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Doberman Hepatitis
Role of Immunological and Genetic Mechanisms

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DOBERMAN HEPATITIS

Role of Immunological and Genetic Mechanisms

Hanna Dyggve

ACADEMIC DISSERTATION

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Abstract

Doberman hepatitis (DH) is a rare and chronic inflammatory condition of the liver. The early diagnosis of DH is difficult, and Dobermans may be affected for months or years before the diagnosis. There is no curative treatment and the prognosis is poor if the condition is associated with clinical signs of liver failure. Unraveling the disease etiology and pathogenesis would enhance the diagnostic approaches, and novel therapies could improve the prognosis for Dobermans. Our studies aimed to identify supporting evidence for the suspected autoimmune background of DH by satisfying more descriptive characteristic risk factors that are described in human autoimmune diseases. We focused on major histocompatibility complex (MHC) class II genes, MHC class II regulatory regions, circulating serum anti-nuclear antibodies (ANA), and possible liver-related autoantigens associated with DH.

We identified a homozygous dog leukocyte antigen (DLA) risk haplotype and allele for DH. DLA-DRB1*00601/DQA1*00401/DQB1*01303 haplotype elevated the risk of DH susceptibility close to 15-fold. A homozygous risk DLA-DRB1*00601 allele was found in all DH dogs, while this allele was present in 56.8% of the healthy controls. DLA-DQA1*00901/DQB1*00101 and allele DLA-DRB1*01501 appear to protect against DH development. Overall, the DLA region showed very low variation among DH patients, with only two different haplotype combinations compared with seven in the control group.

We defined the level of variation of the regulatory promoters in DH patients and controls with the homozygous DLA-DRB1*00601/DQA1*00401/DQB1*01303 haplotype. Also, the potential polymorphism in the DLA-DRAp promoter and DLA-DRA exon 2 was examined. The promoter DLA-DRAp and exon 2 were identical to the dog reference sequences in both groups. The promoters DRBp, DQAp, and DQBp were monomorphic, with no explaining variation when comparing DH patients with healthy control Dobermans. DLA-DRB1*00601 allele was associated with DRBp*1, DLA-DQB1*01303 allele with DQBp*6, and DLA-DQA1*00401 allele with DQAp*2. Our result suggests that promoter variants are not associated as risk modifiers for DH in Dobermans with the risk haplotype, but the whole DLA block is associated with DH.

The study revealed ANA in DH patients. ANA were further characterized as anti-histone autoantibodies (AHA) that were significantly elevated in DH. Thus, immune tolerance has failed in DH. Our results suggest that elevated AHA in conjunction with high alanine aminotransferase (ALT) in a Doberman with or without clinical signs of hepatitis support the DH diagnosis. A negative serum AHA result does not rule out DH, and AHA is not a suitable biomarker for disease progression.

We described two novel liver-related target autoantigens in DH. These could be identified as dehydrogenase enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and alcohol dehydrogenase (ADH). DH dogs had significantly elevated serum IgG immunoreactivity towards GAPDH and ADH antigens compared with controls, and the disease was associated with both autoantibodies. This provides evidence for an antigen-driven autoimmune process and elucidates the previously unknown DH pathogenesis.

Overall, this thesis provides new insights into the suspected autoimmune etiology and pathogenesis of DH. DLA system haplotype and allele and DLA promoter variants as risk modifiers are characterized. The role and diagnostic use for three novel IgG autoantibodies and autoantigens are discussed. We conclude that the study contributes to the development of useful biomarkers that could be used to improve the currently difficult early diagnosis of DH in Dobermans with elevated ALT levels. Our findings combined with the previous observations in DH support the theory that DH has an autoimmune origin.

Tiivistelmä

Dobermannin hepatiitti (DH) on etenevä, krooninen ja tulehduksellinen maksasairaus, mitä esiintyy erityisesti nartuilla. Etenevän tulehduksen seurauksena maksasolut tuhoutuvat ja korvautuvat sidekudoksella. Sairaus on perinnöllinen, mutta periytymismekanismia ei toistaiseksi vielä tunneta. Taudin varhaisdiagnosointi on vaikeaa, sillä hepatiitti voi olla pitkään täysin oireeton ja sairauteen ei ole olemassa parantavaa hoitoa. Koiran ennuste on huono, mikäli tilaan liittyy maksasairauden kliinisiä oireita. Taudin etiologian ja patogeneesin selvittäminen helpottaisi erotusdiagnostiikkaa ja uudet hoitomuodot voisivat myös parantaa sairaiden koirien eliniän ennustetta.

Tämä tutkimuksen tavoitteena oli tutkia koiran leukosyyttiantigeeni- eli DLA-geenejä ja näiden säätelyalueita, sillä geneettinen riski autoimmuunisairauksiin liittyy vahvasti näihin geenialueisiin. Autoimmuunisairaudessa elimistön puolustusreaktio kohdistuu virheellisesti omia kudoksia vastaan. Verinäytteistä määritettävät autovasta-aineet auttavat diagnostiikassa ja viittaavat taudin autoimmuunitaudin luonteeseen. Määritimme verinäytteistä hepatiittipotilaiden tumavasta-aineita sairauden oireettomassa ja oireellisessa vaiheessa sekä etsimme DH-tautiin liittyviä vasta-aineita, jotka tunnistavat maksan autoantigeenejä.

Havaitsimme yhteyden DLA luokka II-alueen geenien ja sairauden välillä. Koirilla, joilla DLA riskihaplotyyppi DLA-DRB1*00601/DQA1*00401/DQB1*01303 esiintyi homotsygoottisena (molemmissa kromosomeissa) oli 15-kertainen sairastumisriski Dobermannin hepatiittiin. Kaikki sairaat koirat kantoivat homotsygoottisena riskialleelia DLA-DRB1*00601. Kontrolleissa tämän suhteen homotsygootteja oli vain 56.8%. Haplotyypillä DLA-DQA1*00901/DQB1*00101 ja alleelilla DLA-DRB1*01501 oli suojaava vaikutus heterotsygoottisena. Kaiken kaikkiaan DLA-alueella oli hyvin vähäinen vaihtelu DH-potilailla, ja vain kaksi erilaista haplotyypin yhdistelmää verrattuna seitsemään kontrolliryhmässä.

Selvitimme selittäisivätkö DLA geenien säätelyalueen muutokset sitä miksi homotsygootit DLA-DRB1*00601/DQA1*00401/DQB1*01303 kantajat sairastuvat, sillä osalla terveistä koirista oli myös riskihaplotyyppi. DRAp säätelyaluetta ei oltu aiemmin dokumentoitu koirilla ja sen vaihtelua myös tutkittiin. DLA-DRAp-säätelyalue ja DLA-DRA eksoni 2 olivat identtiset koiran referenssigeenisekvenssin kanssa. DLA-DRB1*00601 alleeli assosioitui DRBp*1 alleelin kanssa, DLA-DQB1*01303 alleeli DQBp*6:n kanssa, ja DLA-DQA1*00401 alleeli DQAp*2:n kanssa. Vertailussa DLA-geenien säätelyalueet eivät selitä sairastuvuutta, mutta koko DLA-geenialue näyttää assosioituvan hepatiitiin Dobermanneilla.

DH-potilailla havaittiin kohonneet seerumin tumavasta-ainetasot ja jatkotutkimuksissa nämä tunnistettiin vasta-aineiksi tuman histoneja vastaan (AHA). Tämä viittaa immuunitoleranssin murtumiseen. Tulokset osoittavat, että kohonnut AHA yhdessä korkean maksaperäisen alaniiniaminotransferaasin (ALAT) tason kanssa oireettomilla tai oireellisilla hepatiittipotilailla tukee DH-diagnoosia. Negatiivinen seerumin AHA-tulos ei sulje pois DH:ta eikä AHA ole sopiva merkkiaine sairauden etenemiselle.

DH:ssa kaksi maksaan liittyvää autoantigeenia voitiin tunnistaa dehydrogenaasientsyymeiksi, glyseraldehydi-3-fosfaattidehydrogenaasi (GAPDH) ja alkoholidehydrogenaasi (ADH). DH-koirilla oli merkittävästi kohonnut seerumin immunoreaktiivisuus näitä entsyymejä kohtaan ja sairaus assosioitui vahvasti molempiin autovasta-aineisiin. Löydös antaa näyttöä DH-taudun autoimmuuniprosessista ja selittää aiemmin tuntematonta sairauden patogeneesia.

Tämä väitöskirjatutkimus tuo merkittävää uutta tietoa DH:n epäiltyyn autoimmuunietiologiaan ja patogeneesiin. Tutkimuslöydökset edistävät sairauden varhaisdiagnostiikkaa ja tukevat diagnoosin varmistusta Dobermanneilla, joilla on kohonnut ALAT arvo. Ne myös tarjoavat pohjan uusien hoitomuotojen kehittämiselle.

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Helsinki, 3rd October 2017

Contents

List of original publications

This thesis is based on the following publications:

I **Dyggve H**, Kennedy LJ, Meri S, Spillmann T, Lohi H, Speeti M. Association of Doberman Hepatitis to Major Histocompatibility Complex II.
Tissue Antigens 2011;77:30-5.

II **Dyggve H**, Kennedy LJ, Meri S, Spillmann T, Lohi H, Speeti M. Evaluation of DLA Promoters in Doberman Hepatitis.
Tissue Antigens 2011;78:446-50.

III **Dyggve H**, Meri S, Spillmann T, Jarva H*, Speeti M*. Antihistone Autoantibodies in Dobermans with Hepatitis.
*Shared last authorship.
J Vet Intern Med 2017; doi:10.1111/jvim.14838.

IV **Dyggve H**, Jarva H, Spillmann T, Speeti M*, Meri S*. Identification of Glyceraldehyde-3-phosphate and Alcohol Dehydrogenases as Autoantigens in Doberman Hepatitis.
*Shared last authorship.
Scand J Immunol 2017;86:156-64.

These publications are referred to in the text by their Roman numerals and are reprinted with the kind permission of their copyright holder.

Author´s contributions

I, II The author participated in the experimental design and recruited, performed clinical examinations, and collected samples from healthy dogs and three subclinical DH (SDH) dogs. The author studied the genealogical relationships of the controls and cases. The author extracted genomic DNA from 34 DH liver samples, performed PCR and sequencing, participated in DLA allele identification (I), and conducted promoter allele assignment (II). The author interpreted the results under the guidance of Hannu Rita, Hannes Lohi, and her supervisors. The author wrote and revised the manuscript.

III The author participated in the experimental design and recruited, performed clinical examinations, and collected samples from healthy dogs, three SDH dogs, and one clinical DH (CDH) dog. The author prepared the indirect immunofluorescence assay, Line immune assay, and the samples for the enzyme-linked immunosorbent assay (ELISA) study. The author interpreted the data under the guidance of Hannu Rita, Hanna Jarva, and her supervisors. The author wrote and revised the manuscript.

IV The author participated in the experimental design and recruited, performed clinical examinations, and collected samples from healthy dogs, three SDH dogs, and one CDH dog. The author conducted the Western blot assay and prepared gels for the proteomics study and samples for ELISA. The author interpreted the data under the guidance of Hannu Rita, Hanna Jarva, and her supervisors. The author wrote and revised the manuscript.

Abbreviations

ADH	alcohol dehydrogenase
AHA	anti-histone antibody
AIH	autoimmune hepatitis
ALAT	alaniiniaminotransferaasi
ALD	autoimmune liver disease
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AMA	anti-mitochondrial antibody
ANA	anti-nuclear antibody
Anti-LKM-1	liver-kidney microsome type-1 antibody
Anti-LMP	anti-liver membrane antibody
Anti-RNP-A	anti-ribonucleoprotein-A antibody
APC	antigen presenting cell
AST	aspartate aminotransferase
ATP	adenosine triphosphate
CD	celiac disease
CDH	clinical Doberman hepatitis
CIITA	MHC class II trans-activator
CI	confidence interval
DAMP	damage-associated molecular pattern
DC	dendritic cell
DCM	dilated cardiomyopathy
DH	Doberman hepatitis
DLA	dog leukocyte antigen
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
IFN- γ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell

IP	immunoprecipitation
KC	kupffer cell
LD	linkage disequilibrium
MAMP	microbial-associated molecular pattern
MHC	major histocompatibility complex
NFAT	nuclear transcription factor of activated T lymphocytes
NK cell	natural killer cell
NKT cell	natural killer T cell
OPD	o-phenylenediamine dihydrochloride
OR	odd's ratio
PAMP	pathogen associated molecular pattern
PBC	primary biliary cholangitis
PCR	polymerase chain reaction
PRR	pathogen-recognition receptor
PSC	primary sclerosing cholangitis
PTM	post-translational modification
Q	glutamine
R	arginine
RA	rheumatoid arthritis
RT	room temperature
SDH	subclinical Doberman hepatitis
SE	shared susceptibility epitope
SMA	smooth muscle antibody
Th	T helper cell
TG-2	transglutaminase-2
TGF-β	transforming growth factor beta
TLR	Toll-like receptor
TNF-α	tumor necrosis factor alpha
Treg	regulatory T cell
UDCA	ursodeoxycholic acid

1 Introduction

Autoimmune diseases are increasingly prevalent in dogs and humans. These conditions occur when self-tolerance is lost in genetically predisposed individuals encountering possible environmental triggers. As a consequence, adaptive immune mechanisms develop autoantibodies towards self-proteins. Dogs share the living environment and many autoimmune disorders with humans. These include, among others, different endocrine (diabetes mellitus, hypothyroidism, hypoadrenocorticism, polyglandular autoimmune syndrome type II), hematologic (hemolytic anemia, thrombocytopenia), skin (pemphigus, epidermolysis bullosa), muscle (myasthenia gravis), neurologic (narcolepsy), or joint and renal (systemic lupus erythematosus) -associated conditions. Immune mediated liver diseases have been suggested (Andresson & Sevelius 1992, Weiss et al. 1995, Poitout et al. 1997, Boisclair et al. 2001) but autoimmune liver diseases (ALDs) have not been previously confirmed in dogs.

Different approaches to the underlying mechanisms in Doberman hepatitis (DH) have been proposed over the years. DH has been suggested to be a primary copper retention disease (Mandigers et al. 2004), however, it has been speculated that increased copper concentrations could be an incidental finding in chronic hepatitis in Dobermans (Crawford et al. 1985, Thornburg 1998). One author suggested that elevated copper content in the liver is due to hepatitis and can be lowered with corticosteroids (Speeti et al. 1999). Suspicion for an autoimmune background for DH was raised based on mononuclear cell infiltrates in the liver (Speeti et al. 1998) and an aberrant expression of major histocompatibility complex (MHC) class II antigens on hepatocytes, correlating with the degree of inflammation in the liver (Speeti et al. 2003).

Since the etiology and pathogenesis of this devastating and rare disease have been unclear, its diagnosis and treatment have been difficult. Early diagnosis seldom occurs because dogs may long be asymptomatic and the clinical signs are non-specific for DH. Suspicion of DH can be raised when serum enzyme alanine aminotransferase (ALT) levels are elevated for several months. However, a liver sample is always required for diagnosis confirmation. Dobermans with DH present clinical signs late in the course of the disease and taking a liver biopsy is risky. To date, only supportive treatment options have been available, and despite treatment the prognosis is poor.

This thesis aimed at exploring supporting research-based evidence for the suspected autoimmune background of DH. Other goals were to identify possible genetic associations and serological biomarkers to help to diagnose this fatal disease earlier, to provide clues to the etiology and pathogenesis of the disease, and to provide a basis for the development of appropriate targets for therapy.

2 Review of the literature

2.1 Doberman hepatitis (DH)

The Doberman breed, particularly females, is at increased risk for the development of a distinct and severe form of progressive hepatitis and cirrhosis known as Doberman hepatitis (DH) (Crawford et al. 1985, Thornburg 1998, Speeti et al. 1999, Mandigers et al. 2004). DH was first described in the early 1980s by Meyer and colleagues (Meyer et al. 1980), but to date remains a disorder of unknown etiology and poorly understood pathogenesis. The condition usually affects middle-aged dogs, and the high incidence in the Doberman breed suggests a genetic predisposition. The best way of identifying dogs affected by subclinical DH (SDH) is routine laboratory measurement of serum liver-associated variables. A marked increase in the ALT serum level is detected before an increase in alkaline phosphatase (AP) (Speeti et al. 1996). For a definitive diagnosis of DH, a liver biopsy is recommended after obtaining several ALT-readings that are at least threefold above the upper reference limit (Speeti et al. 1998).

Macroscopically, the liver surface looks normal in SDH, but in the advanced clinical stage of DH (CDH), the whole liver is markedly distorted (**Fig. 1)**.

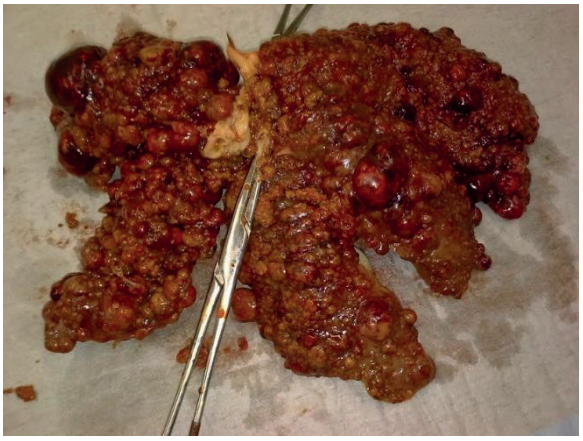

Figure 1 *An example of nodular cirrhosis in a postmortem CDH dog © Dyggve*

Histologically, DH is characterized by a predominant mononuclear inflammation in the parenchymal and portal areas of the liver and, as the disease progresses, by cirrhosis with features of scarring and bridging necrosis changes (**Fig. 2**) resulting in loss of liver function (Speeti et al. 1998, Terraciano et al. 2000). MHC class II antigens were shown to be expressed by the hepatocytes and in conjunction with the lymphocyte infiltration (Speeti et al. 2003).

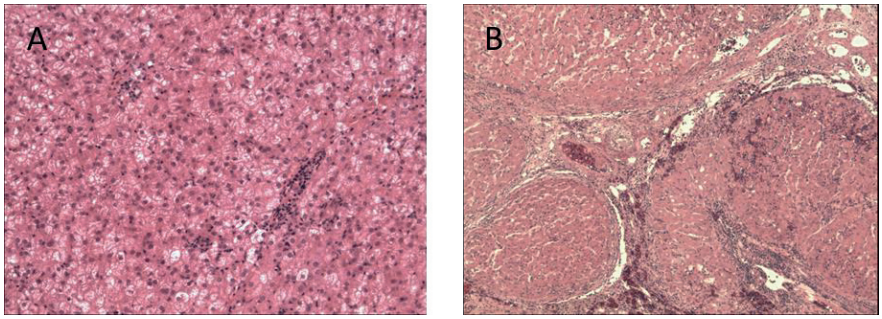

Figure 2 *Histopathological features of DH. (A) Moderate chronic diffuse portal and lobular lymphocytic hepatitis in a SDH dog. Magnification 100x. (B) Macronodular chirrosis in a CDH patient (same dog as in figure 1), magnification 40x. Courtesy of ELTDK/Patologia/Pernilla Syrjä.*

An increase in hepatic copper levels is detected (Van den Ingh et al. 1988, Speeti et al. 1998, Thornburg 1998). In healthy dogs, copper levels may be up to 500 µg/g dry weight (Rolfe & Twedt 1995, Van den Ingh et al. 2006). Copper levels may also be normal in DH (Crawford et al. 1985, Thornburg 1998, Speeti et al. 2003). An elevated copper concentration (up to 1000 µg/g dry weight) without histological findings of hepatitis has also been reported (Thornburg et al. 1990). In copper storage hepatopathy, as seen in Bedlington Terriers and West Highland White Terriers, the minimum copper level believed to cause damage is 2000 µg/g dry weight (Thornburg et al. 1990). In SDH, this level is generally less, ranging from 430 to 2157 µg/g dry weight (mean copper concentration 1097 µg/g dry weight) (Speeti et al. 1999) and copper accumulation appears to be associated with hepatic inflammation (Speeti et al. 1999, Mandigers et al. 2004).

The therapeutic management of DH has been challenging since the disease etiology remains obscure. Corticosteroids were found to decrease tissue inflammation and copper accumulation to some extent (Speeti et al. 1999), but a corticosteroid regimen could not prevent the progression of CDH (Crawford et al. 1985). One study demonstrated liver histopathology improvement in a group of five DH patients treated with D-penicillamine

for four months. D-penicillamine has metal chelator but also anti-inflammatory and immunosuppressive properties that could have improved the histopathology of the DH patients (Mandigers et al. 2005).

2.2 Overview of innate and adaptive immunity in the liver

The hepatic immune system controls the complex balance between immunity, tolerance, and autoimmunity in liver tissue (Doherty 2016). The liver also controls systemic innate immunity through the biosynthesis of various soluble pathogen-recognition receptors (PRRs) and complement components (Gao et al. 2008).

The liver is continuously challenged with different alimentary tract-derived harmless antigenic loads via the portal vein. The antigenic load may also comprise pathogenic infections, toxins, or malignant cells requiring fast immunosurveillance (Ivernizzi 2013). The liver must provide protection against transformed and metastatic liver cells and pathogens, while simultaneously tolerating harmless self and foreign antigens. This task is accomplished with various innate and adaptive immune cells specialized in detecting and capturing pathogens from the liver bloodstream (Jenne & Cubes 2013). The liver consists of parenchymal cells (hepatocytes and cholangiocytes) and non-parenchymal cells and in particular liver sinusoidal endothelial cells and hepatic stellate cells. The key effector cells for immunoregulation and defense in the liver tissue are lymphoid (B cells, $CD4^+$ and $CD8^+$ T cells, natural killer [NK] cells, natural killer T [NKT] cells, and innate lymphoid cells [ILCs]) and non-lymphoid cells (Kupffer cells [KCs] and dendritic cells [DCs]) (Ivernizzi 2013).

Innate immunity in the liver

Strong innate immunity predominates in the liver, with a large number of resident innate immunity cells situated in the organ, like Kupffer cells and NKs (Gao et al. 2008). Innate immunity is the initial and fast response to potentially damaging stimuli such as stress, pathogens, tissue injury, and malignant processes (Janeway & Medzhitov 2002).

Hepatocytes, DCs, NK cells, and KCs produce pro-inflammatory (increase inflammation) and anti-inflammatory (decrease inflammation) cytokines and chemokines that regulate inflammation and immunity in the liver. The hepatocytes and KCs detect microbial-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs) via signal sensing PRRs (Janeway & Medzhitov 2002). When MAMPs and DAMPs are bound, they are phagocytosed and broken down by the hepatocytes and

KCs without the pro-inflammatory mediator production that usually follows the PRR signaling. The rest of the body is thus protected from excessive immune activation by detoxification of gut-derived blood (Robinson et al. 2016).

Innate immune cells have been implicated to play a role in the pathogenesis of immune-mediated hepatic diseases with adaptive immunity mechanisms. The hepatic ILCs and NKT cells have potent immunomodulatory capacities (Baier & Mattner 2014). Evidence also suggests that innate immunity components are involved in liver fibrosis pathogenesis (Gao et al. 2008).

Adaptive immunity in the liver

In the liver, adaptive immunity consists of the humoral immune system derived from B lymphocytes and the cell-mediated immune system derived from T lymphocytes. This subsystem of immunity is composed of highly organized and tailored systemic cells and functions providing protection with diversity and with an immunological memory. B lymphocytes secrete antibodies, and therefore their response is very useful when diagnosing ALDs in people. The T lymphocytes involved comprise $CD4^+$ and $CD8^+$ T cells, and they are categorized to further subsets.

In human ALDs, the adaptive immunity targets hepatocytes and biliary epithelial cells (Shuai et al. 2016). Both innate and adaptive immunity mechanisms are involved in the pathogenic mechanisms of autoimmune hepatitis (AIH). In AIH, the liver damage has been suggested to be mediated by $CD4^+$ T cells under co-stimulatory signal activation. An autoantigen is recognized and presented within a human leukocyte antigen (HLA) class II molecule by an antigen presenting cell (APC) to a naïve $CD4^+$ T helper cell (Th0). Depending on the surrounding cytokine milieu and the autoantigen, Th0 may differentiate further to Th1, Th2, Th17 cells, and regulatory T cells (Tregs). These T cell subsets secrete different cytokines and are distinguished by their capability to induce either cellular (Th1) or humoral (Th2) immune reactions, therefore determining the subsequent immune responses (Kaiko et al. 2008). An association with chronic inflammation of autoimmune diseases has been suggested with Th17 cells (Harada et al. 2009). Upon encounter with an antigen, Th1 cells produce mainly interleukin-2 (IL-2) and interferon gamma (IFN-γ). The latter is associated with tissue damage, as it stimulates $CD8^+$ T cells, promotes the HLA class I expression, and elicits HLA class II expression on hepatocytes (Senaldi et al. 1991). IFN-γ may also activate monocytes and macrophage expression, releasing IL-1 and tumor necrosis factor alpha (TNF-α). IFN-γ enhances antigen presentation by APCs and augments Th1 development. Th2 cells make cytokines IL-4, IL-10, and IL-13, which help B cells maturate into plasma cells and produce autoantibodies. Th0 cells differentiate into

Th 17 cells in the presence of transforming growth factor beta (TGF-β) and IL-6 and produce IL-17, IL-22, TNF-α, and chemokine ligand 20 (Bettelli et al. 2006). Th17 cells have been associated with AIH (Zhao et al. 2011) and the pathogenesis of primary biliary cholangitis (PBC) (Harada et al. 2009). Tregs are T lymphocytes that produce immunosuppressive cytokines IL-10 and TGF-β and promote liver tolerance. An impairment of Tregs may play a role in liver cell destruction, resulting from cytotoxic T cell action, Th1 cytokine release and Th17 cells, recruited monocytes, and macrophages, complement cascade activation or engagement of NK cells by the autoantibody on the hepatocytes (Liberal et al. 2015).

Molecular mimicry and adaptive immunity responses are suggested to have a role in PBC development. An immune attack against bile ducts, involving anti-mitochondrial antibodies (AMAs), $CD4^+$, and $CD8^+$ T cells, and defective Tregs, is probably involved. A cytokine bias toward Th1/Th17 is indicative of immune activation (Webb et al. 2015). The damaged biliary epithelial cells express MHC class I and II molecules and are considered to present antigens to cytotoxic $CD8^+$ and helper $CD4^+$ T cells in the liver (Van den Oord et al. 1986). An infectious antigen within the MHC class II molecule can induce activation of naïve autoreactive T cells, which recognize peptides derived from foreign and self-antigens causing PBC (Cusick et al. 2012).

There is evidence that an altered immune response to pathogens in the pathogenesis of primary sclerosing cholangitis (PSC) exists. After an antigenic stimulus, cholangiocytes release pro-inflammatory mediators, further stimulating immune cells. An inflammatory environment for cholangiocytes is maintained by the interplay between Toll-like receptors (TLRs) and pathogen-associated molecular patterns (PAMPs). The activated TLRs can elevate the expression of IL-6 and promote adhesion molecule release (Xu et al. 2002). Cholangiocytes and immune cells can release TNF and IL-6 and IL-8 and further promote the activation of T cells, macrophages, NK cells, and neutrophils (Liaskou et al. 2014). Evidence has accumulated for an association between the gut microbiota and PSC, and the immune reaction in the liver being connected to an immune response in the gut (Tabibian et al. 2014). Recently, a portion of memory T cells in the gut and the liver was shown to be of the same clonal origin, reacting to similar triggers. This portion was especially high in patients with PSC and concomitant inflammatory bowel disease (IBD) (Henriksen et al. 2016).

2.3 Autoimmune disease

2.3.1 Definition of autoimmune disease

An autoimmune disease is a condition in which tissue damage is induced by an abnormal T cell or antibody reaction to a normal body part. The contemporary characteristics for a human autoimmune disease are divided into direct and indirect evidence and circumstantial proof such as that described in Witebsky´s modified postulates (Rose & Bona 1993, Rose & McKay 2014a). The direct evidence comprises an autoimmune reaction reproduced in a normal recipient by transferring a pathogenic antibody, whereas the indirect evidence is that a similar condition is reproduced in experimental animals. Many human diseases do not meet direct or indirect criteria, but are characterized by the word "autoimmune" if they have a strong circumstantial support. An autoimmune disease can be suspected when a patient has an abnormal expression of MHC class II antigens on the affected organ, an association with MHC class II genes, a lymphocytic infiltration of the target organ, or high levels of relevant serum autoantibodies. Other meaningful factors include a favorable response to immunosuppressants, female gender bias, positive family history of the disease, or concurrent association with other autoimmune diseases in the same patient or their family.

2.3.2 Human autoimmune liver diseases (ALDs)

In people, the main three types of ALDs are autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) (formerly called primary biliary cirrhosis), and primary sclerosing cholangitis (PSC). These variant conditions can overlap. The target of the autoimmune attack is hepatocytes in AIH and bile ducts in PBC and PSC. The clinical presentation and the serological and histological features have distinct characteristics (Washington 2007). The underlying pathogenic mechanisms in ALDs remain poorly understood (Grant & Liberal 2016). A complex genetic background has been implicated in ALD pathogenesis. In all of these conditions, treatment aims to reduce inflammation and prevent cirrhosis.

2.3.2.1 Autoimmune hepatitis (AIH)

AIH is a progressive inflammatory disease, first described in a group of young women in the 1950s (zum Büschenfelde 2003). The initial trigger of liver autoimmunity remains elusive. Female patients are more predisposed than males, but the disease can manifest in both genders and affect all ages.

The clinical presentation of AIH can differ from mild or severe manifestation to fulminant hepatic failure (Krawitt 2006). AIH is further classified into AIH-1 and AIH-2, depending on the produced autoantibody patterns. In general, other abnormal serological characteristics found in AIH include elevated ALT, aspartate aminotransferase (AST), and IgG, except in children, the elderly, or the fulminant cases where IgG can be normal (Zachou et al. 2013). Typical histological characteristics of AIH is interface hepatitis with with a portal and periportal monocellular infiltrates, and clusters of plasma cells. Fibrosis is detected in all but the mildest variants of the disease. Recently, emperipolesis and rosette formation were found to be even better histological predictors of AIH than the classical histological features of interface hepatitis and plasma cells (de Boer et al. 2015).

AIH remains a therapeutic challenge and, if left untreated, usually progresses to liver failure, requiring transplantation (Manns et al. 2015). The conventional treatment regime for AIH is corticosteroids with or without azathioprine (European Association for the Study of the Liver 2015). Approximately 10% of the AIH patients do not respond to standard treatment (Gleeson & Heneghan 2011), and this is more common in AIH-2 than AIH-1 patients (Zachou et al. 2013). Alternative immunosuppressive treatment options, such as cyclosporine for the replacement of azathioprine (Malekzadeh et al. 2001) and budesonide for the replacement of prednisone (Manns et al. 2015) and mycophenolate mofetil (Park et al. 2016), have been attempted with an encouraging outcome.

2.3.2.2 Primary biliary cholangitis (PBC)

PBC is a slowly progressing chronic cholestatic autoimmune disease that destroys the biliary epithelial cells in the liver. The exact cause of PBC remains unidentified, but the loss of liver tolerance is suggested to originate from environmental exposure to xenobiotics, which in combination with a genetic predisposition leads to an immune attack (Bowlus et al. 2016). Middle-aged women are more likely to develop PBC than men (Kaplan & Gerswin 2005). The PBC diagnosis is based on at least two of three of the

following characteristics: the presence of serum AMAs, elevation of liver enzymes, and in particular serum AP, and histologic findings in the liver that are compatible with the presence of lymphoid infiltrate and injury in bile duct walls. Ursodeoxycholic acid (UDCA) is approved for the treatment of PBC, but the response can vary. The UDCA nonresponders have an increased risk of disease progression and are more likely to require liver transplantation (Selmi et al. 2011).

2.3.2.3 Primary sclerosing cholangitis (PSC)

PSC is a chronic cholestatic and biliary tract liver disease. The majority of patients are male and are diagnosed at approximately 40 years of age, but PSC may occur also in children (Hirschfield et al. 2013). The disease progresses slowly, and the clinical course can vary from asymptomatic to fluctuating symptoms or symptomatic. Many PSC patients are diagnosed with IBD, most of which have ulcerative colitis (Ponsioen 2013). The PSC diagnosis is based on clinical, laboratory, and image findings. Laboratory results can include elevated AP and ALT (Karlsen et al. 2014). Most PSC patients have evidence of autoantibodies. In the early stages of PSC, a liver biopsy can be nonspecific and not informative to the diagnosis (Rabiee & Levy 2014). As the disease progresses, intra- and extrahepatic bile duct stricture and gradually cirrhosis and liver damage occur (Hirschfield et al. 2013). Magnetic resonance cholangiopancreatography has been regarded as the first degree diagnostic step for diagnosis of PSC (Dave et al. 2010) but the gold standard for visualizing the biliary tract and treating extrahepatic biliary obstruction is endoscopic retrograde cholangiopancreatography (Tischendorf et al. 2007). A liver biopsy can be helpful with suspected small duct PSC or when excluding an overlapping AIH condition (Björnsson et al. 2002). UDCA is used as a medical option, but, unfortunately, to date, no established medical treatment has proven to prolong survival in PSC, and liver transplantation remains the only viable modality (Tischendorf et al. 2007). PSC patients with elevated immunoglobulin G4 (IgG4) levels have a significant response to corticosteroid treatment (Karlsen et al. 2014).

2.4 Major histocompatibility complex (MHC) class II

2.4.1 Overview of MHC class II genes

The MHC class II gene region is very polygenic and polymorphic.The human MHC class II gene region is located on chromosome six and is referred to as the human leukocyte antigen (HLA) class II region. Three loci, HLA-DR, HLA-DQ, and HLA-DP, exist in humans (Rock 2016). The dog MHC class II is located on chromosome 12 and is termed the dog leukocyte antigen (DLA) class II region. This region includes the loci DLA-DRA1, DLA-DRB1, DLA-DQA1, and DLA-DQB1.

A genetic feature of the MHC is extensive linkage disequilibrium (LD) across the complex compared with the rest of the genome (Matzaraki et al. 2017).

2.4.2 Role of MHC class II genes in antigen presentation

Because MHC class II genes are very polymorphic and polygenic, they create a large MHC class II molecular diversity. MHC class II genes express high levels of intraspecies variation in the peptide-binding regions, which are within exon 2 of the MHC class II genes (Meyer & Thomson 2001). MHC class II genes are crucial in shaping the T cell repertoire. The antigen bound and presented by an MHC class II molecule is determined by the MHC class II binding site structure. The nature of the peptide-binding groove is, therefore, important for disease susceptibility. This diversity is essential in maintaining homeostasis and tolerance of the immune system. The expression of MHC class II is tightly regulated and necessary for a proper functioning adaptive immune system. MHC class II proteins are usually constitutively expressed on thymic epithelial cells and professional APCs such as DCs, macrophages, and B cells. Under inflammatory signals, MHC class II expression may also be induced on cells other than immune cells (Ting & Baldwin 1993). T cells are unable to act directly with structures beneath the cell surface of APCs, but can recognize their antigens bound to MHC class II molecules. MHC class II molecules present peptide fragments that are derived from endocytosed extracellular molecules, but also from cytosolic self-proteins (Suri et al. 2006). Cytoplasmic particles are degraded by autophagy, and the antigens are then presented to $CD4^+$ T cells by MHC II molecules (Münz 2016). MHC class II molecules also determine which antigenic peptides an individual can present to naïve $CD4^+$ T lymphocytes and stimulate an immune

response (Brown et al. 1988). The MHC class II molecule density on the cell surface is crucial for antigen presentation and the intensity of the immune response (Murphy et al. 2008a).

It has been suggested that an abnormal MHC class II expression by parenchymal cells could cause autoimmune diseases. In humans, aberrant MHC class II expression is seen on the cell surface of bile duct epithelium in PBC and PSC (Ballardini et al. 1984, Broomé et al. 1990).

2.4.3 Human leukocyte antigen (HLA) class II gene association in ALDs

HLA class II has been associated with ALDs (Shiina et al. 2004). ALD studies suggest that HLA region alleles are strongly associated with both AIH and PSC and relatively weakly with PBC. HLA haplotypes and alleles have been associated with both risk and protection from ALDs.

AIH is strongly associated with HLA alleles DRB1*0301 and DRB1*0401 (Hennes et al. 2008). HLA-DRB1*0701 allele has been linked with a more aggressive form of AIH and a worse overall prognosis (Rose & MacKay 2014b). In PBC, T cells with risk-conferring allele HLA-DRB1*0801 demonstrated a high-affinity response towards a dehydrogenase subunit, and this response did not occur with subjects with a protective allele DRB1*1101 (Chow et al. 2013). The most associated susceptibility MHC class II haplotypes for PSC are HLA-DRB1*1501-DQB1*0602, HLA-DRB1*1301-DQB1*0603, and HLA-DRB1*0301-DQB1*0201 (Rose & MaKay 2014c), while haplotypes HLA-DRB1*04-DQB1*0302 and HLA-DRB1*0701-DQB1*0303 have been reported to confer protection (Ferri et al. 2016).

Although the HLA class II association is the most thoroughly described genetic predisposition to ALDs, genome-wide association studies have identified other risk loci outside the HLA area in AIH (de Boer et al. 2014).

2.4.4 Dog leukocyte antigen (DLA) class II genes

The DLA gene region is well characterized and has been demonstrated to be highly polymorphic (Kennedy et al. 1999). DLA class II molecules are expressed as heterodimeric transmembrane glycoproteins consisting of α and β polypeptides. The DLA-DQA1 and DLA-DRA1 genes encode the α chains, and genes DLA-DQB1 and DLA-DRB1 encode the β chains. The α and β chains both consist of two domains (α1, α2 and β1, β2). The α1 and β1 domains form a groove that functions as an antigen or peptide-binding site (**Fig. 3**). This groove structure is encoded by DLA class II gene exons 2 and is open at both ends, which allows binding of long peptide antigens. The dog DRA locus seems to be functionally monomorphic (Wagner et al. 1995), and therefore, the allele DLA-DRB1 is important to the DR heterodimer structure. Otherwise, the DLA heterodimers show a large genetic variation. The amino acid polymorphism is clustered in and around the antigen-binding site, shaping the groove and affecting antigen recognition, binding, and presentation.

DLA class II haplotypes and alleles have been associated with disease susceptibility in immune-mediated diseases in dogs. These include, for example, diabetes mellitus (Kennedy et al. 2006a), canine rheumatoid arthritis (Ollier et al. 2001), lymphocytic thyroiditis (Kennedy et al. 2006b & c), immune-mediated hemolytic anemia (Kennedy et al. 2006d), hypoadrenocorticism (Hughes et al. 2009), systemic lupus erythematosus (SLE) (Wilbe et al. 2009), exocrine pancreatic insufficiency (Tsai et al. 2013), and chronic superficial keratitis (Jokinen et al. 2011).

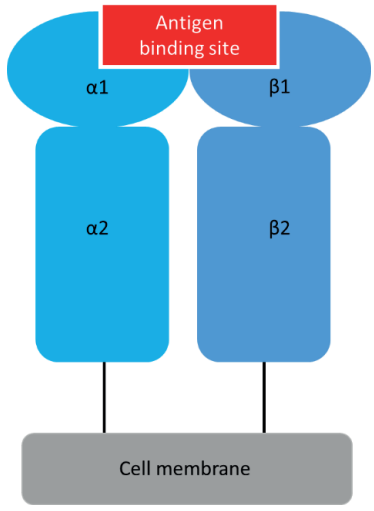

Figure 3 *DLA class II as a schematic illustration.*

2.5 Promoters in transcriptional control of MHC class II genes

The regions of the MHC class II promoter in the regulation of MHC class II expression is well described in mice and men. MHC class II gene expression is mainly controlled at the transcription level with the proximal MHC class II promoters, transcription factors, and an essential co-activator, the MHC class II trans-activator (CIITA, also called MHC2TA) (Berggren & Seddon 2005).

The MHC class II promoter comprises conserved DNA sequences, containing a W-box (including the S-box), the X-box (including the overlapping boxes X1 and X2), the Y-box, and some locus-specific DNA sequences like the T-box of the DQA promoter (Benoist & Mathis 1990, Ting & Trowsdale 2002, Berggren & Seddon 2005) (**Fig. 4**). An enhanceosome complex assembles on specific areas of the proximal promoter. These areas have been recognized within the promoters, as CIITA is recruited onto MHC class II promoters by protein-protein interplay with many components of an enhanceosome complex (Leimgruber et al. 2009). Once bound, CIITA interacts with basal transcription machinery components and initiates transcription of the target genes (**Fig. 4**).

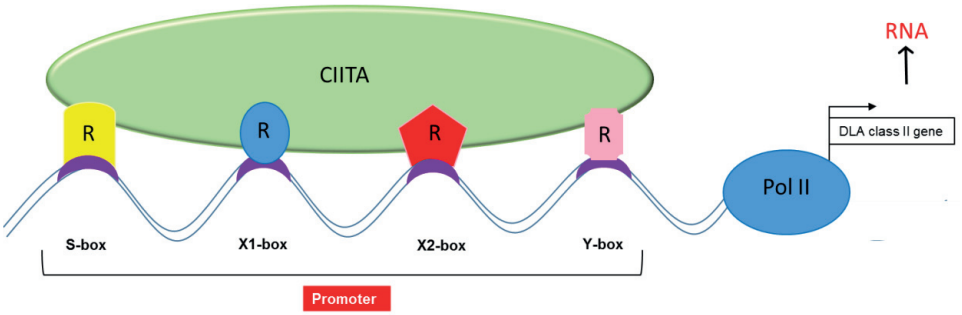

Figure 4 *A schematic representation of the MHC class II gene transcription regulation. The MHC class II promoter region is recognized by different regulatory factors (R) that bind to the boxes S, X1, X2, and Y. This enhanceosome complex recruits the master regulator CIITA by protein-protein interaction. Transcription of the gene is initiated once CIITA is bound. Modified from Handunnetthi et al. (2010).*

Transcription of MHC II genes is under complex regulation, thus also making it a target for genetic variations. Polymorphism in the HLA promoter or enhancer areas has been described in vitiligo and SLE, both regarded as autoimmune diseases in humans (Caillat-Zucman 2017). Mutations at the W-box led to decreases in CIITA binding (Muhlethaler-Mottet et al. 2004). A deletion or replacement of the X or Y box reduces the MHC class II expression in B cells (Tsang et al. 1990), while deletion of the S-box resulted in a minor reduction in expression (Tsang et al. 1988). Variation of a few base pairs in the S-X region was shown to affect HLA-DQB1 expression (Beaty et al. 1995). Genetic polymorphism of the HLA-DQB1 X-box was linked with changes in protein binding pattern and activity in transcription (Andersen et al. 1991). X-box polymorphism has an effect on the level of expression of HLA-DRB1 genes (Singal & Qiu 1996). Y-box polymorphism was demonstrated to control the cytokine-mediated expression of HLA-DRB1 genes (Sindwani & Singal 2001).

2.6 Autoantibodies and autoantigens

Antibodies that react towards self-proteins are referred to as autoantibodies. Normally, the immune system has the capacity to discriminate self-proteins from non-self. To maintain self-tolerance and prevent autoantibody production, autoreactive B cells are eliminated at two checkpoints. The first checkpoint is the bone marrow, where many autoreactive B cells are killed or become anergic as they mature (MacKay et al. 2000). In the peripheral second checkpoint, the actions are based on defective activation signals given to the lymphocyte when it encounters a self-antigen. Usually, this leads to anergy or apoptosis of the autoreactive B cells (Mackay et al. 2000).

At times, the immune system fails to identify the body´s own harmless constituents, leading to pathological autoantibody production. Autoantibodies may directly bind and injure target organs in an organ-specific autoimmune disease such as myasthenia gravis, pemphigus (Elkon & Casali 2008), and celiac disease (CD) (Kalliokoski et al. 2015).

Many autoantibodies are associated with the development of an autoimmune disease, although most autoantibodies are not described to be pathogenic. Autoantibodies have important roles in the study of autoimmune disease processes (Van Gaalen et al. 2005). Autoantibodies might be elevated long before the onset of the autoimmune disease, and specific autoantibody profiling is a biomarker and an opportunity for diagnosis. Autoantibodies are also used to monitor disease progression in humans (Gregorio et al. 2002). IgG autoantibodies with a high-affinity towards self-antigens can reflect pathologic processes, where disturbed functions in cell clearance, antigen-receptor signaling, or cell effector functions occur (Elkon & Casali 2008).

Many autoantigens are enzymes whose active site is often targeted by autoantibodies (Banga & McGregor 1991). What triggers the immune system to react to autoantigens remains a mystery. There may be several triggering factors that result in loss of natural tolerance and induction of autoantibody formation. Possible triggers for autoimmune responses may be provided by impaired apoptosis and clearance deficiency, molecular mimicry with microbial antigens, and post-translational modification of self-proteins, resulting in immunological cross-reactive responses to homologous autoantigens.

Evidence indicates impaired clearance of apoptotic cells in SLE. This deficit was suggested to participate in chronic inflammation formation by providing intitating autoantigens and triggering an autoimmune reaction towards nuclear antigens (Mahajan et al. 2016). Another mechanism is molecular mimicry, where a foreign antigen shares sequence or structural similarities with self-peptides, as suggested in PBC (Cusick et al. 2012). In CD, transglutaminase-2 (TG-2) is a target autoantigen. TG-2 is capable of catalyzing post-translational modification via deamidation of glutamine from gliadin.

Deamidation augments the affinity and binding of gliadin peptides towards HLA. This process elicits a stronger antigen presentation and inflammation and results in secretion of CD-specific autoantibodies towards TG-2 (Molberg et al. 1998).

2.6.1 Role of autoantibodies and autoantigens in human ALDs

Based on the different serological patterns, AIH can be subdivided into two variants: AIH type-1 with high anti-nuclear antibodies (ANA) and/or smooth muscle antibodies (SMA), and AIH type-2 with high titers of antibodies towards liver-kidney microsome type-1 (anti-LKM-1) or antibodies against liver cytosol type-1 (Zachou et al. 2004). Antibodies to soluble liver antigen in AIH-1 are associated with a more severe disease, therefore having prognostic value (Czaja & Manns 2010). These autoantibodies are not pathognomic for AIH, but comprise an essential part of the diagnostic workup. Various antibodies with limited importance have been reported in AIH, including autoantibodies to histone (Chen et al. 1998).

Most of the liver autoantigens in human ALDs are enzymes of key importance for natural cell homeostasis (Bogdanos & Dalekos 2008). Some of the main target-autoantigens in AIH are active enzymes of the human hepatic and non-hepatic xenobiotic metabolism. The recognized target autoantigens in AIH-1 include alpha-enolase, actin, catalase, cathepsin G, lactoferrin, and high-mobility group non-histone chromosomal proteins (Zachou et al. 2004). A member of the hepatic P450 enzyme family, cytochrome P450 2D6, has been recognized as the major target-autoantigen in AIH-2 (Dalekos et al. 2002).

In PBC, AMAs are directed against a mitochondrial part, pyruvate dehydrogenase complex, named lipoic acid (Selmi et al. 2011).

PSC is associated with multiple autoantibodies, but none of them allows the diagnosis alone. There is evidence of antinuclear cytoplasmic antibodies, anti-Saccharomyces cerevisiae antibodies, SMA, ANA, and also anti-LKM1 in patients with PSC (Hov et al. 2008). Recently, an association was found between antibodies against glycoprotein 2 and large bile duct diseases, with poor patient survival (Jendrek et al. 2017).

2.6.2 Anti-histone antibody (AHA)

Histones are a group of alkaline intracellular proteins found in all eukaryotic cell nuclei. They arrange and pack reversible complexes with DNA into nucleosome structures. Histones are extensively modified after protein biosynthesis and are therefore significant in gene regulation (Shechter et al. 2007).

In humans, high AHA levels are described in different autoimmune conditions such as AIH-1 (Chen et al. 1998, Czaja & Manns 2010), PBC (Penner 1987), rheumatoid arthritis (RA) (Sokolove et al. 2002), and induced or spontaneous SLE (Brinet et al. 1988). Anti-histone antibodies have been reported to be common in dogs with SLE (Bremer et al. 2015).

Histone release has been suggested to have a role in autoimmune disease (Chen 2014). A recent study showed that when histones were released into extracellular space by damaged and activated cells, a significant pro-inflammatory or even toxic effect of histones followed (Allam et al. 2014). In SLE, histone is not only a direct autoantigen but may also augment the destructive autoimmune processes (Yu & Su 2013).

2.6.3 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) autoantigen

GAPDH is necessary for the welfare of all normally functioning cells, as it plays a major role in glycolysis (Quail & Yeoh 1995). Besides adenosine triphosphate (ATP) production, GAPDH displays distinct properties when linked to different cellular localizations. It is also localized in polyribosomes (Nagy & Rigby 1995), the Golgi, the nucleus, the endoplasmic reticulum, and the cell membrane (Tristan et al. 2011). It takes part in iron metabolism (Quail & Yeoh 1995), histone biosynthesis (Zheng at al. 2003), and execution of cell death in oxidative stress when located in the nucleus (Hara et al. 2006). Levels of GAPDH in mitochondria rise in stress conditions and GAPDH controls the permeabilization process of the pro-apoptotic apoptosis (Tarze et al. 2007). Other proposed functions include intracellular membrane trafficking (Tisdale 2001), receptor-mediated cell signaling (Harada et al. 2007), and DNA integrity maintenance in oxidative stress (Azam et al. 2008).

Anti-GAPDH IgG was demonstrated in the cerebrospinal fluid of multiple sclerosis (MS) and SLE patients. In MS, the antibodies are suspected to inhibit glycolysis and promote the degeneration of neuron axons (Kölln et al. 2010). Anti-GAPDH autoantibodies were demonstrated in 47% of SLE patients, and GAPDH was proposed to

be both a nuclear and cytoplasmic target autoantigen (Takasaki et al. 2004). One suggestion is that the autoimmune response would be towards foreign GAPDH through molecular mimicry, as this enzyme is also present on the cell surfaces of bacteria, viruses, and parasites (Takasaki et al. 2004). GAPDH in Brucella may induce the formation of anti-GAPDH antibodies in sheep, cattle, and mice (Rosinha et al. 2002).

Anti-GADPH autoantibodies have been found in Dobermans with dilated cardiomyopathy (DCM). The Doberman breed is predisposed to DCM, and the prognosis in this breed is less favorable than in other breeds. An autoimmune origin for DCM has been speculated (Buse et al. 2008). The fact that some Doberman dogs develop DCM but others DH suggests that additional factors in the development of immune disease are needed.

2.6.4 Alcohol dehydrogenase (ADH) autoantigen

ADHs are a group of enzymes, each member having a specific role and function corresponding to their different form. ADH enzymes are present in the liver, gastrointestinal tract, and kidney. The important function of ADH is oxidation of endogenous alcohol produced by gut micro-organisms, ethanol or alcohols from the diet, or substrates from steroid and bile acid metabolism (Cederbaum 2012). The active enzyme forms a dimeric molecule composed of different subunits. Each ADH monomer has a structural zinc domain that maintains structural stability by binding a co-enzyme, and a zinc-binding domain involved in catalytic events (Höög & Ostberg 2011). Patients with an alcoholic liver disease have elevated levels of anti-ADH autoantibodies that correlate with disease severity. ADH was also demonstrated as a target antigen in AIH (Lin et al. 2013).

Dogs benefit from ADH enzymes when metabolizing, for example rotten fruit and when dealing with gastrointestinal flora fermentation. It was also suggested that ADH enzymes developed in dogs to participate in novel metabolic pathways involved in the metabolism of catecholamines, bile acids, arachidonic acid, serotonin, histidine retinoids, and leukotrienes (Hernández-Tobías et al. 2011).

2.6.5 Open questions in Doberman hepatitis

Neither the etiology nor the pathogenesis of Doberman hepatitis are known. Both copper accumulation and autoimmune background have been suggested. Knowing the etiology would be important for the diagnosis and treatment of the disease. Dobermans with DH present clinical signs late in the course of the disease and the current diagnostic tests are nonspecific indicators of liver damage.

Upregulation of MHC class II antigens in the liver suggests an immune system involvement in the disease, but before this thesis work no clear indication of autoreactivity in DH has been reported. Therefore this work aimed at exploring a possible autoimmune background of DH. For this, possible genetic associations, novel autoantibodies and serological biomarkers were searched for both to shed light on the pathogenesis and to help to diagnose the disease at an early stage.

3 Aims of the study

The overall objective of our study was to explore supporting evidence for the suspected autoimmune background of DH and to elucidate the disease pathogenesis. Further aims were to identify possible genetic associations and serological biomarkers to facilitate the diagnosis of this fatal disease.

Specific hypotheses examined were as follows:

1. DLA class II antigens are associated with DH.

2. A variation in promoter areas of homozygous DLA-DRB1*00601/DQA1*00401/DQB1*01303 Dobermans explains why some dogs become affected with DH and others do not.

3. DH patients have circulating serum anti-nuclear autoantibodies.

4. Liver-related autoantigens are associated with DH.

5. Autoantibodies could be used as diagnostic biomarkers for DH.

6. The finding of autoantibodies could provide clues to the etiology and pathogenesis of the disease.

4 Summary of material and methods

4.1 Animals and samples (I-IV)

In total, 53 Dobermans with a diagnosis of SDH or CDH were included in the two case groups (**Fig. 5**). Thirty SDH dogs were clinically healthy and had been monitored for several months, with repeatedly elevated ALT of at least three times the upper reference limit (18–77 U/L). Twenty-three CDH cases presented with hepatitis symptoms and markedly elevated ALT, AP and bilirubin values. The mean ALT concentration was 783.4 U/L (range, 256-1575 U/L) in SDH group and 851.4 (range, 470-1157 U/L) in the CDH group (reference range 18-77 U/L). The mean ALP concentration in the SDH group was 622 U/L (range, 134-1797 U/L) and 1711 U/L (range 403-5347 U/L) in the CDH group (reference range 33-215 U/L). Follow-up samples were collected from nine SDH patients during a period of seven months to up to four years (mean 18 months, median 12 months).

The DH diagnosis was confirmed in all DH dogs by histopathological examination with hematoxylin and eosin stains demonstrating mononuclear cell infiltration in the parenchymal and portal areas of the liver and distinct degrees of increased fibrous tissue and necrosis. Among the SDH dogs, eight had slight fibrosis and of these dogs, mild bridging necrosis was found in seven and one had multifocal apoptotic cells. Eleven SDH dogs were euthanized for reasons other than DH, and the postmortem liver histology remained consistent with SDH. Hepatocyte necrosis and moderate to marked fibrosis was present in the CDH cases. The liver samples were taken postmortem from all CDH dogs.

Rubeanic acid stains revealed excessive copper in all DH dogs, based on granular cytoplasmic staining in the hepatocytes. Copper accumulation was located in the centrolobular and parenchymal area and associated with inflammation. The copper was scored with a classification from 0 to 5, as earlier described by van den Ingh et al. 1988. In the SDH group, four dogs were grade 1, 11 dogs grade 1-2, 14 dogs grade 2, seven dogs grade 2-3, and four dogs grade 3. In the CDH group, three dogs were grade 1, two dogs grade 2, 11 dogs grade 2-3, seven dogs grade 3, and two dogs grade 4. The copper was associated with inflammation focally in the centrilobular region in SDH dogs. In CDH dogs, copper was mainly associated with inflammation in periportal and bridging necrosis areas. Quantification of copper with flame atomic absorption spectrometry was available for 11 SDH dogs with a median of 792 µg/g dry weight liver (dwl) (range 430-1886 µg/g dwl) and seven CDH dogs with a median of 1490 µg/g dwl (range 630-2430 µg/g dwl).

To establish a control group, ethylenediaminetetra-acetic acid (EDTA) -preserved blood and serum samples were collected from healthy Dobermans with no

immunosuppressive medication or signs of liver failure. In total, blood samples were collected from 58 dogs, 46 of which were included in the studies. The remaining 12 dogs were excluded because of abnormal ALT readings. The diagnostic evaluation of the control Dobermans consisted of a complete physical examination by the same examiner (HD), hematology, serum biochemistry, and urinalysis at the clinical University Small Animal Hospital, Faculty of Veterinary Medicine, Helsinki, Finland. Histopathological confirmation of the absence of DH was obtained for 4 of 17 (4/17) control dogs.

Whole venous blood in EDTA, serum and biopsy samples were gathered from client-owned Finnish Dobermans during 1981–2010 and stored at −20°C. For the analysis, the samples were thawed and analyzed on the same day. The dogs did not receive immunosuppressive medication before sampling.

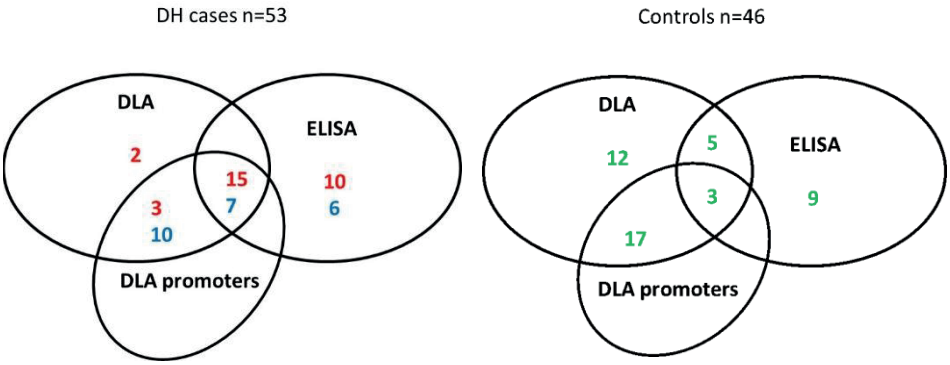

Figure 5 *Overlapping DH cases (n=53) and controls (n=46) in ELISA, DLA, and DLA promoter studies (I-IV). SDH cases are indicated in red, CDH cases in blue, and controls in green.*

4.1.1 Selection of dogs for the DLA study (I)

For the DLA study, 20 SDH and 17 CDH dogs were included in the case groups. In the SDH group, DNA was extracted from 18 liver and two EDTA blood samples. For the clinically ill dogs, DNA was extracted from liver tissue taken postmortem.

As the control samples, EDTA-preserved blood samples were included in the study from 37 healthy Dobermans aged over ten years. Samples from dogs aged over ten years were collected to have the lowest possible genetic risk for DH. Based on the literature review, the youngest reported DH patient was aged 1.5 years, and the oldest was 11 years (Crawford et al. 1985, Fuentealba et al. 1997).

The genealogical relationships were examined with pedigree analysis using GenoPro genealogy software (www.genopro.com) to ensure that the dogs were not closely related. The pedigrees were obtained from the Finnish Kennel Club's pedigree database KoiraNet (www.jalostus.kennelliitto.fi).

4.1.2 Dogs for the promoter analysis (II)

Fifty-five client-owned Finnish Dobermans, all homozygous for haplotype DLA-DRB1*00601/DQA1*00401/DQB1*01303, were studied. Haplotype data for these individuals were available from Study I. The case group comprised 35 SDH and CDH dogs. Sixteen liver samples and two EDTA blood samples were obtained from dogs with SDH. Seventeen liver samples from CDH dogs were collected postmortem.

As controls, EDTA blood samples from 20 healthy Doberman dogs were enrolled in the study. Control dog inclusion criteria, pedigree information, and sample storage were as described in Study I.

4.1.3 Samples for the indirect immunofluorescence (IIF) assay (III)

For immunohistochemical stainings, serum specimens from eight SDH (seven female and one male) and two CDH (one female and one male) dogs in the case group and ten healthy Doberman control dogs (six females and four males) were evaluated.

4.1.4 Samples for the Line immunoassay (III)

The Line immunoassay included serum samples from nine SDH (seven female and two male) and one CDH (female) dog in the case group and ten healthy Dobermans (six females and four males) in the control group.

4.1.5 Samples for the Western blot assay (IV)

Ten DH and ten control Doberman samples were studied. In the DH group, one CDH dog was clinically ill and euthanized because of advanced liver failure, and nine dogs had

SDH. Also, follow-up serum samples from the nine SDH dogs were included. A liver sample for tissue lysate preparation was obtained from a subclinical DH patient, euthanized for reasons other than DH.

4.1.6 Mass spectrometry for proteomics (IV)

Serum specimens from one female SDH and one control Doberman dog were employed. A liver tissue sample was gained from a female SDH patient, euthanized for reasons other than DH.

4.1.7 Samples for enzyme-linked immunosorbent assay (ELISA) (III, IV)

Serum samples from 25 SDH (20 female and five male) and 13 CDH (11 female and two male) dogs were enrolled in the case group. Each CDH patients had one serum sample included in the study. The median age in the SDH group was 4.9 (range 1.9-8.6) years. In the CDH group, the median age at diagnosis was 6.2 (range 2.5-10.3) years. Seventeen clinically healthy Dobermans (ten females and seven males) were included in the analysis. The median age of the controls was 8.2 (range 2.7-13) years.

4.2 Ethical considerations

Approval from the National Ethics Committee for Animal Experiments in Finland (Licence number ESLH-2009-08997/Ym-23) was obtained for the use of liver biopsies and blood samples from client-owned healthy Dobermans. The owners of both prospectively recruited patients and healthy control Dobermans provided written consent for participation in the study.

4.3 Molecular biology methods (I-IV)

The methods used are described in more detail in the original publications (**I-IV**) attached to this thesis.

4.3.1 DNA isolation (I, II)

Genomic DNA was extracted from liver samples by using the QIAamp® DNA Mini Kit for tissues (Qiagen, Valencia, CA, USA) or from EDTA blood samples with the Puregene® DNA kit (Gentra Systems Inc., Minneapolis, MN, USA). The DNA concentrations were determined with a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the samples were adjusted to 10 ng/µl for polymerase chain reaction (PCR). The DNA samples were stored at -20°C until the next study phase.

4.3.2 PCR and sequencing (I, II)

Study I: DLA loci were amplified with specific exon 2 primers for DLA-DRB, -DQA, and -DQB using a standard PCR protocol and primer sequences as detailed in the "Material and methods" section of Study I.

Study II: The promoter regions for DLA-DRB, -DQA, -DQB, and -DRA were amplified, and PCR was performed as detailed in the "Material and methods" section of Study II. The Primer3 program (http://frodo.wi.mit.edu/primer3) was used to design primers for DLA-DRA with the HLA-DRA as the reference because of the similarity of conserved elements across species.

The PCR products for both studies were purified by EXOsap-IT (USB Corporation, Cleveland, Ohio, USA) and sequenced with an ABI 3730 xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

4.3.3 DLA allele identification (I)

The resulting sequencing data were manually analyzed and compared with an existing consensus sequence in the MatchToolsNavigator program (Applied Biosystems). The corrected sequence was evaluated against a reference sequence library (http://www.ebi.ac.uk/ipd/mhc/index.html) to obtain the DLA haplotypes. Previous knowledge of the DLA allele combinations in Dobermans was used to facilitate the construction of the haplotypes.

4.3.4 DLA promoter allele assignment (II)

To identify different DLA promoter alleles, the sequences were compared with an existing consensus sequence from the Ensembl database (http://www.ensembl.org/index.html) for DLA-DRB, -DQA, and -DQB. The 5´regulatory region of the DLA-DRA gene in dogs was compared with the known promoter sequence in humans and the reference sequence in dogs (**Fig. 8**).

4.3.5 Indirect immunofluorescence (IIF) (III)

The presence of serum ANA was analyzed by IIF on mouse liver cryostat sections. The serum specimens were diluted to 1:10, 1:40, and 1:160. Rabbit anti-dog IgG antibody at 1:200 was used as the unlabeled primary antibody. Alexa-fluor-labeled donkey anti-rabbit IgG in 1:400 was used for signal amplification. All incubations were done in a humid chamber to prevent excess drying. The stained samples were then examined under an Olympus fluorescence microscope.

4.3.6 Line blot analysis (III)

The ANA were further analyzed with a commercial Inno-Lia ANA Update (Innogenetics N.V., Ghent, Belgium) test. This test was used to detect the presence of antibodies against nuclear and cytoplasmic autoantigens. The analysis was developed to discover antibodies against 13 different nuclear and cytoplasmic antigens in human serum. In this assay, recombinant antigens, synthetic peptides, and natural proteins were incubated on separate membrane lines. This test was validated earlier for the use of dog serum in SLE disease (Hansson-Hamlin & Rönnelid 2010). The bands were detected by visual interpretation by the same examiner (HD). The control strip was without serum overlay and otherwise handled similarly to the other strips in the assay.

4.3.7 Detection of autoantigens by Western blot (IV)

Immunoblotting was used to detect which liver protein bands separated and reacted with specific liver antibodies in DH patients and controls. A liver lysate was prepared with

RIPA buffer (Radio immunoprecipitation assay buffer), and the protein amount of the lysate was determined.

Proteins from dog liver lysate were resolved by a reduced gradient SDS-PAGE and blotted onto nitrocellulose membranes. Ponceau red was used for protein visualization, and the membrane was cut into strips. A goat anti-dog IgA was used as the primary antibody and a horseradish peroxidase-conjugated anti-goat IgG as the secondary antibody. The protein bands were observed by enhanced chemiluminescence (ECL). As negative controls, one strip/blot was incubated with blocking buffer in the absence of serum and one strip/blot without the primary antibody. These controls were then treated with a secondary antibody and enhanced with ECL.

The assays were reproduced as described above but with goat anti-dog IgM and IgA as the primary antibodies and donkey anti-goat as the secondary antibody.

4.3.8 Immunoprecipitation (IP) of dog IgA from dog serum (IV)

Immunoprecipitation (IP) was used to isolate and concentrate the potential protein antibodies from dog serum for use in WB with dog liver tissue. Protein A binds dog IgG and protein G to goat IgG.

Sepharose beads (A and G) were prepared first. The beads were washed, centrifuged, and resuspended in PBS. To preclear the serum samples with protein A, the samples were centrifuged and the supernatant was mixed with PBS and protein A Sepharose slurry. The beads were centrifuged, and the supernatant was kept.

To then preclear the dog liver lysate with protein A, the liver lysate was centrifuged, and the supernatant was kept. The supernatant was mixed with PBS and protein A Sepharose slurry. The beads were spun down, and the supernatant was kept for later use.

For binding of goat anti-dog IgA to protein G Sepharose, protein G Sepharose slurry was mixed with PBS and goat anti-dog IgA. The beads were then centrifuged and washed. The beads were mixed with earlier precleared serum for the formation of dog IgA beads. The precleared liver lysate was mixed with dog IgA beads and left rotating overnight.

4.3.9 Mass spectrometry for proteomics (IV)

After an immunoprecipitation method had been applied for isolating protein antigens from the liver sample, gel electrophoresis with a pre-cast Tricine gel and a silver staining

followed. The identified bands were dissected from the gel and treated with trypsin, and the subsequent peptides were profiled by Mass spectrometry analysis. This protocol was used as described and suggested for disease-associated protein identification studies (Meri & Baumann 2001). Mass spectrometry analyses for protein profiling were carried out at the Protein Chemistry and Proteomics Unit at Biomedicum, University of Helsinki, Finland.

4.3.10 ELISA study (III, IV)

Study III: Autoantibodies against histone protein were evaluated with an in-house ELISA assay. Diluted (1:600) serum in duplicate was added onto microtiter wells, previously coated with a dissolved calf thymus histone preparation. After a one-hour incubation at room temperature (RT), the wells were washed with PBS/Tween and a rabbit anti-dog IgG antibody was added in 1:5000 dilution. The plates were washed with PBS/Tween, and a goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate was added. After a one-hour incubation at RT, the wells were washed with PBS/Tween, and the enzyme activity was determined by adding o-phenylenediamine dihydrochloride (OPD) diluted in milliQ water and H_2O_2 (30%). The obtained color reaction was stopped by adding H_2SO_4, and the photometric results were read at an optical density of wavelength 492 nm.

Study IV: The serum samples of DH patients and healthy controls were diluted 1:600 in PBS. The diluted samples were pipetted in duplicate onto GAPDH and ADH coated microtiter wells, and the plates were incubated for one hour at RT. The plates were then washed with PBS/Tween, and the wells were incubated for one hour at RT with rabbit anti-dog IgG at a dilution of 1:5000 in PBS/BSA. After washing with PBS/Tween, an HRP-conjugated goat anti-rabbit IgG antibody at a dilution of 1:2000 in PBS/BSA was added. After final washes with PBS/Tween, a visible color reaction was observed by adding milliQ water, and 5 µl peroxide 30% diluted OPD. The color reaction was terminated by adding H_2SO_4, and the absorbance was measured at a wavelength of 492 nm.

In both studies (**III, IV**), one positive DH sample was chosen as a designated control sample, and put in the same location on each plate. The results were adjusted towards this DH sample for comparison of results. The ratio of the absorbance of the study specimen and the designated control sample was called the absorbance value. All of the measurements were made in duplicate.

4.4 Statistical analyses

The statistical analyses were performed by commercially available statistical software (PASW 18.0 software for Windows; SPSS, Inc., Beijing, China). Differences with $P < 0.05$ were considered significant.

In Study I, allele and haplotype frequencies were calculated and compared between all DH cases and controls using Chi-squared statistics. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated.

In Studies III and IV, Student´s *t*-test was used to compare differences between the groups. Fisher's exact test was applied to calculate the association of the autoantibodies in study groups. The normality assumption was satisfied by visual interpretation of Q-Q Plots. The presentation of the graphical data was prepared by using GraphPad Prism (GraphPad Software, CA, USA). In case of an outlier, the statistical interpretations were carried out with and without the result.

5 Results

5.1 Association of DH with MHC class II genes (I)

We genotyped 37 cases and controls and identified six different DLA-DRB1 and five DLA-DQA1, and –DQB1 alleles, forming a total of seven different DLA class II haplotypes (**Table 1**). The haplotypes were unevenly distributed between the cases and the controls. The two most frequent haplotypes were haplotypes 1 and 3, and these were found in 95% of cases and controls. Altogether 74.3% of the cases and controls carried haplotype 1 (**Table 1**). The DH cases presented only haplotypes 1 and 2, while haplotypes 1-7 were seen in the controls (**Table 1**).

Table 1 *DLA haplotype frequencies in Dobermans with and without DH. The table is modified from Study I*

				Phenotype					
	Haplotype			Cases (n=37)		Controls (n=37)		All (n=74)	
Haplotype number	DRB1	DQA1	DQB1	n	Frequency %	n	Frequency %	n	Frequency %
1	00601	00401	01303	35	94.6	20	54.1	55	74.3
2	00601	05011	00701	2	5.4	1	2.7	3	4.1
3	01501	00901	00101			12	32.4	12	16.2
4	01502	00601	02301			1	2.7	1	1.35
5	028v	00401	01303			1	2.7	1	1.35
6	00201	00901	00101			1	2.7	1	1.35
7	00101	00101	03601			1	2.7	1	1.35
Total	6	5	5			37		74	100

The majority (94.6%) of the affected dogs were homozygous for haplotype 1, DLA-DRB1*00601/DQA1*00401/DQB1*01303, while only 54% of control Dobermans were homozygous (**Table 2**). All dogs presented at least one copy of the haplotype DLA-DRB1*00601/DQA1*00401/DQB1*01303, but we observed a highly significant association of DH with this haplotype in duplication [$P < 0.00005$, OR = 14.9, 95% CI = 3.1–71.7]. The overall heterozygosity of DH dogs was significantly reduced compared with controls (5.40% vs. 45.95%, $P < 0.001$, **Table 2**).

Table 2 *Frequencies of overall DLA-DRB1*00601/DQA1*00401/DQB1*01303 haplotype homozygosity and heterozygosity in DH cases and controls. The table is modified from Study I.*

	Phenotype					
	Cases		Controls			
	n	Frequency %	n	Frequency %	OR	95% CI
Homozygous	35	94.6	20	54.05	14.9	3.1-71.7
Heterozygous	2	5.4	17	45.95		
Total	37	100	37	100		

All cases and only 56.8% of the controls were homozygous for allele DLA-DRB1*00601 ($P < 0.0005$) (**Fig. 6**). Homozygosity for allele DLA-DRB1*00601 showed a strong association with disease ($P < 0.00005$). Allele DLA-DRB1*01501 was in LD with DLA-DQA1*00901/DQB1*00101 (**Table 1**). This allele was present in 32.4% of the controls and did not appear among DH cases (**Fig. 6**).

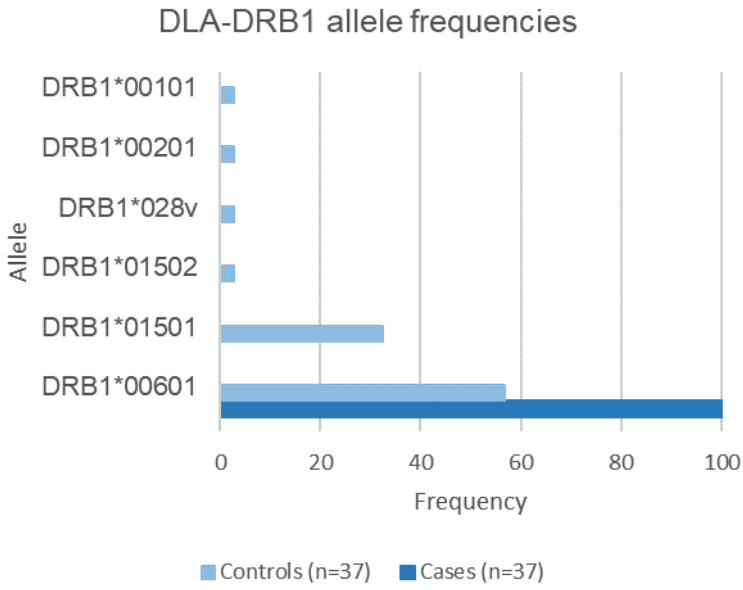

Figure 6 *DLA-DRB allele frequencies in DH cases and controls. DLA-DRB1*01501 allele was present only in control dogs (I).*

DQ heterodimer, DLA-DQA1*009 01/DQB1*00101, was present in 35% of healthy Dobermans ($P < 0.001$), but was not found in any of the DH cases (**Fig. 7**).

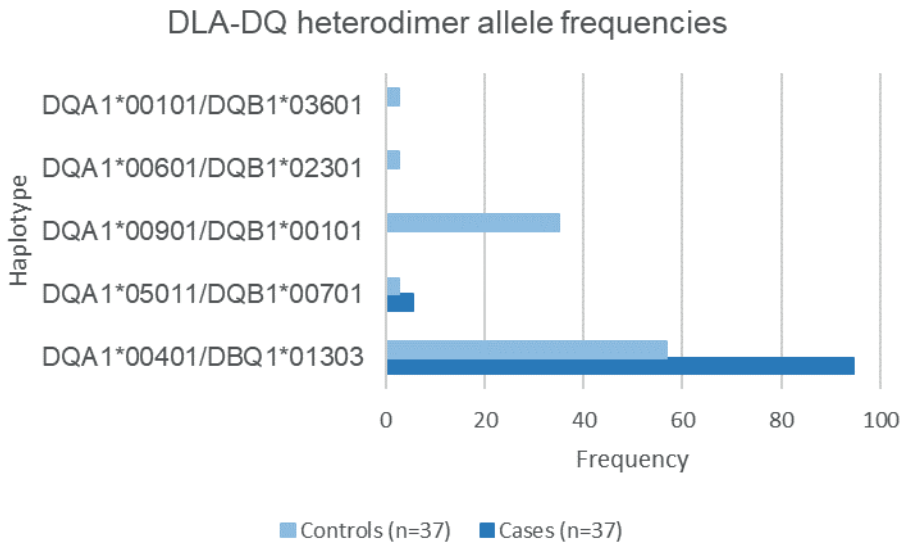

Figure 7 *DLA-DQ heterodimer allele frequencies in DH cases and controls. DLA-DQA1*00901/DQB1*00101 heterodimer was found only in control dogs. This figure is modified from Study I.*

Females were overrepresented (75.7% vs. 24.3%, $P = 0.0056$) in the case group compared with the control group, where the gender distribution was more even (54.1% vs. 45.9 %). The majority (85.4%) of the females presented haplotype 1 (27 cases and 14 controls), while only 14 males carried this haplotype (eight cases and six controls). Haplotype 3 was only seen among controls (seven males and five females). Male controls had more haplotype variation than female controls (6 vs. 3). One male control dog presented a previously unpublished haplotype in the Doberman breed: DLA-DRB1*028v/DQA1*00401/DQB1*01303 (haplotype 5) (**Table 1**).

5.2 Evaluation of regulatory DLA promoters (II)

A strong LD was present between DLA alleles and proximal promoters when analyzing 55 Dobermans with homozygous DLA-DRB1*00601/DQA1*00401/DQB1*01303 haplotype. All 35 patients with DH and 20 control Dobermans with DLA-DRB1*00601 had the same promoter allele DRBp*1, and all Dobermans with DLA-DQA1*00401 had the promoter allele DQAp*2. Only one allele, DQBp*6, was found within the DQB promoter region, and this was in LD with DLA-DQB1*01303. The female gender was overrepresented in both groups (controls 70% and cases 77%).

One allele for each promoter variant DLA-DRBp, -DQAp, and -DQBp were noted. The DLA-DRAp promoter area and exon 2 in all cases and controls were identical to the reference sequences. However, in DLA-DRA exon 1, a G/A mutation at position seven was found, changing the amino acid from isoleucine to valine, both of which are regarded as non-polar and hydrophobic. This variant was observed in all cases and controls. Humans have an isoleucine at this position, and exon 1 is longer than in dogs (**Fig. 8**).

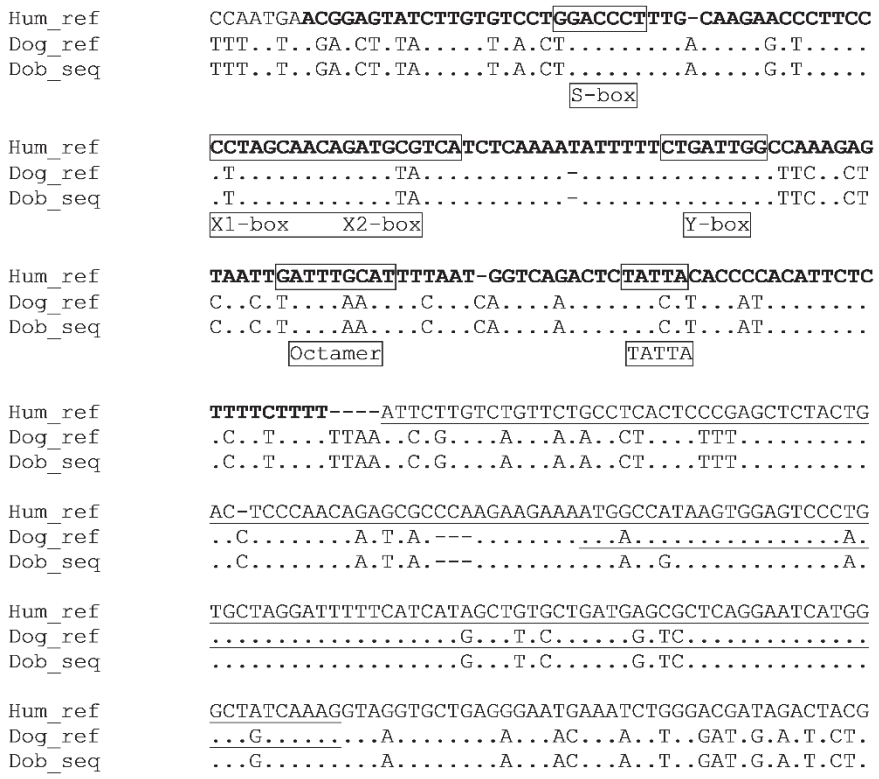

Figure 8 *HLA-DRA promoter and exon 1 sequences compared with general dog reference (Dog_ref, GenBank 481731) and Doberman (Dob_seq) sequences. Exon 1 is underlined. Human promoter is in bold and the conserved elements are indicated with boxes. Identical bases are indicated with dots (II).*

5.3 Detection of anti-nuclear autoantibodies (ANA) by IIF (III)

The immunofluorescence analysis of DH samples demonstrated a typical staining for ANA. Five (5/10) DH serum samples were regarded as positive in the ANA test. Two sera (1 SDH and 1 CDH Doberman) had an ANA titer of 1:40, two subclinical sera a titer of 1:160, and one subclinical serum a titer of > 1:160. All ANA-positive cases showed a homogeneous nuclear pattern (**Fig. 9**). One of ten sera showed a detectable ANA at a titer of 1:10 in the control group.

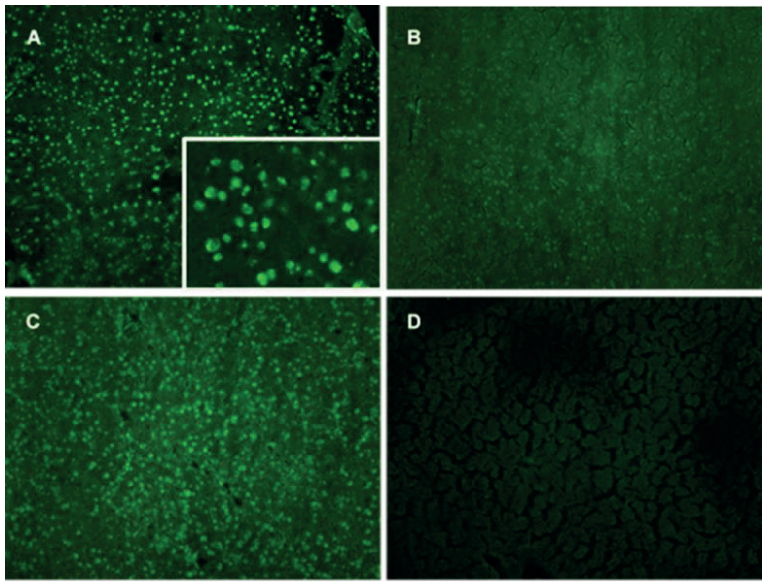

Figure 9 *IIF analysis of autoantibodies at 400 x magnification. The serum specimens were diluted 1:10, 1:40, and 1:160 for detection of antinuclear antibodies. In the case group, 5 of 10 samples were identified as positive in the ANA test. (**A**) 2 of 10 samples showed an ANA titer of 40 (Inset at 1000 x magnification). (**B**) An ANA titer of 160 was present in 2 of 10 samples. (**C**) One DH case had a titer of > 160. (**D**) One sample with non-detectable ANA, dilution 1:10. Representative data are shown (III).*

5.4 Line blot analysis of ANA in serum (III)

In the line blot, four of ten DH samples (three SDH and one CDH dog) revealed clear anti-histone reactivity. None of the control dogs reacted with histones. Anti-RNP-A (anti-ribonucleoprotein-A) was found in one SDH and CDH dog as well as in one control dog. This reactivity does not satisfy the criteria for RNP autoantibodies. Also, one subclinical dog had antibodies towards small nuclear ribonucleoprotein-associated protein B (anti-SmB) and another towards small nuclear ribonucleoprotein-associated protein D (anti-SmD) reactivity. Nonspecific positive reactivity was observed towards natural protein SS-A/Ro60 (**Fig. 10**). Line blot result was also tested by Western blotting with a calf histone preparation (Sigma, Germany), yielding similar results (data not shown).

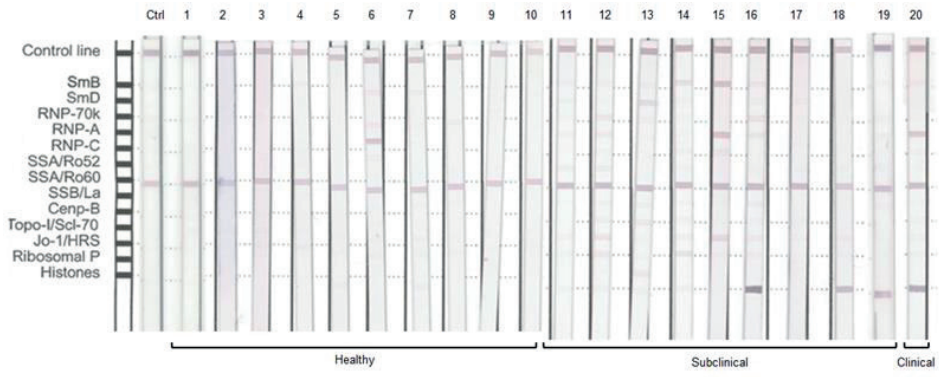

Figure 10 *Line blot analysis of ANA in 10 controls (1-10) and 10 DH cases (11-20) (III).*

5.5 Autoantibodies against histone (III)

The AHA result was above the cut-off value (0.87) in 23 (92%) of 25 SDH cases, and in the clinical stage, in 11 (85%) of 13 dogs. The 17 controls were seronegative. These results showed a significant association between AHA IgG and DH ($P < 0.0005$). The antibody results showed a greater overall variation within the case groups. When comparing the mean absorbance values, both the SDH (1.36 ± 0.60, mean ± SD) and the CDH absorbance values (1.46 ± 0.49) were significantly higher than the values for controls (0.51 ± 0.18, $P < 0.0001$). The difference between the SDH and CDH groups was not statistically significant ($P = 0.29$) (**Fig. 11**). One subclinical case was an outlier with a higher result, and the statistical tests were performed with and without this value. The outcome was the same when the outlier was kept in the analysis. In total, 34 (89.5%) of the 38 dogs with either subclinical or clinical disease presented AHA.

For detecting subclinical or clinical DH, the ELISA assay had a sensitivity of 89.5% (95% CI from 75.20% to 97.06%) and a specificity of 100% (95% CI from 80.49% to 100.00%).

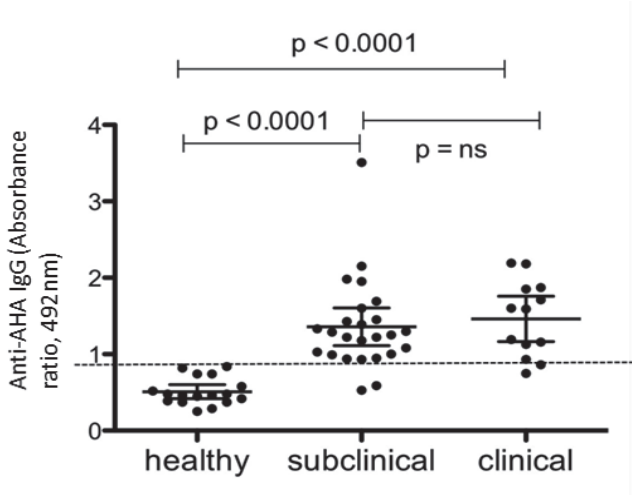

Figure 11 *ELISA results for IgG antibodies against histone comparing DH patients with control dogs in 1/600 dilution. A cut-off value of 0.87 for positivity is indicated with a dotted line. Only significant P-values are reported in the figure (III).*

5.6 Detection of autoantigens by immunoblotting (IV)

Because of a suspected autoimmune etiology of DH, Western blotting was employed first, and sera from ten DH patients and ten controls were used to detect bands representing possible autoantigens in dog liver tissue. Also, nine SDH follow-up sera were analyzed (follow-up time range from seven months to four years). Specific bands were evident on Western blots, where DH patient sera were overlaid on a liver homogenate compared with controls.

With IgG, a band of 37 kDa was detected by five (four SDH and one CDH) initial DH case samples; five SDH samples remained negative.

Three initial samples from SDH dogs reacted weaker, five (four SDH and one CDH) stronger, while one other control dog showed weak reactivity with a band of 39 kDa (**Fig. 12**).

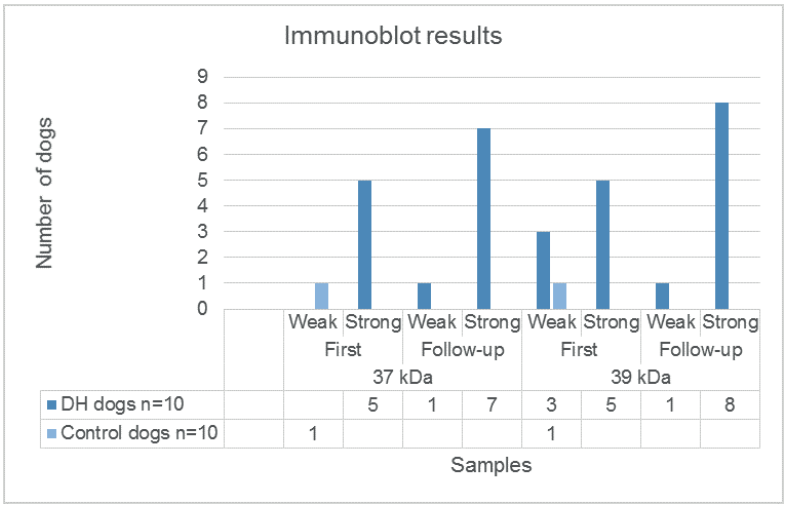

Figure 12 *Immunoblot results for subclinical patients and control dogs. A band of 37 kDa was detected in five (four SDH and one CDH) initial DH case samples. The reaction was enhanced in the follow-up samples, demonstrating seven strong and one weaker reacting subclinical patient. One control dog showed weak reactivity. At 39 kDa, three initial SDH dogs reacted weaker, five (four SDH and one CDH) stronger, while one control dog showed weak reactivity. The reaction increased in the follow-up samples. One SDH sample showed a weaker and eight SDH samples a stronger reactivity. The figure is modified from Study IV.*

IgG immunoreactivity against the putative liver autoantigens increased with disease progression (**Fig. 13**). At 37 kDa, one initially negative dog reacted weakly, and three dogs were clearly positive in the follow-up samples. Overall, in the follow-up samples, seven were clearly positive and one was weaker.

Two SDH dogs were initially negative at 39 kDa. Later, one dog had a weaker and another dog a clear positive reaction in the follow-up samples. Three initially weaker samples were clearly positive in the follow-up. Overall, one SDH sample showed a weaker and eight SDH samples a stronger reactivity. The IgA showed similar immunoreactivities while IgM showed no reactivity against liver autoantigens.

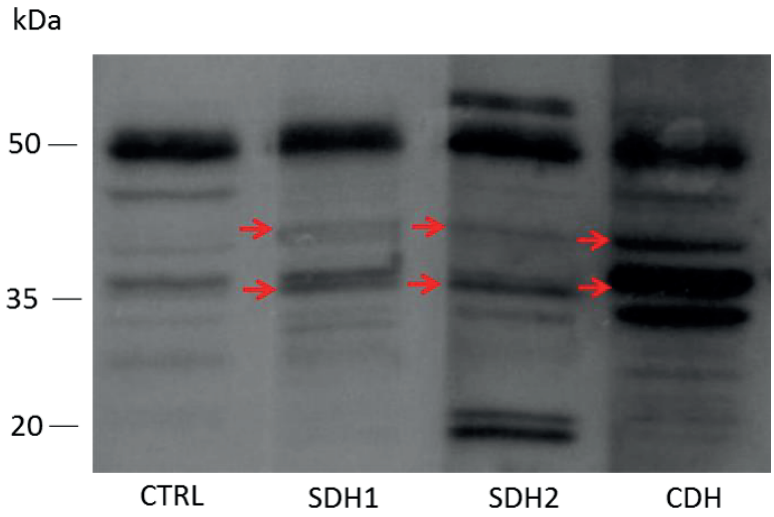

Figure 13 *Protein bands in liver homogenates reactive with serum IgG from DH patients. Representative Western blot analysis (gradient gel) of a healthy Doberman serum (CTRL), and serum from a dog with SDH (SDH 1) and the follow-up sample (SDH 2) taken from the same dog two years later, and a CDH dog. The candidate antigens are shown with red arrows. Molecular weight standards are shown on the left (IV).*

5.7 Immunoprecipitation (IP) (IV)

Using IP with anti-dog immunoglobulin A antibodies bound to Sepharose G beads, dog sera containing autoantibodies and a precleared liver lysate, candidate antigens of the autoantibodies were isolated. Immunoprecipitation demonstrated two bands at 37 kDa and 39 kDa (**Fig. 14**).

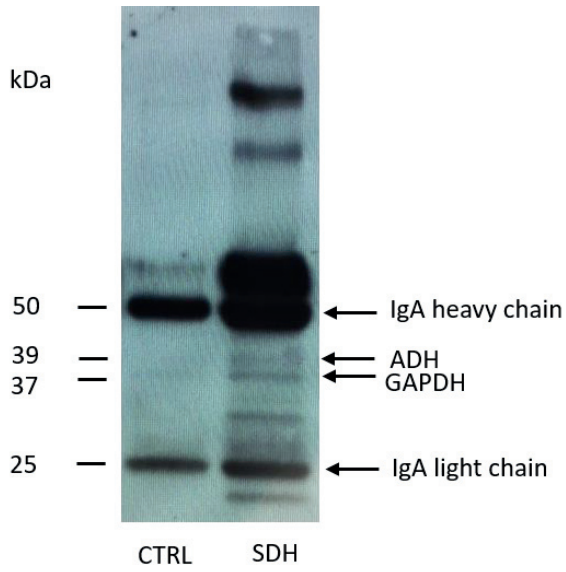

Figure 14 *Immunoprecipitation demonstrated two bands in a liver homogenate that reacted with an SDH patient serum. The bands at 37 kDa (GAPDH) and 39 kDa (ADH) were subjected to mass spectrometric analysis for identification. Test using a serum from a healthy dog is shown on the left (CTRL). Molecular weight standards are shown on the left (IV).*

5.8 Mass spectrometry for proteomics reveals novel autoantigens (IV)

When the specifically immunoprecipitated bands were subjected to mass spectrometry, the proteomic analysis identified the band 37 kDa as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the band at 39 kDa as alcohol dehydrogenase class pi chain precursor isoform 23 (ADH).

5.9 Autoantibodies against GAPDH and ADH (IV)

IgG antibodies against GAPDH and ADH in patient and control sera were analyzed by using an in-house ELISA assay. Antibody levels in patients with SDH (n=25) and CDH (n=13) as well as in healthy controls (n=17) were studied at a serum dilution of 1:600.

The mean absorbance value of samples obtained from SDH Dobermans was 1.64 ± 0.64 and from 13 CDH Dobermans 1.94 ± 0.59. These results were significantly higher than in control Dobermans 1.06 ± 0.35 ($P < 0.0001$). Anti-GAPDH IgG ELISA was above the mean+2SD threshold (1.76) in 36% (9/25) of SDH dogs (eight females and one male) and in 69.2% (9/13) of clinical stage DH dogs (seven females and two males). All controls were seronegative for anti-GAPDH autoantibodies (**Fig. 15**). A correlation was noted between SDH, CDH and the presence of anti-GAPDH IgG as assessed by Fisher's exact test ($P < 0.0005$). There was no difference in anti- GAPDH IgG values between the SDH and CDH groups ($P = 0.08$) (**Fig. 15**).

The ELISA test for anti-GAPDH showed a sensitivity of 47.4% (95% CI from 31% to 64.2%) and a specificity of 100% (95% CI from 80.5% to 100%).

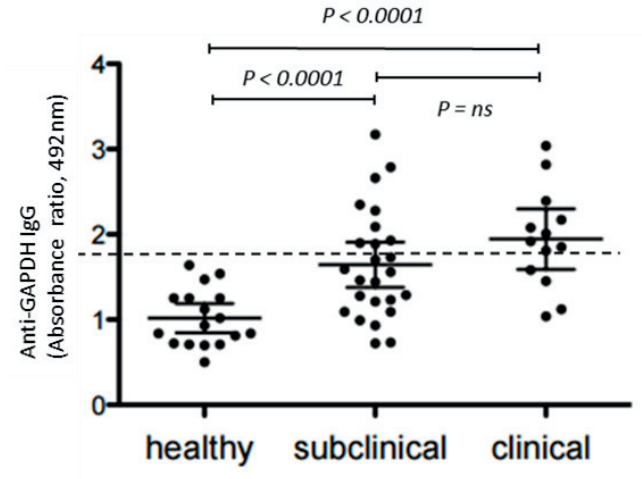

Figure 15 *Results for IgG antibodies against GAPDH comparing DH patients with control dogs in 1:600 dilution. The dotted line represents the cut-off value of 1.76. SDH and CDH results were both significantly higher than the results for controls ($P < 0.0001$). The difference between the two case groups was not statistically significant ($P = 0.08$) (IV).*

The mean absorbance values for the anti-ADH ELISA in SDH dogs (1.35 ± 0.84, mean ± SD) and in CDH dogs (1.44 ± 0.65) were significantly higher than incontrol subjects (0.47 ± 0.17) ($P < 0.0001$) (**Fig. 16**). The ADH antibody value was above the cut-off (0.81) in 72% (18/25) of SDH dogs (15 females and three males) and in 76.9% (10/13) of CDH dogs (eight females and two males).

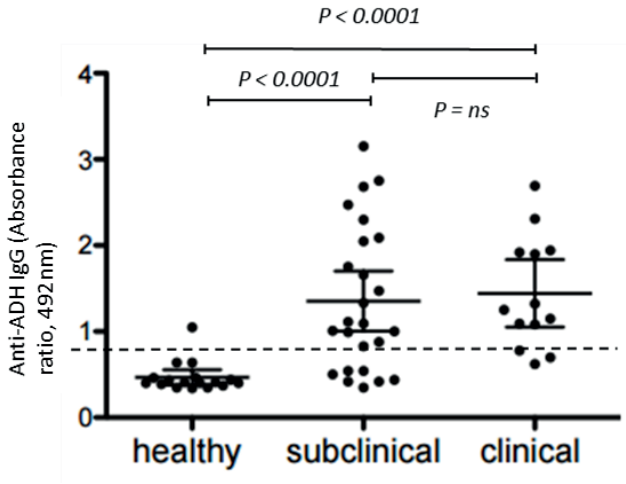

Figure 16 *IgG anti-ADH ELISA results in 1:600 dilution. The dotted line represents the cut-off value of 0.81. The mean absorbance value of control subjects was significantly lower that the values of SDH and CDH dogs (P < 0.0001). The case group comparison was not statistically significant (P = 0.36) (IV).*

In total, ELISA detected serum-specific IgG ADH antibodies in 73.7% (28/38) of dogs with either subclinical or clinical disease. In the healthy group, one dog (1/17) was seropositive. This one control subject was an outlier, and statistical tests were carried out with and without this result. The outcome remained the same when the outlier was kept. A significant association was observed between SDH, CDH and the presence of anti-ADH IgG, as assessed by Fisher's exact test ($P < 0.0005$). No statistical differences were found between the SDH and CDH groups ($P = 0.36$).

Anti-ADH ELISA in patients with DH showed a sensitivity of 73.7% (95% CI from 56.9% to 86.6%) and a specificity of 94.1% (95% CI from 71.3% to 99.9%).

5.10 Autoantibody combinations for AHA, GAPDH and ADH

Different autoantibody combinations were noted in DH patients. Of the cases, 42.1% (16/38, 12 females and four males) showed elevations in all three autoantibodies (AHA, GAPDH, and ADH); 9/25 were SDH dogs (seven females and two males) and 7/13 were CDH dogs (five females and two males). The combination AHA with ADH was found in 31.6% (12/38) of the DH cases. Ten dogs suffered from SDH (eight females and two males) and two females had CDH. AHA was elevated alone in 15.8% (6/38) of patients: four SDH (one female and three male) and two CDH (both female) dogs. GAPDH and ADH were high in one female CDH dog. One female CDH dog had only GAPDH. Two female SDH dogs were seronegative for AHA, GAPDH, and ADH (**Table 3**).

Table 3 *Frequencies of different autoantibody combinations for AHA, GAPDH and ADH in SDH and CDH dogs.*

	DH cases					
	n=38	Frequency %	SDH n=25	Frequency %	CDH n=13	Frequency %
AHA+GAPDH+ADH	16	42.1	9	36	7	53.8
AHA+GAPDH						
AHA+ADH	12	31.6	10	40	2	15.4
AHA	6	15.8	4	16	2	15.4
GAPDH+ADH	1	2.6			1	7.7
GAPDH	1	2.6			1	7.7
ADH						
negative	2	5.3	2	8		
Total	38	100	25	100	13	100

6 Discussion

This work explored the hypothesis that DH is an autoimmune disease. It aimed to assess whether modified Witebsky´s circumstantial evidence for autoimmune disease is fulfilled in DH. Autoimmune disease can be suspected by modified Witebsky´s circumstantial evidence (Rose & Bona 1993, Rose & MacKay 2014a) when affected patients have a statistical association with a certain MHC class II haplotype, a lymphocytic infiltration of the target organ, an aberrant expression of MHC class II antigen on the affected cell, elevated levels of autoantibodies, a favorable response to immunosuppression, a positive family history of the disease, or another autoimmune disease in the same patient or their family. Female predilection is also typical for many autoimmune diseases (MacKay 2000). Previous research has suggested autoimmune disease as the underlying reason for DH, as DH is characterized by mononuclear infiltration (Speeti et al. 1998), abnormal MHC class II antigen hepatocyte expression associated with mononuclear infiltration (Speeti et al. 2003), and a female bias (Crawford et al. 1985, Speeti et al. 1996).

This study focused on MHC class II genes, MHC class II regulatory regions, circulating serum anti-nuclear autoantibodies, and liver-related autoantigens associated with DH.

6.1 MHC class II genes as risk factors in DH (I)

The human MHC class II genotype region is the best described predisposing factor in most autoimmune diseases (Shiina et al. 2004). This study showed a strong association of the homozygous DLA-DRB1*00601/DQA1*00401/DQB1*01303 haplotype with DH, and especially the homozygous DLA-DRB1*00601 allele appears to confer a risk of DH development. Intriguingly, all of the DH cases were homozygous for allele DLA-DRB1*00601, while this allele was homozygous in only 56.8% of healthy dogs. Of the DH dogs, 94.6% were homozygous for haplotype DLA-DRB1*00601/DQA1*00401/DQB1*01303. This DLA haplotype is otherwise rare in the general dog population, to date only found in Dobermans, Rough Collies, and mixed breed dogs from Brazil (Dr. LJ Kennedy, personal communication). In the present study, all Dobermans carried at least one copy of the characteristic “Doberman haplotype” DLA-DRB1*00601/DQA1*00401/DQB1*01303, but when present in duplicate, it elevated the risk of DH susceptibility to nearly 15-fold. The DLA region displayed a remarkably low

variation among DH patients, with only two different haplotype combinations compared with the seven in the control group (**Table 1**).

The haplotype frequencies found in this study are worrisome. In order for a haplotype to persist in the Doberman population, it should be evenly distributed. However, haplotypes 4-7 were rare and at risk of disappearing from the gene pool.

In humans, some homozygous HLA class II alleles are associated with risk susceptibility to autoimmune diseases like AIH-1 and CD (Cassinotti et al. 2009). Certain homozygous HLA class II alleles have been demonstrated to affect the severity of the disease outcome in CD (Karinen et al. 2006), RA (Gonzalez-Gay et al. 2002) and MS (Barcellos et al. 2003). Different dog breeds have been developed as a result of extensive phenotypic selection, in many cases resulting in inbreeding and restriction of genetic diversity. An association with susceptibility to autoimmune diseases and infections was demonstrated with high interbreed variation and low intrabreed variation in dogs (Kennedy et al. 2002).

Different DLA genotypes affect the development of immune tolerance and may thus be a background factor between autoimmune diseases and DLA genes. Homozygous dogs may be more susceptible to pathogens or autoimmune diseases because they are able to respond to a smaller range and variety of antigens than heterozygous dogs. The spectrum of immune reactions is thus more limited in homozygous dogs than in the more advantageous heterozygous dogs. Variations in the MHC class II allele and haplotype frequencies could explain why individual dogs or certain breeds are good or poor responders to certain microbial antigens and why certain dogs are susceptible to autoimmunity and the development of autoantibodies (Tizard 2009).

DQ-heterodimer DLA-DQA1*00901/DQB1*00101 was demonstrated in 35% and allele DLA-DRB1*01501 in 32.4% of control dogs, and they appear to protect against the development of DH. This DQ molecule and DR allele were not found in the patient group and are otherwise rare in the Doberman breed. The DLA-DRB1*01501 allele makes a big contribution to the DR heterodimer structure because of the functional monomorphism of the DRA locus in dogs (Wagner et al. 1995). Other DLA heterodimers demonstrate extensive polymorphism in exon 2.

The encoded MHC class II genes and molecules regulate the development, activation, and homeostasis of $CD4^+$ T cells (Pachot et al. 2005). HLA-DQ genes have been recognized to confer risk of disease development but also to protect against autoimmune disorders (Murphy et al. 2008b). The DLA-DQA1*004/DQB1*013 heterodimer seem to be protective against diabetes (Kennedy et al. 2006a). HLA-DQ molecules have been suggested to be more involved in the repertoire spectrum in the

thymus, and HLA-DR molecules are proposed to be more involved in antigen presentation in the periphery (Altmann et al. 1991).

Human HLA-DRB1 alleles that code a conserved five amino acid sequence QARAA motif at positions 70-74 of the DRβ chain are described as having a shared susceptibility epitope (SE) (Caillat-Zucman 2017). This SE is a component of the third hypervariable region that encodes a functionally necessary part of the human DRB1, being responsible for peptide recognition and binding affinity of the MHC class II antigen binding site. It was hypothesized that alleles carrying this SE could efficiently provide the disease-inducing epitope or could present a disease-inducing peptide themselves (Muller-Hilke & Mitchison 2006).

Current evidence suggests that glutamine (Q) or arginine (R) at position 70 is critical for RA risk in humans. When homozygous, SE has been associated with RA penetrance and therefore disease severity (Holoshitz 2010). In dogs, the risk allele DLA-DRB1*00601 comprises a five amino acid sequence RARAA at positions 70-74 with a predisposing arginine at position 70. This was demonstrated in immune-mediated rheumatic disease in Nova Scotia duck tolling retrievers (Wilbe et al. 2009). Allele DLA-DRB1*00601 was also demonstrated to confer risk of a more aggressive form of chronic hepatitis in English Springer spaniels with an unknown etiology (Bexfield et al. 2012). A relationship with SE sequence QRRAA of the risk DRB allele and AIH in the Chinese population was noted (Ma & Qiu 2001). Our study provide evidence that this is also the case in DH and the RARAA-containing risk allele DLA-DRB1*00601.

The MHC class II association can increase or decrease the risk of disease and dosage effects may occur in complex autoimmune disorders (Cassinotti et al. 2009). One hypothesis for the MHC class II association and the risk of autoimmune disease development is based on differences in the capacity of different allelic variants of MHC class II antigens to present autoantigenic peptides to autoreactive T cells. The second theory for the association suggests that MHC class II alleles shape the T cell selection in the thymus. According to this theory, self-peptides can drive the positive selection of developing T cells that are specific for certain autoantigens (Murphy et al. 2008a). These genes have an important part in the development of T cell tolerance.

6.2 Evaluation of DLA class II promoters in DH (II)

The differential expression of various MHC class II alleles following promoter diversity has been proposed as a possible mechanism in autoimmunity (Tsang et al. 1988). Promoter polymorphism could lead to differential expression of MHC class II molecules on APCs,

change the signal intensity in the immunological synapse, and alter the immune response towards inflammation (Müller-Hilke & Mitchison 2006).

We assessed the level of variation of the proximal promoters of DLA genes in Dobermans with the homozygous risk haplotype DLA-DRB1*00601/ DQA1*00401/DQB1*01303. We studied whether promoter sequence variation in DRBp, DQAp, and DQBp could account for why some homozygous Dobermans become affected with DH and others do not. The promoter region was found to be monomorphic, with no variation when comparing 35 DH patients with 20 healthy control Dobermans. We also examined the potential polymorphism in the DLA-DRAp promoter and DLA-DRA exon 2. The human HLA-DRA genes are monomorphic in their coding region, but demonstrate allelic variability within their proximal promoter (Pinet et al. 1991). The results revealed that promoter DLA-DRAp and DLA-DRA exon 2 were identical in cases and controls. To our knowledge, DLA-DRA promoters have not been investigated before in dogs.

In a previous study, only minor polymorphism was found in the DLA-DRB and DQA promoters, with just two different promoter alleles recognized in each locus in dogs: DRBp*1 and DRBp*2, and DQAp*1 and DQAp*2. All of these four alleles existed in Dobermans, but DRBp*1 and DQAp*2 were the most common. In other breeds, the DQAp*1 allele is more frequently seen than DQAp*2 (Berggren & Seddon 2005). All of our dogs carried promoters DQAp*2 and DRBp*1. The promoter allele DRBp*1 includes a polymorphic site, positioned in the conserved area, the X1-box, with an A–G transversion mutation. The S, X2, and Y boxes and the CCAAT motif were invariant.

Eleven different alleles have been identified within the DLA-DQB promoter region in dogs. Three promoter alleles were associated with allele DLA-DQB1*01303, and DQBp*10–13 have only been found in diabetic dogs (Seddon et al. 2010). All of our Dobermans were associated with the promoter variant DQBp*6.

The promoter DLA-DRAp and DRA exon 2 in cases and controls were identical to the dog reference sequences. Interestingly, in DLA-DRA exon 1, a G/A mutation at position 7 was found, changing the amino acid from isoleucine to valine, both regarded as hydrophobic and non-polar. This variant was noted in all cases and controls. At this position, humans have isoleucine, and the exon 1 is longer than in dogs.

Based on our results, promoter variants are not associated as risk modifiers for DH in Dobermans with the risk haplotype DLA-DRB1*00601/DQA1*00401/DQB1* 01303. This implies that additional factors are needed. Strong LD was noticed between the DLA alleles and proximal promoters. In Dobermans, the DLA-DRB1*00601 allele is associated with the promoter variant DRBp*1, the DLA-DQB1*01303 allele with variant DQBp*6, and the DLA-DQA1*00401 allele with variant DQAp*2. Our study suggests that the whole DLA block is associated with DH. The CIITA-controlled transcription of the MHC

class II genes is well recognized. However, there is proof of additional layers of complexity in the MHC class II expression regulation. These include the proximal and distal elements of the MHC class II region, intergenic regions, chromosomal looping, epigenetic mechanisms, and environmental factors (Handunnetthi et al. 2010).

6.3 Autoantibodies against histone (III)

Our goal for the study was to investigate possible ANA in DH and to further characterize the suspected autoimmune background. Given the challenges in diagnosing DH, we also aimed to determine whether these autoantibodies could be used in the disease diagnostics. The ANA were further identified to AHA, and we were able to demonstrate significantly elevated levels of AHA in both SDH and CDH dogs. Moreover, the healthy control Dobermans were all seronegative.

An immune-mediated background for DH was suspected, based on similar characteristics as for autoimmune diseases in humans. Dobermans have a higher risk for the development of a severe chronic hepatitis, and the affected patients can have a positive family background for DH (Johnson et al. 1982, Speeti et al. 1996). To the best of our knowledge, simultaneous autoimmune diseases with DH have not been reported in the literature. Females are clearly predisposed to DH (Crawford et al. 1985, Speeti et al. 1996). Monocellular infiltrates in the liver parenchyma and portal areas are an apparent histological feature in early DH (Speeti et al. 1998), and expression of MHC class II molecules on hepatocytes, correlating with the degree of inflammation, has been noted (Speeti et al. 2003). DLA class II alleles and haplotypes have been associated with immune-mediated diseases in dogs. Homozygous DLA allele DRB1*00601 is significantly associated as a risk factor for DH (Dyggve et al. 2011). The affected hepatocytes express MHC class II antigens that correlate with the degree of inflammation (Speeti et al. 2003).

The finding of AHA in DH is evidence of failed immune tolerance in DH, a central feature in human autoimmune diseases. High AHA titer in connection with elevated ALT serum levels in a Doberman with or without clinical signs of hepatitis supports DH diagnosis. A negative AHA does not, however, completely exclude DH. AHA is not an appropriate biomarker for disease progression; the difference between the SDH and CDH groups was not statistically significant here (**Fig. 11**). Histone release has been suggested to have a role in autoimmune disease (Chen et al. 2014). Histones have an important nuclear function, but also a significant toxic or pro-inflammatory function when released into extracellular space by activated and damaged cells (Allam et al. 2014). Histones are

suggested to act as a direct autoantigen, but also to magnify harmful autoimmune processes (Yu & Su 2013). The line blot analysis demonstrated nonspecific positive reactivity towards SS-A/Ro60 (**Fig. 10**). This was most probably due to secondary antibody reactivity. The presence of AHA was also confirmed with Far Western blotting, and the outcome was similar to that of ELISA analysis.

An important part of the diagnostic process is the detection of autoantibodies in patients with a suspected autoimmune disease. Two studies have described autoantibodies earlier in DH. Andersson and Sevelius (1992) examined circulating autoantibodies in three DH Dobermans with IIF. One DH dog was ANA positive at a titer of 1:10 and showed a granular nuclear fluorescent staining. No other autoantibodies were found. The medical records of these patients were not given. Another study of different dog breeds, including four Dobermans, showed anti-liver membrane antibodies (anti-LMP) in chronic hepatitis patients (Weiss et al. 1995). Two of the Dobermans had high anti-LMP titers, while the other two were anti-LMP negative. The latter two were on immunosuppressants. A previous study reported AHA to be a common finding in other dog breeds suffering from SLE (Bremer et al. 2015).

6.4 GAPDH and ADH as autoantigens (IV)

The identification of autoantigens is the key to unraveling potential autoimmune etiology and pathogenesis of DH, but also to improving autoantibody tests to diagnose or verify the disease. This study revealed liver-related autoantigens associated with DH and elucidated the DH etiology and pathogenesis. Two dehydrogenase enzymes were identified as target autoantigens: GAPDH and ADH, and DH was significantly associated with both. The finding of these liver-related autoantigens supports the theory that DH hepatocytes have become APCs. In light of our results, hepatocytes with GAPDH and ADH autoantigens associated with MHC class II antigens, are presented to autoreactive $CD4^+$ helper T cells. The GAPDH and ADH autoantibodies of DH Dobermans indicate that $CD4^+$ T cells have been activated, producing cytokines specific for type 2 T helper cells (Th2). These cytokines initiate B cell activation and autoantibody production against the enzymes GAPDH and ADH. We observed that in DH Dobermans with two copies of allele DLA-DRB1*00601 have an elevated risk of developing the disease, compared with heterozygous dogs (Dyggve et al. 2011).

A failure in immune tolerance towards self-proteins predisposes to autoimmunity. What induces the immune system to react to autoantigens remains, however, unclear.

Possible factors for autoimmune responses could include molecular mimicry for microbial antigens, impaired apoptosis, and clearance and post-translational modification (PTM) of self-proteins. In molecular mimicry, self-peptides resemble a foreign antigen structure or sequence. The viral or bacterial antigen within the MHC class II molecule can initiate activation of autoreactive naïve T cells, which recognize peptides from foreign and self-antigens and result in autoimmune disease (Cusick et al. 2012) Molecular mimicry is the supposed background mechanism in PBC (Selmi et al. 2011). Defects in the removal of apoptotic cells are involved in chronic inflammation formation by supplying triggering autoantigens and initiation of autoimmune response towards nuclear autoantigens, as has been shown in SLE (Mahajan et al. 2016). PTM proteins are important for many normal cellular events, but sometimes PTM in self-proteins can influence antigen recognition and also initiate autoimmune responses. PTM may occur for self-proteins in inflammation, apoptosis, or aging. APCs take up these transformed proteins when they are released from the cells. MHC class II molecules present the modified peptide particles to T and B cells that have escaped central tolerance, and these autoreactive lymphocytes may infiltrate tissue and cause autoimmune pathology (Doyle & Mamula 2012). PTM has been demonstrated in CD, where deamination of glutamines to glutamates leads to the generation of peptides that bind strongly to the disease predisposing MHC II molecules (Molberg et al. 1998).

This study presents evidence for an antigen-driven autoimmune process and elucidates the previously unknown DH pathogenesis. It was earlier suspected that DH is mediated by autoimmune mechanisms and the hepatocytes have become APCs. The MHC class II expression was demonstrated to correlate with mononuclear cell infiltration and the severity of DH (Speeti et al. 2003). MHC class II molecules are not normally presented by hepatocytes, but by professional APCs such as dendritic cells, B cells, and macrophages. MHC class II expression can be induced by cytokine stimulation, e.g. IFN-γ secretion by lymphocytes (Ting & Balwin 1991). In DH, $CD4^+$ T helper cells likely become activated when they are presented with GAPDH and ADH peptide particles by MHC class II molecules, which are on the surface of the hepatocytes (**Fig. 17**). Naturally, this does not exclude presentation by local dendritic cells. For specific autoantibody formation, also presentation by GAPDH and ADH-specific B cells is needed (**Fig. 17**).

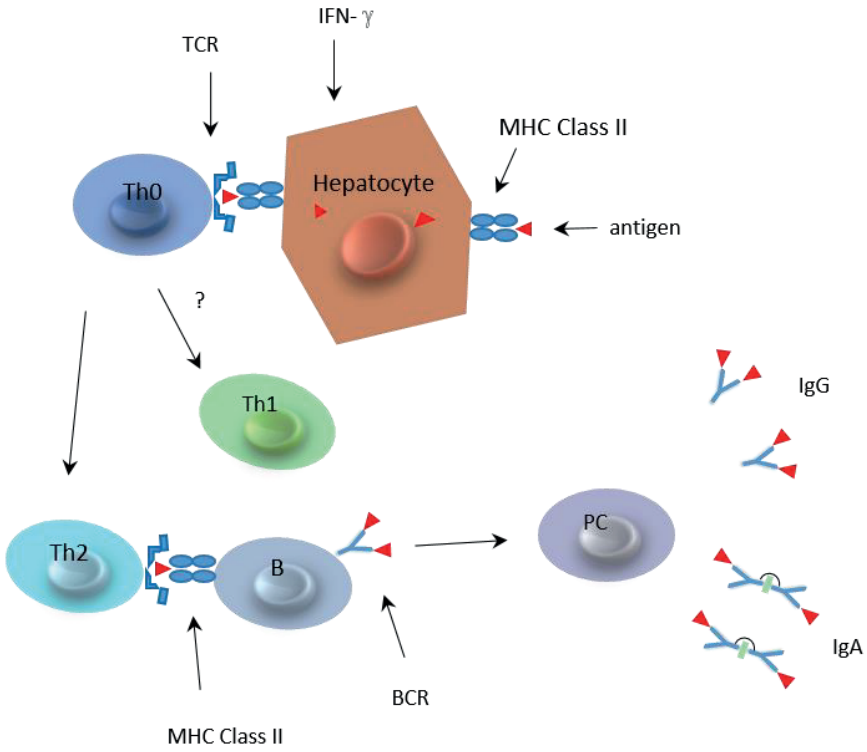

Figure 17 *Suggested key steps for autoantibody production towards hepatocyte antigens in DH. In inflammatory conditions, hepatocytes start abnormally expressing MHC class II antigens on their surface. An autoantigenic peptide (histone, GAPDH, or ADH, as red triangle) is presented by the MHC class II molecule to Th0 T cell receptor (TCR). The activated Th0 cell differentiates to Th2 in a suitable cytokine environment. B cells capture peptides from autoantigens (B cell receptor, BCR) and present these to Th2 cells with a surface antibody receptor. The antigen-specific B cells are proliferated and differentiated into plasma cells (PCs) by Th2 stimulation. PCs produce soluble IgG and IgA antibodies in larger amounts towards histone, GAPDH, or ADH (IV).*

6.5 Witebsky´s modified circumstantial evidence in DH (I, III, IV)

Previous studies on DH have revealed a female predilection (Crawford et al. 1985, Speeti et al. 1996), lymphocyte infiltration in the liver (Speeti et al. 1998), and MHC class II antigens expressed by the hepatocytes in conjunction with the lymphocyte infiltration (Speeti et al. 2003). This thesis provides further evidence for DH being an autoimmune disease of the liver according to the modified Witebsky´s postulates. Significant associations between MHC class II genes and elevated autoantibodies towards the histone and liver-related enzymes GAPDH and ADH were found.

6.6 The role of copper in DH

Copper levels may be up to 500 µg/g dwl in healthy dogs (Rolfe & Twedt 1995, Van den Ingh et al. 2006). In Bedlington terrier copper toxicosis, histological signs of hepatitis were noted when copper levels exceeded 2,000 µg/g dwl (Twedt et al. 1979). In our study, quantification of copper was available for 11 SDH dogs with a median of 792 µg/g dwl (range 430-1886 µg/g two) and seven CDH dogs with a median of 1490 µg/g dwl (range 630-2430 µg/g dwl). Copper was associated with inflammation in the centrilobular region in SDH dogs, and with inflammation in the periportal and bridging necrosis areas in the CDH dogs. Wilson's disease in humans is a metabolic disorder characterized by a marked increase of Cu in the liver. Normally liver copper concentration is less than 50 µg/g dwl in humans, and a 5x normal copper content (250 µg/g dwl) is considered diagnostic for Wilson´s disease (Patil et al. 2013).

In these copper metabolic disorders, hepatocellular copper accumulation exceeds the hepatocyte capability to bind copper into intracellular proteins. Free copper accumulation can exert toxicity through direct oxidative damage and unregulated apoptosis, leading to hepatic injury (Rodriguez-Castro et al. 2015). Semiquantitative identification of abnormal copper levels in the liver is possible with special stains. In Wilson´s disease, copper accumulation was demonstrated histologically in the periportal hepatocytes (Stromeyer et al. 1978).

We suggest that copper accumulation is secondary due to lower liver copper concentrations than in primary copper metabolism disorders, and the association of copper accumulation with mononuclear inflammation and not copper accumulation alone. Also, corticosteroids were found to decrease tissue inflammation and copper accumulation (Speeti et al. 1999). The relationship of copper and DH remain to be elucidated.

6.7 Suggested treatment for DH (I, III, IV)

The treatment of DH has been a challenging, as the etiology and pathogenesis have remained obscure. Corticosteroids have failed to prevent the progression of DH, particularly in CDH patients. The pathogenesis and the autoimmune background of DH are elucidated here. When autoreactive T cells are activated, an autoimmune mode will continue for as long as an autoantigen is available. Based on the suggested pathogenesis, immunosuppressive approaches like cyclosporine and budesonide could be an attractive option to target and inhibit the autoimmune reaction in DH. Cyclosporine suppresses overall immune response, as it inhibits activated T cells from producing interleukin. Cyclosporine forms a complex with cyclophilins, thus preventing the activation of T cells. The formed complex prevents calcineurin from dephosphorylating the nuclear transcription factor of activated T lymphocytes (NFAT). Without dephosphorylation, the cytoplasmic NFAT cannot translocate to the nucleus to start transcription of cytokine IL-2, which is necessary for initiating T cell growth and differentiation (Tedesco & Haragism 2012). Budesonide is a synthetic, non-halogenated glucocorticoid absorbed in the proximal gastrointestinal tract. It has a 90% first-pass metabolism in the liver, a much greater glucocorticoid receptor affinity than prednisone, and a low systemic bioavailability minimizing systemic effects. Budesonide has been reported to reduce inflammatory symptoms and improve liver function in patients with AIH (Wiegand et al. 2005). Cyclosporine and budesonide could be promising immunosuppressive therapy options for Dobermans suffering from SDH. Prospective clinical trials are warranted to test this hypothesis.

There is an increasing interest in prospective pharmacotherapy in AIH. To minimize tissue damage, antioxidant treatment could be used as an adjunctive intervention (Czaja 2016). Antioxidant treaments could also be used in dogs with hepatitis (Webb & Twedt 2008).

7 Limitations of the study

These studies have some limitations, which might have influenced their results and interpretation.

In the DLA study (**I**), the control dogs did not have histopathological confirmation of liver health. With the long asymptomatic phase of DH, it is possible that subclinical cases were included as control dogs. However, the samples were collected from dogs aged over ten years to ensure the lowest possible genetic risk for DH. However, the oldest reported DH patient was 11 years (Fuentealba et al. 1997). The control dogs went through a complete physical examination, hematology, serum biochemistry, and urinalysis. The dogs were only included in the control group when no abnormalities were found; those with increased serum ALT activity were excluded.

One potential limitation of an association study is the probability of a false-positive relationship between genes and markers. The inheritance patterns were studied with pedigree analysis to verify that the dogs were not closely related (**I, II**). A limitation of the study was that three unrelated dogs were revealed to be grandfathers of dogs in the case group: dogs A and B had three descendants, and dog C had two descendants. However, all of these cases had different parents.

In the AHA, GAPDH, and ADH studies (**III, IV**), two samples were outliers, which might have influenced the results of the statistical analysis. In the AHA ELISA study (**III**), one SDH dog and one control dog were outliers. The statistical tests were carried out with and without these outliers; the outcome remained virtually the same whether or not these cases were included. In the last two studies, the differences in the female/male ratio between the DH and control groups may have had an influence on the data. This is, however, unlikely because also male dogs were positive for AHA, GAPDH, and ADH among the SDH and CDH dogs. Another limitation was that WSAVA standardized evaluation of histopathology could not be performed on the older samples. The grading of histopathologic findings for hepatitis was, however, based on Knodell´s method (Knodell et al. 1981, Speeti et al. 1998). Also, the histopathological confirmation was not available for all control dogs. Nonetheless, these dogs were considered clinically healthy, showing no increases in serum ALT or AP activity at the time of sampling, and their health status was followed for two years after sampling, with no clinical evidence emerging for SDH or CDH including no elevation of serum ALT or AP activity.

In study **IV**, IP was made only with IgA and this could have influenced the result. However, the ELISA assays were carried out with both IgA and IgG. IgG was clearly producing stronger results.

8 Future prospects

The etiology of DH has long been controversial. This study is the first to provide research-based evidence of an autoimmune liver disease in dogs. An MHC class II gene association was found in patients with DH. Furthermore, it has not previously been shown that autoantibodies are significantly elevated in this form of chronic hepatitis. Understanding the biological pathways in immune-mediated diseases is pivotal for identifying appropriate targets for new therapy options. These results combined with previous knowledge about DH shed light on the etiology and pathogenesis, and provide tools for treatment trials in DH with novel approaches.

Today, DH is a rare disease and this poses difficulties for large treatment trials. One of the reasons could also be that Doberman breed is less popular. However, the prevalence of SDH is unknown and the clinical disease might return when breeding affected dogs. Further studies into the prevalence of SDH with population-wide analysis of AHA, GAPDH, and ADH autoantibodies in Dobermans and their associations with increases in serum ALT activity are therefore warranted. Follow-up studies in SDH dogs and their offspring would allow the course of the disease and its penetration to be clarified. Prospective treatment trials in CDH and SDH dogs are warranted using cyclosporine and budesonide and with follow-up examinations of ALT activites, autoantibody titers, and histologic changes by repeated minimally invasive liver biopsies. Liver elastography could be used to assess disease severity. This minimally invasive imaging technique could be advantageous for disease monitoring and diagnostics.

Further research is required to identify the exact immune mechanisms in DH. Immunohistochemistry would identify discrete distribution and localization of different cellular components in DH. It also remains to be investigated whether autoantibodies have a functional consequence in DH and in possible cell-mediated immunity against the same target antigens and peptides.

In humans, a new field of research is proteogenomics, which combines high-throughput mass spectrometry and protein sequencing to reveal the immunopeptidome presented by the MHC class II molecules (Caillat-Zuchman 2017). Proteogenomics in dogs would enhance our knowledge of the peptides presented by DLA molecules on the hepatocyte cell surface.

Future studies should be aimed at identifying the role of intestinal microbiota and intestinal permeability in the gut-liver axis in DH. An abnormal alteration in gut microbiome composition aggravates the intestinal barrier integrity, leading to an increased passage of gut-derived products, affecting gut and liver biology. These products might even serve as heteroantigens, inducing autoimmunity.

Mice are often used as models to represent human autoimmune conditions. However, no single mouse model can completely replicate human conditions. Dogs share their living environment with humans and assessing the validity of DH as a spontaneous novel disease model for human ALDs would therefore be justified.

9 Conclusions

1. Homozygous DLA class II haplotypes and alleles confer a risk for and from DH. Homozygous three-locus haplotypes DLA-DRB1*00601/DQA1*00401/ DQB1*01303 increase the risk for DH to almost 15-fold. The homozygous DLA-DRB1*00601 allele appears to confer a risk for DH development. DQ-heterodimer DLA-DQA1*00901/DQB1*00101 and DLA-DRB1*01501 appear to protect against DH. DH shows a polygenic pattern of inheritance, but the observed DLA class II association suggests a role for the immune system in the disease development.

2. Promoter variants are not associated as risk modifiers for DH in Dobermans with the risk haplotype DLA- DRB1*00601/DQA1*00401/DQB1*01303, but the whole DLA block is associated with DH. The promoter DLA-DRAp and exon 2 are identical to the dog reference sequences in DH patients and controls. Strong LD was demonstrated between the DLA alleles and their promoters. The DLA-DRB1*00601 allele is associated with the promoter variant DRBp*1, the DLA-DQB1*01303 allele is associated with the variant DQBp*6, and the DLA-DQA1*00401 allele is associated with the variant DQAp*2.

3. ANA was found in DH patients. ANAs were further characterized as autoantibodies towards histone that were significantly elevated in DH patients. Our results show that immune tolerance has failed in DH. Elevated AHA in conjunction with elevated ALT in a Doberman with or without clinical signs of hepatitis support the DH diagnosis.

4. Two novel liver-related target autoantigens in DH were described. These could be identified as dehydrogenase enzymes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and alcohol dehydrogenase (ADH). DH dogs had significantly elevated serum IgG immunoreactivity towards GAPDH and ADH antibodies relative to controls, and the disease was significantly associated with both autoantibodies. This provides evidence for an antigen-driven autoimmune process and elucidates the DH pathogenesis.

5. The diagnosis of DH is suggested to include the following: subsequent, at least threefold ALT levels, a positive test for AHA, anti-GAPDH, and anti-ADH antibodies, and a compatible DH liver histology. We suggest that all three

autoantibodies be tested, as no single autoantibody is specific for the diagnosis of DH. Screening these autoantibodies in CDH patients could help in making the diagnosis when a liver biopsy is too risky for the patient. A negative serum autoantibody result does not rule out DH. AHA, anti-GAPDH and anti-ADH autoantibodies are not suitable biomarkers for disease progression.

6. This study provides research-based evidence that DH is a T cell-mediated autoimmune disease of the liver. The results may provide a potential tool to differentiate DH from hepatitis of another etiology in Dobermans.

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