Unraveling the Genetics of Lymphocytic Thyroiditis

Using the Dog as a Model

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Abstract

The domestic dog, with its unique genetic structure formed by domestication and recent breed creation, has been proven to be an excellent model for mapping disease genes. In this thesis, we use the dog as a model to investigate the prevalence and genetic background of canine lymphocytic thyroiditis (CLT), one of the most common immune related diseases in dogs. The canine phenotype has been carefully characterized and suggests a strong comparative value to human Hashimoto's disease because of similar etiology, clinical signs and disease progression. Therefore, our aim was to gain increased knowledge about CLT and its relevance to human autoimmune thyroid disease, to benefit both dogs and humans.

In our first study (paper I) we screened two birth cohorts (3-4 and 6-7 years old, respectively) for elevated serum levels of autoantibodies to thyroglobulin (TgAA) and thyroid stimulating hormone (TSH) and could estimate a very high prevalence of CLT in the giant schnauzer and the hovawart breeds. Next, we aimed at unraveling the genetic background of CLT in the same breeds, using two different approaches; a candidate gene approach followed by a genome-wide association analysis. Using the first approach we obtained evidence that *DLA* class II polymorphisms can function both as a genetic risk factor predisposing for the disease as well as a protective factor against the disease. Using genome wide association we identified two additional strong susceptibility loci located on chromosome 11 and X. Both regions harbour genes with known immune-regulatory functions and implicates three genes involved in NF-κB pathway. Given the reported role of NF-κB in many human autoimmune diseases, our results suggest CLT as an excellent genetic model for human thyroiditis.

Keywords: CLT, lymphocytic thyroiditis, hypothyroidism, dog, Hashimoto's disease, animal model, genome wide association mapping, MHC.

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To my dad,
for always believing in me

and to Dan,
for always being there

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Ferm, K.**, Björnerfeldt, S., Karlsson, Å., Andersson, G., Nachreiner, R. & Hedhammar, A. (2009). Prevalence of diagnostic characteristics indicating canine autoimmune lymphocytic thyroiditis in giant schnauzer and hovawart dogs. *J Small Anim Pract* 50(4), 176-9.

II Wilbe, M., **Sundberg, K.**, Hansen, I.R., Strandberg, E., Nachreiner, R.F., Hedhammar, Å., Kennedy, L.J., Andersson, G. & Björnerfeldt, S. (2010). Increased genetic risk or protection for canine autoimmune lymphocytic thyroiditis in Giant Schnauzers depends on DLA class II genotype. *Tissue Antigens* 75(6), 712-9.

III **Sundberg, K.**, Kamgari, N., Ratnakumar, A., Ahlgren, K.M., Lobell, A., Truvé, K., Strandberg, E., Kämpe, O., Andersson, G., Hedhammar, Å., Pielberg, G., Lindblad-Toh, K. Genome wide-association mapping identifies major susceptibility loci for Canine Lymphocytic Thyroiditis. Manuscript.

Abbreviations

ADAMTSL	ADAMTS-like protein 1
AITD	autoimmune thyroid disease
BB/W-rat	BioBreeding/Worcester rat
BCOR	co-repressor of B-cell lymphoma 6
BCL-6	B-cell lymphoma 6
BNC2	basonuclin2
bp	base pair
CHV	canine hepatitis C virus
CI	confidence interval
CLT	canine lymphocytic thyroiditis
CNTLN	centlein
CS	cornell special strain
CTLA-4	cytotoxic T lymphocyte antigen-4
CYBB	cytochrome-b b-chain
DLA	dog leukocyte antigen
DMRTA1	DMRT-like family A1
DMRTA2	DMRT-like family A2
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELAVL2	embryonic lethal, abnormal vision, Drosophila-like 2
ELISA	enzyme-linked immunosorbent assay
FCI	Fédération Cynologique Internationale
FREM1	FRAS1 related extracellular matrix 1
HCV	hepatitis C virus
HLA	human leukocyte antigen
IFN-a	interferon alpha
IIT	interferon induced thyroiditis

IL-8	interleukin-8
kb	kilo bases
LANCL3	lanc-like protein 3
LD	linkage disequilibrium
LincRNA	long intergenic non-coding RNA
Lyp	lymphoid-specific phosphatase
MAF	minor allele frequency
Mb	mega bases
MHC	major histocompatibility complex
MTAP	methylthioadenosine phosphorylase
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFIB	nuclear factor I/B
NOD	non-obese diabetic
OMIA	Online Mendelian Inheritance in Animals
OS	obese strain
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PLD	Polish lowland dog
PTPN22	tyrosine phosphatase nonreceptor 22
QQ-plot	quantile-quantile plot
RNA	ribonucleic acid
RPGR	retinitis pigmentosa GTPase regulator
SAT	spontaneous autoimmune thyroiditis
SCID	severe combined immunodeficiency
SKC	the Swedish Kennel Club
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism
T_3	triiodinethyronine
T_4	thyroxine
Tg	thyroglobulin
TgAA	thyroglobulin autoantibodies
TILRR	toll-like/interleukin-1 receptor regulator
TPO	thyroperoxidase
TSH	thyroid stimulating hormone
TSHR	thyroid stimulating hormone receptor
TSPAN7	tetraspanin 7
WSS	Welsh springer spaniel
XK	Membrane transport protein XK

Introduction

The dog as an animal model for human disease

The domestic dog (*Canis familiaris*) is truly man's best friend. Not only has it been our loyal companion for at least 15 000 years (Savolainen *et al.*, 2002; Vila *et al.*, 1997; Wayne *et al.*, 1997), but it can also help us to better understand the genetic background of many of our common diseases. Animal models have long been used to study basic biological functions or to identify genetic or environmental risk factors for disease development. Traditional model organisms such as the mouse and the rat have been, and still are, valuable tools for such research. However, high quality genome sequences for several domesticated species have recently been made available, adding important opportunities for comparative research in species closer to humans in terms of anatomy, physiology and genetics. The work in this thesis is based on genetic and epidemiologic studies of a common and naturally occurring disease in the dog, canine lymphocytic thyroiditis.

The remarkable dog genome

It is believed that the dog's ancestor, the grey wolf (*Canis lupus*), coexisted with human in a mutually beneficial relationship, and that the first selection of wolves was based on the individuals non-aggression towards human (McGreevy & Nicholas, 1999). Eventually, man began to select and breed dogs possessing desirable behavioral- (hunting, guarding, retrieving) and morphological (size, scull shape, coat color and texture) characteristics, creating different types of dogs. But it was only recently, not more than 200 years ago, that the actual breed creation and establishment of strict breed standards took place. Today, the domesticated dog population is divided into more than 350 breeds recognized by the international canine

organization Fédération Cynologique Internationale (FCI), each representing a closed population. The large number of breeds and breed variants implies breeding in genetically small populations. Also, common to many breeds is a founder event involving only a few individuals, the continuous use of popular sires and systematic inbreeding, further increasing genetic drift and loss of heterozygosity (Lindblad-Toh *et al.*, 2005; McGreevy & Nicholas, 1999).

The restrictive breeding strategies have created a reduced heterozygosity within breeds. However, the degree of genetic variation across breeds is extensive, much higher than the variation between human populations. Of the total existing genetic variation in dogs, 27% is observed between breeds, in humans the corresponding figure between populations is only 5-10% (Parker *et al.*, 2004).

Indeed humans have, from the genome of the grey wolf distilled most of all possible morphologic and behavioral variation, divided it and enriched it within more than 350 different genetic isolates and created the most diverse of all domesticated species, the domesticated dog. An event by some referred to as 'one of the greatest genetic experiments ever' (Ostrander & Wayne, 2005).

Fewer markers, fewer individuals

The unique population- and breed history of the dog have shaped its genome to offer great advantages for mapping traits. As a part of the Dog genome project linkage disequilibrium (LD) and haplotype blocks within and across breeds was defined (Lindblad-Toh *et al.*, 2005). It was shown that within breeds haplotypes are large (0.5-1 Mb) and LD are 10-100 times more extensive than in human, reaching over several megabases. It was also found that large portions (up to 100 kb) of the haplotypes are shared across breeds, suggesting that genetic risk factors may be shared between breeds. In contrast, haplotypes across breeds are shorter and LD extends only tens of kilobases.

The extensive haplotype structure of the dog allows mapping with far fewer markers compared to humans (Figure 1) (Lindblad-Toh *et al.*, 2005). While human studies require 300 000 to a million markers, 10 000 to 50 000 markers are sufficient to perform whole genome associations studies in the dog (Karlsson *et al.*, 2007; Lindblad-Toh *et al.*, 2005; Sutter & Ostrander, 2004).

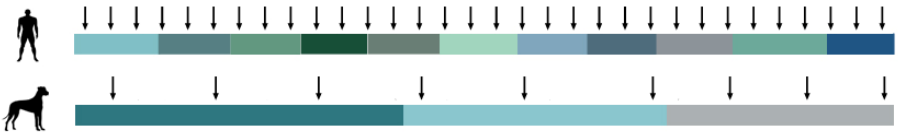

Figure 1: The extensive linkage disequilibrium in the canine genome allows mapping with fewer markers than in humans.

The low genetic variation within breeds also reflects the enrichment of a few strong genetic factors, allowing efficient mapping with fewer individuals than in human populations, which are much more diverse. Lindblad-Toh and colleagues (2005) predicted that 20 cases and 20 controls should be sufficient for efficient mapping of recessive traits. For dominant traits 50 cases and 50 controls should be sufficient, and for a complex trait, 100 cases and 100 controls should identify alleles conferring a 5 fold increased risk (Lindblad-Toh *et al.*, 2005). Several successful studies identifying genetic risk factors for both monogenic and complex diseases later proved this prediction to be correct (Olsson *et al.*, 2011; Wilbe *et al.*, 2010; Karlsson *et al.*, 2007; Salmon Hillbertz *et al.*, 2007).

Common disease, common genes, shared environment

As a consequence of the reduced heterozygosity, domesticated dog breeds suffer from an excess of inherited diseases such as cancers, diabetes, epilepsy, cardiovascular and autoimmune diseases, all of which are also common in humans (OMIA, 2012; Karlsson & Lindblad-Toh, 2008; Lindblad-Toh *et al.*, 2005; Ostrander *et al.*, 2000). Clinical manifestation and disease progression is often similar between the two species. The database OMIA (Online Mendelian Inheritance in Animals) reports more than 500 genetic diseases in dogs, the majority indicated as potential models for human diseases (OMIA, 2012). Furthermore dogs, like humans, receive extensive medical care and reliable clinical diagnoses, which constitute another valuable benefit in genetic disease mapping. Most purebred dog populations also have extensive pedigree records in which to trace familiar genetic diseases.

The genomes of dog and human are very similar. Comparative studies between the human, mouse and dog genome show that dog to human nucleotide sequence similarity is higher compared with the similarity

between dog and mouse. Dog have roughly the same number of genes (20 000, compared to 21 000 in humans), and the difference is mainly due to gene-family expansion. Also, a large proportion of genes identified in dog are orthologous to human genes (Lindblad-Toh *et al.*, 2005), further strengthening the comparative value of the dog model.

But humans and dogs do not only share a common history of several thousand years, disease spectra, medical treatment and a large proportion of our genetic makeup. We also share a common environment. Dogs live, unlike more traditional model organisms such as mouse and rat, close to humans and often share their indoor environment as well as exercise and food habits with their fellow humans. Certainly, the dog offers the research community unique opportunities as a model organism for mapping both Mendelian and complex diseases.

Autoimmune thyroid disease

Autoimmune thyroid disease and lymphocytic infiltration of the thyroid gland can result in two opposite clinical manifestations, in humans referred to as Hashimoto's disease and Grave's disease. In the former, lymphocytic infiltration in the thyroid leads to apoptosis and *hypo*thyroidism, while in the latter it leads to stimulation of thyroid hormone production and *hyper*thyroidism. Hyperthyroidism, or Grave's disease, is common in humans (Wang & Crapo, 1997) while extremely rare in dogs. Therefore this thesis focuses on hypothyroidism although at times referring also to Grave's disease, as the two diseases in humans share some common genetic background (reviewed in Tomer, 2010a).

Hypothyroidism in humans and dogs

The Japanese specialist Hashimoto Hakuru first described human autoimmune hypothyroidism in 1912. The disease was named Hashimoto's thyroiditis and was the first autoimmune disease ever to be described. Today, it is one of the most common autoimmune diseases (Jacobson *et al.*, 1997) affecting up to 2% of the general population (Wang & Crapo, 1997) and women ten times more frequently than men (Tunbridge & Vanderpump, 2000). The occurrence of canine lymphocytic thyroiditis (CLT) and its striking similarities to human Hashimoto's disease was first described in a purebred colony of Beagle dogs in 1968 (Beierwaltes & Nishiyama, 1968). Since then, the disease has been extensively reported in

different breeds and is now considered to be one of the most common endocrine diseases also in dog.

As in human, CLT is characterized by the presence of activated T-lymphocytes and thyroidal infiltration of lymphocytes, plasma cells and macrophages (Gosselin *et al.*, 1982), leading to an acute loss of thyroid follicular cells and eventually inability to produce sufficient amounts of the thyroid hormones triiodinethyronine (T_3) and thyroxine (T_4). As the thyroid hormone titers decrease, overt hypothyroidism arises and clinical symptoms start to appear. The thyroid hormones are crucial for maintaining a normal body metabolism, therefore symptoms of hypothyroidism can be widely variable and in both human and dog include metabolic (weight gain, exercise intolerance, weakness, cold intolerance), dermatologic (hair loss, dry skin) and psychological (lethargy, depression) alterations (Mussig *et al.*, 2012; Burman & McKinley-Grant, 2006; Ai *et al.*, 2003; Dixon *et al.*, 1999). In dogs, common clinical signs include lethargy, obesity, hair loss and poor coat quality (Dixon *et al.*, 1999) (Figure 2).

Figure 2. Pictures of the same CLT-affected hovawart dog, before (left) and after (left) medical treatment. Note poor coat quality, lethargic appearance, less bright markings and weight gain in the untreated dog (left). Photo: Dr Elisabeth Dietchi.

The clinical diagnosis of both human and canine hypothyroidism is commonly based on the presence of appropriate clinical signs as well as increased serum concentrations of thyroid stimulating hormone (TSH) and decreased T_4 concentration. Moreover, circulating autoantibodies to specific thyroid antigens are present during the course of disease progression. Today, no cure or modifying drug for autoimmune hypothyroidism exist, thus both human and canine patients are dependent on life-long L-thyroxine substitution therapy.

Canine lymphocytic thyroiditis; a model for Hashimoto's disease

CLT etiology, clinical signs and disease progression perfectly mimic human Hashimoto's disease. The disease develops spontaneously and in high frequency. As a consequence of low genetic variation and breeding within closed populations discussed earlier, the genetic risk factors for CLT have been enriched in certain breeds, theoretically offering a more simple and straightforward mapping strategy than in human. Studies from USA report high incidence of hypothyroidism in a variety of breeds, such as Doberman pinchers, Great Danes, poodles, Irish setters, miniature schnauzers, boxers, golden retrievers, dachshunds, Shetland sheepdogs, Pomeranians, cocker spaniels and Airedale terriers (Milne & Hayes, 1981; Nesbitt *et al.*, 1980). In Sweden, veterinary care insurance data have indicated the highest prevalence of CLT in giant schnauzer and hovawart, but also golden retriever, Doberman pincher, American cocker spaniel, boxer and Rhodesian Rigeback have a higher than average CLT-prevalence (Egenvall *et al.*, 2000) suggesting these breeds to be excellent study populations.

Although many similarities, also some differences exist between the human and canine form of autoimmune hypothyroidism. In human, the disease is ten times more frequent in women than men (Tunbridge & Vanderpump, 2000), whilst neither sex nor neutering status have been observed to influence CLT prevalence in the dog (Dixon *et al.*, 1999; Beierwaltes & Nishiyama, 1968). Also, in human two major antigens are prevalent; thyroglobulin (Tg) and thyroperoxidase (TPO). Both proteins play key roles in thyroid hormone synthesis; Tg as the precursor of thyroid hormones and TPO as the enzyme catalyzing iodination of Tg-associated tyrosine. In dogs, the major CLT antigen is Tg (Graham et al., 2007), although autoantibodies to TPO can also be found (Skopek et al., 2006).

Hypothyroidism in other animal models

Several rodent models for autoimmune hypothyroidism exist. Some models, like the non-obese diabetic mouse (NOD mouse) and the BioBreeding/Worcester rat (BB/W rat) develop the disease spontaneously (Bernard *et al.*, 1992; Yanagisawa *et al.*, 1986), whereas in others, *e.g.* the severe combined immunodeficiency mouse (SCID mouse) and the nude mouse, the disease requires inducement by injection of autoantigens (*i.e* thyroglobulin) (reviewed in Volpe *et al.*, 1993).

A domestic animal model extensively used for the study of spontaneous autoimmune disease is the Obese strain (OS) chicken, developed at Cornell

University by R.K. Cole (Cole, 1966). Cole noticed that about 1% of females of the Cornell Special strain (CS) showed symptoms of spontaneous autoimmune thyroiditis and started to selectively breed from them. Today, after many decades of selective breeding, the strain shows a nearly 100% incidence of spontaneous autoimmune thyroiditis (SAT), independent of sex. The systematic selection for the SAT phenotype is thought to have resulted in homozygosity within the disease-causing loci. Symptoms of SAT include small body size and high body weight, long silky feathers and small comb (Figure 3). Many studies of the OS chicken have focused on the immunology and pathology of SAT (reviewed in Wick *et al.*, 2006), but ongoing efforts at Uppsala University involves studies also of the genetic background of the disease (personal communication, Dr. Susanne Kerje).

Figure 3. An obese strain (OS) chicken (left) showing typical signs of hypothyroidism: small size, small comb, long silky feathers and subcutaneous and abdominal fat deposits (therefore referred to as "obese"). To the right, a healthy white leghorn chicken. Photo: Dr Susanne Kerje.

Genetics of autoimmune thyroid disease

Today, there is abundant evidence for a strong genetic background of AITD both in human and dog. Early reports of familiar occurrence of both Grave's and Hashimoto's disease date back almost half a century (Hall & Stanbury, 1967). More recent studies have shown that over 30% of patients with Graves' or Hashimoto's disease have a family history of AITD (Villanueva *et al.*, 2003). Several twin studies have also reported a higher concordance rate in monozygotic twins compared to dizygotic twins for both Grave's disease

and Hashimoto's disease, further highlighting the genetic importance of disease susceptibility (Brix *et al.*, 2001; Brix *et al.*, 2000).

In dogs, the first report of familial thyroiditis was described in 1968, in a breeding colony of beagle dogs (Beierwaltes & Nishiyama, 1968) later followed by a study in a family of borzoi dogs (Conaway *et al.*, 1985). Furthermore, the fact that prevalence of CLT differs substantially between breeds, strongly supports a genetic background of the disease also in dog (Milne & Hayes, 1981; Nesbitt *et al.*, 1980).

Human AITD susceptibility genes

The strong epidemiologic evidence for a genetic background to AITDs has encouraged investigators to identify and characterize AITD susceptibility genes by use of linkage-, candidate gene- and more recently, whole genome association analysis. To date, three immune regulatory and two thyroid specific genes have been reported to have an effect on human AITD. Most susceptibility genes are common to both diseases, while some are specific for Grave's disease. However, to date, no genes specific for Hashimoto's disease have been reported.

General immune-regulatory genes

The first locus to be linked to human AITD susceptibility was the major histocompatibility complex (*MHC*), which encodes the human leukocyte antigen (*HLA*) proteins. They are cell-surface antigen-presenting proteins, thus playing a key role in immune response activation. The *HLA* region is highly polymorphic and several haplotypes have been associated with AITD susceptibility in humans. The strongest association reported has been to the *HLA-DRB1*0301* allele at the *DRB1* locus and an increased frequency of the *HLA-DR3* haplotype which carries the *DRB1*0301* allele have been reported in both Grave's and Hashimoto's disease patients (Levin *et al.*, 2004; Zamani *et al.*, 2000; Moens *et al.*, 1978). These studies have been further supported by studies in transgenic mice expressing either *HLA-DRB1*0301* or *DRB1*0201* and immunized with both human or mouse thyroglobulin (Tg). Only *DRB1*0301* transgenic mice developed severe thyroiditis, providing strong evidence for *DRB1* polymorphism as a genetic risk factor for thyroiditis (Kong *et al.*, 1996). Additionally, *HLA-DR5* and *DQB1*0301* have been linked to Hashimoto's disease (Wu *et al.*, 1994; Farid *et al.*, 1981). Recently, sequencing of the HLA-DRB1 gene and structural modeling studies of the antigen binding pockets identified a

specific molecular structure to be strongly associated to both AITD and type1 diabetes. An arginine at position 74 in the HLA-DRβ1 chain resulted in a unique pocket structure with a stronger binding to the antigen (Menconi *et al.*, 2010).

Another gene predisposing to general thyroid autoimmunity rather than to one specific disease, is the cytotoxic T lymphocyte antigen-4 (*CTLA-4*). The association of *CTLA-4* to Grave's disease has been consistent over different ethnic groups, such as Caucasians (Allahabadia *et al.*, 2001; Kotsa *et al.*, 1997; Yanagawa *et al.*, 1995), Japanese (Akamizu *et al.*, 2000), Chinese (Yanagawa *et al.*, 1995) and Koreans (Marron *et al.*, 1997) and to Hashimoto´s disease in Caucasians (Kotsa *et al.*, 1997) and Japanese (Akamizu *et al.*, 2000). A more recent study reported that the G allele of the *CTLA-4* A/G_{49} SNP is associated with decreased function of CTLA-4, and thus an impaired inhibitory effect of CTLA-4 on T-cell proliferation (Ban *et al.*, 2003a). The *CTLA-4* gene has also been linked to two additional autoimmune disorders in human; Addison's disease (Donner *et al.*, 1997a) and type 1 diabetes (Donner *et al.*, 1997b), suggesting that *CTLA-4* polymorphism induces a general susceptibility to autoimmunity (Kristiansen *et al.*, 2000).

Another negative regulator of T-cell activation implicated in human organ specific and systemic autoimmune disease is the lymphoid-specific phosphatase (Lyp), encoded by the protein tyrosine phosphatase nonreceptor 22 gene (*PTPN22*). A SNP in this gene, changing a cytosine residue into a thymine at position 1858, inhibits the protein's ability to suppress T-cell activation and was first found to be associated to type 1 diabetes in human (Bottini *et al.*, 2004). Since then, the same allele has also been associated with both types of AITDs (Criswell *et al.*, 2005; Velaga *et al.*, 2004), familial systemic lupus erythematosus (SLE) (Kaufman *et al.*, 2006; Kyogoku *et al.*, 2004) and rheumatoid arthritis (Begovich *et al.*, 2004).

Apart from the three genes mentioned above, all representing a shared susceptibility to Grave's and Hashimoto's disease, one additional immune regulatory gene specific to Grave's disease has been identified; the *CD40* gene (Tomer *et al.*, 2002a). This gene encodes a B-cell surface receptor involved in activation of antigen presenting cells.

Thyroid specific genes

Thyroglobulin (Tg) is a protein produced by the thyroid epithelial cells, and serves as a precursor and storehouse of thyroxine (T4) and triiodothyronine (T3). Also, thyroglobulin is one of the major autoantigens of human AITDs

and therefore represents an appealing candidate gene for both Grave's and Hashimoto's disease. Indeed, two separate linkage analyses confirmed a strong association between the *Tg*-locus and familial AITDs (Tomer *et al.*, 2002b; Sakai *et al.*, 2001). Later, this association was confirmed and strengthened in both human and murine studies (Ban *et al.*, 2003b).

A characteristic and specific feature of Grave's disease is the production of autoantibodies to the thyroid stimulating hormone receptor (*TSHR*). Dechario and colleges identified *TSHR* as a disease-specific susceptibility gene for Grave's disease (Dechairo *et al.*, 2005).

Canine AITD susceptibility genes

In dog, the only known genetic risk factor associated to hypothyroidism disease susceptibility is the dog leukocyte antigen class II (*DLA II*), the dog equivalent to human leukocyte antigen (HLA) class II. In 2006, Kennedy and colleagues identified and reported a *DLA* haplotype in Doberman pincher dogs, *DLA-DRB1*01201/DQA1*00101/DQB1*00201*, to be almost twice as frequent in affected dogs compared to unaffected dogs. They also suggested it to be a rare haplotype and supposed it unlikely to be associated with hypothyroid disease in other breeds (Kennedy *et al.*, 2006a). However, the same investigators later reported association to CLT also in Rhodesian ridgebacks and English setters carrying the same *DQA1* allele (*DQA1*00101*). Notably, CLT affected boxers included in that study did not have an increased frequency of *DQA1*00101*, suggesting different *DLA* class II susceptibility alleles/haplotypes in different breeds (Kennedy *et al.*, 2006b). *DLA* class II polymorphism as a genetic risk factor for CLT will be further discussed in paper II of this thesis.

Environmental risk factors

The identification of several AITD susceptibility genes shows the relevance of genetic components of both Hashimoto's and Grave's disease in humans, as well as for CLT in dogs. Nevertheless, studies in human report concordance rates of monozygotic twins well below 1 (Brix *et al.*, 2001; Brix *et al.*, 2000), suggesting an important role also for environmental risk factors. Through studies of monozygotic twins it has been calculated that 79% of the susceptibility to develop Grave's disease is attributable to genetic factors, leaving the remaining 21% to be explained by environmental factors (Brix *et al.*, 2001).

Indeed, several environmental risk factors have been identified in human AITD patients. The major environmental triggers include pregnancy, medications, stress, smoking, iodine intake, selenium deficiency as well as viral and bacterial infections (reviewed in Prummel *et al.*, 2004). Here we will focus on three of the most well documented environmental triggers of AITD; iodine intake, interferon alpha treatment and hepatitis C virus infection.

Iodine intake

Iodine is necessary for thyroid hormone production. Thus the impact of iodine intake on human thyroid function has been extensively reported. The general belief has long been that severe to moderate iodine deficiency may induce hypothyroidism (Hetzel, 1987) and that excessive iodine intake is well tolerated without any apparent side effects (Pennington, 1990). However, more recent studies have concluded that the association between iodine intake of a population and the occurrence of thyroid diseases is U-shaped, with an increased risk from low as well as high iodine intake (Bulow Pedersen *et al.*, 2002).

Iodine induced hypothyroidism in genetically susceptible animal strains have also been investigated. In the autoimmune prone non-obese mouse strain (NOD)-$H2^{h4}$ 5% of individuals develop hypothyroidism spontaneously. However, if excessive iodine is added to the drinking water more than 60% of individuals develop the disease (Rasooly *et al.*, 1996). In dogs, Castillo and colleagues reported a highly significant increase of serum TSH together with decrease of free T_4 in puppies fed a high iodine diet, compared to puppies fed a normal control diet (Castillo *et al.*, 2001). The same investigators also report a large difference of iodine content in commercial dog feeds, and suggest that dogs fed a high iodine commercial diet may develop hypothyroidism.

Interferon alpha treatment

Interferons are a group of distinct proteins that possess antiviral and antitumoral activity. They belong to the large family of cytokines and are released by host cells in the presence of pathogens such as virus, bacteria, parasites or tumor cells. Their function is to trigger inflammatory and antiviral responses by activating immune cells (*i.e* macrophages and natural killer cells) and up-regulate antigen presentation to T lymphocytes.

Interferons are divided into two major subgroups; type I and type II. Interferon alpha (IFN-α) is a type I interferon widely used as a treatment against hepatitis C virus (HCV) infection in human (reviewed in Deutsch & Hadziyannis, 2008). Indeed, it is successful; remission of HCV occurs in nearly 50% of patients given a combination of IFN-α and ribavirin (another anti-viral drug)(Fried *et al.*, 2002). However, interferon treatment is also associated with many side effects of which interferon induced thyroiditis (IIT) is among the most common (Prummel & Laurberg, 2003). Studies have shown that up to 40% of IFN-α treated HCV patients develop thyroid autoantibodies and up to 15% develop clinical hypothyroidism (reviewed in Tomer *et al.*, 2007), suggesting that pharmacological doses of IFN-α may induce a general activation of the immune system and trigger autoimmunity. However within the IFN-α treated HCV patient group, non-autoimmune thyroiditis is as commonly reported as autoimmune thyroiditis (Tomer, 2010b). These findings suggest also a direct effect of IFN-α on thyrocytes and encouraged Mandac and colleagues to propose a new classification of IIT; autoimmune IIT and non-autoimmune IIT (Mandac *et al.*, 2006). The direct effect of IFN-α on thyrocytes was further supported by Caraccio *et al* who reported a significantly decreased expression of key enzymes involved in iodine uptake and thyroid hormone production when IFN-α was cultured with human thyroid follicular cells (Caraccio *et al.*, 2005). Also, a recent study reports that IFN-α treatment of cultured thyrocytes increased the expression of Tg, TSHR and TPO, also up-regulating antigen presentation and cytokine pathways, as well as increased thyrocyte apoptosis (Akeno *et al.*, 2011). This suggests a direct toxic effect of type I interferon on the thyroid, as a complementary mechanism to the immune effects.

To summarize, the strong association between IFN-α and thyroid disease points out IFN-α as one of the environmental factors capable of triggering the onset of AITD in genetically susceptible individuals. Furthermore, high endogenous IFN-α levels may also trigger naturally occurring thyroiditis (Prummel & Laurberg, 2003).

Hepatitis C virus infection

Although a number of viral and bacterial infections have been implicated in the pathogenesis of human AITDs (reviewed in Eschler *et al.*, 2011; Desailloud & Hober, 2009; Tomer & Davies, 1993) perhaps the most extensively investigated is hepatitis C virus infection (HCV). Studies have shown that presence of thyroid autoantibodies is significantly more frequent in patients with HCV infection compared to controls, also in patients not

receiving IFN-α treatment (Tran *et al.*, 1993). In a large study of 630 chronic HCV patients it was reported that both hypothyroidism and thyroid autoimmunity were more common in HCV patients compared to controls, even in the absence of IFN-α (Antonelli *et al.*, 2004). Further, HCV RNA can be detected in the thyroid glands of patients having chronic HCV infection (Bartolome *et al.*, 2008). Also, it has been shown that HCV proteins are able to bind to thyroid cells and up-regulate secretion of the pro-inflammatory cytokine IL-8 (Akeno *et al.*, 2008).

No closely related animal homolog to the human HCV virus has been identified until just recently, when Kapoor and colleagues reported the discovery of a novel hepatitis C virus in dogs, the CHV. Comparative phylogenetic analyses of CHV showed that it is the most genetically similar animal virus homolog of HCV. Interestingly, the estimated divergence time of HCV and CHV was only 500-1000 years, well after the domestication of the dog, suggesting a potential zoonotic origin of the virus (Kapoor *et al.*, 2011). Although the distribution of CHV in the over all dog population is currently not known, it will be exciting to see if future studies will find an association also between CHV and CLT.

Two main hypothesis regarding potential mechanisms in which infection induces AITDs exists; the molecular mimicry (Wucherpfennig, 2001) and the by-stander activation theory. While the first hypothesis proposes that the pathogen expresses a part of a protein mimicking self antigens (*i.e* Tg), causing a cross-activation of auto-reactive T- or B-cells, the latter suggests that infection of certain tissues induces local inflammation through cytokine secretion, and in that way activates T-cells.

A theoretical consequence of a virus ability to induce autoimmunity is the potential triggering effect of vaccination. Indeed, this theory has been extensively claimed and counter claimed in the literature (reviewed in Wraith *et al.*, 2003). Also in the dog, vaccinations have been argued as a potential trigger of autoimmune thyroid disease. Scott-Moncrieff investigated whether routine vaccination induced antibodies against thyroglobulin in dogs, by vaccinating five dogs with a multivalent vaccine and a rabies vaccine, five dogs only with rabies vaccine and compared with five dogs that were not vaccinated. A significant increase of TgAA was seen in rabies-vaccinated dogs, compared with control dogs (Scott-Moncrieff *et al.*, 2002). However, in a follow up study in which also postmortem examination of the thyroid glands were performed, the investigators failed to find any association between vaccination and thyroiditis, since the highest rate of thyroiditis (3 of 5 dogs) was actually found in the control group (Scott-Moncrieff *et al.*, 2006).

Current breeding strategies for CLT and advantages of susceptibility gene identification

Today, few breed clubs have adopted breeding strategies to reduce CLT prevalence within the breed. To simply avoid affected animals and their close relatives in breeding have not been effective in reducing disease prevalence. Therefore, identifying susceptibility gene variants for CLT would benefit affected dogs and responsible dog breeders. Such new knowledge might lead to the development of a genetic test to use as a breeding tool in order to reduce CLT prevalence, as a complement to present diagnostic tools to get an earlier diagnose, or to guide treatment based on genetic makeup. Further, identification of the genetic background of CLT would enable us to better understand the disease mechanism behind CLT and therefore, develop better treatment strategies.

Aims of the thesis

The overall aim of this thesis was to;

Unravel the genetic background of lymphocytic thyroiditis in dogs and increase the knowledge about its relevance to human Hashimoto's disease.

The specific aims were to;

I. Evaluate the diagnostic criteria and estimate the prevalence of CLT in two dog breeds, previously indicated as high-risk breeds.

II. Investigate DLA class II as a potential genetic risk factor for CLT.

III. Identify additional genetic risk loci for CLT using whole genome association mapping.

Summary of Present Studies

Material and Methods

In this section, a brief description of material and methods used in paper I-III is presented.

Subject recruitment and sampling procedures

All dogs included in paper I-III were privately owned pet dogs. Through access to the Swedish Kennel Club (SKC) registry, potential study subjects were selected based on breed and age. Owners were contacted through mail and were invited to have their dog included in the study. Also, a website describing CLT, common clinical symptoms, disease progression and sampling procedures was produced in order to facilitate information to dog owners and veterinarians. Information about the study and further recruitment of subjects was also achieved by visiting breed club meetings, dog competitions and dog shows. A close cooperation with veterinarians, the SKC doping commission and the Swedish University Animal Hospital laboratory also enabled targeted sampling of dogs previously diagnosed as CLT-affected. All dogs were purebreds belonging to the Scandinavian population, with the exception of a few importees.

For all dogs, EDTA-blood and serum samples were collected. Trained clinicians or veterinarians collected all samples after owners consent, following ethical approval protocols (Dnr C139/9, Ethical board for experimental animals in Uppsala, Sweden). Accompanying all samples were a dog-owner questionnaire surveying clinical status and environmental factors potentially influencing dog disease status, as well as registration number and phenotypic information. DNA was extracted using QIAamp

DNA Blood Mini/Midi Kit or QiaSymphony DNA midikit (Qiagen, Valencia, CA).

Dog material

Blood and sera from 600 giant schnauzer and 255 hovawart dogs were collected between 1999 and 2012. Also, a number of individuals from three additional breeds; Welsh springer spaniel (WSS), boxer and Polish lowland dog (PLD) were sampled (Table 1).

Table 1. Total number of collected samples within different breeds.

Breed	Blood and sera samples	Classified as cases	Classified as controls
Giant Sch.	597	142	102
Hovawart	255	47	87
WSS	50	15	10
Boxer	15	10	2
PLD	43	18	12

WSS Welsh springer spaniel, PLD Polish lowland dog.

Diagnostic criteria and phenotypic characterization (paper I-III)

Inclusion criteria for cases and controls were based on serological measurements of thyroid stimulating hormone (TSH), free thyroxine (fT4) and autoantibodies to thyroglobulin (TgAA). Concentration of TSH was measured by using a commercially available immunoradiometric assay kit (Diagnostic Products Corporation, Los Angeles, CA, USA) previously validated for canine serum samples (Paradis *et al.*, 2003). TgAA were assessed using ELISA following the manufacturers' instructions (Oxford Biomedical Research) and using the same diagnostic cutoff as previously defined (Nachreiner *et al.*, 1998).

Dogs were classified into three different categories as follows; cases, borderline cases and controls, as shown in Table 2.

Table 2. Diagnostic criteria for cases, borderline cases and controls.

	Case	Borderline case	Control
TSH (mU/L)	≥40	40 < TSH ≥ 30	≤ 25
TgAA (%)	≥200	200 < TgAA ≥150	≤ 100
fT4 (pmol/l)			5-25
Age (years)			≥ 7

TSH thyroid stimulating hormone, TgAA autoantibodies to thyroglobulin, fT4 free thyroxine.

Estimation of CLT prevalence (Paper I)

To evaluate the prevalence of canine lymphocytic thyroiditis (CLT) in the Swedish populations of giant schnauzer and hovawart dogs and to study disease progression within these breeds, paper I was performed as a birth cohort study including only 3-4 and 6-7 year old dogs, respectively. In total 236 giant schnauzers (3-4 years n=105, 6-7 years n=131) and 95 hovawarts (3-4 year n=47, 6-7 years n=48) participated. CLT status was investigated through serological screening of TSH and TgAA as described previously. Dogs with TSH serum levels ≥40mU/L and/or TgAA serum levels ≥200% of the negative control were considered CLT-affected (Table 2).

Identification of CLT risk loci (Paper II and III)

DLA sequencing

Using a candidate gene approach, paper II was designed to evaluate MHC class II genotype as a predisposing genetic risk factor for development of CLT in giant schnauzer dogs. A comparative sequence analysis of the entire polymorphic exon 2 of *DLA-DRB1, -DQA1* and *-DQB1* was performed in 30 controls, 74 cases and 28 borderline cases, using flanking intronic and locus-specific primers. For comparative purposes, 48 hovawarts (cases, n=23, controls n=20, borderline case n=5) were also sequenced. After PCR amplification and subsequent sequencing, the obtained sequences were matched to a reference sequence library to provide the matching DLA allele. Odds ratios, risk ratios, confidence interval and p-values for each allele and haplotype were calculated using χ^2 test.

Genome-wide association analysis

In order to identify novel susceptibility loci for CLT, paper III was designed as a genome-wide association study, using the canine high density SNPchip (Illumina) comprising more than 174,000 evenly spaced and validated SNPs (Vaysse *et al.*, 2011). In total, 122 giant schnauzers unrelated at parental level and strictly classified as cases (n=74) and controls (n=48) were included in the sample set. PLINK software (Purcell *et al.*, 2007) was used to perform a standard case-control analysis as well as assess genomic inflation and population stratification. After frequency and genotype pruning (call rate >75%) 117,064 SNPs and all 122 individuals were included in subsequent analysis. The final dataset was shown to be non-stratified using a QQ-plot (λ=1.03), where the observed values deviate from expected values at a -logp of 4.0. This was therefore used as a significance threshold.

Targeted re-sequencing

To further investigate the associated regions and to search for causative mutations, a 385 K custom-designed sequence capture array from Roche NimbleGen was used for targeted re-sequencing of nine giant schnauzer dogs classified and selected based on phenotype (case n=3, control n=3) as well as genotype (homozygous risk n=7, homozygous control n=1, heterozygous n=1). In total 4.1 Mb on chromosome 11 (position 39,000,000 - 41,000,000 and 42,600,000 - 44,7000,000) was targeted. The obtained sequences were aligned to CamFam2.0 (Lindblad-Toh *et al.*, 2005) using Burrows-Wheeler Alignment Tool (BWA) (Li & Durbin, 2009).

SNP- and indel detection

SNP-calling was performed using SAMtools (Li *et al.*, 2009). To investigate putative insertions, deletions or copy number variants differing between dogs carrying the risk versus control haplotype, we used Seqscoring (Truve *et al.*, 2011) to calculate the ratio between average coverage in dogs carrying risk versus control haplotype. Seqscoring software (Truve *et al.*, 2011) using the SiPhy (Garber *et al.*, 2009) 29 mammals conservation set (Lindblad-Toh *et al.*, 2011) was also utilized to score SNPs according to their degree of conservation. SNPs located within or maximum 5 bp away from a conserved element were selected for further genotyping in a larger sample set. Genotyping was performed using a custom designed VeraCode GoldenGate assay (Illumina).

Homozygosity and association to litter size

Based on the oscillating association on chromosome 11 we wanted to examine the region for evidence of a selective sweep. By searching for regions of reduced heterozygosity we revealed a 291 kb region of heavily reduced heterozygosity (minor allele frequency, MAF = 0-0.02) at position 40,091,793-40,382,938 bp. This region of reduced heterozygosity covers the first half of the gene encoding centlein (*CNTLN*), a centrosomal protein important for meiosis (Makino *et al.*, 2008). In order to investigate whether this potential sweep signal is specific for giant schnauzers or exists also in other breeds, we examined the degree of heterozygosity in the surrounding genomic region (chromosome 11 position 39-41 Mb) in a number of other breeds using genotype data from the 170 k Illumina SNP array (Vaysse *et al.*, 2011). It was shown that hovawart, English setter and weimaraner showed a similar pattern of reduced heterozygosity over the *CNTLN* gene. Another long region of reduced heterozygosity (30 kb) covering most of the *BNC2* gene at position 39,540,307-39,930,544 bp was identified in German shepherd, Dalmatian and Brittany spaniel. Breeds being heterozygous over the region were Border collie, Golden retriever, Eurasier, Irish wolfhound, Rottweiler, shar-pei and Greenland sledge dog.

Since the reduced heterozygosity was found in many breeds, we hypothesized that selection was being employed for more generally desirable factors, generating selective sweeps in many breeds. Such selection could potentially explain the high frequency of thyroiditis observed in many breeds. We hypothesized that selection for a large litter size might be common. This hypothesis was also attractive based on the biological influence of centlein during meiosis (Makino *et al.*, 2008), and the presence of several genes expressed in ovary and uterus in this region. Using the Swedish Kennel club registration data for all births, we examined the mean litter size for 14 breeds (all breeds examined were large or medium sized breeds as litter sizes in small dogs are generally lower than in large dog breeds) for 22 consecutive years and calculated an overall mean litter size per breed. Breeds were then grouped based on the presence of *BNC2*- (giant schnauzer, hovawart, English setter, Weimaraner) or *CNTLN* sweeps (German shepherd, Dalmatian and Brittany Spaniel) or for being heterozygous across the region (Border collie, Golden retriever, Eurasier, Irish wolfhound, Rottweiler, shar-pei and Greenland sledge dog). As a secondary test we examined the fraction of dogs within each of 14 breeds that carried at least one copy of the 'selected' haplotype. We then performed a correlation between this fraction and the overall mean litter size per breed.

Main findings

In this section, a brief description of obtained results from paper I-III is presented.

High prevalence of CLT in giant schnauzer and hovawart dogs (paper I)

In paper I we identified an extraordinarily high prevalence of CLT in giant schnauzer and hovawart dogs, suggesting a strong genetic predisposition to CLT within these breeds. In total, 16% of giant schnauzers and 13% of hovawarts had indications of CLT, with or without clinical signs of hypothyroidism. As expected, CLT prevalence was higher in the older cohort (6-7 years) than in the young cohort (3-4 years) where only a few individuals had showed any clinical signs of CLT or received a CLT diagnosis at the time of sampling (Figure 4). However, measurement of serum TSH and TgAA in this young group revealed signs of CLT (positive TgAA or increased TSH) in more than 10 % of dogs, suggesting a high prevalence of subclinical CLT within the age group where most breeding dogs are being used.

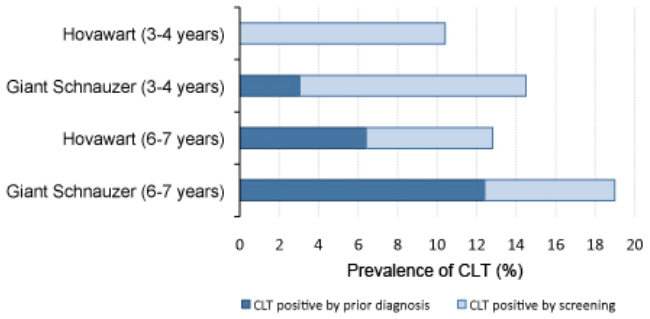

Figure 4. Prevalence of CLT within different breed and age categories. Dark blue bars represent the percentage of dogs already diagnosed with CLT at the indicated age; *i.e* cases of overt hypothyroidism, while light-blue bars indicate the proportion of dogs with subclinical CLT. The total length of the bars represents the total prevalence of CLT (with and without clinical signs) within each breed and age group.

Identification of risk- and protective *DLA*-haplotypes (paper II)

In paper II we obtained evidence that *DLA* class II polymorphism can function both as a genetic risk factor predisposing to the disease, and as a protective factor against the disease. In total eight *DRB1* alleles, four *DQA1* alleles and six *DQB1* alleles, organized into nine different haplotypes were identified in the giant schnauzer. The frequencies of two specific haplotypes differed markedly between cases and controls. The *DLA-DRB1*01201/ DQA1*00101/DQB1*00201* haplotype was found in 18.2% of cases, while only in 3.3 % of controls and the *DLA-DRB1*01301/ DQA1*00301/ DQB1*00501* haplotype was found in 35.0% of controls while only in 15.5% of cases (Table 3). Odds ratios for the risk and protective haplotypes were 6.5 (99% CI 1.0-194) and 0.3 (99% CI 0.14-0.85) respectively, and if including borderline cases, the OR for the risk haplotype increased to 6.6.

Table 3. *DLA-DRB1, -DQA1* and *-DQB1* haplotype frequencies among all giant schnauzer samples, and for the study population (cases and controls). Bold numbers indicate significant differences between cases and controls.

Haplotype	All samples (132 dogs)	Cases (74 dogs)	Controls (30 dogs)
*DRB1*DQA1*DQB1	% (no[a])	% (no[a])	% (no[a])
*00101*00101*00201	25.0 (66)	29.1 (43)	20.0 (12)
*01201*00101*00201	15.2 (40)	**18.2 (27)**	**3.3 (2)**
*00601*00401*01303	17.0 (45)	15.5 (23)	20.0 (12)
*01301*00101*00201	7.2 (19)	8.8 (13)	6.7 (4)
*01301*00301*00501	22.0 (58)	**15.5 (23)**	**35.0 (21)**
*02301*00301*00501	8.7 (23)	8.1 (12)	8.3 (5)
*00901*00101*008011	2.3 (6)	2.0 (3)	3.3 (2)
*01501*00601*02201	1.9 (5)	1.4 (2)	3.3 (2)
*02001*00401*01301	0.8 (2)	1.4 (2)	0 (0)

[a] Two chromosomes per individual

When comparing allele frequencies separately, we observed a higher frequency in CLT cases compared to controls for all three alleles found in the risk haplotype. However, *DRB1*01201* was exclusively carried on the risk haplotype, while the other two alleles were also present on non-risk haplotypes. This indicates that it may be a *DRB1* allelic effect, rather than a haplotypic effect, causing the increased genetic predisposition in dogs carrying the *DRB1*01201* allele. In contrast, the protective effect was not detected when comparing the three alleles on the protective haplotype separately, suggesting that these three alleles act together to obtain a protective effect.

In the hovawart, six *DLA-DRB1* alleles were found. The *DLA-DRB1*01201* allele had a total allele frequency of 48%, while the additional five alleles were rare or moderately frequent with allele frequencies ranging between 1.0-18.4%.

Strong association to chromosome 11 and X (paper III)

The genome-wide association analysis identified a strong association to chromosome 11 and X, both with raw p-values of 1.5 x 10^{-6} (Figure 5). The QQ-plot indicated a –log (p) value above 4.0 to be genome-wide significant at the 5% level. Assessment of the genomic inflation factor indicated very limited stratification within our sample set, with a lambda-value of 1.03.

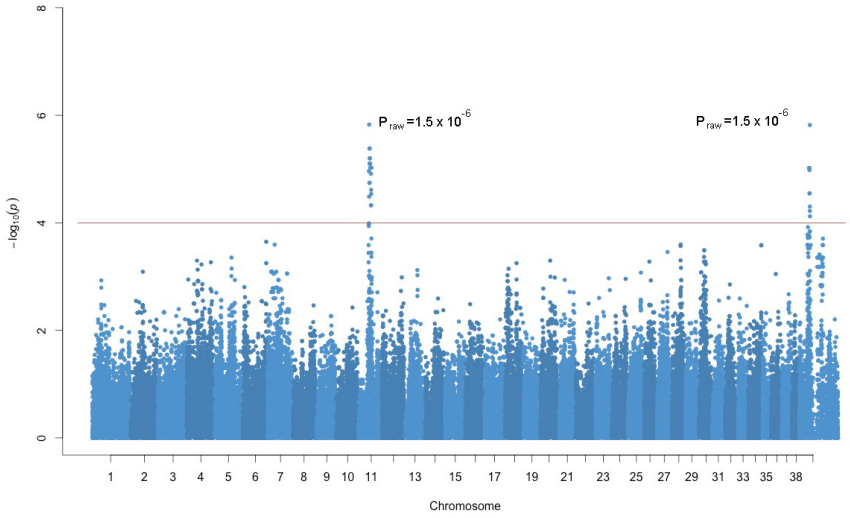

Figure 5. Genome-wide association analysis of 74 cases and 48 controls identified strong susceptibility loci located on chromosome 11 and X. A -logp value of 4.0 were used as a threshold for genome-wide significance as indicated by red horizontal lines.

Chromosome X

The associated region on chromosome X encompassed a 2.3 Mb wide region and was separated into peaks within or close to the genes *LANCL3*, *XK*, *CYBB*, *OTC*, *TSPAN7* and *BCOR*, respectively (Figure 6). Four SNPs flanking the *BCOR* gene represented the strongest association within the locus (p_{raw}= 1.5x10^{-6}, OR= 4.3). *BCOR* encodes the protein Co-repressor of B-cell lymphoma 6. It is a co-repressor of *BCL6*, an evolutionarily conserved zinc-finger protein known to suppress transcription of genes involved in inflammation and lymphocyte differentiation (Shaffer *et al.*, 2000). Also, a recent study reports that BCL6 and NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) act as opposite regulators of the innate immune response (Barish *et al.*, 2010). Other interesting

candidate genes within the chromosome X locus are *TSPAN* 7 (encoding the leukocyte cell surface protein tetraspanin 7) and *CYBB* (encoding the b-chain of cytochrome b). Tetraspanins are known to interact with molecules of the immune system, such as integrins, immunoreceptors and the major histocompability complex (Tarrant *et al.*, 2003). CYBB is involved in microbicidal oxidase in phagocytes (Pollock *et al.*, 1995) and different mutations in this gene has previously been linked to chronic granulomatous disease (Rabbani *et al.*, 1993; Bolscher *et al.*, 1991; Dinauer *et al.*, 1989; Teahan *et al.*, 1987).

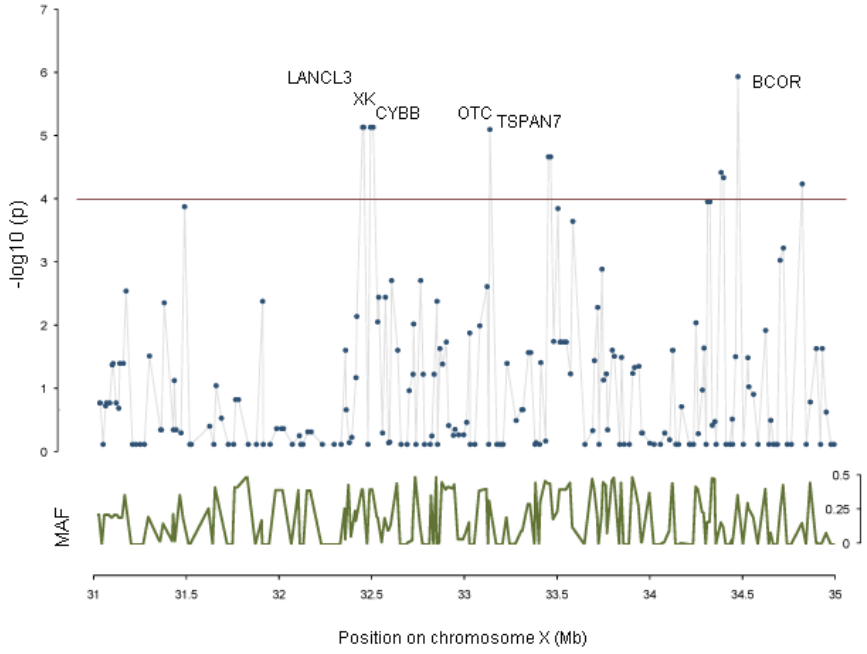

Figure 6. The associated region on chromosome X. The most consecutive association, also including the strongest SNP (p-value = 1.5 x 10^{-6}) mapped to the *BCOR*-gene, known to interact with genes important for inflammation and lymphocyte differentiation.

Chromosome 11

The associated region on chromosome 11 stretched 8.4 Mb (Chr11: 37.36-45.75 Mb) and was separated into several association peaks (Figure 7). The strongest signal came from a single SNP located downstream of *FREM1* (position 38.193.521, p_{raw}=1.5x10^{-6}), a gene with two alternatively spliced transcript variants. The shorter transcript, called Toll-like/interleukin-1 receptor regulator (TILRR), is a co-receptor of the interleukin 1 receptor

family and thought to contribute to the control of inflammatory response activation through the NF-kB pathway (Zhang *et al.*, 2010). However, the largest consecutive signal, represented by ten genome-wide significant SNPs, mapped to the flanking regions of *BNC2* (basonuclin2). It is an extremely conserved zinc-finger protein thought to act as a transcription factor (Romano *et al.*, 2004; Vanhoutteghem & Djian, 2004). Interestingly, recent data suggests that BNC2 also act as a suppressor of NF-kB activity (Li *et al.*, 2011). The interferon alpha genes, represented by a single SNP at position 43.807.665 ($p_{raw}=1.2x10^{-5}$) is also possible candidate genes, since high IFN-α levels are known to trigger autoimmune thyroid disease both through direct effect on the thyrocytes and through immune response activation (Mandac *et al.*, 2006). Furthermore, a long intergenic non-coding RNA (lincRNA) is located just upstream of *BNC2* . LincRNAs are known to have an effect on gene expression both through association with chromatin-modifying complexes and by recruitment of transcription factors (Khalil *et al.*, 2009) and thus are potential regulators of disease development.

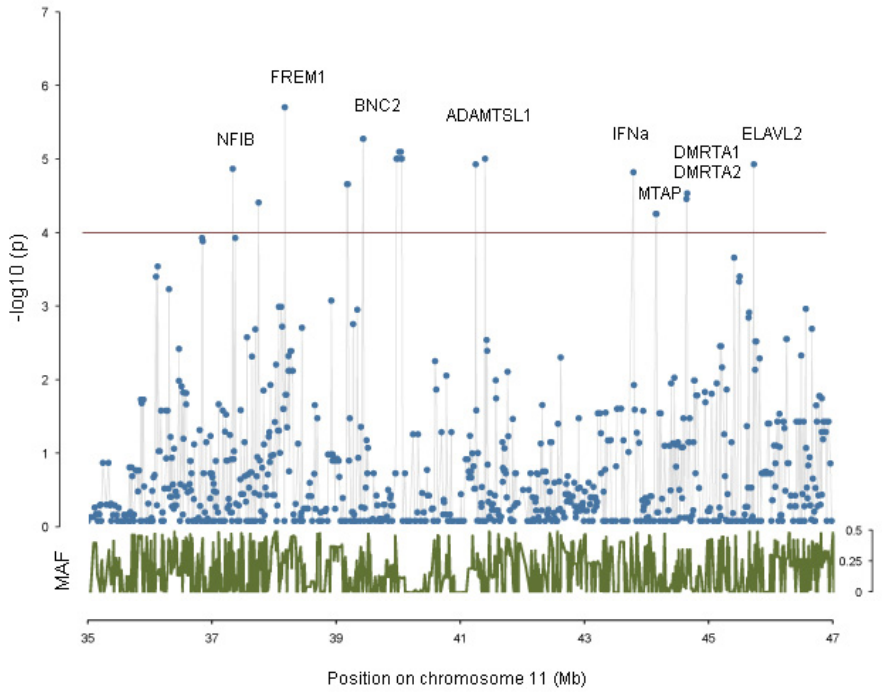

Figure 7. The associated region on chromosome 11. The strongest signal was to a SNP downstream of *FREM1*, while the most consistent association mapped to the flanking region of *BNC2*. Both genes are known regulators of the NF-kB pathway.

Targeted re-sequencing identified >9000 SNPs and a 9.5 kb insertion within the targeted region. Currently we are investigating a number of SNPs located within the promoter and coding sequences of *FREM1*, *BNC2* and *CNTLN* for allele-specific expression. Also, the large indel (insertion/deletion) located within the interferon alpha region, is further investigated in a larger sample set.

Homozygozity and a potential connection to litter size

We identified a drop in minor allele frequency in the close vicinity of our strong association on chromosome 11 (Figure 8b). The homozygous region was 291 kb and covered most of the gene encoding centlein (*CNTLN*). A similar pattern of homozygosity was also recognized in hovawart, English

setter and Weimaraner. A different homozygosity pattern instead covering the *BNC2* gene was found in German shepherd, Dalmatian and Brittany spaniel, while border collie, golden retriever, Eurasier, Irish wolfhound, rottweiler and Greenland sledge dog were heterozygous across the region. Average litter size was statistically different for all groups, although strongest when comparing the heterozygous group to the *CNTLN*-group ($p < 0.0001$) (Figure 8b-d).

As a secondary test we examined the fraction of dogs within each of 14 breeds that had at least one copy of the 'selected' haplotype and performed a correlation between this fraction and the overall mean litter size per breed. An R^2- value of 0.73 was seen. Although being performed in a limited material, these observations suggest that selection for increased litter size within certain breeds has caused a reduced variation within this region. Whether this selection has influenced also CLT susceptibility remains to be further investigated.

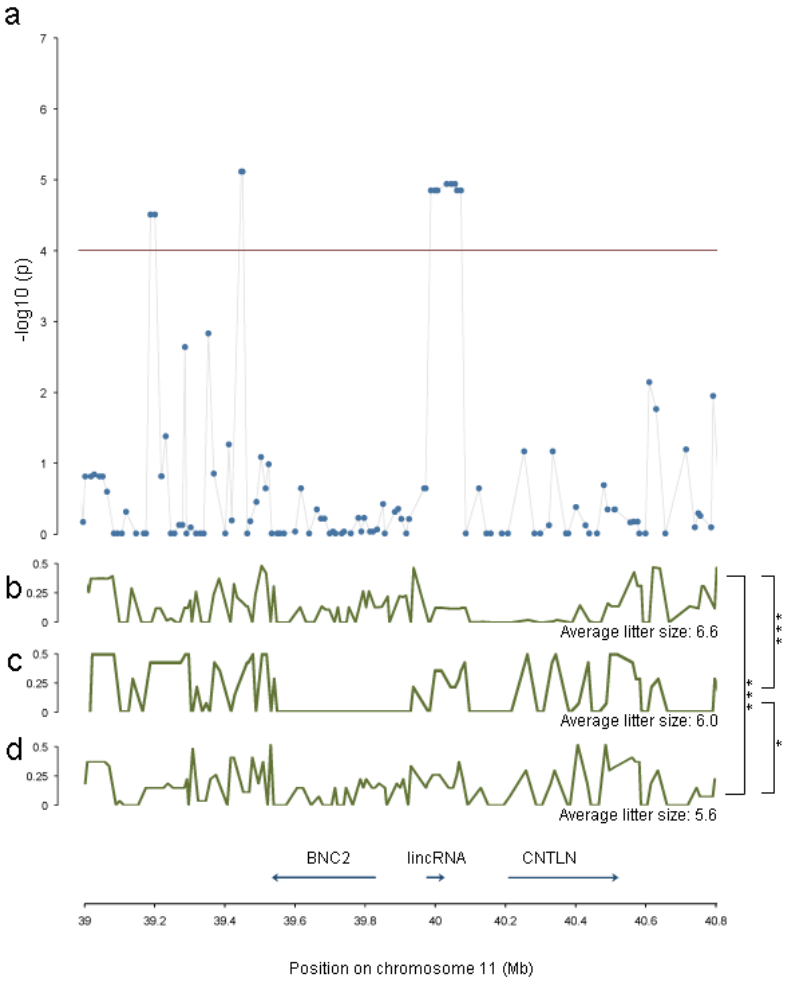

Figure 8. A zoom in on chromosome 11, illustrating the association across *BNC2, lincRNA* and *CNTLN* (a). Green lines illustrate the minor allele frequency (MAF) in giant schnauzer (b), dalmatian (c) and golden retriever (d) across the region.

General discussion

The purpose of this thesis was to estimate the prevalence of canine lymphocytic thyroiditis and to gain knowledge about the genetic background of the disease. Here the results obtained in paper I-III are discussed in a more general perspective.

Prevalence of CLT

Canine lymphocytic thyroiditis (CLT) is considered to be one of the most common endocrine diseases in dog, affecting multiple breeds in high frequency. Characteristics for modern dog breeding have long been the use of a limited number of breeding dogs, frequent use of popular sires and strong selection for desirable traits. This breeding strategy, intended to fix a desirable trait within the population, has unfortunately also enriched genetic risk factors for specific diseases to the extent that almost all breeds exhibit their 'own' diseases.

For diseases that develop late in life, many dogs may have been bred prior to disease onset, thereby contributing to the high disease frequency. This is in fact the case with CLT, in which clinical signs usually develop late. As seen in paper I, few dogs show clinical signs at the age of 3-4 years, even though many are affected by subclinical CLT. According to data from the Swedish Kennel Club registry, the majority of giant schnauzers are 2-3 years old when they are first used in breeding, therefore able to produce affected offspring before CLT is diagnosed. Based on the results from this study we proposed screening of individuals within high-risk breeds prior to breeding. Today the Swedish breed club of giant schnauzer dogs has adopted this strategy in order to try to reduce CLT prevalence within the breed.

Insurance data has previously indicated the giant schnauzer and the hovawart to be high-risk breeds for the disease (Egenvall *et al.*, 2000), showing at least 6 times increased risk compared to the overall Swedish dog population. Although providing important epidemiologic information such as breed- differences and age distribution, insurance data stands short when it comes to estimating CLT prevalence. CLT is not a costly disease to diagnose, therefore economic compensation is not usually claimed. Consequently, CLT diagnoses registered at the insurance company are probably underestimating the true prevalence. In paper I we therefore based our prevalence estimation on serological screening of roughly 230 giant schnauzers and 100 hovawarts, which should provide a more accurate view. Still, our study has limitations. We have sampled only 22-38% of dogs born within each birth-cohort. Consequently, this may result in estimation errors. Also, a certain degree of relatedness could be seen both within and between cohorts.

Consequences of high CLT prevalence within the study cohort

The high prevalence of CLT within the giant schnauzer breed had consequences on sampling strategies for our subsequent genetic studies. Even though high disease prevalence should be beneficial when hunting for disease-causing alleles, it might have been more straightforward to perform the study in a breed showing a slightly lower CLT prevalence, since we found it challenging to find enough healthy controls to match our cases. Many dogs, believed to be old healthy controls, had to be excluded due to increased TSH concentrations. To investigate if the increased TSH was due to age rather than genetic risk factors for CLT, we measured TSH, TgAA and free T4 concentrations in ten German shepherd dogs, a breed not commonly affected with CLT. All dogs were above ten years of age and had serum concentrations well within our strict criteria for healthy controls, suggesting that increased TSH in older giant schnauzer dogs is not a sign of an aging thyroid but rather a sign of forthcoming disease. The high genetic predisposition to CLT in giant schnauzers was also well illustrated in paper III, by the high frequency of the risk haplotype within our study population. Eighty-one percent of cases were homozygous for the risk haplotype across *FREM1*, *BNC2* and *INF-α*, while no cases were homozygous for the protective haplotype. The risk haplotype was common also in the control group, where 38% of dogs were homozygous. Together, these observations explain the extraordinary high prevalence of CLT within the breed, as well as the difficulties identifying suitable controls.

CLT classification

The classification of cases and controls established in paper I and applied in paper I-III proved to be well suitable. Strong genetic risk factors, both the DLA and the loci on chromosome 11 and X were identified based on those criteria. However, in a bigger sample set and by using a breed showing not quite as high CLT prevalence we might have been able to apply even more stringent criteria for cases and controls. Also, by analyzing the data divided into subsets of different diagnostic characteristics, age at onset, gender etc we might have been enabled to assess genetic modifiers and possible gender differences.

DLA- risk and protective haplotype

In paper II we identified the *DLA-DRB1*01201/ DQA1*00101/DQB1 *00201* haplotype to be a risk factor for CLT, while the *DLA-DRB1*01301/ DQA1*00301/ DQB1*00501* haplotype instead was found to have a protective effect against development of the disease. Assessment of all the different alleles separately suggested the *DLA-DRB1*01201* as a major risk allele for CLT development in giant schnauzer dogs. This allele has also been correlated with the development of hypothyroid disease in Doberman pinchers (Kennedy *et al.*, 2006a). The Swedish giant schnauzer population appeared to be moderately genetically diverse with four DRB1 alleles in relatively high frequencies and with an additional five rare alleles, whereas hovawart appears to exhibit lower genetic variation at the MHC.

The major difference in MHC polymorphism between the giant schnauzer and the hovawart was the fact that within the hovawart sample set, one specific haplotype, the *DLA-DRB1*01201/DQA1*00401/DQB1 *013017*, was present in a frequency of 48%, indicating that hovawart is a breed with less divergence within the DLA region, compared to the giant schnauzer. This haplotype contains the *DLA-DRB1*01201* allele as found in the giant schnauzer risk haplotype, potentially explaining the high frequency of CLT within the breed. However, these results must be interpreted cautiously, since the number of hovawarts in the study was rather limited.

As expected, the association to DLA was not identified in the genome wide association analysis, but only through the candidate gene approach. This is

because the DLA region is extremely polymorphic, and the SNP density of the SNPchip is not sufficient to fully explore the genetic variation within the DLA region, thus direct sequencing is more efficient.

Genome-wide association analysis

In paper III, strong susceptibility loci were identified using genome-wide association analysis in only 74 cases and 48 controls, even lower than the predicted number of individuals needed to map a complex trait (Lindblad-Toh *et al.*, 2005) but in concordance to other recent studies (Olsson *et al.*, 2011; Wilbe *et al.*, 2010). This highlights the unique features of the dog genome and the great opportunities it offers in the hunt for disease causing mutations. The associated region on chromosome 11 was wide, showed several signals and harbors a large number of candidate genes, thus making interpretation of the association more difficult. Linkage disequilibrium across the region is extensive, illustrated by the high proportion of individuals (78.6%) showing an un-broken haplotype all the way from *FREM1* (at 38.1 Mb) to *IFN-α* (at 43.8 Mb).

Litter size vs hypothyroidism

The possible connection between litter size and thyroid autoimmunity observed in paper III is somewhat controversial, since hypothyroidism is thought to negatively influence reproduction both in human (Matalon *et al.*, 2001) and dog (Johnston, 1980). On the other hand, thyroid function has been reported to be elevated during pregnancy in humans (Krajewski & Burman, 2011) and hypothyroidism frequently debut in conjunction with pregnancy (reviewed in Gaberscek & Zaletel, 2011), suggesting a potential link between reproduction, increased thyroid activity and an autoimmune reaction. The two genes located within the potential sweep, *BNC2* and *CNTLN,* both are expressed in uterus. Centlein is a centrosomal protein important during meiosis (Makino *et al.*, 2008) and *BNC2* have been reported to be essential for reproduction in zebrafish (Lang *et al.*, 2009).

Also in domestic and wild birds, a connection between thyroid stimulating hormone (TSH) and reproduction is well known, since the TSH-receptor (TSHR) is an established regulator of photoperiod control of reproduction in birds. Increased TSH is a known trigger of seasonal breeding in birds (Nakao *et al.*, 2008; Yoshimura *et al.*, 2003), suggesting that an increased TSH concentration would reduce the strict regulation of

seasonal reproduction found in natural populations. Interestingly, recent whole genome sequencing of the chicken revealed a selective sweep, present in all domestic chickens, within the locus of thyroid stimulating hormone receptor (TSHR) (Rubin *et al.*, 2010). Two of the most striking differences between the domestic chicken and its ancestor, the red jungle fowl, are the absence of seasonal breeding and an extraordinary increased egg production.

Hypothetically, selective breeding for larger litter size in certain breeds of dogs might have influenced genes important for thyroid autoimmunity, located within the selected haplotype.

FREM1, BNC2 and BCOR and their role in NF-kB pathway

Although a large number of genes are present in the associated regions identified in our genome-wide association study, a striking pattern between the most strongly associated genes *FREM1*, *BNC2* (both on chromosome 11) and *BCOR* (chromosome X) emerges. Each member of this gene triplet is known to have an impact on the NF-kB pathway (nuclear factor kappa-light-chain-enhancer of activated B cells), which plays an important role in the regulation of apoptosis and inflammation as well as innate and adaptive immunity. Incorrect regulation of NF-κB has been linked to several inflammatory and autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, type1 diabetes, inflammatory bowels disease, SLE and AITDs (reviewed in Kurylowicz & Nauman, 2008).

FREM1, the gene located close to the strongest peak of genetic association on chromosome 11 (p-value $1.5x10^{-6}$) has two alternative transcripts. The shorter transcript is called Toll-like/interleukin-1 receptor regulator (TILLR), is highly conserved, widely expressed and functions as an enhancer of interleukin 1-regulated inflammatory responses through activation of NF-kB and inflammatory genes (Zhang *et al.*, 2010).

BNC2, also located within the chromosome 11 region and represented by the largest consecutive association, encodes the zinc-finger protein basonuclin2, an extremely conserved protein thought to act as a transcription factor (Vanhoutteghem & Djian, 2004). In a recent paper, BNC2 was also reported to significantly suppress NF-kB activity (Li *et al.*, 2011).

BCOR, located within the strongest association on chromosome X, encodes a co-repressor of BCL6 (B-cell lymphoma 6) (Huynh *et al.*, 2000). BCL-6 has an important function in inflammation (Shaffer *et al.*, 2000) and can either promote or inhibit apoptosis (Kurosu *et al.*, 2003; Albagli *et al.*,

1999). Moreover, BCL-6 and NF-kB were recently reported to mediate opposite regulation to the innate immune response (Barish *et al.*, 2010). In summary, the biological functions of these three genes indicate their potential role in a common pathway. Together with the high association found to their genomic location, this makes them strong candidate genes for the development of thyroid autoimmunity.

The interferon alpha region

In paper III we also identified a SNP strongly associated to CLT phenotype (p_{raw}= 1.2x10^{-5}) at position 43.807.665, within the cluster of interferon alpha genes. Resequencing of the region indicated a potential insertion/duplication, 9.5 kb in size. If this insertion duplicates IFN-α regulatory elements, or even entire IFN-α genes, it might lead to an increased endogenous IFN-α concentration and potential susceptibility to autoimmune thyroid disease, as discussed earlier. However, working with the interferon region in dog has proven to be more challenging than expected. First, there seems to be a problem with the genome assembly for this region, since canine IFN-α genes are aligned to 'chromosome unknown' (which contains sequences that can not be confidently aligned to a specific chromosome). Second, the interferon region is not well annotated in the dog, illustrated by interferon genes annotated as genes with introns, even though interferon genes are known to be intron-less. Further more, it is not possible to measure IFN-a concentrations in dog sera using human assays. For regions such as this, it is important to develop better methods to assemble and to detect copy number variants as well as annotation strategies, *e.g.* combine mRNA-sequence data from dog with better alignments of other species.

Conclusions

Overall, the results of this thesis contribute to an increased knowledge of the prevalence and genetic background of canine lymphocytic thyroiditis (CLT). It also highlights the advantages of using the dog as a model for mapping complex traits. More specifically, the main conclusions are as follows:

- Giant schnauzer and hovawart are extreme high-risk breeds for canine lymphocytic thyroiditis (CLT), with an overall prevalence of 16 and 13 percent in the Swedish populations. Subclinical CLT is prevalent in 3-4 year old dogs, while overt hypothyroidism is more common in older dogs.

- DLA class II polymorphisms can generate increased risk for CLT, but also provide protection against the disease. The *DLA-DRB1*01201/DQA1*00101/DQB1*00201* haplotype was shown to be a strong genetic risk factor for CLT development in giant schnauzer dogs, conferring a 6.5-fold increased risk. On the contrary, the *DLA-DRB1*01301/DQA1*00301/DQB1*00501* haplotype conferred a 3.3-fold decreased risk for CLT development in giant schnauzers.

- Additional risk loci for CLT in giant schnauzer are located on chromosome 11 and X and include strong candidate genes involved in immune regulation and inflammation. The strongest associations were identified close to *FREM1* and *BCOR*, both are which are linked to Nf-κB activation.

Future prospects

Genetic risk factors

Even though we have made significant progress towards understanding the genetic contribution to the etiology of CLT, we still have a long way to go. Within the closest future lies the challenge to identify disease-causing mutations within the associated regions on chromosome 11 and X. Therefore, we are currently expanding the targeted re-sequencing to further investigate the associated region on both chromosomes and in additional dogs. We are also currently investigating SNPs located within the coding sequences and promoter regions of *FREM1*, *BNC2* and *CNTLN* for allele-specific expression in different tissues, by examining SNPs in the transcript that tag the different alleles. Also, the potential 9.5 kb insertion/deletion within the *IFN-α* region needs to be genotyped in a larger sample set.

Further, a crucial part of the future of this project is the functional characterization of the identified candidate mutations. It is important to plan these experiments based on the nature of the candidate mutation *i.e.* coding mutation (causing a change in the protein), non-coding mutation (may affect expression level or degradation of mRNA) and regulatory mutation in elements outside the transcripts (promoters and enhancers/silencers). This can be performed by using molecular cell biology techniques such as cell-cultures, over-expression/silencing of proteins or promoter-assays. The functional results should be compared to molecular genetic data derived from mRNA sequencing (for instance from lymphocytes and thyroid tissue). Furthermore, methods like Chromatin Immunoprecipitation-sequencing (Chip-seq), Electrophoretic Mobility Shift Assay (EMSA) etc., would provide answers about signaling pathways, transcriptional regulatory networks and other proteins involved in disease development. The information about functionality of the causative mutations would take us

one step closer to the ultimate goal of developing new strategies for diagnosing and more effectively treating the disease in both dogs and humans.

Next in the pipeline lies the identification of additional risk loci through use of other high-risk breeds for CLT. We have already performed a genome-wide association analysis in 30 cases and 30 controls from the hovawart breed and identified an association to a novel CLT locus, confirmed and strengthened by meta-analysis together with a second breed. Re-sequencing of that region is ongoing. If sampling additional breeds, the golden retriever with a proposed increased risk of 2.8 (Egenvall et al., 2000) and an annual registration number >2000 individuals is an appealing candidate breed.

In parallel to revealing more about the genetic background of CLT and the effects of the risk factors to the development of the disease, we are planning to use the gained knowledge to reveal comparative information relevant for human Hashimoto's thyroiditis. We have gathered a list of genes from the various mapping projects of canine immunological- and immune-mediated diseases ongoing in our laboratory, as well as genes indicated as counterparts in the pathways that canine candidate genes are playing a role in and added a selection of genes known to have a role in immune system in general. We are planning to re-sequence all the known transcripts of these roughly 1500 genes, as well as potential regulatory elements located in the non-coding parts of the genes of interest. This strategy is slightly adapted from a general and nowadays widely used exome sequencing and we believe it is more relevant since most of the causative mutations we expect to be regulatory and/or rare variants.

Environmental risk factors

Also the environmental triggers of CLT etiology will be more easily assessed once we have identified the genetic risk factors. Then, more thorough and unbiased analyses could be done in dogs classified both by clinical phenotype (affected, un-affected) as well as genotype (risk, non-risk). Attractive theoretical risk factors to investigate in the dog would be canine hepatitis C virus infection, vaccination, iodine intake and pregnancy.

Practical implications

Decrease disease prevalence

After identifying the disease-causing mutations for CLT, development of a genetic test would be beneficial for breeders of high-risk breeds, to avoid breeding genetically susceptible individuals. However, such a genetic test has its limitations. First of all; CLT is a complex trait, although in the giant schnauzer it appears to be highly heritable. In other breeds, however, this might not be the case. A genetic test for a complex trait will lead to a certain proportion of dogs being excluded from breeding for carrying genetic risk factors, but never developing the disease themselves, because of avoidance of genetic triggers. Also, if not all genetic components are identified, there is a risk of including genetically susceptible dogs not detected by the test. Second; Genetic risk factors might not be the same in all breeds and all populations, therefore a genetic test applied in one breed might not be used in another. Third; genetic tests involving the DLA class II region needs some consideration. Since DLA class II polymorphism is crucial for maintaining a diverse immune response and to sustain viral and bacterial infections, one needs to nourish that genetic diversity, even though avoiding homozygosity of the risk variant. Fourth, and perhaps the most important in the giant schnauzer breed; to employ a genetic test for CLT in a high-risk breed such as the giant schnauzer might also be challenging due to the high frequency of risk alleles within the population. Since the majority of dogs carry the risk alleles, excluding all such dogs from breeding would be disastrous, as a very small number of dogs would be left. Instead, while not currently acceptable by breed clubs, introducing some novel alleles from a different breed might be the biologically most viable option.

Healthier dogs, healthier humans

Today, no treatment or cure is available for autoimmune hypothyroidism, but patients are dependent on life-long L-thyroxine substitution therapy. It is a synthetic variant of the lacking thyroxine and even though most patients answer well to the treatment, this drug does not stop or cure the disease but only treats the symptoms.

However, unraveling the genetic background of CLT and Hashimoto's disease might lead to drugs designed to slow down, stop or even prevent the disease. Perhaps the most extensively studied fields within human medicine are those investigating possible disease therapies. Therefore, healthcare tailored to suit the genetic makeup of the patient is no longer a science

fiction, but will soon be available for several diseases, possibly also for patients suffering from CLT and Hashimoto's disease. Inhibition of NF-kB and its related genes have been extensively investigated as therapeutic targets for inflammatory and autoimmune diseases (reviewed in Gilmore & Garbati, 2011). Hypothetically, a drug designed to specifically suppress TILRR-activity might soon be investigated as treatment for CLT in dogs and, if it is identified as a risk factor also in human AITD, for patients suffering from Hashimoto's disease.

Populärvetenskaplig sammanfattning

Hunden är människans bästa och närmsta vän på många sätt. Vi lever i samma miljö, äter i stort •sett samma mat och drabbas också i stor utsträckning av samma ärftliga sjukdomar. Cancer, diabetes, epilepsi, hjärt-kärlsjukdomar och autoimmuna sjukdomar förekommer också hos hund, och många gånger är sjukdomens uppkomst, utveckling och kliniska symtom mycket lika människans. Genom att studera den genetiska orsaken till hundens sjukdomar kan vi därför lära oss mycket även om människas motsvariga sjukdomar. Den här avhandlingen beskriver studier av autoimmun sköldkörtelrubbning, även kallad lymfocytär thyroidit (LT) hos hund.

Lymfocytär thyroidit

Lymfocytär thyroidit tillhör de organspecifika autoimmuna sjukdomarna och drabbar både hundar och människor. Hos människa kallas den Hashimoto's sjukdom, efter den japanska specialist som första gången karaktäriserade sjukdomen år 1912. Att sjukdomen är autoimmun och organspecifik innebär att kroppen felaktigt uppfattar sitt eget organ som främmande, vilket leder till aktivering av immunologiska celler och nedbrytning av kroppsegen vävnad. Sköldkörteln är en liten, fjärilsformad körtel som sitter på halsens framsida, strax under struphuvudet. Här bildas tyroxin (T_4) och trijodtyronin (T_3), två viktiga hormon som styr kroppens ämnesomsättning. Tillväxt, värmereglering, fettförbränning, hjärtfrekvens, fertilitet och många andra kroppsfunktioner styrs av T_3 och T_4. Producerar kroppen för lite sköldkörtelhormon ses symtom över nästan hela kroppen. Trötthet, ökad vikt, sänkt hjärtfrekvens, frusenhet och håravfall är vanliga symtom både hos hund och människa.

Förekomsten av lymfocytär thyroidit (LT) hos hund och dess slående likheter med Hashimoto's sjukdom beskrevs för första gången 1968 i en flock beaglehundar. Sedan dess har sjukdomen rapporterats som frekvent förekommande i många raser och anses idag vara en av de vanligaste endokrina sjukdomarna hos hund. I Sverige är de mest frekvent drabbade raserna riesenschnauzer och hovawart, men också golden retriever, dobermann, cocker spaniel, boxer och rhodesian ridgeback har rapporterats vara högriskraser.

Idag finns inga avelsstrategier för att reducera sjukdomen, förutom att undvika att använda sjuka hundar och dess nära släktingar i avel. Men eftersom LT oftast debuterar i medelålders eller äldre hundar, har denna strategi inte varit effektiv. Sjuka hundar har redan hunnit producera sjuka avkommor. Med kunskap om de genetiska orsakerna till LT skulle det vara möjligt att utveckla ett genetiskt test och på så vis få tillgång till ett mer effektivt avelsverktyg. I förlängningen skulle sådan kunskap också kunna leda till effektivare diagnoser och skräddarsydda mediciner.

Syftet med studierna

Syftet med denna avhandling var att undersöka den genetiska orsaken till lymfocytär thyroidit hos hund, vilket även skulle kunna vara till nytta för människor med Hashimoto's sjukdom. Mer specifikt var målet att:

- Uppskatta frekvensen av LT i riesenschnauzer och hovawart, två raser tidigare indikerade som högriskraser.
- Undersöka DLA klass II som en potentiell genetisk riskfaktor för LT.
- Identifiera ytterligare genetiska riskfaktorer genom en så kallad helgenom-associationsanalys.

Sammanfattning av studierna

I vår första studie (artikel I) ville vi undersöka frekvensen av LT i riesenschnauzer och hovawart. För att också få en bild över sjukdomens utveckling och jämföra frekvensen i olika ålderskategorier samlade vi in information och blodprover från två olika årskullar. Hundarna var vid provtillfället 3-4 respektive 6-7 år gamla (födda 1995 och 1992). Genom att mäta blodets koncentration av sköldkörtelhormon (T4), sköldkörtel-

stimulerande hormon (TSH) och förekomst av autoantikroppar mot thyroglobulin (TgAA) kunde vi uppskatta förekomsten av LT till 16 % hos riesenschnauzer och 13 % hos hovawart. Väldigt få hundar i den yngre kategorin hade utvecklat symtom och fått en LT-diagnos vid tiden för provtagning, men enligt våra mätningar befann sig mer än 10 % av de unga i ett tidigt skede av sjukdomen. Detta är en viktig iakttagelse, eftersom de flesta hundar som används som avelsdjur är i denna ålder eller ännu yngre. Risken är därför stor att en hund som senare i livet utvecklar LT kan komma att användas i avel. Detta kan delvis förklara den höga frekvensen sjukdom i dessa två raser.

I vår nästa studie (artikel II) undersöktes genetiska riskfaktorer för LT genom en så kallad kandidatgen-strategi. Tidigare studier har rapporterat ett starkt samband mellan Hashimoto's sjukdom hos människor och variation i HLA klass II gener. Dessa gener kodar för protein vars funktion är att presentera olika ämnen i kroppen för immunförsvarets celler. Upptäcker immuncellerna ett främmande ämne (tex en bakterie eller ett virus) bundet till en HLA-molekyl aktiveras ett immunsvar. Eftersom ett samband hade setts till Hashimoto's sjukdom var vår hypotes att hundens motsvarighet till HLA, vilken kallas DLA, kan ha inverkan även på LT. Genom att jämföra den genetiska sekvensen i tre DLA klass II gener hos 30 sjuka och 74 friska riesenschnauzer kunde vi konstatera att en viss kombination av dessa gener gav en starkt ökad risk att utveckla LT, medan en annan variant av DLA klass II generna istället gav ett ökat skydd mot sjukdomen.

Vi hade nu identifierat en god studiepopulation och sett att våra diagnostiska kriterier var starka nog att hitta genetiska riskfaktorer inom riesenschnauzer. Härnäst ville vi undersöka hela arvsmassan i jakt på fler gener med inverkan på sjukdomen, eftersom det sedan tidigare är känt att både Hashimoto's sjukdom och LT styrs av flera gener. Genom att förutsättningslöst jämföra arvsmassan hos 74 sjuka och 48 friska individer i en så kallad helgenoms association kunde vi identifiera två regioner med stark koppling till sjukdomen, den ena lokaliserad på kromosom 11 och den andra på kromosom X. Båda de associerade områdena innehåller gener som tidigare rapporterats ha en stor inverkan på reglering av immunförsvaret. De två starkaste signalerna sågs intill generna *BCOR* (kromosom X) och *FREM1* (kromosom 11). Intressant är att de båda generna är involverade i en och samma signalkaskad, NF-κB, som sedan tidigare är känd för sina viktiga inflammatoriska och immunologiska effekter. Framtida studier syftar till att identifiera den eller de exakta mutationer som ger uppkomst till sjukdom, och att förstå den funktionella effekten av dessa mutationer.

Slutsatser i korthet

Resultaten från denna avhandling bidrar med viktig kunskap om förekomsten och den genetiska bakgrunden till lymfocytär thyroidit hos hund. Vidare visar den tydligt fördelarna med att använda hund i genetisk sjukdomsforskning. Mer specifikt kunde följande slutsatser dras:

- Riesenschnauzer och hovawart är raser med hög förekomst av LT, cirka 16 respektive 13 % av hundarna drabbas. I yngre hundar (3-4 år) är subklinisk LT vanligt, medan fullt utvecklad sjukdom är vanligare hos äldre hundar.

- En viss variant av DLA klass II generna kan ge ökad risk att utveckla LT, medan en annan variant ger ett ökat skydd mot sjukdomen.

- Ytterligare genetiska riskfaktorer identifierades på kromosom 11 och X. Flera av generna i området är involverade i NF-κB signalkaskaden, ett välkänt reglerelement för immunologiska och inflammatoriska sjukdomar hos människa. Detta tyder på att hund är en mycket god modell för studier av Hashimoto's sjukdom.

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