Canine immune-mediated disease

Studies of epidemiology, genetics, and autoantibodies

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Abstract

Immune-mediated diseases in dogs constitute a large group of disorders with the common attribute that they are caused by dysfunctions in the immune system. Broadly they can be classified into immunodeficiency disorders, allergies, and autoimmune diseases. Earlier studies have suggested that dogs of the breed Nova Scotia duck tolling retriever (NSDTR) are at increased risk of developing immune-mediated disorders. Two disorders have received particular attention: immune-mediated rheumatic disease (IMRD) and steroid-responsive meningitis-arteritis (SRMA). The major aims of this thesis were to 1) determine the incidence of immune-mediated disease in NSDTRs, 2) identify genetic risk factors for IMRD and SRMA, and 3) investigate the occurrence of different autoantibodies in dogs with autoimmune disease.

Data from Agria Pet Insurance were used to estimate incidence of disease. Incidence of different immune-mediated diseases in NSDTRs was compared with that in other dog breeds. In general, the incidence for autoimmune diseases was three times higher in NSDTRs compared to other breeds. For IMRD and SRMA, the incidences were >10 times higher.

Both IMRD and SRMA are complex genetic diseases and several genetic loci associated with the respective disease have been identified by our research group. We performed a detailed analysis of these loci and found 11 genes with altered gene expression associated with IMRD or SRMA. The majority of these genes were associated with either IMRD or with SRMA only, but one gene (*AP3B2*) showed association to both diseases.

Autoantibodies are important hallmarks of autoimmune diseases, including IMRD. In the studies presented in this thesis, different methods were used to identify the autoantibody targets in dogs that were positive for antinuclear autoantibodies. Both previously reported and new canine autoantibodies were found. The interleukin enhancer-binding factors 2 and 3 (ILF2 and ILF3) appear to be common autoantibody targets in IMRD patients. These autoantibodies have not previously been described in dogs and have the potential to be used as diagnostic markers for canine systemic autoimmune disease.

Keywords: Antinuclear antibodies, autoimmunity, dog, gene expression, ILF2, ILF3, immune-mediated rheumatic disease, Nova Scotia duck tolling retriever, steroid-responsive meningitis-arteritis, systemic lupus erythematosus.

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Immunmedierade sjukdomar hos hund – epidemiologi, genetik och autoantikroppar

Sammanfattning

Immunmedierade sjukdomar hos hund består av en stor grupp av olika åkommor. Gemensamt är att de orsakas av defekter i immunförsvaret. Generellt kan de delas in i immunbristsjukdomar, allergier och autoimmuna sjukdomar. Tidigare studier har antytt att hundar av rasen Nova Scotia duck tolling retriever (kallade tollare) har en ökad risk att utveckla immunmedierade sjukdomar. Det är framförallt två sjukdomar som uppmärksammats hos rasen: immunmedierad reumatisk sjukdom (IMRD) och steroid-responsiv meningit-arterit (SRMA). De huvudsakliga syftena med denna avhandling var att 1) beskriva incidensen av immunmedierade sjukdomar hos tollare, 2) identifiera genetiska riskfaktorer för IMRD och SRMA hos tollare och 3) undersöka förekomst av olika autoantikroppar hos hundar med autoimmun sjukdom.

Data från Agria Djurförsäkring användes för att uppskatta sjukdomsincidens. Incidensen av olika immunmedierade sjukdomar hos tollare undersöktes och jämfördes med incidensen hos hundar av andra raser. Autoimmuna sjukdomar var tre gånger vanligare hos tollare jämfört med andra hundraser. Incidensen för IMRD och SRMA var >10 gånger högre.

Både IMRD och SRMA är komplexa genetiska sjukdomar. Flera regioner i arvsmassan associerade med dessa sjukdomar har tidigare identifierats. Vi utförde en detaljerad uppföljningsanalys av dessa regioner och hittade 11 gener associerade med IMRD eller SRMA som visade förändrat genuttryck. De flesta av dessa gener var associerade med bara en av sjukdomarna, men en gen (*AP3B2*) var associerad med båda sjukdomarna.

Autoantikroppar är viktiga kännetecken för autoimmuna sjukdomar såsom IMRD. I studierna som presenteras i denna avhandling användes olika metoder för att detektera de målstrukturer som antinukleära autoantikropparna är riktade mot. Både tidigare beskrivna och nya autoantikroppar hos hund identifierades. Autoantikroppar mot interleukin enhancer-binding factor 2 och 3 (ILF2 och ILF3) var vanligt förekommande hos hundar med IMRD. Dessa autoantikroppar har inte beskrivits tidigare hos hund och har potential att kunna användas som diagnostiska markörer för systemisk autoimmun sjukdom hos hund.

Nyckelord: Antinukleära antikroppar, autoimmunitet, genuttryck, hund, ILF2, ILF3, immunmedierad reumatisk sjukdom, Nova Scotia duck tolling retriever, steroid-responsiv meningit-arterit, systemisk lupus erythematosus.

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Dedication

To my parents

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 Bremer HD*, Vilson Å, Bonnett BN, Hansson-Hamlin H (2015). Disease patterns and incidence of immune-mediated disease in insured Swedish Nova Scotia Duck Tolling Retrievers. *Veterinary Record*, 177 (3), p. 74.

II Wilbe M, Kozyrev SV, Farias FHG, Bremer HD, Hedlund A, Pielberg GR, Seppälä EH, Gustafson U, Lohi H, Carlborg Ö, Andersson G, Hansson-Hamlin H*, Lindblad-Toh K* (2015). Multiple Changes of Gene Expression and Function Reveal Genomic and Phenotypic Complexity in SLE-like Disease. *PLOS Genetics*, 11 (6), e1005248.

III Bremer HD*, Lattwein E, Renneker S, Lilliehöök I, Rönnelid J, Hansson-Hamlin H (2015). Identification of specific antinuclear antibodies in dogs using a line immunoassay and enzyme-linked immunosorbent assay. *Veterinary Immunology and Immunopathology*, 168 (3-4) pp. 233-241.

IV Bremer HD*, Landegren N, Sjöberg R, Hallgren Å, Renneker S, Lattwein E, Leonard D, Eloranta M-L, Rönnblom L, Nordmark G, Nilsson P, Andersson G, Lilliehöök I, Lindblad-Toh K, Kämpe O, Hansson-Hamlin H (2018). ILF2 and ILF3 are autoantigens in canine systemic autoimmune disease. *Scientific Reports,* 8(1), p.4852

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The contribution of Hanna Bremer to the papers included in this thesis was as follows:

I Took major part in planning the study, performed the data analyses, interpreted the results together with co-authors and had the main responsibility for writing the manuscript.

II Sampled and performed phenotypic characterisation of some of the dogs, performed some laboratory work and made minor contributions to the writing of the manuscript.

III Took minor part in planning the study, assembled data and performed the data analyses, interpreted the results together with co-authors and had the main responsibility for writing the manuscript.

IV Took major part in planning the study, sampled some of the dogs, performed phenotypic characterisation of the canine patients together with main supervisor, performed most of the laboratory work, performed the data analyses, interpreted results together with co-authors and had the main responsibility for writing the manuscript.

Related publication not included in the thesis

- Bremer HD, Hillström A, Kånåhols M, Hagman R, Hansson-Hamlin H (2017). Serum C-reactive protein concentrations in Nova Scotia Duck Tolling Retrievers with immune-mediated rheumatic disease. *Acta Veterinaria Scandinavica*, 59(1):21

Abbreviations

ANA	Antinuclear antibodies
ANA^{H}	Antinuclear antibody positive with a homogenous pattern
ANA^{S}	Antinuclear antibody positive with a speckled pattern
CLIFT	*Critidia luciliae* indirect immunofluorescence test
dsDNA	Double stranded DNA
DYAR	Dog years at risk
ELISA	Enzyme-linked immunosorbent assay
GWAS	Genome-wide association study
HEp-2	Human epithelial-2
IIF	Indirect immunofluorescence
ILF2	Interleukin enhancer-binding factor 2
ILF3	Interleukin enhancer-binding factor 3
IMRD	Immune-mediated rheumatic disease
MHC	Major histocompatibility complex
NFAT	Nuclear factor of activated T-cells
NSDTR	Nova Scotia duck tolling retriever
OR	Odds ratio
RBMX	RNA-binding motif protein, X chromosome
RNP	Ribonucleoproteins
RR	Relative risk
SLE	Systemic lupus erythematosus
Sm	Smith antigen
SNP	Single nucleotide polymorphism
SRMA	Steroid-responsive meningitis-arteritis

1 Introduction

The immune system consists of a wide number of tissues, cells, and molecules that protect the body against foreign substances. The mechanisms that protect the body, the immune response, also have the potential to cause tissue injury and disease. Immune-mediated disorders develop when the immune system either fails to respond adequately (immunodeficiency), responds with increased magnitude against foreign substances that are not by themselves harmful (allergy), or responds to self-molecules (autoimmune disease). These varying disorders affect both humans and animals, including dogs. The studies included in this thesis are primarily focused on dogs with signs of autoimmune disease, specifically dogs of the breed Nova Scotia duck tolling retriever (NSDTR). This breed has previously been reported to be affected by a number of immune-mediated diseases at unknown frequencies (Hansson-Hamlin & Lilliehöök, 2013; Hansson-Hamlin & Lilliehöök, 2009; Anfinsen *et al.*, 2008; Hughes *et al.*, 2007; Redman, 2002; Burton *et al.*, 1997). In this thesis, the incidence of different immune-mediated diseases in NSDTRs is presented and it is shown that this breed is predisposed to autoimmune diseases. Several genetic risk factors for immune-mediated disease in NSDTRs were identified. Finally, canine sera were investigated for the occurrence of different autoantibodies – important diagnostic markers – and autoantibodies not previously described in dogs were discovered.

2 Background

2.1 The immune response and immune-mediated disorders

The immune response can be divided into the innate and the adaptive immune response (Abbas *et al.*, 2017). The innate immune response is the body's first response against a foreign substance. It is fast, unspecific, and has no memory. It consists of physical and chemical barriers (*e.g.* skin and antimicrobial substances), mechanical processes (*e.g.* cilia in airways), some blood proteins (*e.g.* components of the complement system), normal microbial flora, and some types of immune-cells and their products (*e.g.* phagocytic cells). If a foreign substance is not kept back or eradicated by the innate immune response, it will encounter the adaptive immune response. The adaptive immune response is specific and can respond to an infection with increased magnitude. It is rather slow, but has memory, and if infected again by the same pathogen, the response will be rapid. The main components of the adaptive immune response are lymphocytes and their products, such as cytokines and antibodies. Antibodies work in many ways to combat infections, by, for example, neutralizing toxins, opsonisation, and complement activation (Lu *et al.*, 2017).

There are several ways in which immune-mediated disorders may be classified (Day, 2008; McGonagle & McDermott, 2006; Pedersen, 1999). Broadly, they can be classified into immunodeficiency, allergy, and autoimmune disease. Sometimes immune system neoplasia is also included. Immuno-deficiency diseases are characterized by an absent or compromised immune response. Allergies are caused by hypersensitivity to environmental factors. Allergic diseases, in particular with skin involvement, are common disorders in dogs (Wiles *et al.*, 2017; O'Neill *et al.*, 2014b). Autoimmune diseases develop as a loss of tolerance against self-molecules.

A primary or idiopathic autoimmune response develops in the absence of an underlying cause (Day, 2008). A secondary autoimmune reaction is more common and can develop as a response to, for example, infections, neoplasia, and certain drugs. In reality, it can be difficult to differentiate between primary and secondary autoimmunity, since underlying factors may remain unidentified. Indications of a primary autoimmune aetiology are: association to the major histocompatibility complex (MHC) class II genes, lymphocytic infiltration of target organs, presence of autoantibodies, and response to immunosuppressive drugs, although these factors alone do not prove that a disease is autoimmune (Rose & Bona, 1993).

2.2 Autoimmune diseases in dogs and humans

Autoimmune diseases can be organ specific or systemic, involving many organ systems. An example of an organ specific autoimmune disease that affects both dogs and humans is myasthenia gravis (Meriggioli & Sanders, 2009; Dewey *et al.*, 1997). Systemic lupus erythematosus (SLE) is the classic example of a systemic autoimmune disease, well described in both humans (D'Cruz *et al.*, 2007) and dogs (Fournel *et al.*, 1992; Bennett, 1987; Grindem & Johnson, 1983; Lewis *et al.*, 1965). One of the hallmarks of SLE is the occurrence of autoantibodies directed at different structures in the cell nucleus. These diverse autoantibodies are called antinuclear antibodies, commonly referred to as ANA (Tan, 1989).

The diagnosis of autoimmune and other immune-mediated diseases is based on history, clinical signs, exclusion of other disorders, and for some diseases, specific tests. In human medicine, a diagnosis of an autoimmune disease is often based on a set of standardised criteria involving symptoms and laboratory abnormalities, as for example in human SLE (Petri *et al.*, 2012; Hochberg, 1997; Tan *et al.*, 1982). A consistent definition of a disease facilitates research and makes comparisons of different studies feasible. However, there is often considerable overlap between different immune-mediated and autoimmune diseases with respect to diagnostic findings and clinical signs (McGonagle & McDermott, 2006), and individual patients are not always easy to classify. In veterinary medicine, well-established disease criteria for autoimmune diseases are often lacking, which is a research limitation.

2.2.1 Epidemiology

In epidemiology, disease frequency is measured to identify populations at increased risk for a disease; this can indicate a genetic predisposition. Increased

awareness of disease frequency and predisposition in different dog breeds can assist in breeding and research prioritisations (O'Neill *et al.*, 2014a). Disease frequency (morbidity) can be measured as prevalence (the proportion of existing cases in a population), as incidence proportion, and as incidence rate. Incidence rate is the number of new cases per population at risk in a given time period (Rothman, 2012).

Many large and population based studies estimating the disease frequency of autoimmune disorders in humans are published, but equivalent studies in dogs are scarce. As separate entities, autoimmune disorders can be considered rare, but as a group they are common with an approximate prevalence of 5% in humans (Hayter & Cook, 2012; Jacobson *et al.*, 1997). Estimates of frequencies for autoimmune diseases in dogs are harder to obtain, partly because of the lack of standardised criteria, as discussed earlier. A recent study by Wiles *et al.* (2017) estimated the overall prevalence of disease in UK purebred dogs from owner reported data. The proportion of autoimmune disorders reported in that study was 1.67%. This is likely an underestimate due to underreporting of minor conditions and because some autoimmune disorders are probably included in other disease categories. In humans, autoimmune diseases are more common in females than in males (Hayter & Cook, 2012; Whitacre *et al.*, 1999; Jacobson *et al.*, 1997). Although suggested by some (Pedersen, 1999; Quimby *et al.*, 1980), a female predisposition is not as obvious in dogs (Sundburg *et al.*, 2016); this may at least partly be explained by neutering practises. Autoimmune diseases often co-exist with other autoimmune diseases in humans (Cooper *et al.*, 2009) and both human and canine patients with autoimmune disease also have a higher risk of developing some types of cancers (Keller, 1992; Pettersson *et al.*, 1992). Clustering within families also occurs. This is also evident in dogs where some breeds and lines appear to have a higher incidence of autoimmune disease than others (Wiles *et al.*, 2017; Day, 2008; Pedersen, 1999). Epidemiological studies can provide important information about risk factors and predisposition, but it should be noted that they do not prove what cause disease, since association is not the same as causation.

2.2.2 Genetics

Autoimmune diseases are more common in certain dog breeds and in some families (Wiles *et al.*, 2017; Pedersen, 1999; Day & Penhale, 1992), strongly suggesting underlying genetic factors. Most autoimmune diseases segregate within families, but not according to a Mendelian inheritance pattern typical for monogenic disorders. Instead, the inheritance pattern for most autoimmune diseases is complex, in which changes (*i.e.* mutations) in multiple genes

contribute to disease susceptibility, and environmental factors contribute to, or trigger, development of disease (Marson *et al.*, 2015; Rosenblum *et al.*, 2015). The majority of diseases in dogs are complex (O'Neill *et al.*, 2014b).

The MHC genes are strongly associated with autoimmune disease and were the first genes to be identified as risk factors for autoimmunity (Rosenblum *et al.*, 2015; Gough & Simmonds, 2007). These groups of highly polymorphic genes are also called human leucocyte antigen (HLA) or dog leucocyte antigen (DLA). The MHC genes consist of three classes. The class II genes have been associated with many human autoimmune diseases as well as with various canine diseases with a suggestive autoimmune aetiology, including immune-mediated rheumatic disease (IMRD) in NSDTRs (Wilbe *et al.*, 2009), hypothyroidism (Wilbe *et al.*, 2010b; Kennedy *et al.*, 2006b; Kennedy *et al.*, 2006c), and hypoadrenocorticism, also called Addison's disease (Massey *et al.*, 2013; Hughes *et al.*, 2010). The MHC class II genes can be considered common risk factors for autoimmunity. They code for proteins that play a crucial role in the adaptive immune response against foreign substances, and they are also important for development of tolerance against self-molecules (Abbas *et al.*, 2017). How genetic variations in the MHC genes contribute to development of autoimmune disease is not fully understood (Rosenblum *et al.*, 2015). In dogs, there are three polymorphic class II genes – *DRB1*, *DQA1*, and *DQB1* (Kennedy *et al.*, 2001; Kennedy *et al.*, 2000).

By genome-wide association studies (GWAS) many more genetic loci than the MHC associated with complex autoimmune diseases have been identified, mainly in humans (Marson *et al.*, 2015; Parkes *et al.*, 2013), but also in dogs (Bianchi *et al.*, 2015; Wilbe *et al.*, 2010a). The majority of the associated genetic variants are located in non-coding regions close to or within genes and may have effect on gene regulation (Farh *et al.*, 2015; Marson *et al.*, 2015). Many of the candidate genes identified are preferentially expressed in immune cells and play a role in the development and regulation of the immune response.

In addition to genetic factors, several environmental factors that likely contribute to or trigger development of autoimmune disease have been identified in humans, some of which, such as infections and UV-light, probably also play a role in canine disease (Rosenblum *et al.*, 2015; Costenbader *et al.*, 2012; Day, 2008). Vaccination may also be a triggering factor in genetically susceptible individuals but are rare complications considering the large number of vaccines administrated (Toplak & Avcin, 2009; Day, 2008).

Many of the genetic and environmental risk factors are still to be discovered, and little is known about how these actually interact to cause autoimmune diseases in dogs. Today it is difficult to estimate the risk for an individual to develop a complex disease, and no single genetic test can be used to predict

disease accurately, since multiple genetic changes contribute to disease development (Jostins & Barrett, 2011). Therefore, it is impossible to give breeding advice that applies to all autoimmune diseases and all breeds. Many factors have to be considered, such as the prevalence and severity of disease, and the size of the population. Even for a specific disease in one breed, it is often difficult to give any other breeding advice than not to breed from diseased animals.

Identification of genetic risk factors

Many different approaches can be undertaken to identify genetic risk factors for disease. In candidate gene studies, a possible association between the disease and one or a limited number of genes is searched for. The choices of genes may be based on previous studies or on the function of the gene if it suggests disease involvement. Genetic variation in the candidate gene is then compared between cases and controls, with the aim of finding genotypes or haplotypes associated with disease.

The publication of the canine genome sequence (Lindblad-Toh *et al.*, 2005), in combination with a rapid development in the field of genomics, has opened up for new ways to search the whole genome for genetic risk factors. In GWAS, thousands to millions of single nucleotide polymorphisms (SNPs) across the whole genome are analysed in cases and controls, in order to find genetic variants associated with disease. The first successful GWAS for a complex canine disease was published in 2010 (Wilbe *et al.*, 2010a). Since then, several regions in the genome associated with other complex canine diseases have been identified (some are reviewed in: van Steenbeek *et al.*, 2016; Lequarre *et al.*, 2011). Even though GWAS is a powerful methodology to find associated regions, these regions are large, often containing several genes. After identification of associated regions, other methods such as fine-mapping of the associated regions are necessary to narrow down the region of association, and targeted resequencing to identify actual genes and variants that may be involved in disease (Marson *et al.*, 2015). Other methods than GWAS, such as whole exome and whole genome sequencing will in the future most likely help to identify more genetic risk factors for different diseases in dogs and humans (Sayyab *et al.*, 2016; van Steenbeek *et al.*, 2016).

Genetic association analyses do not prove that a gene variant is causing disease. Functional studies focussing on the effects of genetic variation can provide further clues into the mechanism underlying disease (Marson *et al.*, 2015). One way is to study the effect that different genetic variants have on gene expression in different cell lines or tissues.

Genetic studies in dogs

The dog population has gone through two bottleneck events during its history, the first when a limited number of wolfs gave rise to the domestic dog, and more recently during breed creation (Lindblad-Toh *et al.*, 2005). The effect of a bottleneck is illustrated in Figure 1. Modern dog breeding has resulted in many different dog breeds with very large phenotypic variations. Although the diversity at species level is extensive, the genetic variation within breeds is relatively small, which is a consequence of bottlenecks, founder effect (a small number of dogs are the founders of each breed), structured inbreeding, and overuse of popular sires (Ostrander *et al.*, 2017; Leroy, 2011). This breeding strategy has resulted in the great diversity of morphological and behavioural traits we see in dogs today, but it has also resulted in drawbacks. Disease causing mutations, which are uncommon in the general dog population, can become common in a breed. It is since long recognised that dogs of certain breeds are at high risk for some diseases. Some of these inherited disorders are related to conformation, while others are not related to the breed standards (Summers *et al.*, 2010; Asher *et al.*, 2009). Overall, purebred dogs have a higher prevalence for some disorders than crossbreds (O'Neill *et al.*, 2014b; Bellumori *et al.*, 2013).

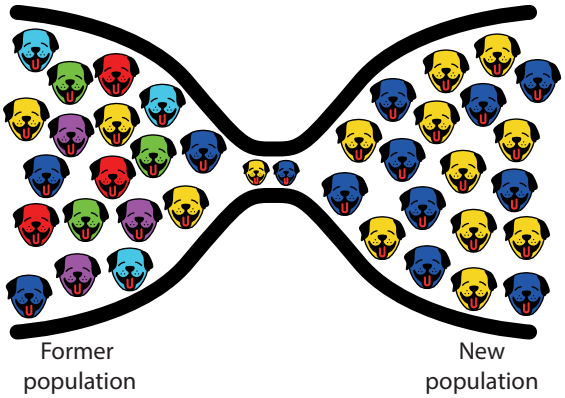

Figure 1. A bottleneck leads to reduced genetic diversity.
Image: Jens Bremer

The limited genetic diversity within dog breeds is, however, an advantage when performing genetic association studies. The recent breed creation has led to dogs having long haplotype blocks compared to humans. Haplotypes are genetic segments that are inherited together. As a consequence of this, fewer markers are needed for disease mapping in dogs compared to humans (Lindblad-Toh *et al.*, 2005). Less individuals are also needed, around 100 cases and 100 controls often provide enough statistical power to map a complex disease in dogs

(Karlsson & Lindblad-Toh, 2008), in comparison to the thousands of individuals that are needed for similar studies in humans.

Dogs suffer from many of the same, or similar, diseases as humans. Genetic association studies are technically simpler to perform in dogs than in humans, and diseases that are rare in humans, can be common in one dog breed. Since dogs live in the same environment as humans, they are exposed to some of the environmental factors that may influence disease onset and progression in both species. All of these described properties make the dog a suitable genetic model for human disease, and results from canine studies can be used to search for risk factors in human disease, in a targeted approach (Shearin & Ostrander, 2010; Karlsson & Lindblad-Toh, 2008).

2.2.3 Autoantibodies

One important characteristic of autoimmune disease is the occurrence of autoantibodies directed at self-molecules called self-antigens or autoantigens. Autoantibodies can be directly pathogenic, for example, by forming immune-complexes that can deposit in different tissues and cause inflammation, and by exerting functional effects on receptors (Elkon & Casali, 2008). However, autoantibodies are not always pathogenic, but can be useful as markers of the tissue destruction and disease status. Testing for autoantibodies is an important diagnostic tool in the investigation of a possible autoimmune disease. Occurrence of autoantibodies can provide support for an autoimmune aetiology (Rose & Bona, 1993) and, in some instances, serve as prognostic markers (Scofield, 2004). Further, the presence or absence of different autoantibodies can be used to stratify patients into subgroups. This can be valuable in the clinical handling of patients, but also in research, for example in genetic studies. Autoantibodies to double stranded DNA (dsDNA) can serve as an example of an autoantibody of diagnostic and prognostic importance in human medicine. These autoantibodies are more specific for SLE than ANA, included as one of the diagnostic criteria for SLE (Tan *et al.*, 1982) and implicated in the pathogenesis of lupus nephritis (Rahman & Isenberg, 2008; Koffler *et al.*, 1967). Further, the autoantibody titres can correlate with disease activity and be predicative of disease exacerbations (Terborg *et al.*, 1990; Swaak *et al.*, 1986). Autoantibodies to dsDNA can also be present before onset of disease (Arbuckle *et al.*, 2003).

In veterinary medicine, autoantibodies have been identified in a variety of disorders with a suspected autoimmune aetiology, for example hypoadreno-corticism (Boag *et al.*, 2015), hypothyroidism (Dixon & Mooney, 1999), diabetes mellitus (Davison *et al.*, 2008), and masticatory masseter myositis (Wu

et al., 2007; Shelton *et al.*, 1987), but the frequency and diagnostic importance of these autoantibodies vary between the diseases. Routine autoantibody testing is only performed in a limited number of disorders, such as SLE or SLE-related disorders (ANA), hypothyroidism (thyroglobulin autoantibodies), and myasthenia gravis (acetylcholine receptor autoantibodies). While autoantibody analyses really have acquired a central role in the diagnosis of and research on human autoimmune diseases, the relevance for many of these analyses needs to be further explored in veterinary medicine.

Antinuclear antibodies

Antinuclear antibodies are a diverse group of autoantibodies directed at different nuclear antigens (Tan, 1989). Some autoantibodies directed at other cell compartments than the nucleus are also included within this group for historical reasons (Chan *et al.*, 2015). Presence of high titres of ANA is a sensitive, but unspecific, marker for SLE in both humans and dogs (Smee *et al.*, 2007; Monier *et al.*, 1992; Bennett, 1987; Tan *et al.*, 1982). These antibodies are also commonly present in other systemic rheumatic diseases (Satoh *et al.*, 2007; Hansson-Hamlin *et al.*, 2006), but they have also been reported at lower frequency in other canine disorders with a suspected immune-mediated aetiology (Dyggve *et al.*, 2017; Bohnhorst *et al.*, 2002; Bohnhorst *et al.*, 2001), as well as in some infections such as leishmaniasis and *Ehrlichia canis* (Smith *et al.*, 2004; Lucena *et al.*, 1996). Healthy dogs and humans can infrequently and generally at low titres also have circulating ANA (Tan *et al.*, 1997; Bennett & Kirkham, 1987). The prevalence of ANA in healthy individuals appears to increase with age in humans (Ramos-Casals *et al.*, 2003; Tomer & Shoenfeld, 1988) a phenomenon that has not yet been studied in dogs.

Autoantibodies analyses

The standard method to detect ANA in humans and dogs is by indirect immunofluorescence (IIF) microscopy (Bennett & Kirkham, 1987; Coons *et al.*, 1950). Briefly described, cells are fixed on glass slides and then incubated with patient sera. If autoantibodies are present in the sera, they will bind to the cell substrate. Different cell substrates can be used, but the human epithelial-2 (HEp-2) cells are routinely used in human diagnostics (Chan *et al.*, 2015) and are also commonly used in veterinary diagnostics (Bell *et al.*, 1997; Hansson *et al.*, 1996). Fluorescent-dye conjugated secondary antibodies are then added and will bind to the autoantibodies (Figure 2). The slides are examined in fluorescence microscopy.

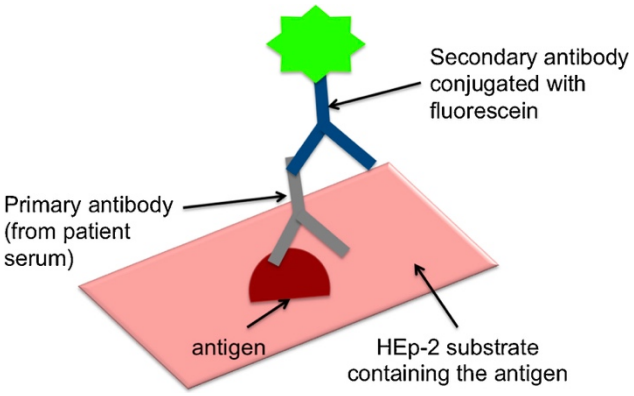

Figure 2. Principle of indirect immunofluorescence using HEp-2 as substrate (Di Cataldo *et al.*, 2016), with permission.

Two distinct IIF patterns can be identified in canine sera with HEp-2 cells as substrate – homogenous and speckled (Hansson *et al.*, 1996). In the homogenous pattern (ANA^{H}), the chromosomal region of mitotic cells is stained, while the speckled pattern (ANA^{S}) shows negative chromosomal staining surrounded by positive nucleosome staining (Figure 3). These two patterns are also major ANA patterns in humans, but other patterns occur (Chan *et al.*, 2015). In human diagnostics, different ANA patterns are associated with reactivity to different nuclear antigens, which can be indicative of different autoimmune disorders (von Mühlen & Tan, 1995). In veterinary medicine, the knowledge about the association between ANA patterns, the specific autoantigen reactivity, and clinical signs, is limited. One study reported that ANA positive dogs that display a homogenous ANA pattern by IIF are likely to have SLE, involving multiple organs, while dogs with a speckled ANA pattern more likely have SLE-related disease displaying primarily musculoskeletal signs (Hansson-Hamlin *et al.*, 2006).

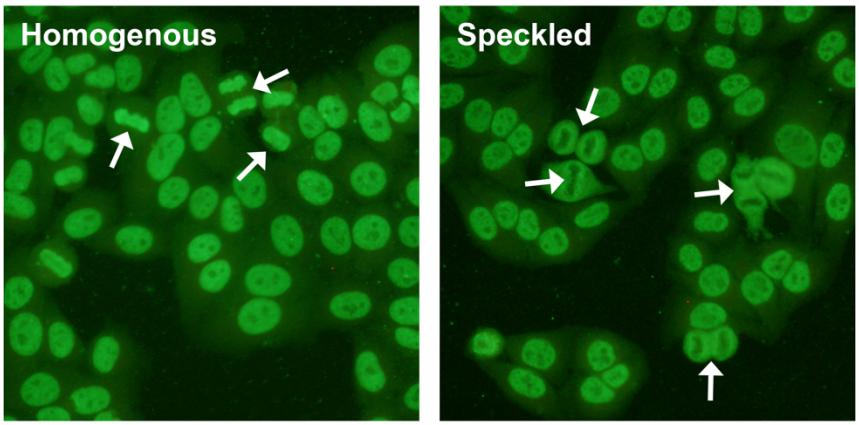

Figure 3. Picture illustrating the two distinct immunofluorescence patterns identified in dogs with HEp-2 cells as substrate. Arrows pointing at mitotic cells. From paper III (Bremer *et al.*, 2015).

With IIF microscopy, the exact autoantibody target (the autoantigen) cannot be determined. To identify the autoantigen, other methods such as enzyme-linked immunosorbent assay (ELISA) and line blot techniques can be used. These methods test for specific ANA and are commonly used in human diagnostics (Murdjeva *et al.*, 2011; Tozzoli *et al.*, 2002) but not in routine veterinary diagnostics. Testing for specific ANA in individuals that are positive for ANA by IIF is a crucial step in the diagnosis of autoimmune diseases in humans, since different specific ANA are associated with different autoimmune disorders. Several specific ANA have also been identified in dogs (Hansson-Hamlin & Rönnelid, 2010; Welin Henriksson *et al.*, 1998; Fournel *et al.*, 1992; Monier *et al.*, 1992; Thoren-Tolling & Ryden, 1991; Hubert *et al.*, 1988; Monier *et al.*, 1988; Costa *et al.*, 1984; Monier *et al.*, 1980; Monier *et al.*, 1978), but little is known about the clinical and pathological relevance of these specific ANA in dogs. The occurrence of some specific ANA that in more recent years have been shown to be of importance in human medicine, has not been investigated in dogs. Previous to the studies included in this thesis, this area of veterinary medicine has not received much attention in the last 10-20 years.

Discovery of novel autoantigens

Different approaches can lead to the discovery of novel autoantigens targeted by autoantibodies (Burbelo & O'Hanlon, 2014; Satoh *et al.*, 2007). These can be discovered by a hypothesis driven approach, where testing for autoantibodies to one or a number of candidate autoantigens is performed. Other methods allow for a broader and more unbiased screening. One of these methods, which

successfully has been used in human studies to identify novel autoantigens, is protein arrays (Dalin *et al.*, 2016; Landegren *et al.*, 2016; Landegren *et al.*, 2015). These arrays contain thousands of full-length proteins printed on a glass slide, making simultaneous screening for a large number of autoantigens possible. The proteins are usually recombinant proteins expressed in yeast or insects cells, with a protein tag that enables purification and detection (Duarte & Blackburn, 2017).

2.3 Immune-mediated disease in the Nova Scotia duck tolling retriever

The NSDTR is a small retriever dog originating from Canada (Figure 4). The breed came to Sweden in 1984 (Svenska Tollarklubben, 2017a) and has since gained in popularity in the Scandinavian countries. By the end of 2017, 4212 NSDTRs were registered by the Swedish Board of Agriculture, representing 0.47% of the registered Swedish dog population (Swedish Board of Agriculture, 2018). The Swedish NSDTR breed club is the largest of its kind in the world (Svenska Tollarklubben, 2017b). In comparison to many other countries, the breed is quite common in Sweden, making it an ideal country to study disorders affecting the breed. Although popular and generally considered a healthy breed (The Swedish Kennel Club, 2014), there have been several reports suggesting that dogs of this breed are at increased risk of developing immune-mediated disorders, in particular immune-mediated rheumatic disease (Hansson-Hamlin & Lilliehöök, 2009) and steroid-responsive meningitis-arteritis (Hansson-Hamlin & Lilliehöök, 2013; Anfinsen *et al.*, 2008; Redman, 2002). It has been proposed that the high incidence of immune-mediated disease observed in this breed is due to bottle necks caused by two canine distemper outbreaks in Canada in the beginning of the 20th century. Hypothetically, dogs that survived and repopulated the breed had particularly reactive or strong immune systems allowing them to survive, but now making their offsprings prone to develop immune-mediated disease (Hughes *et al.*, 2010; Wilbe *et al.*, 2010a). In 2002, Hansson-Hamlin, initiated the "Toller Project" and started collecting clinical data and blood samples from NSDTRs with immune-mediated disease. The aim was to clinically characterize and identify biomarkers and genetic risk factors for these diseases in NSDTRs.

Figure 4. A Nova Scotia duck tolling retriever.
Photo: Jens Bremer

2.3.1 Immune-mediated rheumatic disease

The clinical features of immune-mediated rheumatic disease (IMRD) in NSDTRs was described in 2009 by Hansson-Hamlin and Lilliehöök. The disease most often affects middle-aged dogs and is characterised by chronic stiffness, mainly after rest, and pain from multiple joints, indicative of non-erosive polyarthritis. Affected dogs sometimes show other clinical signs such as muscle pain, skin problems, and fever. A majority of cases are positive for ANA by IIF. Improvement of clinical signs can often be obtained with glucocorticoid treatment. The occurrence of autoantibodies, response to immunosuppressive treatment, and genetic association to the MHC class II genes, suggest that IMRD is an autoimmune disease (Hansson-Hamlin & Lilliehöök, 2009; Wilbe *et al.*, 2009). The clinical and diagnostic findings of IMRD are overlapping with SLE, and some other systemic rheumatic diseases, and it can therefore be referred to as an SLE-related or SLE-like disease.

2.3.2 Steroid-responsive meningitis-arteritis

Steroid-responsive meningitis-arteritis (SRMA) has been reported in several dog breeds including NSDTRs (Hansson-Hamlin & Lilliehöök, 2013; Tipold & Schatzberg, 2010; Tipold & Jaggy, 1994). It is a disease primarily affecting young dogs. The typical acute form is characterized by fever, lethargy, intense neck pain and stiffness, as well as general signs of inflammation, such as increased number of white blood cells and increased concentrations of acute-phase proteins in sera. Analysis of cerebrospinal fluid reveals presence of

inflammatory cells but absence of identifiable infectious organisms (Lowrie *et al.*, 2009). Increased concentrations of immunoglobulin A in serum and cerebrospinal fluid can sometimes also be detected (Maiolini *et al.*, 2012; Lowrie *et al.*, 2009). As the name implies, the clinical signs of the condition respond to glucocorticoids (which is a steroid). Improvement of clinical signs is normally rapid after treatment has been initiated, but recurrence of signs is common (Biedermann *et al.*, 2016; Hansson-Hamlin & Lilliehöök, 2013; Lowrie *et al.*, 2009). Antinuclear antibodies are not detected in NSDTRs with SRMA (Hansson-Hamlin & Lilliehöök, 2013). Although the immune system is likely to be involved in the pathogenesis, the exact cause of disease is unknown (Tipold & Schatzberg, 2010). It is not clear if SMRA is a primary autoimmune disease or a secondary autoimmune reaction to unknown stimuli.

2.3.3 Epidemiology

Previous studies of immune-mediated diseases in NSDTRs are mainly case-reports or case-control studies (Hansson-Hamlin & Lilliehöök, 2013; Wilbe *et al.*, 2010a; Hansson-Hamlin & Lilliehöök, 2009; Wilbe *et al.*, 2009). Such studies are important in highlighting a problem in a breed but do not give information about disease frequency. To measure the disease frequency, information about the population at risk is necessary, information that can be difficult to obtain. However, in a previously published study, disease frequency was estimated in a limited number of Norwegian NSDTRs. The study identified nine dogs that were, or had been affected, by SRMA, resulting in a prevalence of 2.5% (Anfinsen *et al.*, 2008). To compare, the prevalence of SRMA has been estimated in UK dogs of many different breeds to be 0.15% (Wiles *et al.*, 2017). Although several studies suggest that NSDTRs are at increased risk of immune-mediated diseases, no previous studies have been able to show that such is the case. To show that dogs of a specific breed are at increased risk, a comparison of the disease frequency in the breed of interest to other dog breeds is necessary.

2.3.4 Genetics

Early observations in NSDTRs suggested that IMRD and SRMA are genetic disorders, but observations did not support monogenic inheritance, instead complex inheritance patterns were suspected (Hansson-Hamlin & Lilliehöök, 2009; Wilbe *et al.*, 2009; Anfinsen *et al.*, 2008). By a candidate driven approach, Wilbe *et al.* (2009) showed that IMRD are associated with a particular MHC class II haplotype, and also that general homozygosity in the MHC class II region increased the risk of IMRD. No association between SRMA and MHC class II

was found. In 2010, IMRD and SRMA were the first complex canine disorders to be successfully mapped by a GWAS (Wilbe *et al.*, 2010a). Five candidate loci, located on chromosomes 3, 8, 11, 24, and 32 were identified. The loci on chromosome 3, 8, 11, and 24 were associated with IMRD and the locus on chromosome 32 showed association to both IMRD and SRMA. All of the identified regions contain strong candidate genes for follow-up studies. Interestingly, several of the candidate genes are involved in the nuclear factor of activated T-cells (NFAT) pathway. The NFAT family is a group of transcription factors important for T-cell activation and regulation, and is also important for the function of many other immune cells (Muller & Rao, 2010).

3 Aims

The major aim of this study was to increase the knowledge of disease frequency, genetic risk factors, and occurrence of autoantibodies in dogs with immune-mediated disease, specifically, in dogs of the breed NSDTR.

The specific aims were to:

- investigate the overall incidence of disease and describe the general disease pattern in NSDTRs,
- determine the incidence of immune-mediated disease in general, and that of IMRD and SRMA in particular, and, to test the hypothesis that NSDTRs are predisposed to these disorders,
- perform resequencing of genetic loci previously identified as associated with IMRD and SRMA in NSDTRs in order to identify risk genotypes and haplotypes, then evaluate how these are associated with expression of candidate genes,
- investigate if particular sub-phenotypes of IMRD based on ANA pattern (ANA^{H} and ANA^{S}) are associated with different genetic variants,
- identify both new and known canine autoantibodies with several different methods, and
- investigate if different ANA patterns (ANA^{H} and ANA^{S}) are associated with different autoantibodies.

4 Comments on materials and methods

Detailed methods are described in the respective papers. Following are some comments on the materials and one the most important methods.

4.1 Study populations

Study I was a retrospective cohort including dogs insured by Agria Pet Insurance. The study population included all dogs insured before one year of age during the years 1995 to 2006 and consisted of 445,336 dogs of which 2,890 were NSDTRs.

The studies II-IV were retrospective case-control studies including privately owned dogs, with confirmed (NSDTRs only) or suspected immune-mediated disease (dogs of other breeds than NSDTRs), and healthy control dogs. Study IV also included human patients and controls. Samples from NSDTRs had been collected during the years 2002 and 2016 to be included in different research studies as part of the "Toller Project". Sampling was coordinated by Hansson-Hamlin, and performed by researchers involved in the studies, or by different veterinarians throughout the country. Some Finnish dogs were also included in study II.

Patients with IMRD displayed musculoskeletal signs with shifting lameness, stiffness, and pain from multiple joints upon manipulation. Clinical signs had been apparent for at least 14 days before inclusion and no other cause of the clinical signs was suspected. Dogs affected with IMRD were tested for ANA with IIF, and ANA-positive dogs were divided into two groups, depending on the type of ANA pattern, ANA^{S} and ANA^{H}. Dogs with SRMA had typical clinical signs of SRMA and showed resolution of clinical signs after glucocorticoid treatment. Analysis of cerebrospinal fluid was performed in some cases.

4.2 Measuring disease frequency (paper I)

Disease frequencies were assessed using data on number of veterinary visits registered by the Agria Pet Insurance's database. This database has been validated for epidemiological studies and is described in detail elsewhere (Egenvall *et al.*, 1998). Following is a brief description of the database. If the cost for a veterinary visit exceeds the deductible limit, the main cause of the veterinary visit is registered as a diagnostic code in the database. The diagnostic code is assigned by the attending veterinarian from a standard registry.

Unfortunately, the standard registry is not perfect or complete, specific codes for some disorders, including IMRD and SRMA are lacking. To overcome this problem, we selected other diagnostic codes that were likely to represent these disorders. To describe the general disease pattern in NSDTRs, diagnostic codes were grouped into different categories based on organ systems and disease processes. Incidence rates were calculated by taking the number of veterinary visits, divided by the time at risk. The rates were multiplied by 10,000 and presented as number of cases per 10,000 dog years at risk (DYAR). If a dog had more than one claim for the investigated disease or disease category, only the first claim was counted. Incidence rates for NSDTRs were compared with incidence rates for other dog breeds, to estimate the incidence rate ratio/relative risk (RR). Two groups were established for comparison, the first consisting of all dogs except NSDTRs, the other of all retrievers except NSDTRs. These were then compared to the NSDTRs.

4.3 Genetic studies (paper II)

4.3.1 Sequencing and analysis of MHC class II

The polymorphic exon 2 of MHC class II was sequenced for each of the *DRB1*, *DQA1*, and *DQB1* genes in ANA-positive IMRD cases and healthy control dogs. Cases were subdivided based on ANA pattern as ANA^S or ANA^H (six cases were not possible to classify due to lack of sera). Sequences were analysed and haplotypes and genotypes were assigned to each dog. The total number of cases and controls carrying a specific allele or genotype was compared with cases and controls not carrying it, using a 2x2 contingency table. The total number of homozygous dogs was also compared in cases and controls. Odds-ratios (OR) and p-values (Yates' chi-square test) were calculated for each allele, haplotype and genotype.

4.3.2 Re-sequencing of associated loci, SNP selection, and genotyping

To identify candidate variants associated with IMRD and/or SRMA, the five regions on chromosome 3, 8, 11, 24, and 32 that were previously found to be associated with IMRD and SRMA (Wilbe *et al.*, 2010a) were re-sequenced in four IMRD cases, two SRMA cases, and three healthy control dogs using NimbleGen capture and Illumina sequencing. The data was analysed with various tools to discover variants (SNPs, indels, and structural changes) in the genomic sequence between IMRD, SRMA, and healthy controls. From this re-sequencing data, 308 SNPs from the five loci were chosen for genotyping in all the available cases and controls. The SNPs were selected based on the following criteria: difference in allele frequency between cases and controls, positioned in either protein coding region, 5' UTR or 3' UTR, and located within non-coding conserved elements. The 308 SNPs were genotyped by GoldenGate Genotyping Assay. Association analyses were performed using PLINK (Purcell *et al.*, 2007). Healthy controls were compared with each of the different groups: SRMA cases, all IMRD cases, and the two subgroups; IMRD ANA^S and IMRD ANA^H. Conditional analyses were also performed where ANA^S dogs homozygous for the MHC class II risk haplotype, ANA^H dogs homozygous for MHC class II, and ANA^H dogs with the identified risk allele were included respectively. The SNPs that showed the highest association to disease were selected for investigation of its effect on gene expression.

4.3.3 Expression studies

Peripheral blood from 167 healthy NSDTRs were collected for gene expression studies of the candidate genes. Genomic DNA and RNA were extracted from the samples and genotyping of 51 SNPs was performed with either pyrosequencing or Sanger sequencing. Gene expression was measured by real-time PCR and normalised to the reference gene *TBP*. Gene expression levels were then correlated with genotype and haplotypes using one-way ANOVA.

4.4 Autoantibody investigations

4.4.1 Indirect immunofluorescence (paper II-IV)

Canine sera were tested for ANA by IIF microscopy as previously described (Hansson *et al.*, 1996). Analyses were performed by the staff at the Clinical Pathology Laboratory, University Animal Hospital, SLU, or at Euroimmun, Lübeck, Germany. Monolayers of HEp-2 cells were used as substrate. The glass

slides were examined by fluorescence microscopy and considered positive at a titre of ≥1:100. The visible nuclear pattern (ANA^H or ANA^S) was registered for each dog positive for ANA.

4.4.2 Enzyme-linked immunosorbent assay and line immunoassay (paper III)

We screened canine sera for reactivity to 18 antigens known to be associated with autoimmune disease in humans. Four different enzyme-linked immunosorbent assays (ELISA) and a line immunoassay (Euroline ANA profile 5), originally developed for detection of human ANA, were used. The analyses were performed at Euroimmun by automated systems according to the manufacturer's instructions, with the exception of the secondary anti-human antibody that was exchanged for an anti-dog antibody. We used the highest observed value obtained from a serum in the healthy group as a cut-off value and compared the difference in positive cases between ANA^H and ANA^S groups with Fisher exact probability test (two tailed).

4.4.3 Protein array screen (paper IV)

The HuProt™ Human Proteome Microarray v2 (CDI Laboratories) was used to search for novel autoantibodies. The arrays contain approximately 17,000 proteins printed on a glass slide. We modified the human protocol to work with canine sera. Briefly described, the arrays were incubated with 1:200 diluted canine sera from 12 IMRD patients and nine healthy controls, followed by incubation with a fluorophore conjugated anti-dog antibody. The arrays were scanned using a microarray scanner. To search for increased autoantibody signals in IMRD patients, we used a cut-off of mean + 3SD (log-transformed data) based on the healthy controls.

4.4.4 Radio-ligand binding assay (paper IV)

To validate our findings from the protein array screen, and to screen more dogs for autoantibodies to the candidate autoantigens, we used radio-ligand binding assays. Recombinant proteins were expressed *in vitro* in the presence of ^{35}S-labeled methionine and immunoprecipitated with canine patient and control sera. Serum antibodies were immobilised to protein A Sepharose and radioactivity was measured in a microbeta counter. Autoantibody index was calculated as ((sample value mean-negative control)/(positive control-negative control))x100. Cut-off values were calculated from healthy controls as mean+5SD (mean+7SD for the RBMX antigen).

5 Results

5.1 Disease frequency in Nova Scotia duck tolling retrievers (paper I)

5.1.1 Overall disease frequencies

Of the 2890 NSDTRs included in the study, 51% had at least one veterinary visit during the study period. The incidence rate for veterinary visits was 1300 cases per 10,000 DYAR, somewhat higher (RR 1.1, p=0.0075) compared to all other breeds combined, but similar to other retrievers (RR 0.96, p=0.091). The most common causes of veterinary visits in NSDTRs were injuries, gastrointestinal disease, and locomotor disorders.

5.1.2 Frequencies and relative risk of immune-mediated disease

The overall incidence rate for immune-mediated (named immunological in paper I) disorders was 44 cases per 10,000 DYAR, approximately the same as in other breeds and other retrievers. Immune-mediated disorders did not belong to one of the most common causes of veterinary visits in NSDTRs. Immunological disorders consisted of three subgroups of disorders; allergic, autoimmune, and various (other immune-mediated disorders not possible to classify as allergic or autoimmune, *e.g.* the diagnosis "immunological changes whole animal"). Autoimmune disorders, and disorders belonging to the group "various immunological disorders", were significantly more common in NSDTRs than in other breeds (RR 2.7, p=0.00025 and RR 3.9, p=0.00011, respectively). The incidence for neurological disorders (not belonging to the group immune-mediated disease) was also higher in NSDTRs than in other breeds (RR 1.7, p<0.0001), in particular neurological infections/inflammations (RR 9.0, p<0.0001).

The incidence rate for disorders representing IMRD was estimated to 6.8 cases per 10,000 DYAR in NSDTRs, 18 times higher than in other breeds and 30 times higher than in other retrievers. For SRMA, the incidence rate was 20 cases per 10,000 DYAR, 12 times higher than in other breeds.

5.2 Genetic studies in Nova Scotia duck tolling retrievers (paper II)

5.2.1 Association of the MHC class II genes with IMRD

By sequencing 122 dogs for MHC class II polymorphism, five different haplotypes, forming 10 genotypes were identified. Haplotype 2 (*DRB1**00601/ *DQA1**005011/*DQB1**02001) was strongly associated with IMRD ANA^S (OR=9.7 and $p<0.0001$ compared with controls), with an even higher OR in homozygote individuals (genotype 2, OR=21 and $p<0.0001$). No association was found at the haplotype or genotype level for $ANA^{H,}$ but at the allelic level a significant association was identified for *DQA1**00601 (OR=5.1, p=0.00017). A general homozygosity regardless of haplotype was also associated with ANA^H (OR=5.1 and p=0.0016 including all genotypes).

5.2.2 Additional genes associated with IMRD and SRMA

Resequencing of the other five genetic risk loci discovered by GWAS (Wilbe *et al.*, 2010a) identified 426 SNPs and 88 indels with a potential functional effect. Of these, 308 SNPs following the risk haplotype patterns, were genotyped in the entire sample set to identify risk variants associated with SRMA and ANA positive IMRD, including the two sub-phenotypes ANA^H and ANA^S. The five different loci showed association to different, but also overlapping, phenotypes. For example, the strongest association on Chr 11 was with all IMRD cases, while the risk locus on Chr 32 showed two independent signals, one to SRMA and one to ANA^S. The SNPs that showed the highest association to disease were chosen to study their effect on gene expression. Functional changes associated with the risk haplotypes were identified in 11 genes located on the five chromosomes. Significant changes in gene expression were identified for 10 genes. In addition, a non-synonymous SNP in *HOMER2* was associated with the IMRD ANA^H risk haplotype. A non-significant trend towards lower mRNA expression levels of this gene was also observed in the risk haplotype. The results are summarized in Table 1.

Table 1. *Genes associated with IMRD and SRMA phenotypes. The strongest association to a phenotype marked with bold "**+**", regular "+" means the gene is associated with a particular phenotype. Modified from paper II (Wilbe et al., 2015).*

chromosome	SNP ID	gene/phenotype	gene effect	all ANA	ANA^S	ANA^S+DLA	ANA^H	ANA^H+DLA	SRMA
11	11:67537177	*PTPN3*	down	**+**	+	+	+	+	-
24	24:36087012	*WFDC3*	up	+	-	-	+	**+**	-
32	32:24542001	*BANK1*	up	+	+	**+**	-	+	-
	32:24827518	*DAPP1*	up	-	-	-	-	-	**+**
	32:24827518	*LAMTOR3*	up	-	-	-	-	-	**+**
	32:24827518	*DDIT4L*	up	-	-	-	-	-	**+**
	32:24827518	*PPP3CA*	up	-	-	-	-	-	**+**
3	3:57484486	*AP3B2*	up	**+**	+	-	-	-	+
	3:57432981	*WHAMM*	up	**+**	+	-	+	+	-
	3:57546568	*HOMER2*	nsSNP (Thr->Ala)[1]	+	-	-	+	**+**	-
8	8:68708503	*VRK1*[2]	up	+	-	-	+	**+**	-

1. We observed also a trend towards down-regulation of *HOMER2* in the risk haplotype, although it did not reach statistical significance due to small sample size.
2. The strong genetic association signal with SRMA on chromosome 8 was not associated with VRK1 expression levels.

While some of the genes showed association to only one phenotype, others were shared between IMRD ANA^{H} and IMRD ANA^{S} phenotypes. There was little overlap between the genes associated with IMRD and SRMA, but one gene, *AP3B2*, was associated with both IMRD ANA^{S} and SRMA (Figure 5).

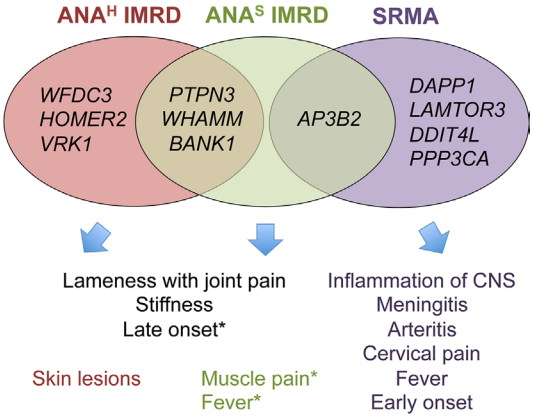

Figure 5. Venn diagram showing overlap between clinicopathological features and associated genes. From paper II (Wilbe et al., 2015).

5.3 Autoantibodies in dogs

5.3.1 Specific antinuclear antibodies and ANA pattern (paper III)

Sera from 240 dogs with suspicion of autoimmune disease and sera from 27 healthy control dogs were screened for specific ANA. Of the 240 sera, 210 had been selected because they were ANA positive and 30 were ANA negative. Of the 210 ANA positive samples, 141 (67%) samples were classified as ANA^{S} and 68 (32%) as ANA^{H} (one sample was not possible to classify and excluded from further analyses). The patient sera, as well as sera from 27 healthy control dogs, were evaluated and compared with regards to specific ANA reactivity. Dogs in the ANA^{H} group, frequently had high levels of dsDNA and nucleosome autoantibodies, detected by ELISA and the line immunoassay. Because previous investigations had yielded inconclusive results about dsDNA autoantibodies in dogs, these results were validated with *Crithidia luciliae* indirect immunofluorescence test (CLIFT), a method with high specificity for detecting dsDNA autoantibodies in humans. With CLIFT, dsDNA autoantibodies were confirmed in 10 out of the 39 samples that were positive in the ELISA and/or line immunoassay. Dogs in the ANA^{S} group, frequently had high levels of

autoantibodies to ribonucleoproteins (RNP) and to Smith antigen (Sm). In individual dogs, we also found autoantibodies to SS-A, SS-B, Jo-1, Scl-70, and PCNA. In conclusion, we found that the types of ANA pattern are associated with reactivity to particular nuclear antigens.

5.3.2 Identification of interleukin enhancer-binding factors 2 and 3 as canine autoantigens (paper IV)

By a protein array screen, we identified interleukin enhancer-binding factor 2 (ILF2) as a potential autoantigen in IMRD patients. Autoantibodies to ILF2 were identified in seven of 12 IMRD patients, but only in patients with ANA^S. To confirm the results from the protein array screen that ILF2 is a common autoantigen in IMRD ANA^S patients, we performed a radio-ligand binding assay on the same sera as well as on sera form a larger cohort. Sera from a total of 29 IMRD ANA^S patients and 121 dogs from other breeds with ANA^S were screened. Autoantibodies to ILF2 were present in 27/29 (93%) of sera from IMRD ANA^S patients and in 49/121 (40%) sera from ANA^S dogs of other breeds than NSDTRs (Figure 6). The ILF2 autoantibodies were only present in ANA^S sera. We observed a strong breed difference regarding ILF2 autoantibodies.

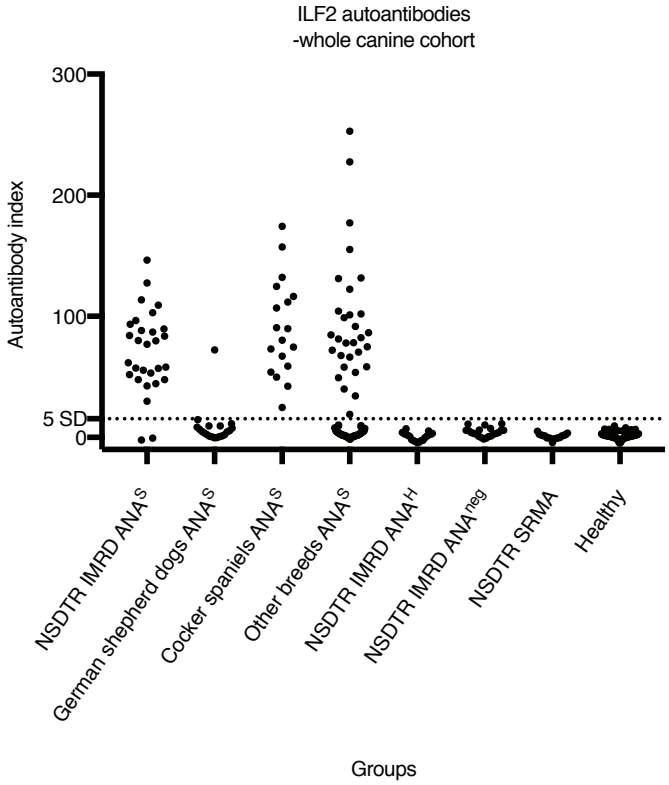

Figure 6. Radio-ligand binding assay was used to validate ILF2 as an autoantigen in the discovery and in an extended cohort. From paper IV (Bremer *et al.*, 2018).

The ILF2 protein is expressed as a heterodimeric complex with the protein interleukin enhancer-binding factor 3 (ILF3). Because of this functional interaction between ILF2 and ILF3, and because ILF2 autoantibodies have been reported to occur together with ILF3 autoantibodies in mice and humans (Kuroda *et al.*, 2006; Satoh *et al.*, 1999), we screened the canine samples for ILF3 autoantibodies. A majority (87%) of the samples that were ILF2-positive were also ILF3-positive. Cross-reactivity studies showed that this was not due to cross-reactivity. We also identified ILF2 and ILF3 autoantibodies in five human patients with either SLE or Sjögren's syndrome.

The sera were also tested for autoantibodies to RNA-binding motif protein, X chromosome, RBMX (also known as hnRNP G), previously described as an autoantigen in dogs. These autoantibodies were only detected in 19 of 119 (16%) sera from ANA^{S} dogs and were only present in German shepherd dogs, cross-breeds, and in one dog of unknown breed.

6 Discussion and future perspectives

The work presented in this thesis builds on previous studies of immune-mediated diseases in NSDTRs (Hansson-Hamlin & Lilliehöök, 2013; Wilbe *et al.*, 2010a; Hansson-Hamlin & Lilliehöök, 2009; Wilbe *et al.*, 2009). The original idea was to focus only on this breed, but the investigations were extended to include dogs of other breeds (paper III and IV) and also humans (paper IV). Although this work focuses on studies of immune-mediated diseases in NSDTRs, it also provides information relevant to the larger research fields of autoimmune diseases in dogs and humans.

6.1 Nova Scotia duck tolling retrievers are predisposed to autoimmune diseases

As hypothesised, and as suggested but not actually shown by others (Hansson-Hamlin & Lilliehöök, 2013; Hansson-Hamlin & Lilliehöök, 2009; Anfinsen *et al.*, 2008; Redman, 2002), NSDTRs were here shown to be predisposed to autoimmune disorders, particularly to IMRD and SRMA (paper I). Information about which disorders are high-risk in a breed can be of value to veterinarians, dog breeders and owners, and researchers. Based only on the results from paper I, it is not possible to draw conclusions about the causes of the identified predispositions in NSDTRs. A breed predisposition can reflect a genetic component, but can in some cases be explained by lifestyle factors or owners' attitude to seek veterinary care. It should be noted that NSDTRs were compared to all other dog breeds combined, and to all other retrievers combined, but not to every single breed separately. Individual breeds might have similar, or higher, incidence rates for autoimmune disease, as NSDTRs. Considered together with results from previous studies, showing that genetic factors contribute to IMRD and SRMA (Wilbe *et al.*, 2010a; Wilbe *et al.*, 2009), it is strongly indicated that

the high relative risk observed for these two disorders reflect a genetic predisposition in NSDTRs.

The incidence rates for IMRD and SRMA were >10 times higher in NSDTRs than in other dog breeds combined. Even so, they were lower than for several other conditions. Overall, morbidity in NSDTRs was slightly higher compared to all other breeds but similar to retrievers from other breeds. One of the purposes of describing the whole disease pattern in NSDTRs was to rank disorders in terms of how common they are. This information is helpful, because it is sometimes difficult to interpret incidence for a disease, unless it is done in comparison to incidences of other diseases. The information about relative risk and incidence should be considered together, because preventive measures ideally should be directed at disorders that are common, severe, and high-risk. Although being one of the primary aims with paper I, it turned out to be difficult to estimate exact incidence rates for IMRD and SRMA, mainly because of the problem with diagnostic classification. A review of diagnostic codes assigned to the NSDTRs included as IMRD cases in the case-control studies (paper II and IV), showed that these dogs often receive the diagnostic code lameness. Lameness was not included in the IMRD category, because it represents a large number of different disorders. The incidence rates for IMRD and SRMA in paper I are therefore likely underestimates of true incidence, and insurance data might not actually be the best way to assess disease frequency for these diseases. Other attempts have been made to estimate the disease frequency of IMRD and SRMA in NSDTRs, by owner questionnaires, but these unpublished studies suffer from other drawbacks such as lack of validation, participation bias, and an ill-defined population at risk.

The overall disease description in NSDTRs presented in paper I provides novel information that should be of interest to different stakeholders. The study is based on insurance data, which benefits from containing information about both the disease events, and the background population (O'Neill *et al.*, 2014a; Egenvall *et al.*, 2009). A recent report in Sweden stated that approximately 9 of 10 dogs are insured (Agria Pet Insurance, 2017; Novus, 2017). An estimated 40% of these dogs are insured by Agria Pet Insurance (Agria Pet Insurance, personal communication, 2018). Thus, the dogs insured by Agria represent a large proportion of the entire Swedish dog population, and the insurance database contains data from sufficient number of dogs to provide power to gain significant results. One of the major drawbacks with insurance data concerns the problem of diagnostic accuracy. The Agria Pet Insurance database has been validated, and the agreement between the database and medical record was considered fair (Egenvall *et al.*, 1998) but it can be assumed to vary between different diagnoses. Another problem is lack of diagnostic codes for some

disorders. A new, hopefully improved, diagnostic registry has recently been released, which might solve some of these issues (Svensk Djursjukvård, 2017).

6.2 IMRD and SRMA are associated with different but overlapping sets of genes

It was previously shown that MHC class II genes are strong genetic risk factors for IMRD but not SRMA (Wilbe *et al.*, 2009). In addition to the MHC, five genetic loci associated with either IMRD or SRMA had been identified (Wilbe *et al.*, 2010a). Paper II builds on these previous findings. A more detailed analysis of the associated loci than what was previously done was performed. Unlike the previous studies, only ANA-positive IMRD cases were included. The IMRD cases were further subdivided into two sub-phenotypes, based on the ANA pattern, in order to identify potential risk factors associated with different sub-phenotypes. In paper II, it is shown that the haplotype 2 (*DRB1**00601/*DQA1**005011/*DQB1**02001), which was previously found to be strongly associated with IMRD, is only associated with IMRD ANA^S and not with IMRD ANA^H. A similar haplotype (*DRB1**00601/*DQA1**005011/*DQB1** 00701) has been associated with other canine disorders, including immune-mediated haemolytic anaemia in multiple breeds (Kennedy *et al.*, 2006a) and chronic hepatitis in English Springer Spaniels (Bexfield *et al.*, 2012). No MHC class II haplotypes or genotypes were found to be significantly associated with IMRD ANA^H, which might be due to lack of power. A general homozygosity in MHC class II region was, however, significantly associated with IMRD ANA^H.

The other genetic regions showed a complex pattern of association where some regions showed association to more than one disease or sub-phenotype. In order to identify functional changes associated with disease, expression studies of the genes in the associated regions were performed in blood from healthy NSDTRs. Gene expression between dogs with risk and non-risk haplotypes were compared. Eleven genes showed altered expression associated with one or more of the risk haplotypes (Table 1 and Figure 5). The SRMA risk haplotypes were associated with altered expression of one gene on chromosome 3 (*AP3B2*) and four genes on chromosome 32 (*DAPP1*, *LAMTOR3*, *DDIT4L*, and *PPP3CA*). Altered expression of *AP3B2* was also associated with IMRD ANA^S risk. Three genes (*PTPN3*, *WHAMM*, and *BANK1*) showed altered expression in both IMRD ANA^H and IMRD ANA^S risk haplotypes and three genes (*WFDC3*, *HOMER2*, and *VRK1*) showed association to only IMRD ANA^H. The altered gene expression in HOMER did not reach statistical significance but a non-synonymous SNP in this gene was associated with the risk. Some of the identified genes are well-studied and have been associated with various human

autoimmune diseases, such as *BANK1* (Muhali *et al.*, 2013; Rueda *et al.*, 2010; Kozyrev *et al.*, 2008). Other genes not previously described in autoimmunity provide good candidates for follow-up studies.

To identify the genetic variants causing altered gene expression further functional studies are necessary, since the identified SNPs are not necessarily functional, but rather in linkage disequilibrium with the functional SNP. One way to do this is by using transient transfection in different cell lines with reporters carrying either the risk or non-risk SNP. The effect of potentially disease-causing coding SNPs could be studied by cell culture methods overexpressing the genes containing the mutations.

The results from study II throw light on the genetic complexity of immune-mediated disease in dogs. Variations in multiple genes contribute to disease, where some gene variants are shared between diseases and sub-phenotypes, while others appear to be specific for only one phenotype. In addition to the risk factors identified in paper II, there are probably unidentified genetic, epigenetic, and environmental risk factors that contribute to development of IMRD and SRMA in NSDTRs. Furthermore, a future challenge will be to establish how these risk factors functionally interact to cause disease. Modern methods such as whole genome and transcriptome sequencing of affected dogs, could potentially provide further clues into the genetics underlying IMRD and SRMA. Although our understanding of how genetic factors contribute to the development of these diseases have increased greatly since they were first noticed in NSDTRs, a lot is still to be learned and understood. A future priority is to determine which combination of genetic risk factors that give the highest risk of developing IMRD and SRMA. To do this, recruitment of more diseased dogs would be needed to provide enough power to gain statistically significant results.

6.3 Several autoantigens identified in ANA-positive dogs

Paper III and IV aimed at finding autoantibody targets in ANA-positive IMRD NSDTRs and also in dogs of other breeds. Several different methods, originally developed for detection of human autoantibodies, were adapted to work with canine sera. The frequencies of different ANA patterns were described and the speckled pattern was the common pattern, in agreement with earlier studies (Hansson-Hamlin *et al.*, 2006; Hansson *et al.*, 1996). Similarly to what is already well established in humans (Chan *et al.*, 2015), different ANA patterns were found to be associated with reactivity to different autoantigens. Results from previous studies already suggested that this was the case (Hansson-Hamlin & Rönnelid, 2010; Welin Henriksson *et al.*, 1998; Fournel *et al.*, 1992; Monier *et al.*, 1992; Monier *et al.*, 1988; Monier *et al.*, 1980) but it has not been shown as

clearly before. In most of the older studies, different substrates, such as rat liver or mouse blood smears, were used for ANA detection. With these substrates, the type of pattern can be difficult to determine and false positive results might be a problem (Hansson *et al.*, 1996). They have therefore largely been replaced by HEp-2 cells.

In paper III nucleosomes and dsDNA were found to be common autoantibody targets in ANA^{H} sera, and RNP/Sm to be targets in many ANA^{S} sera, in agreement with findings in humans (Chan *et al.*, 2015). In humans, dsDNA and Sm autoantibodies show high specificity for SLE (Petri *et al.*, 2012; Satoh *et al.*, 2007; Tan *et al.*, 1982). RNP autoantibodies are associated with mixed connective tissue disease (Sharp *et al.*, 1972) but might also occur in other systemic rheumatic diseases. Both RNP and Sm autoantibodies have previously been described in dogs (Hansson-Hamlin & Rönnelid, 2010; Welin Henriksson *et al.*, 1998; Fournel *et al.*, 1992; Hubert *et al.*, 1988; Costa *et al.*, 1984) but it is difficult to compare the frequency of positive cases between our present study and previous studies, mainly because the inclusion criteria and methods of autoantibody detection differ. Previous studies of dsDNA autoantibodies in dogs have yielded inconclusive results (Monestier *et al.*, 1995; Fournel *et al.*, 1992; Monier *et al.*, 1992). In a portion of the dogs these findings were validated with CLIFT, a highly specific method for dsDNA autoantibody detection in humans (Enocsson *et al.*, 2015; Haugbro *et al.*, 2004), supporting the conclusion that dsDNA autoantibodies exist in dogs.

One of the major limitations of study III was that a large representative control group was lacking. This led to difficulties establishing proper cut-off values. Still, many of the positive samples showed high reactivity, well above cut-off. In a number of ANA-positive dogs, mainly in the ANA^{S} group, the autoantigen could not be identified by the targeted approach used in paper III. A screening of a large number of sera from NSDTRs with the same methods described in paper III (unpublished data), revealed negative results for known autoantibodies in the majority of ANA^{S} sera from IMRD patients. This led us to study IV, where a broad screening of autoantibodies in IMRD patients with focus on the ANA^{S} group was performed. The chances of finding the autoantigens using protein arrays with recombinant human proteins were fair, since it was known that these patients have autoantibodies that cross-react with human proteins (HEp-2 cells) and that autoantibodies are often directed at conserved proteins (Utz *et al.*, 2000). Although there are limitations with this technology that can lead to false negative results (Duarte & Blackburn, 2017), a good candidate autoantigen, ILF2, was found, and was further validated as a canine autoantigen with an independent method in a larger cohort of dogs. Indeed, the prevalence of autoantibodies directed against ILF2, and the associated protein

ILF3, was very high in ANA^S sera from several dog breeds, including NSDTRs. Autoantibodies to RBMX, previously described as an important autoantigen in dogs with ANA^S (Soulard *et al.*, 1993; Soulard *et al.*, 1991), were found at a lower frequency. A clear breed difference in autoantibody reactivity was observed which indicates, and also supports by the findings in study II, that the genetic background is important for the autoantibody development. The ILF2, ILF3, and RBMX proteins belong to the same complex, but autoantibodies to RBMX were found only in German shepherd dogs, mixed breeds, and in one dog of unknown breed, while ILF2/ILF3 autoantibodies were found in NSDTRs, cocker spaniels, and other dogs breeds. ILF2 and ILF3 are expressed in many tissues where they function as transcription factors crucial for expression of for example interleukin-2 and interleukin-13 (Kiesler *et al.*, 2010; Shi *et al.*, 2007; Zhao *et al.*, 2005; Corthesy & Kao, 1994; Kao *et al.*, 1994). Why tolerance is lost against these proteins is unknown but intriguing.

Preliminary analyses of combined data from paper III and IV, together with some additional unpublished data, show that in almost all ANA-positive dogs, a specific reactivity can be identified. Autoantibodies to nucleosomes/dsDNA are found in most ANA^H sera, and autoantibodies to either ILF2/ILF3, RBMX, or RNP/Sm can be found in almost all ANA^S sera. It is possible, that testing for these autoantibodies can replace, or be an additional test to, the conventional ANA test. The conventional ANA test is laborious and somewhat subjective since it is manually interpreted (Satoh *et al.*, 2007), and other methods may be more suitable for large scale testing. However, more work is required to elucidate if these autoantibodies have a role as diagnostic markers. As discussed earlier, testing for specific ANA is important in the diagnosis of human systemic autoimmune disease, but investigation of autoantibodies in canine systemic autoimmune disease is mainly restricted to the IIF-ANA test. More specific test in dogs could be an aid in the diagnosis, and potentially help in monitoring of patients. To study the potential clinical use of the autoantibodies described in this thesis, large and well characterised cohorts of dogs with different autoimmune manifestations would be required. Dogs with other inflammatory but non-autoimmune disorders could also be included as disease controls. A limitation of the current work is the lack of clinical data from dogs of other breeds besides NSDTRs. Also, the inclusion criteria for IMRD and SRMA were quite broad, to facilitate collection of a large enough material. Although the veterinary records were assessed by either myself or other veterinarians from our research group, different veterinarians investigated the dogs, and different tests were used to diagnose and to rule out other causes of disease. Ideally, a standardised set of tests should have been performed in all cases, but this was not possible due to the retrospective nature of the studies.

6.4 Can IMRD be separated into two diseases based on the ANA pattern?

In this thesis, it is shown that the two different sub-phenotypes of IMRD based on the ANA pattern correlate with different autoantibodies and different genetic risk factors. Some clinical signs also appear to be more common in one of the sub-phenotypes; for example, skin lesions are more frequently present in IMRD ANA^{H} dogs, which has also been reported previously (Hansson-Hamlin & Lilliehöök, 2009). However, in our experience, IMRD cannot clearly be separated clinically into two diseases based on the ANA pattern. Autopsies of IMRD-affected dogs could potentially reveal different underlying aetiologies or pathogenesis, but these dogs are rarely euthanized as a consequence of disease, at least not in the acute phase of disease. To predict severity of disease and response to treatment is difficult at disease onset, and it would be of value with a prognostic marker for IMRD. It needs to be further studied if the ANA pattern, or specific autoantibodies, maybe in combination with some genetic risk factors, can give insight into the prognosis. In this work patients were subdivided on the basis of ANA pattern. Future studies could aim at finding association between different genetic variants and specific canine autoantibodies.

7 Conclusions

- The overall incidence of disease in NSDTRs is similar to the incidence in other retrievers but slightly higher than in all other breeds combined.
- Nova Scotia duck tolling retrievers are predisposed to autoimmune and neurological disorders in general and to IMRD and SRMA in particular.
- A particular MHC class II haplotype, *DRB1**00601/*DQA1**005011/ *DQB1**02001, is associated with IMRD ANA^S.
- A general homozygosity in the MHC class II region is associated with IMRD ANA^H.
- Eleven genes associated with IMRD and/or SRMA show altered functional changes, of which four are associated with only SRMA, one with both SRMA and IMRD ANA^S, three with both IMRD ANA^S and IMRD ANA^H, and three with only IMRD ANA^H.
- Autoantibody reactivity to dsDNA and nucleosomes are associated with ANA^H. Reactivity to RNP/Sm is associated with ANA^S.
- The autoantibody reactivity varies between dogs of different breeds.
- The proteins ILF2 and ILF3 are common autoantigens in ANA^S dogs.

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Popular science summary

Immune-mediated diseases are caused by a defective immune system. These are common diseases that affect both humans and animals including dogs. Broadly they can be divided into immunodeficiency disorders, allergies, and autoimmune diseases. The research presented in this thesis primarily focuses on autoimmune diseases in dogs. In autoimmune disease, the immune system that is meant to protect the body from foreign substances, such as infections, attacks the body's own cells and tissues. This leads to organ damage and clinical signs of disease. Dogs of the breed Nova Scotia duck tolling retrievers, often called tollers, are affected by an autoimmune disease called immune-mediated rheumatic disease (IMRD) and another immune-mediated disease called steroid-responsive meningitis-arteritis (SRMA). In Sweden, both these diseases are often referred to as "Toller disease". IMRD affects middle aged dogs. Dogs are often between two and six years of age at disease onset. The main clinical signs are lameness that can shift between legs, and general stiffness which is often most apparent after the dog has been resting. SRMA is a disease primarily affecting young dogs. The main clinical signs are high fever and intense neck pain caused by meningitis.

This thesis deals with three different areas; epidemiology (paper I), genetics (paper II), and autoantibodies (paper III and IV). In paper I the frequencies of different immune-mediated diseases including IMRD and SRMA in tollers were studied and compared to the frequencies in other dog breeds. Data were assembled from Agria Pet Insurance, which holds information about the cause of a dog visiting a veterinarian. In agreement with what has long been suspected, but not actually shown before, IMRD, SRMA, and autoimmune disease in general were found to be more common in tollers than in other dog breeds combined. These diseases were not particularly common even in tollers, however. The most common causes for tollers to visit a veterinarian were injuries, gastrointestinal disease (such as diarrhoea and vomiting), and clinical signs related to the locomotor system (such as lameness).

It was previously shown that IMRD and SRMA in tollers are complex genetic diseases. This means that mutations in several genes cause disease, probably in combination with unknown environmental factors. Some regions in the genome associated with IMRD or SRMA have been identified. In paper II we present an analysis of these regions where we found that genetic variants in 11 genes were associated with disease. Most of these genes were associated with only IMRD or SRMA, but one gene showed association to both diseases. All the genes are good candidates for follow-up studies. Further studies are needed to investigate how variations in the genes contribute to disease, and to find the exact disease-causing mutations.

Antibodies are an important part of the immune system. In many ways they protect the body against infections. In autoimmune diseases the antibodies are wrongly directed at the body's own proteins, which can cause disease. These autoantibodies are markers of autoimmune disease and can be used for diagnosis. In paper III and IV we aimed at identifying the autoantibody targets and to discover new autoantibodies in dogs. We found both new and well-known autoantibodies. Two proteins, ILF2 and ILF3, were common targets of autoantibodies in IMRD patients. These autoantibodies have not been described in dogs previously and have the potential to be used as markers for systemic autoimmune disease.

Populärvetenskaplig sammanfattning

Immunmedierade sjukdomar orsakas av defekter i immunsystemet och är vanliga sjukdomar hos både människor och djur inklusive hundar. Översiktligt kan de delas in i immunbristsjukdomar, allergier och autoimmuna sjukdomar. Den forskning som presenteras i denna avhandling berör framförallt autoimmuna sjukdomar hos hund. Vid en autoimmun sjukdom attackeras kroppens egna celler och vävnader av immunförsvaret, vilket leder till skador på organ och kliniska tecken på sjukdom. Hundar av rasen Nova Scotia duck tolling retriever (tollare) kan drabbas av en autoimmun sjukdom som kallas immunmedierad reumatisk sjukdom (IMRD) och en annan immunmedierad sjukdom som heter steroid-responsiv meningit-arterit (SRMA). I Sverige benämns ofta båda dessa sjukdomar som tollarsjuka. IMRD drabbar framförallt medelålders hundar som oftast är mellan två och sex år när sjukdomen debuterar. De vanligaste sjukdomstecknen är hälta som kan vandra mellan olika ben och stelhet som oftast är mest tydligt efter att hunden har vilat. SRMA är en typ av hjärnhinneinflammation som framförallt drabbar unga hundar. Feber och kraftig nacksmärta är de vanligaste tecknen på sjukdom.

Forskningen som denna avhandling bygger på kan delas in i tre delar; epidemiologi (studie I), genetik (studie II) och autoantikroppar (studie III och IV). I studie I undersöktes frekvensen av olika immunmedierade sjukdomar hos tollare i jämförelse med frekvens hos andra hundraser. Försäkringsdata från Agria Djurförsäkringar användes; dessa data innehåller bland annat information om orsaker till veterinärbesök. IMRD, SRMA och autoimmuna sjukdomar generellt visade sig vara vanligare hos tollare än hos andra hundraser. Det har tidigare misstänkts men inte kunnat visas förrän nu. Trots den ökade förekomsten hos tollare, var dessa sjukdomar inte särskilt vanliga. De vanligaste orsakerna till att en tollare fick veterinärvård var skador, magtarmbesvär (såsom kräkningar och diarré) och kliniska tecken kopplade till rörelseapparaten (såsom hälta).

Det har tidigare visats att IMRD och SRMA är komplexa genetiska sjukdomar. Det betyder att mutationer i flera gener, troligtvis tillsammans med okända miljöfaktorer, orsakar sjukdom. Flera regioner i arvsmassan som är associerade med IMRD och SMRA har hittats. I studie II genomförde vi en analys av dessa regioner och fann 11 gener som var associerade med sjukdom. De flesta av dessa gener var bara associerade med en av sjukdomarna, men en gen var associerad med både IMRD och SRMA. Ytterligare studier behövs för att identifiera de specifika sjukdomsorsakande mutationerna och fastställa hur dessa verkar för att orsaka sjukdom.

Antikroppar är en viktig del av immunförsvarets skydd mot infektioner. Vid autoimmuna sjukdomar kan antikropparna felaktigt riktas mot kroppens egna proteiner. Dessa autoantikroppar är markörer för autoimmun sjukdom och kan användas som ett hjälpmedel för att ställa diagnos. Studie III och IV syftade till att identifiera de målstrukturer som autoantikropparna är riktade mot och hitta nya autoantikroppar hos hund. Vi fann både tidigare beskriva och nya autoantikroppar. Autoantikroppar mot två proteiner, ILF2 och ILF3, var vanligt förekommande hos hundar med IMRD. Dessa autoantikroppar har inte tidigare beskrivits hos hund och har potential att användas som markörer för systemisk autoimmun sjukdom.

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