver copper granules

A spatial relationship was found between the inflammatory reaction and Cu accumulation in DH. In the subclinical stage of the disease, when inflammation was most prominent in the parenchymal area, Cu accumulations were also observed in the same location. The grades for these parameters were 3.2 and 2.6, respectively. In the periportal area, only a small proportion of the limiting plates were destroyed and inflammation was present. Cu was seldom seen in these areas. Bridging necrosis and inflammation were also rare findings in the subclinical stage. Only a small amount of Cu was present in two samples. In the periportal and bridging necrosis areas, inflammation and Cu were scored as 1.8 and 0.5 and 0.5 and 0.8, respectively.

In the clinical stage of DH, inflammation and Cu were mainly located in the periportal and bridging necrosis areas. The mean grades for inflammation and Cu in the different areas were the following: parenchymal area 1.9 and 1.4, periportal area 3.3 and 1.8, and bridging necrosis area 3.4 and 2.4.

# 8.3.6. Relationship of mononuclear inflammation to MHC class II expression in hepatocytes

In all biopsy samples, diffuse inflammation with multifocal clusters of mononuclear inflammatory cells in the parenchymal area was observed. When evaluated by density of cells, the mean value for inflammation was 2.2 (SD 0.8, scale 0-3). The mean value in the same area for MHC class II expression in hepatocytes was graded as 1.9 (SD 1.5, scale 0-5). In the periportal region, the mean index for mononuclear cell infiltration was 1.4 (SD 0.6, scale 0-3). MHC class II expression in hepatocytes was seen in seven of 15 DH cases. The mean value for expression was graded as 0.8 (SD 1.2, scale 0-4). Bridging necrosis was noted in five biopsies. Weak MHC class II expression in hepatocytes was present adjacent to bridging necrosis areas in three DH samples.

When numerically evaluating the lobular area consisting of parenchymal, periportal, and bridging necrosis areas, a correlation was observed between MHC class II expression in hepatocytes and mononuclear cell inflammation (p<0.05). Upon dividing the lobular area into subsections, the positive correlation was found to be contained within the periportal area (p<0.05) and between hepatocytes adjacent to the bridging necrosis area and mononuclear cell inflammation in bridging necrosis areas (p<0.05).

In autopsy samples, the level of cell infiltration was graded as 1.6 (SD 0.6, scale 0-3) in the parenchymal area. The mean level of MHC class II expression was graded as 2.4 (SD 1.3, scale 0-5). Mean values for mononuclear cell infiltration in periportal and bridging necrosis areas were 2.3 (SD 0.7, scale 0-3) and 1.9 (SD 0.9, scale 0-3), respectively. Mean values for MHC class II expression in these areas were 2.9 (SD 1.2, scale 0-4) and 2.8 (SD 1.2, scale 0-3), respectively.

A positive correlation between inflammation and MHC class II expression in hepatocytes was observed in the lobular area (p<0.05), in the parenchymal area (p<0.05), and between hepatocytes adjacent to bridging necrosis areas and mononuclear cell inflammation in bridging necrosis areas (p<0.05).

#### 8.3.7. Comparison of rubeinic acid and rhodanine stains for copper detection

In 19 of 40 liver samples (47.5%), rubeinic acid demonstrated a stronger positive reaction than rhodanine to Cu. In 19 cases (47.5%), the stains were equal, and in two of 40 cases (5%) rhodanine gave a stronger stain.

#### 8.3.8. Evaluation of hepatic copper concentrations

In control Dobermans, hepatic Cu levels ranged from 27 to 352 (mean 226)  $\mu$ g/g dw. In dogs in the subclinical stage of DH, hepatic Cu content ranged from 430 to 2157 (mean 1097)  $\mu$ g/g dw and in dogs euthanized because of DH from 630 to 4155 (mean 1768)  $\mu$ g/g dw. When between different groups were compared, Cu content in controls was found to be significantly lower than in dogs in the subclinical stage of DH or those euthanized because of DH. Furthermore, Cu content was significantly greater in the end-stage than at the beginning of the disease.

## 8.4. Effect of corticosteroid treatment in DH

Seven dogs with DH for whom both biopsy and autopsy samples were available were treated with a small dose of a corticosteroid drug (II). While the medication scheme varied among the dogs, it was obvious that these treatment programs failed to halt progression of the disease.

Fourteen Dobermans were euthanized because of advanced chronic hepatitis. Seven of these dogs had received prednisolon (0.1-0.5 mg/kg/day) for 4 to 5 months. In untreated, euthanized Dobermans, the mean values for MHC class II expression in hepatocytes in lobular, parenchymal, and periportal areas and in regions adjacent to bridging necrosis areas were 10.3 (SD 2.6), 3.3 (SD 1.4), 3.4 (SD 1.1), and 3.6 (SD 0.8), respectively. A thick band of hepatocytes adjacent to the bridging necrosis areas was also recorded to express MHC class II. In Dobermans treated with corticosteroids before euthanization, scattered hepatocytes or small groups of hepatocytes expressed MHC class II. The mean values for MHC class II expression in hepatocytes in lobular, parenchymal, and periportal areas and in regions adjacent to bridging necrosis areas were lower than in untreated animals, being 6.9 (SD 2.6), 1.6 (SD 1.1), 2.9 (SD 0.9), and 2.4 (SD 1.0), respectively. Thus, prednisolon treatment had significantly decreased

the hepatocytes' ability to express MHC class II in the lobular area (p<0.05), the parenchymal area (p<0.05), and region adjacent to bridging necrosis areas (p<0.05). Among untreated Dobermans, the correlation (rs=0.82) between MHC class II expression in/on hepatocytes and mononuclear cell infiltration in the lobular area was significant (p=0.024). Among Dobermans treated with corticosteroids before euthanasia, the correlation was also positive (rs=0.33) but not statistically significant (p=0.47).

# 9. **DISCUSSION**

DH is a chronic liver disorder which most often affects female Dobermans (Johnson et al. 1982, Crawford et al. 1985, Fiorito 1985, Cornelius 1989a). Although the disease can be detected during routine screening of biochemical profile before a surgical procedure, it is usually diagnosed when the dog already has clinical signs of liver failure (Meyer et al. 1980, Doige and Lester 1981, Johnson et al. 1982, Crawford et al. 1985, van den Ingh et al. 1988, Cornelius 1989a, Fuentealba et al. 1997). The prognosis is poor in the clinical stage, and despite treatment with corticosteroids, the dog's condition will deteriorate requiring euthanasia (van den Ingh et al. 1988, Rothuizen 1997).

While reports have been published on Dobermans with clinical signs of hepatitis, scant information is available on dogs in the subclinical stage. The most commonly analyzed liver enzymes are ALT and AP, both of which are typically elevated in DH (Meyer et al. 1980, Doige and Lester 1981, Johnson et al. 1982, Thornburg et al. 1984, Crawford et al. 1985, van den Ingh et al. 1988, Thornburg 1988, Cornelius 1989a, Röcken et al. 1991, Fuentealba et al 1997). No data are available, however, on which enzyme is superior in diagnosig Dobermans in the subclinical stage. In addition, follow-up studies on how biochemical parameters change during disease progression are practically nonexistent.

Since the prognosis of DH in the clinical stage is very poor, detection of affected individuals in the asymptomatic subclinical stage is essential. Identifying these dogs is beneficial both for the breed as for individual dogs. The high prevalence of chronic hepatitis in Dobermans suggests a genetic background (Johnson et al. 1982, Thornburg et al. 1984, Hardy 1985). Dogs in the subclinical stage can easily be used for breeding. If DH is considered a hereditary disease, testing Dobermans for the disease before mating would lower the health risk of future offspring.

The dog itself will also benefit from early diagnosis of DH because it will probably prolong survival time. Environmental factors may also affect DH development, although specific effects are unknown (Crawford et al. 1985). Such environmental factors as nutrition and future medication can be tailored according to the dog's health

status. Pregnancies and unnecessary operations should be avoided. If an operation is needed, consideration must be given to the underlying disease.

# 9.1. Serum parameters

Serum ALT, AP, and total bilirubin levels were evaluated in dogs diagnosed in the subclinical stage of DH. These parameters were compared with those obtained from dogs in the clinical stage to evaluate changes during disease progression.

Based on our findings, dogs with subclinical DH can be identified by regular testing of ALT and AP. The procedure can be performed during annual vaccination visits, and the benefits will far outweigh any costs. However, it should be noted that elevated liver enzyme values are not pathognomonic of DH and can be observed in many other disorders affecting the liver (Dillan 1985, Twedt 1998). Definitive diagnosis should therefore always be based on a liver sample.

Based on our results, ALT is the best screening test for DH. Although both ALT and AP values are routinely evaluated in suspected cases of DH, ALT is the first liver enzyme to rise. Unlike ALT, AP levels can be normal in the initial stages of the disease. Therefore, an evaluation of AP alone might result in the misdiagnosis of a patient with DH.

Although ALT is an effective enzyme for screening dogs with subclinical DH, it cannot be used for predicting disease progression from the subclinical to the clinical stage, because ALT values can be abnormal for several months or even years before a dog has any clinical signs. During this period the values may fluctuate frequently. In this study, despite fluctuation, ALT values did not return to the normal range. In addition, no significant difference was present in serum ALT levels between the subclinical and clinical stages of DH.

According to the literature, normal ALT activity does not rule out the possibility that a dog has chronic hepatitis (Fuenteable et al. 1997). Our findings do not, however, support this assumption. We found ALT levels to decrease as clinical signs became

more severe. However, before euthanasia, none of the Dobermans with obvious signs of advanced liver failure, such as ascites, jaundice, and severe cahexia, had ALT levels in the normal range. This indicates that normalization of serum ALT concentration is rare even in the end-stages of the disease in Dobermans. Our finding is supported by previously published results (Meyer et al. 1980, Doige and Lester 1981, Johnson et al. 1982, Thornburg et al. 1984, Crawford et al. 1985, van den Ingh et al 1988, Thornburg 1988, Cornelius 1989a, Röcken et al 1991, Fuentealba et al 1997). In the clinical stage of the disease, only three of 74 blood samples (0.04 %) had ALT values in the normal range.

AP levels may be increased for several months or years even though the dog is asymptomatic. Similarly to ALT, AP is not a very good prognostic indicator for disease progression at the individual level despite AP values at group level being significantly higher in the clinical stage of DH than in the subclinical stage. While AP is elevated throughout diskase, it is common for values to fluctuate from sample to sample. The increase in serum AP concentration might be due to increased alteration in parenchymal structure and induction of AP from hepatocytes and bile duct cells. The elevation in AP values seen in this study could also be due to the corticosteroid treatment that some of the dog received.

Total bilirubin values are not routinely analyzed in Dobermans with hepatitis, especially if dogs are not clinically icteric (Meyer et al. 1980, Crawford et al. 1985, Cornelius 1989a, Röcken et al. 1991). Based on our results, however, serum total bilirubin levels should also be routinely tested in dogs suffering from subclinical or clinical DH. Although this value is not used in screening dogs with subclinical DH, it can provide additional insight into progression of the disease after DH is confirmed by liver biopsy. Total bilirubin is a better indicator than ALT or AP for predicting disease progression from the subclinical DH. Total bilirubin values stayed within the normal ranges for several months or years despite increased ALT and AP values. However, when dogs exhibited clinical signs of liver failure, total bilirubin levels were invariable abnormal. In comparing total bilirubin values between the subclinical and clinical stages, the differences were significant. It is therefore advisable in re-examinations of patients to check serum total bilirubin levels in addition to the liver enzymes previously mentioned.

Because corticosteroid treatment can normalize total bilirubin values of dogs in the clinical stage of DH (Center 1996b), the value of this measurement in treated dogs is limited.

DH can be divided into subclinical and clinical stages based on clinical and biochemical results (Speeti 1998). In addition, serum biochemical values are indicative of progression of DH when no treatment is used. Once continuously elevated ALT and AP values are confirmed by liver biopsy to be caused by DH, the biochemical profile of the patient should be re-evaluated at least twice a year to keep abreast of disease progression of the. Increased serum ALT concentration followed by increased AP but normal bilirubin, is typical of the subclinical stage. Abnormal bilirubin can predict the advancement of DH to the clinical stage.

The results here can be used on a practical level when examining dogs with clinical signs resembling those of DH. First, a dog with elevated serum AP but normal ALT is probably suffering from something other than DH. This suggestion is further supported if the total bilirubin value is normal. Second, if a Doberman with clinical signs has both increased ALT and AP values, but consecutive serum total bilirubin concentrations are normal, it is this author's opinion that the dog's clinical signs are most likely not due to DH. This conclusion is based on the findings that in the clinical stage of DH total bilirubin values were always abnormal. In addition, the liver has been shown to easily be secondarily affected by extrahepatic diseases. In these reactive hepatopathies, bile acids are usually normal, but definitive diagnosis is based on a liver biopsy which reveals histological findings different from those in DH. It is also noteworthy that clinically affected Dobermans with increased ALT and AP values but consecutive normal total bilirubin values may show characteristic histological findings of subclinical DH in biopsy samples. This merely indicates that this patient with subclinical DH has a concurrent disease which is actually the reason for the clinical signs.

# 9.2. Diagnosis of subclinical DH

Definitive diagnosis of DH should be based on the results of a liver biopsy. Previous research examining histological changes of the liver has focused on patients with

clinical signs of liver failure (Meyer et al. 1980, Doige 1981, Johnson et al. 1982, Crawford et al. 1985, Fiorito 1985, van den Ingh et al. 1988, Cornelius 1989a, Fuenteable et al. 1997). Our study comprised histological descriptions of 18 dogs with subclinical DH., the Histological findings in all these dogs differed from those described in the literature regarding Dobermans with clinical signs of liver failure. In the subclinical stage of DH, the most prominent feature was mononuclear inflammation in the parenchymal and portal areas. Also typical of subclinical DH is that the limiting plate composed of a layer of single hepatocytes surrounding the portal areas was mostly intact. This finding differs from those previously published which assume that limiting plate necrosis is an important aspect in chronic hepatitis of Dobermans (Johnson et al. 1982, Sarli 1992). Our results also indicate that the inflammatory process starts in the centrolobular area. Our observations resemble those of Thornburg (1998), who reported the presence of inflammatory cells in the central area. However, in this article, the first inflammatory cells had been noted in the walls of hepatic veins and around nearby hepatocytes.

The diagnosis of subclinical DH here was based on four criteria. First, dogs were clinically asymptomatic. No signs of liver failure or any other disease were observed when blood samples were taken or at the time of liver biopsy. Second, dogs had consecutive ALT values which were at least three times the normal upper value. We recommend that at least a three fold elevation in ALT values be recorded in consecutive blood samples before a liver biopsy is performed on an otherwise healthy Doberman. This is because with only mildly elevated ALT levels the histological findings will be so minor that a definitive diagnosis probably cannot be made. Third, inflammation composed of mononuclear cells was present in liver tissue. Fourth increased hepatic Cu content was observed by special stains.

DH was shown in this study to be a progressive disease. The literature has described dogs with lobular hepatitis without bridging necrosis or cirrhosis that undergo spontaneous remission. Their increased ALT values can return to normal in 4 to 10 weeks (Thornburg 1988). Here, ALT value was measured 3 to10 (mean 6) times in all 18 dogs in the subclinical stage of DH. Altogether 108 blood samples were evaluated. In all samples, ALT was abnormally high, and in 93%, it was three times the normal value.

Autopsy liver samples were evaluated for nine out of the 18 Dobermans diagnosed in the subclinical stage of DH. The time between biopsy and autopsy samples ranged from 3.5 to 65 months. By comparing the histological lesions of biopsy and autopsy samples from the same dog, liver destruction was clearly seen to have progressed in every dog. These observations indicate that when a Doberman has consistently elevated ALT values and biopsy reveals portal inflammation, diffuse to multifocal parenchymal inflammation, and increased copper levels the dog is suffering from subclinical DH. Because the disease is progressive in nature, the dog may get clinical signs and histological examination will then reveal features typical of chronic hepatitis, including piecemeal and bridging necrosis, fibrosis, and cirrhosis.

A scoring system, commonly used in human medicine to compare lesions in different samples from patients suffering from viral hepatitis, was used for the evaluation of histological lesions in dogs with DH. This method, a modified version of the system created by Knodell (1981), allowed for effective evaluation of progression of histological lesions in the subclinical stage of DH. This scoring system is not widely used in veterinary medicine. However, an evaluation system where different lesions have different numerical values based on their severity is the only objective way of determining whether a disease is progressing (Desmet et al. 1994, Brunt 2000, Jevon 2001). Its usefulness is also obvious when evaluating the possible effect of different medications and environmental agents, such as nutrition and stressful events, on the progression of DH.

Subclinical DH was diagnosed in 21 of 23 Dobermans with continuously elevated ALT values. In the liver biopsies of two dogs, no inflammation or Cu was observed; histological findings were instead dominated by vacuolization of liver cells. These two dogs may have had some extrahepatic disorder causing the elevation in liver enzyme values. Alternatively, DH may have been in a very early stage, with histological features not yet fulfilling the criteria established for diagnosis of subclinical DH. Based on these two Dobermans with elevated ALT values, the diagnosis of subclinical DH should always be based on the findings in liver biopsy.

Since morphological findings, such as the marked piecemeal necrosis typical of CAH were absent from all of our biopsies, we preferred to use the term Doberman hepatitis

for this entity. The absence of prominent periportal inflammation in biopsy samples of dogs with chronic hepatitis has also been noted by others who criticize the use of the term 'chronic active hepatitis' for dogs (Sevelius 1995).

# 9.3. Role of copper in DH

Copper is an essential trace element for humans as well as dogs, but excessive amounts can be very harmful (Linder and Hazegh-Azam 1996, Richter 2003). Cu concentration in the body is therefore strictly controlled. Normal canine liver Cu levels are assumed to be under 400  $\mu$ g/mg dw, and at this level, Cu cannot be detected histologically by special stains (Twedt et al. 1979, Thornburg et al. 1985, Rolfe and Twedt 1995). In DH, increased hepatic Cu content has been reported both by measuring liver Cu levels and by histological staining, but its role in this disease remains unknown (Doige and Lester 1981, Johnson et al. 1982, Thornburg et al. 1984, Crawford et al. 1985, Hardy 1986, van den Ingh et al. 1988, Cornelius 1989a). These concentrations have been measured mainly from dogs in the clinical stage of DH. Liver Cu content was also observed in this study. However, contrary to previous reports where Cu was detected mainly in the clinical stage of DH, here it was recorded in both the subclinical and clinical stages of DH.

Three theories as to why liver Cu content is elevated in DH predominate. The most common of these is that increased hepatic Cu content is caused by chronic intrahepatic cholestasis (Johnson et al. 1982, Cornelius 1989b, Sarli 1992, Center 1996b). This suggestion is based on findings from the clinical stage of the disease. In this stage, increased hepatic Cu content has been noted around the portal area or in the periphery of the pseudolobulus surrounded by bridging necrosis areas. Bile plugs in the liver tissue and increased serum bilirubin values are other common findings in the clinical stage.

Our observations from the subclinical stage of DH do not support the cholestatic theory. Increased liver Cu content was always detected already in the subclinical stage. In this stage, Cu was located in the parenchymal area, whereas periportal areas were mainly intact. Bridging necrosis areas were absent in the subclinical stage.

Bile plugs are considered signs of intrahepatic cholestasis because their formation arises from impaired bile flow (Johnson et al. 1982, Fiorito 1985). Our findings indicate that intrahepatic cholestasis is not present in the subclinical stage of DH. Bile plugs were absent in all but one Doberman in the subclinical stage. It is furthermore supposed that bile plugs in the canaliculus may vanish during the laboratory processing of the liver tissue. However, since intracanalicular bile plugs were observed in only 5.8% of samples in the subclinical stage but 100% of samples in the clinical stage, laboratory processing is unlikely to be the reason for the absence of bile plugs in subclinical DH. The lack of bile plugs in subclinical dogs is supported by other studies (Thornburg et al. 1984, Fuentealbe et al. 1997).

Chronic intrahepatic cholestasis as a cause for increased Cu in DH has also been suggested as a result of DH being compared with chronic cholestatic liver disorders, such as primary biliary cirrhosis (PBC), in humans (Johnson et al. 1982, Sarli 1992). In PBC, the main targets for destruction are medium-sized bile ducts. The damage to bile ducts eventually leads to intahepatic cholestasis (Kaplan 1993). Comparing PBC with DH, Cu accumulation is in the periportal area, rather than the parenchymal area, and accumulation is typically observed in the later stages of the disease, whereas in DH Cu levels are elevated already in the subclinical stage. Therefore, the reason for Cu accumulation is different in these two liver disorders.

Elevated serum bilirubin level is also considered a sign of cholestasis (Meyer et al. 1980, van den Ingh et al. 1988) since bilirubin is secreted via bile and reduced bile flow causes the elevation in bilirubin values. However, 94% of the Dobermans in the subclinical stage had total bilirubin levels in the normal range. This indicates that hyperbilirubinemia is a rare finding at the beginning of DH, contradicting the cholestatic theory of Cu accumulation in DH.

Cu accumulation in DH has also been suggested to occur because of a primary metabolic defect based on a study of two Dobermans with subacute hepatitis. Increased hepatic Cu was observed in the centrolobular area and no signs of intrahepatic cholestasis were present (Thornburg 1984). In humans, Wilson's disease, and in dogs, Bedlington Terriers' Cu toxicosis, are disorders of primary Cu metabolism. In both diseases, Cu secretion from hepatocytes is abnormal, resulting in progressive Cu accumulation in the liver (Twedt 1997, Subramanian et al. 2002, Richter 2003). Hepatic destruction usually does not occur until Cu concentration has exceeded the hepatocytes' ability to bound free Cu. Unbound Cu causes damage to hepatocytes, inducing inflammation (Brewer 2000, Richter 2003). In Bedlington Terriers, hepatic damage is not evident until Cu levels exceed 2000  $\mu$ g/mg dw (Thornburg 2000).

Our observations do not support the hypothesis that DH is a primary Cu metabolic disease such as the one seen in Bedlington Terriers. Mononuclear infection in liver tissue was obvious despite Cu concentrations being lower than 1500 µg/mg dw. Also, according to the literature, hepatic Cu concentrations in DH rarely reach the concentrations reported in Bedlington Terriers (van den Ingh et al. 1988, Sevelius et al. 1993). Other differences between DH and primary Cu metabolic diseases also exist. D-penicillamine is widely used to treat Wilson's disease and Cu toxicosis in Bedlington Terriers. It chelates with Cu in the blood and tissues, causing secretion of Cu into the urine (Twedt 1997). D-penicillamine is effective for preventing chronic hepatitis and cirrhosis in both of these primary Cu metabolic disorders (Subramanian et al. 2002, Richter 2003). However, its effect on DH has thus far not been well studied (Crawford et al. 1985, Center 1996).

Finally, Cu in DH has been argued to have no clinical relevance, merely an incidental finding. This is based on a report of five of 35 Dobermans with chronic hepatitis having either normal hepatic Cu concentration or no detected Cu in rhodanine-stained sections (Thornburg 1998). In contrast to the above, in our study, elevated hepatic Cu concentration was observed using a special stain in every sample obtained from 18 dogs with subclinical DH and in 20 out of 21 samples from dogs with clinical DH. In the control group, no Cu accumulation was detected in histological examination. Hepatic Cu concentration was also quantitatively measured in both the subclinical and the clinical stages of DH as well as in control Dobermans. Cu appeared to increase as the disease progressed; in control Dobermans, concentrations did not exceed 400  $\mu$ g/mg dw. In our opinion, elevated hepatic Cu concentrations in DH are not, therefore,

incidental. In fact, elevated hepatic Cu content was one of the criteria for diagnosing subclinical DH in this study. A later study on subclinical DH also noted consistently elevated hepatic Cu content (Mandigers 2000).

Our result showed that increased hepatic Cu content was associated with tissue inflammation. A spatial relationship was observed between these two parameters. Moreover, the finding that corticosteroid treatment alone can decrease hepatic Cu content in DH supports the theory that a connection exists between hepatic tissue inflammation and Cu accumulation in DH (Center 1996b). By reducing tissue inflammation, corticosteroids most probably also decrease the Cu content of the liver. Corticosteroid treatment may explain why in some published research the copper levels in DH have been recorded within the normal range. We assume that Cu accumulate in hepatocytes because mononuclear inflammation has disturbed the movement of Cu from hepatocytes to the bile canaliculus.

Two histological stainings for Cu, rubeinic acid and rhodanine, were compared. Based on our results, the former seems to be more sensitive to Cu-positive granules. Rubeinic acid stain is therefore routinely used by the author to diagnose subclinical DH when histologically evaluating the possible existence of increased Cu in the liver section is important.

# 9.4. MHC class II expression in hepatocytes and etiology of DH

Information regarding the etiology of DH is sparse. Here, etiological background of DH was analyzed based on findings of abnormal MHC class II expression in hepatocytes in different stages of DH. In this evaluation, a classification similar to that in humans suffering from chronic hepatitis was used.

MHC class II molecules are normally expressed by professional antigen-presenting cells (APCs), including B-lymphocytes, macrophages, and dendritic cells. In dogs, MHC class II expression is also observed in T-cells without activation of the cells (Doveren 1985, Tizard 1996). While hepatocytes typically do not express MHC class II antigens

(Spengler et al. 1988, Tizard 1996), here, in 87% of the biopsies taken in the subclinical stage of DH and in 100% of autopsy cases, MHC class II antigens were present in hepatocytes.

MHC class II expression by non professional APCs can be due to microbes, drugs, toxins, or autoimmunity. Adenovirus-1 is the best-known viral cause of chronic hepatitis in dogs. Canine adenovirus-1 infections in dogs have not, however, been diagnosed in Finland for several years. This microbe cannot therefore be considered a stimulus for MHC class II expression in DH in this study. Other viruses have also been suggested as etiological factors in DH (Rothuizen 1997). Although a virus as a primary source of antigen for MHC class II expression in DH cannot be ruled out, some findings contradict a viral etiology. When a virus infects a cell, the antigen derived from the virus is mainly presented by MHC class I molecules, not MHC class II molecules, to Tcells (Tizard 1996). If DH were a primary viral hepatitis, corticosteroid treatment would be contraindicated because corticosteroids suppress the immune response necessary for effective elimination of the virus. Dobermans in the subclinical stage of chronic hepatitis may be on a corticosteroid treatment for long periods without deterioration of health status. Furthermore, Dobermans with clinical signs of liver failure can show temporary improvement with corticosteroid treatment. Lastly, if DH were a primary viral hepatitis, other breeds would be expected to contract this infection at the same However, chronic hepatitis is far more common in frequency as Dobermans. Dobermans than in most other breeds.

Leptospires are a potential agent for causing chronic hepatitis in Dobermans. However, leptospirosis is diagnosed in Finland in only a few cases annually, mainly in dogs that have travelled abroad. Therefore, this microbe is an unlikely antigen source for MHC class II in hepatocytes of DH-positive dogs of this study.

Drugs, toxins, or environmental chemicals can also stimulate MHC class II expression. Hepatocytes have the ability for the pinocytosis of drugs and toxins from the portal blood. However, a drug or a toxin as a noxious stimulus in DH was ruled out on the basis of several findings. First, dogs with elevated liver enzymes and subclinical DH were not taking medication and had no history of toxic exposure prior to the recording of elevated liver enzyme values or at the time of liver biopsy. Second, for stable MHC class II molecules, continuous antigen exposure is necessary, otherwise the molecule would dissolve into parts and vanish (Germain and Hendrix 1991). In our study, MHC class II expression in hepatocytes was observed both in the biopsy and autopsy samples of the same 10 dogs. The time between biopsy and autopsy specimens varied from 3.5 to 65 months. During this interval MHC class II expression in hepatocytes persisted and got stronger. These dogs were not on any kind of medication, except possibly for corticosteroids during follow-up. These observations indicate that the stimulus for MHC class II expression in hepatocytes was permanent, thereby ruling out drugs as a causative agent. Third, in the subclinical stage of DH, expression of the MHC class II in hepatocytes was noted in the centrolobular area, whereas periportally, the hepatocytes appeared negative for expression. If the antigen arrived through the portal vein, the first hepatocytes to be affected would be around the portal area. This finding further discounts toxins as a potential cause of DH.

In humans, aberrant expression of MHC class II in various tissues has been implicated in the pathogenesis of autoimmune diseases (Rask et al. 1991, Nepon 1993, McDevitt 1998). The finding that MHC class II molecules are expressed in hepatocytes in DH is therefore a strong indicator of an autoimmune background for this disease. Since the major function of MHC class II is to present antigens to T-cells, in DH this antigen is most likely an autoantigen of hepatocytic origin.

Other evidence also favours the autoimmune nature of DH. First, females are more susceptible than males to DH (Johnson et al. 1982, Crawford et al. 1985, Fiorito 1985). Likewise, in human autoimmune diseases, a female preponderance is common. Second, Dobermans seem to have a genetic predisposition to chronic hepatitis. An association between different diseases, especially those autoimmune in nature, and MHC class II is well established in human diseases (McDevitt 1998). Thus, in DH, genetic predisposition is also most likely associated with MHC class II genes. Third, antibodies against liver-specific protein have previously been observed in two of four Dobermans examined that suffered from chronic hepatitis (Weiss et al. 1995).

# 9.5. MHC class II expression in hepatocytes and pathogenesis of DH

At present, no effective treatment exists for DH. Understanding of the pathogenesis of the disease is therefore essential for development of treatment protocols. Suggestions made here about the pathogenesis of DH are based on findings of mononuclear inflammation in the liver tissue and a significant correlation between inflammation and MHC class II expression in hepatocytes. Another important observation is that different stages of the disease.

Within the immune system, MHC class II has a dual function. In the thymus, it is involved in the deletion of T-cells capable of reacting with self-antigens (Janeway et al. 1999a). This deletion is, however, imperfect. All living beings have autoreactive T-cells that are capable of reacting with autoantigens. In the periphery, MHC class II has a major role in presenting antigens to CD4+ T-cells (Mellins 1992, Tizard 1996 Day 1999). The results obtained in this study suggest that DH is an autoimmune liver disease that affects genetically susceptible Dobermans. The susceptibility is based on the type of MHC class II molecules that the individual produces. Dobermans whose MHC class II antigen complex interacts, with the help of co-stimulatory signals, with the T-cell receptor on T-cells, causing their activation, are prone to development of DH.

In humans, an inducing agent is generally needed to initiate the autoimmune response (Czaja 2002). An environmental agent, e.g. a microbe, is assumed to be responsible for molecular mimicry, where the protein fragment from an infectious agent presented to the immune system will closely resemble a part of a self-protein. As a result of molecular mimicry, the activated immune system will not only attack a protein fragment from an infectious agent but also the protein fragment of a self-protein.

An environmental trigger could also cause the initial mononuclear cell infiltration in DH. Cytokines produced by these cells then further induce expression of MHC class II in hepatocytes. This suggestion is based on findings in two Dobermans for which both biopsy and autopsy samples were obtained and mononuclear cell infiltrations were

present. While MHC class II expression was not observed in the biopsies, it was noted in autopsies of both dogs.

The target cell in DH remains obscure (Thornburg 1998). Here, in 87% of biopsies taken in the subclinical stage of DH and in 100% of autopsies, MHC class II expression was seen in hepatocytes. MHC class II expression in hepatocytes is a persistent phenomenon, and expression seems to increase as the disease progresses. Hepatocytes of control Dobermans did not express MHC class II. This expression in DH indicates that in hepatocytes a specific antigenic peptide has become bound to the MHC class II molecule. Hepatocytes appear to be the target cells of this disease.

In the present study, a correlation was observed between the extent of mononuclear cell inflammatory reaction and expression of MHC class II in hepatocytes. Certain cytokines are known to induce expression of MHC class II genes in non lymphatic cells. Moreover, bile duct epithelial cells, despite obvious portal inflammation, only in few cases and weakly expressed MHC class II. Although bile duct epithelial cells do not normally express MHC class II, in primary biliary cirrhosis and sclerosing cholangitis, both classified as autoimmune diseases in humans, they do so (Ballardini et al. 1984, Broome et al. 1990). These findings indicate that in DH cytokines produced by mononuclear inflammatory cells induce an aberrant expression of MHC class II in hepatocytes but not in bile duct epithelial cells.

In biopsies, MHC class II expression was primarily seen in the cytoplasm of hepatocytes, while in autopsy samples, expression was most prominent on cell surfaces. These findings indicate that as long as the MHC class II molecule and antigen are in the cytoplasm, the hepatocyte is safe from an immune system response. When the MHC class II:autoantigen complex reaches the cell surface, however, the immune system recognizes the complex and becomes activated. The lymphocytes that react with hepatocytes expressing MHC class II on their cell surfaces appear to be CD4+ T-cells. In autopsy samples, the most prominent MHC class expression on cell surfaces of hepatocytes was adjacent to piecemeal and bridging necrosis areas. This finding suggests that CD4+ T-cells were activated and had started an immune attack against hepatocytes. This is known as "MHC class II restricted activation of T-cells" (Janeway

et al 1999b). These T-cells appear to be autoreactive, suggesting that DH is a T-cellmediated disease.

Dogs with subclinical DH can remain asymptomatic for several months or years, despite plasma levels of their liver enzymes being elevated. It is therefore probable that after the initial induction of the autoimmune reaction by an environmental agent a subsequent stressful stimulus, such as pregnancy, enhances liver destruction.

Although CD4+ T-cells are responsible for initiating the immune reaction, they are not capable of causing the final destruction of hepatocytes. Instead, after the interaction between the MHC class II:autoantigen complex on hepatocytes and T-cell receptors on CD4+ T-helper cells, the T-helper cells become either Th1- or Th2-cells. These cells are differentiated by the cytokines they secrete (Day 1999). Th1-cells are mainly responsible for activating macrophages. They can also activate CD8+ cytotoxic T-cells. Th2-cells, by contrast, are the most effective activators of B-cells (Janeway et al. 1999). Therefore, in DH, the final mechanism causing the destruction of hepatocytes depends on the type of cytokines secreted by activated T-helper cells (Fig 5).

Figure 5. Hypothesis for the pathogenesis of DH.

A. An environmental trigger (e.g. a microbe) causes the initial mononuclear cell infiltration.

B. Cytokines produced by the inflammatory cells induce the expression of MHC class II genes of hepatocytes in individuals who are genetically suspeptible to DH. The environmental agent is responsible for molecular mimicry in which the activated immune system will attack not only protein fragments from infectious agents but also fragments of native proteins.

C. While the MHC class II molecule and autoantigen are in the cytoplasm, the hepatocyte is safe from an immune system response. However, when the MHC class II:autoantigen complex reaches the cell surface, the immune system will recognize the complex and become activated.

D. The lymphocytes that react with hepatocytes expressing MHC class II molecules on their cell surfaces are CD4+ T-cells.

E. After the interaction between the MHC class II:autoantigen complex on hepatocytes and the T-cell receptors on CD4+ T-helper cells, the T-helper cells become activated and start to secrete cytokines. The final mechanism causing the destruction of hepatocytes depends on the type of cytokines secreted by activated CD4+ T-helper cells as well as the types of cells (macrophages, B-cells, or CD8+ cytotoxic T-cells) stimulated.




## **10. CONCLUSIONS**

Dobermans with subclinical chronic hepatitis can be identified by regular testing of serum ALT and AP levels. ALT was the best screening parameter for DH because it was the first liver enzyme to rise. Unlike ALT, AP levels can be normal in the initial stages of the disease. Serum ALT and AP values can be abnormal for several months or years before a dog shows clinical signs. During the subclinical and clinical stages the ALT and AP values frequently fluctuate. Despite fluctuation, enzyme values of dogs here did not return the normal range. The normalization of serum ALT concentration is rare even in the end-stages of the disease.

ALT and AP are not good prognostic indicators of disease progression from the subclinical to the clinical stage. Total bilirubin is a better indicator. In dogs with subclinical DH, total bilirubin levels were normal for several months or years despite increased ALT and AP values. When dogs exhibited clinical signs of liver failure, the total bilirubin level was invariably abnormal. Total bilirubin values should therefore be routinely tested in dogs from suffering either subclinical or clinical DH.

A Doberman who has consistently elevated ALT values and whose liver biopsy reveals mononuclear inflammation in the parenchymal and portal areas, absent or minimal piecemeal and bridging necrosis, and increased Cu levels is suffering from subclinical DH. The disease is progressive in nature, leading to histological alterations typical of for chronic hepatitis, including piecemeal and bridging necrosis, fibrosis, and cirrhosis.

Increased Cu levels of the liver were noted in both the subclinical and the clinical stages of DH. Cu was initially observed in the parenchymal area, and in the later stages of the disease, adjacent to piece meal– and bridging necrosis areas. By measuring Cu levels in different stages of the disease, Cu content was found to increase with disease progression.

In the subclinical stage, no signs of chronic cholestasis were present despite increased hepatic Cu levels. Total bilirubin values were normal. The Cu was mainly situated in the parenchymal areas, and the periportal areas were intact. Bile plugs were not detected. Thus, chronic cholestasis as a reason for Cu accumulation in the liver in DH is unlikely.

Increased hepatic Cu content was associated with tissue inflammation in the liver. Cu accumulation was assumed to be due to tissue inflammation and not vica versa. Cu is proposed to accumulate in hepatocytes because mononuclear inflammation disturbs the movement of Cu from hepatocytes to the bile canaliculus.

MHC class II, a protein expressed by professional APCs, presents processed antigens to T-cells. Hepatocytes, which normally do not express MHC class II molecules, do so in DH. The MHC class II expression by non professional APCs can be due to microbes, drugs, toxins, or autoimmunity. In DH, drugs and toxins were ruled out. The most probable explanation for abnormal MHC class II expression by hepatocytes in DH is autoimmunity, with the presented antigen being an autoantigen of hepatocytic origin. However, primary viral infection cannot be completely ruled out.

The MHC class II expression in hepatocytes strongly indicates that hepatocytes are the target cells of DH. An environmental trigger, e.g. microbe, could cause the initial mononuclear cell infiltration. Cytokines produced by these cells then further induce expression of MHC class II molecules in hepatocytes. An environmental agent probably causes molecular mimicry, whereby the protein fragment of an infectious agent presented to the immune system closely resembles part of a self-protein. As a result, the activated immune system will not only attack the protein fragment of an infectious agent but also the protein fragment of a self-protein. As long as the MHC class II molecule and autoantigen remain in the cytoplasm, the hepatocyte is safe from an immune system response. When the MHC class II:autoantigen complex reaches the cell surface, however, the immune system recognizes the complex and becomes activated.

Lymphocytes that react with hepatocytes expressing MHC class II on their cell surfaces are CD4+ T-cells. This indicates that these T-cells are autoreactive and that DH is a Tcell-mediated disease. Although CD4+ T-cells are responsible for starting the immune reaction, they are not capable of causing the final destruction of hepatocytes. The final mechanism causing the death of hepatocytes depends on the types of cytokines secreted by the activated CD4+ T-helper cells and the types of cells are stimulated. These cells can be macrophages, B-cells, or cytotoxic T-cells.

The high prevalence of chronic hepatitis in Dobermans indicates a genetic predisposition, which is somehow associated with MHC class II genes. Dobermans whose MHC class II antigen complex interacts, with the help of co-stimulatory signals, with the T-cell receptor on T-cells, causing their activation, are prone to the development of DH.

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