

Symmetrical Onychomadesis and Hypothyroidism in the Gordon Setter and English Setter

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Til Andreas, Mikkel, Jesper og Mats

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List of papers

Paper I:

Ziener M.L., Bettenay S.V., Mueller R.S.

Symmetrical Onychochomadesis in Norwegian Gordon and English setters.

Vet Dermatol (2008) 19: 88-94

Paper II:

Ziener M.L., Nødtvedt A.

A treatment study of canine symmetrical onychomadesis, (symmetrical lupoid onychodystrophy), comparing fish oil and cyclosporine supplementation in addition to a diet rich in omega-3 fatty acids.

Acta Vet Scand (2014) 56: 66. doi: 10.1186/s13028-014-0066-y

Paper III:

Wilbe M., Ziener M.L., Aronsson A., Harlos C., Sundberg K., Norberg E., Andersson L., Lindblad-Toh K., Hedhammar A., Andersson G. & Lingaas F.

DLA class II alleles are associated with risk for canine symmetrical lupoid onychodystrophy (SLO).

PLoS One (2010) doi: 10.1371/annotation/6f88cccd-2f41-464d-904b-24356b5d87c9

Paper IV:

Dahlgren S., Ziener M.L., Lingaas F.

A genome-wide association study identifies a region strongly associated with Symmetrical Onychomadesis on chromosome 12 in dogs.

(Submitted to *Animal Genetic*, 2015)

Paper V

Ziener M.L., Dahlgren S., Thoresen S.I., Lingaas F.

Genetics and epidemiology of hypothyroidism and symmetrical onychomadesis in the Gordon setter and the English setter

(Submitted to *Canine Genetics and Epidemiology*, 2015)

The papers will subsequently be referred to by their Roman numerals (I-V).

Summary

Symmetrical onychomadesis (SO), also called symmetrical lupoid onychodystrophy, is a severe claw disease frequently seen in the Gordon Setter (GS) and English Setter (ES). Affected dogs will initially show sudden onset of lameness and licking of paws, progressing during some months to sloughing of all claws. The disease is painful and affects the dog's quality of life. Hypothyroidism (HT) is one of the most common endocrine diseases reported in dogs. In most cases, the disease is considered to be the result of an autoimmune attack of the thyroid gland. Thyroid hormones are involved in several energy demanding processes in the body and subnormal hormone levels in dogs may cause a variety of clinical signs such as dullness, weight gain, lethargy, and skin changes. Both SO and HT are suspected to be complex diseases where several genes and environmental factors affect disease development. Epidemiologic studies of the two diseases in GS and ES could reveal triggering factors for disease development.

The main aims of this thesis were to describe epidemiology, clinical pathology, treatment, prognosis and genetic risk factors for SO and HT in the GS and ES. Additionally, we wanted to investigate whether there were any clinical or genetic associations between SO and HT.

Symmetrical onychomadesis in GS and ES was not described in the literature prior to this study. The clinical signs and histopathological changes found in claw biopsies in the GS and ES diagnosed with SO in the present studies were similar to what has been described previously in dogs with SO from other breeds. The prevalence of SO in 104 unrelated GS was found to be 12.6 % in dogs with a mean age of 9.2 years, and 8.9 % in a sample of 291 eight years old GS. An increased prevalence (34.3 %) was found in 101 dogs with at least one SO affected sibling. This finding indicates that SO has a high heritability. The prevalence of HT in the unrelated, 9.2 years old GS was 5.8 % and in the sample of eight years old GS it was 2.7 %. An increased prevalence of HT was not seen in the dogs with SO affected siblings (4.3 %).

An epidemiologic survey was performed among GS and ES with and without SO to gain more insight into possible trigger factors for SO and the prognosis of the disease. Mean age at onset of SO was 4.3 years (range 1- 9 years), with no sex differences detected in disease occurrence. In 75 % of the 86 affected dogs the first symptoms of SO occurred between April and September. There were only 11 % of the dogs that recovered from the disease and got

normal claws back. Recurrent onychomadesis occurred in 31 % of the affected dogs and in 58 % of the affected dogs the claws ended up being dystrophic. All dogs were used for hunting prior to developing disease, but after the occurrence of SO 26 % of the affected dogs were retired as hunting dogs. Symmetrical onychomadesis was reported as the cause of euthanasia in 12 out of 73 deaths (16 %).

A treatment study was performed in GS and ES with SO to compare the effect of using cyclosporine versus omega-3 supplementation. All dogs were fed the same diet that contained a high amount of omega-3. All dogs improved their claw quality during the six months treatment trial, but no statistical differences were observed among the two groups.

A candidate gene approach was used to study genetic risk factors associated with SO and/or HT in the GS and the ES. The dog leukocyte antigen (DLA) genes DRB1, DQA1 and DQB1 were genotyped based on a proposed autoimmune etiology behind the two diseases. DLA genes code for the major histocompatibility complex (MHC), which present antigens for T cells in the immune system. Homozygosity for the most common DLA haplotype (DRB1*01801/DQA1*00101/DQB1*00802) in the GS was significantly associated with SO in one study and with protection for HT in another study. The DLA haplotype DRB1*02001/DQA1*00401/DQB1*01303 was associated with protection for SO in GS. The DLA haplotype in the GS with the highest OR associated with HT was DRB1*00103/DQA1*00101/DQB1*00201. The DLA haplotype DRB1*10201/DQA1*00101/DQB1*00201 was associated with increased risk of HT in ES, and the DLA haplotype (DRB1*00601/DQA1*005011/DQB1*00701), only present in 3.3 % of the ES population, was associated with protection of HT in ES. The DLA allele DQB1*00201 was associated with HT in both GS and ES. The DLA allele DQA1*00101 was associated with SO in GS and HT in ES. This DLA allele (DQA1*00101) was also present in all ES with SO. In summary, one DLA haplotype in the GS was associated with both increased risk for one presumed autoimmune disease (SO) and decreased risk for another autoimmune disease (HT). This indicates a genetic complexity of this group of diseases and suggests that potential marker assisted selection of breeding animals should be applied only after consideration of all prevalent autoimmune diseases in one breed.

The purpose of a genome wide association study (GWAS) is to identify regions within the entire genome that harbor possible genetic variants causing the studied phenotype. An associated single nucleotide polymorphism (SNP) found by GWAS, could either directly affect the phenotype, but in most cases it would be closely linked with genetic variants associated with the phenotype. A GWAS for SO in GS identified a region on chromosome 12 with twelve statistically significant SNPs, covering 3.3 mega base (Mb) pairs. No associated SNPs were located within the DLA-genes DRB1, DQA1, DQB1, but the associated region covered the DLA region. Many of the SNPs were inherited together as haplotypes. The haplotype with the highest OR associated with SO was made up by three of the significant SNPs and covered 0.5 Mb pairs. These haplotypes containing significant SNPs from the GWAS could be used to calculate a genetic risk index for SO in GS and ES. Such risk estimates could assist breeding, and contribute to a reduction of the incidence of SO in the GS and ES. However, breeding advice based on associated genetic markers should be performed with caution, and it is crucial to account for potential unfavorable correlations to other diseases. It is also important to note that the exact genes responsible for development of SO are still not known. We believe that resequencing the identified candidate region in cases and controls will be crucial to identify genetic variants that underlie the development of SO. A GWAS of hypothyroid GS and ES should also be performed to look for significant SNPs for HT as well and to compare the location with the SO GWAS results.

This thesis resulted in several important conclusions. Firstly, that GS has a high prevalence of SO. The disease has a negative impact on quality of life and hunting abilities for affected GS and ES. A diet with high amount of omega 3 together with cyclosporine or omega 3 as supplementation improved the claw health in GS and ES with SO. Associations to breed-specific DLA haplotypes and DLA alleles were found for both SO and HT in the GS and for HT in ES. The results from the GWAS confirmed an association between SO and the DLA region on chromosome 12. There were not found any common genetic risk factors for SO and HT in this thesis.

Sammendrag

Kloløsning er en sykdom som har vært kjent hos gordonsetter og engelsksetter siden 1980-tallet. Gordonsetteren økte sterkt i popularitet utover tidlig 2000 tallet og da økte også antall tilfeller av kloløsning. Kloløsning er en smertefull sykdom som gir hundene redusert livskvalitet og fører ofte til at hundene har problemer med å jakte. Dette studiet ble startet for å øke kunnskapen om kloløsning og hypotyrose hos gordonsetter og engelsksetter. Kloløsning har blitt kalt både «symmetrical lupoid onychodystrophy» og «symmetrical onychomadesis» og begge navnene blir brukt i dette studiet. Kloløsning arter seg ved at hundene blir halte, slikker seg på klørne og i løpet av noen måneder faller de aller fleste klørne av. Det oppstår ofte sekundær infeksjoner der hvor klørne løsner og sykdommen er smertefull for de fleste hunder. De klørne som vokser ut er ofte misdannede og flisete (dystrofiske). Hundene har ikke tegn på andre hud- eller systemiske sykdommer som kan forklare kloløsningen. Hypotyrose er en av de vanligste endokrine lidelsene hos hund. Sykdommen er som regel forårsaket av et autoimmunt angrep på thyroidea som fører til at kjertelen blir ødelagt. Thyroidea hormoner er involvert i mange energikrevende prosesser i kroppen og lave nivåer fører til nedstemthet, vektøkning og pelsforandringer hos hund. Både kloløsning og hypotyrose er mistenkt for å være komplekse sykdommer der flere gener og miljø faktorer påvirker utviklingen av sykdommene.

Målet i denne studien var å beskrive epidemiologi, klinisk patologi, behandling og prognose ved kloløsningen og hypotyrose hos gordonsettere og engelsksettere. Det var også ett mål å avdekke genetiske risikofaktorer hos gordonsettere og engelsksettere med kloløsning og/eller hypotyrose ved å utføre flere studier av kandidatgenene DRB1, DQA1 og DQB1. Disse DLA genene ble valgt fordi både kloløsning og hypotyrose er mistenkt for å være autoimmune sykdommer og assosiasjoner mellom autoimmune sykdommer og DLA gener er påvist før hos hund. DLA genene koder for MHC som har til oppgave å presentere antigen på overflaten av celler slik at T celler gjenkjenner antigenet og setter i gang en immunrespons. DLA genene ligger tett koblet på kromosom 12 hos hund og nedarves derfor som haplotyper. Videre ønsket vi å finne det området på genomet som var assosiert med kloløsning for å kunne lokalisere de genene som påvirker utviklingen av sykdommen. Det siste målet var å undersøke om det var en klinisk eller genetisk sammenheng mellom kloløsning og hypotyrose hos gordonsetter og engelsksetter.

I det første studiet ble det påvist at gordonsetterne og engelsksetter hadde de samme symptomene ved kloløsning som er beskrevet hos andre raser. Biopsiene fra affiserte klør hos setterne viste også de samme histopatologiske forandringene som biopsiene fra andre raser hadde vist før. Prevalensen på kloløsning hos 104 gordon settere som ikke var i slekt og som var i gjennomsnitt 9,2 år var 12,6 %. I ett utvalg på 291 åtte år gamle gordon settere var prevalensen av kloløsning på 8,9 %. Den høyeste prevalensen av kloløsning (34,3 %) ble funnet hos 101 hunder som hadde ett eller flere kullsøsken med kloløsning. Den store forskjellen i prevalensen av kloløsning mellom beslektede og ubeslektede individer tyder på at arv spiller en betydelig rolle for utvikling av kloløsning. Prevalensen på hypotyrose hos de ubeslektede gordonsetterne med en gjennomsnittsalder på 9,2 år var på 5,8 %. Til sammenligning var prevalensen på hypotyrose på 2,7 % hos de åtte år gamle gordonsetterne, mens søsknene til hunder med kloløsning ikke viste noen tendens til økt forekomst av hypotyrose (4,3 %). Det ble foretatt en epidemiologisk undersøkelse blant gordonsetterne og engelsksetterne med og uten kloløsning. Undersøkelsen viste at hundene var 4,3 år (1-9 år) når de fikk sykdommen og at like mange hannhunder som tisper fikk kloløsning. Symptomene på kloløsningen startet mellom april og september hos 75 % av de 86 hundene som hadde kloløsning. Prognosen ved kloløsning var forholdsvis dårlig og bare 11 % av hundene fikk tilbake normale klør etter første tilfelle med kloløsning. Hos 31 % var det gjentatte tilbakefall med kloløsning og hos 58 % ble klørne dystrofiske, men de løsnet ikke flere ganger. Alle hundene ble brukt som jakthunder før kloløsningen, men 26 % av hundene ble pensjonert som jakthunder etter at de hadde fått diagnosen. Kloløsning ble oppgitt som grunnen til avliving hos 12 av de 73 hundene som var avlivet når undersøkelsen ble foretatt (16 %). Det ble foretatt ett behandlingstudium hos gordonsetterne og en engelsksetter med kloløsning der behandlingseffekten av omega tre tilskudd ble sammenlignet med den immundempende medisinen cyclosporin. Alle hundene fikk samme fôr mens studien pågikk med ett høyt innhold av omega tre. Alle hundene fikk bedre klokkvalitet i løpet av behandlingsperioden, men det var ikke signifikant forskjell mellom behandlingalternativene.

Resultatene fra kandidatgenstudiene for kloløsning hos gordonsetter viste at det å være homozygot for den mest vanlige DLA haplotypen hos gordonsetter (DRB1*01801/DQA1*00101/DQB1*00802) ga økt risiko for å utvikle kloløsning i ett DLA

studie og beskyttelse mot hypotyroser i ett annet DLA studie. DLA haplotypen (DRB1*02001/DQA1*00401/DQB1*01303) var forbundet med beskyttelse mot å utvikle klølsning hos gordonsetter. DLA haplotypen forbundet med høyest risiko for å utvikle hypotyroser hos gordonsetteren var DRB1*00103/DQA1*00101/DQB1*00201. DLA haplotypen DRB1*10201/DQA1*00101/DQB1*00201 var forbundet med økt risiko for å utvikle hypotyroser hos engelsksetter og DLA haplotypen DRB1*00601/DQA1*005011/DQB1*00701 var forbundet med beskyttelse for å utvikle hypotyroser i samme rase. DLA allelet DQB1*00201 var forbundet med økt risiko for å utvikle hypotyroser hos både gordonsetter og engelsksetter. DLA allelet, DQA1*00101, var forbundet med økt risiko for å utvikle klølsning hos gordonsetter og hypotyroser hos engelsksetter. Alle engelsksettere med klølsning hadde dette DLA allelet. Etter sammenligning av resultatene fra DLA studiene blir konklusjonen derfor at ved utvalg av avlsdyr bør det ikke legges ukritisk vekt på DLA -varianter som kan være assosiert med en sykdom uten å ta hensyn til at rasen kan ha flere immunmedierte sykdommer knyttet til DLA haplotyper/alleler. Det kan se ut til at den påviste assosiasjonen mellom DLA gener og klølsning og/eller hypotyroser, kan være sterkt påvirket av andre gener som er tett koblet til DLA genene.

Det ble også utført en helgenomsanalyse (GWAS) på gordonsettere og engelsksettere med klølsning for å lete etter områder på genomet som inneholder potensielle sykdomsframkallende gener. Det ble identifisert ett område på kromosom tolv, som dekket 3,3 Mb par, med tolv signifikante SNP-er. Ingen av de tolv SNP-ene lå i DLA genene DRB1/DQA1/DQB1, men de var spredd over det området der DLA genene er lokalisert. De tolv SNP-ene var tett koblete og det vil si at de ble nedarvet sammen som haplotyper. Den haplotypen som var forbundet med den høyeste økte risikoen for å utvikle klølsning inneholdt tre signifikante SNP-er og dekket et område på 0,5 Mb par. Informasjon om de assosiert SNP-ene kan bli et hjelpemiddel til en mer effektiv seleksjon mot klølsning hos gordonsetter og engelsksetter, selv om det enda ikke er kjent hvilke funksjonelle gener som påvirker utviklingen av sykdommen. Målet for framtiden er å finne ut mer om hvilke gener som påvirker utviklingen av klølsning. Sekvensering av det aktuelle området på kromosom tolv hos gordonsettere med og uten klølsning, kan være med på å avsløre hvilke gener som spiller en avgjørende rolle ved utvikling av sykdommen. Det bør også utføres en GWAS på

gordonsettere og engelsksettere med hypotyreoidisme for se hvor på genomet signifikante SNP-er for denne sykdommen er lokalisert.

Det er gjort flere viktige funn i dette arbeidet. Det første er at kloløsning har en høy forekomst hos gordonsetteren. Sykdommen påvirker hundenes livskvalitet og jaktegenskaper negativt. En anbefalt behandlingsmetode for kloløsning er å fôre med et høyt innhold av omega tre og samtidig gi tilskudd av omega tre eller cyclosporin. Det ble påvist sammenhenger mellom spesifikke DLA haplotyper og DLA alleler for kloløsning og hypotyreose hos gordonsetter, og tilsvarende sammenhenger ble også påvist for hypotyreose hos engelsksettere for hypotyreose. Resultatene fra GWAS'en på kloløsning hos gordon setter og engelsk setter påviste en assosiasjon mellom kloløsning og en region på kromosom 12 som dekker 3,3 Mb par og inneholder blant annet DLA genene. Det ble ikke påvist noen felles genetiske risikofaktorer for kloløsning og hypotyreose i dette studiet.

Abbreviations

ANA:	Antinuclear antibodies
CI:	Confidence interval
DLA:	Dog leukocyte antigen
ES:	English Setter
FT4:	Free thyroxine
GS:	Gordon Setter
GWAS:	Genome wide association study
HLA:	Human leukocyte antigen
HT:	Hypothyroidism
MAF	Minor allele frequencies
Mb:	Mega base
MDS:	Multi dimensional scaling
MHC:	Major histocompatibility complex
mRNA:	Micro ribonucleic acid
NESK:	Norwegian English Setter Club
NGK:	Norwegian Gordon Setter Club
NKK:	Norwegian Kennel Club
SLE:	Systemic lupus erythematosus
SLO:	Symmetrical lupoid onychomadesis
SNP:	Single nucleotide polymorphism
SO:	Symmetrical onychomadesis
TgAA:	Thyroglobulin autoantibodies
TNF:	Tumor necrosis factor
TT4:	Total thyroxin
TSH:	Thyroid stimulating hormone

Terminology

Gene:	One defined area of DNA that give rise to a RNA chain that code for a protein
Haplotype:	A collection of closely located alleles on a chromosome usually inherited together
Linkage disequilibrium:	Non- random association of alleles that are located closely together on a chromosome
Onychodystrophy:	Malformation of the claw (1)
Onycholysis:	Separation of claw from the underlying corium but with continuing proximal attachment
Onychomadesis:	Sloughing of claws
Paronychia:	Inflammation of the claw fold
Polymorphism:	Describes a locus with several alleles
QQ plot:	Probability plot, Q stands for quantile
Single nucleotide polymorphism:	A single base pair mutation at a specific locus in a DNA sequence

Introduction

The Gordon Setter and the English Setter

The Gordon Setter (GS) and the English Setter (ES) are hunting dogs primarily used for bird hunting. They were considered a single breed until 1874, when they were divided into two separate breeds (2). The ES was the first breed registered in the Norwegian Kennel Club (NKK) (1898). Both breeds have been very popular in Norway since the foundation of the Norwegian English Setter Club (NESK) in 1907 and the Norwegian Gordon Setter Club (NGK) in 1916. Around 1960, several ES were outcrossed with GS to improve the hunting ability of the Norwegian GS breed. The common genetic background of the two breeds is reflected through their shared appearance in conformation and colours (Fig 1). The GS are traditionally black and tan coloured (Fig 1A), but some GS are white coloured with black and tan (Fig 1B). English Setters are traditionally white coloured with black or brown ticking (Fig 1C), but some individuals are white with black and tan (Fig 1D), looking similar to the GS in Fig 1B. The common genetic background of the GS and ES is also expressed through their shared predisposition to the same diseases. Generally, these breeds are known to be quite healthy, with a life expectancy of around ten to twelve years. However, certain diseases including hypothyroidism (HT) (3) and symmetrical onychomadesis (SO) (4) are described as prevalent in both breeds. Lymphocytic HT in dogs is an autoimmune disease and SO is suspected to be autoimmune in origin, therefore the following introduction includes a section on the immune system and a section on autoimmune diseases in general.



Figure 1. A: Gordon Setter, black and tan, B: Gordon Setter, black and tan with large white areas, C: English Setter, white with red ticking, D: English Setter, black and tan Photo: Ziener M.

The Immune System

Innate and adaptive immune systems

The immune system comprises an innate and an adaptive part (5). The innate immune system is non-specific and present at birth, while adaptive immunity develops following antigen exposure (5). The most important cells of the innate immune system are neutrophils, macrophages, dendritic cells, natural killer cells, eosinophils, basophils and mast cells. These cells have receptors that recognize and bind foreign antigens. Activation of the innate immune system results in production of eicosanoids and cytokines and an ensuing rapid inflammatory response (5, 6). Failure of the immune system, such as an inability to regulate the inflammatory response, can cause development of systemic autoimmune diseases and cancer (7). The innate immune system has lately gained increased attention in research into the pathogenesis of both autoimmune diseases and cancer (5, 8).

The adaptive immune system involves lymphocytes (B and T cells) that are found in immune tissues like bone marrow, thymus, lymph nodes and spleen (9). These cells produce antigen-

specific receptors. On first time exposure to a new antigen there is a lag phase of several days to weeks before the adaptive immune system can mount an effective immune response (5). However, this system has a memory mechanism that makes the body able to recognize the antigen on subsequent encounters, and then produce a rapid and much more effective response (5). The responses of the adaptive immune system are divided into cell-mediated immunity and humoral immunity (10). Different types of T cells are the most active cells in the cell mediated immune response, while different types of B cells are the most active cells in the humoral immune response (11, 12). Antigens are presented to the immune system by the major histocompatibility complex (MHC) on cell surfaces (13). The MHC is a set of cell surfaces proteins encoded by MHC class I and MHC class II genes (8). MHC I is present on every nucleated cell in the body. MHC I presents endogenous antigen to cytotoxic T cells, thereby activating mainly the cell mediated immune response (Fig 2).

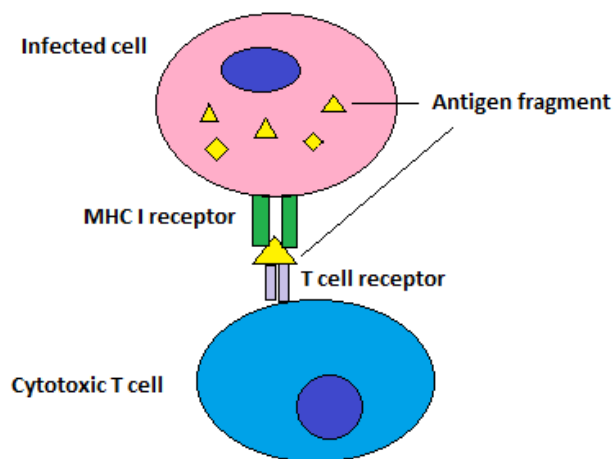


Figure 2: An Infected cell presents antigen to cytotoxic T cell by MHC I (figure drawn by Ziener ML)

MHC II is present on antigen presenting cells such as macrophages (13). These cells' main task is to phagocytize exogenous antigen and present it to T helper cells. As a result, either the humoral or the cell mediated immune response is activated (Fig 3). T helper 1 cells stimulate the cell mediated immune response involved in the delayed hypersensitivity reaction and macrophage activation (11). T helper 2 cells stimulate B cells to transform into plasma cells which produce immunoglobulins (humoral immune response). Immunoglobulins bind antigen in body fluids, thereby making phagocytosis of antigen by macrophages more

effective (14). Activated B cells can become either antibody producing plasma cells or memory cells (12).

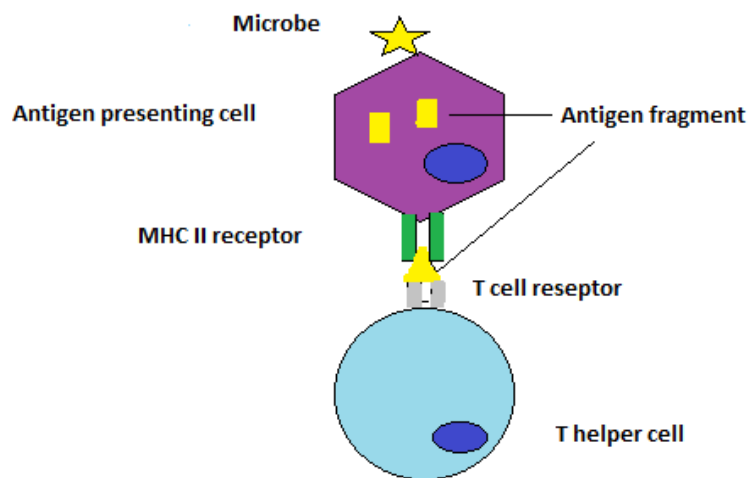


Figure 3: An antigen presenting cell presents antigen to T helper cell by MHC II
(figure drawn by Ziener ML)

Self-tolerance

The MHC helps the immune system to differentiate between self-antigen and non-self-antigen (8). For instance, T cells are only activated if the antigen peptide is presented by MHC class I or II (13). Self-tolerance is crucial to prevent the occurrence of autoimmune diseases (7). It is part of the adaptive immune system and mainly developed at a young age. The development of self-tolerance takes place in the bone marrow and the thymus in two processes called negative and positive selection. In negative selection the lymphocytes with receptors for self-antigen are eliminated (15), and in positive selection the lymphocytes that bind with the right affinity to MHC II are stimulated for further replication (15).

Immunogenetics

Immunogenetics is the study of genetic factors controlling an individual's immune response (8). Genes coding for T cell receptors and immunoglobulins use gene recombination, somatic mutations and gene conversions to achieve the tremendous amount of variation needed to defend the body against a wide variation of pathogens (8). T cell receptors, immunoglobulins and MHC are members of the same immunoglobulin superfamily (Fig 4) (8).

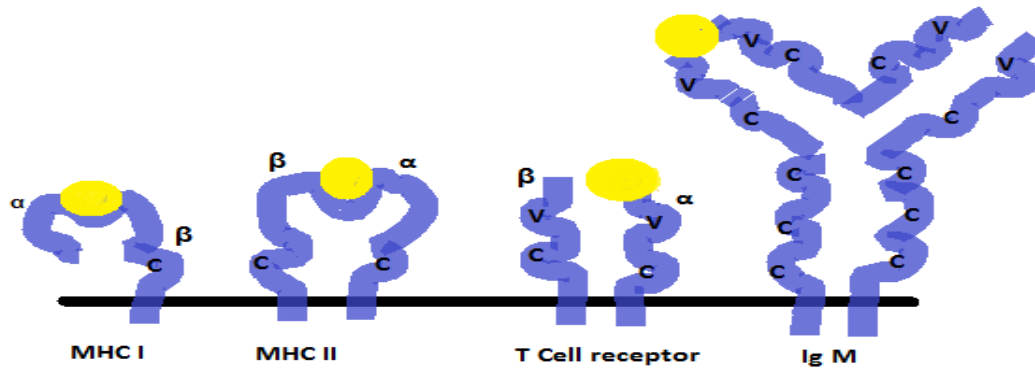


Figure 4: Molecular structure of MHC I, MHC II, T cell receptor and immunoglobulin M. C=constant region, V=variable region, α =alfa chain, β = beta chain (figure drawn by Ziener ML)

The MHC complex is divided into three regions on the genome; MHC I, II and III. These regions are some of the most gene dense areas of the genome, containing several immunogenes in addition to other genes (8). In humans, MHC genes are called human leukocyte antigen (HLA) while in dogs they are called dog leukocyte antigen (DLA). The HLA genes are located on chromosome 6 (8). In dogs, DLA genes class I- III are located on chromosome 12 (Fig 5). A part of the DLA class I genes are also located on chromosomes 7, 18 and 35 (8, 16).

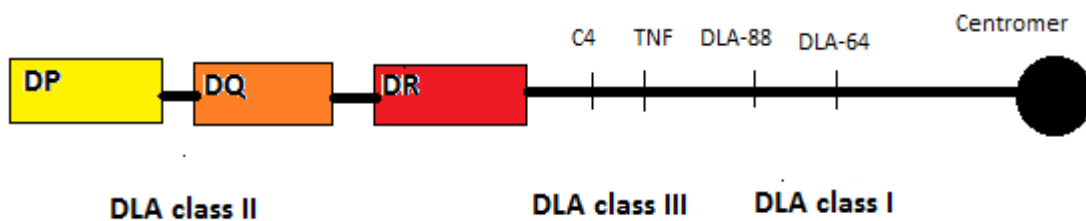


Figure 5: Location and orientation on chromosome 12 of some DLA genes in dogs, covering 3Mb pairs (figure drawn by Ziener ML).

Every mammal inherits one MHC haplotype from each parent. HLA/DLA haplotypes follow Mendelian inheritance and due to the close linkage there are few recombinations in the area (8, 13). The MHC I loci are called A, B, C and contain both highly polymorphic and non-polymorphic loci. All nucleated cells in the body from one individual have the same MHC I (13). The highest grade of polymorphism is seen in the genes coding for MHC II. The MHC II region is called the D region and is divided into three sub regions called DP, DQ and DR. Each of these sub regions contains at least two functional genes A and B (13). The antigen binding site in the MHC is built up by α and β chains (Fig 4). The DPA, DQA and DRA are encoding the α - chain of the MHC receptor. The DPB, DQB and DRB on the other hand encode the β - chain. The allelic variance results in a change of antigen binding at the receptor site by affecting how $\alpha 1$ and $\alpha 2$ chains are folded together. A high diversity and a high variation in the peptide binding region is believed to make individuals less sustainable to disease (8). Most of the allelic variation occurs in DRB1, DQA1 and DQB1 (Fig 6). These genes are therefore traditionally the ones that are studied to reveal associations with immune mediated disease (8). In the region of MHC III there are genes coding for proteins involved in the innate immune system such as tumor necrosis factor (TNF) and complement factors. The MHC III genes in dogs are located between the MHC I and MHC II genes on chromosome 12 (Fig 5) (8).

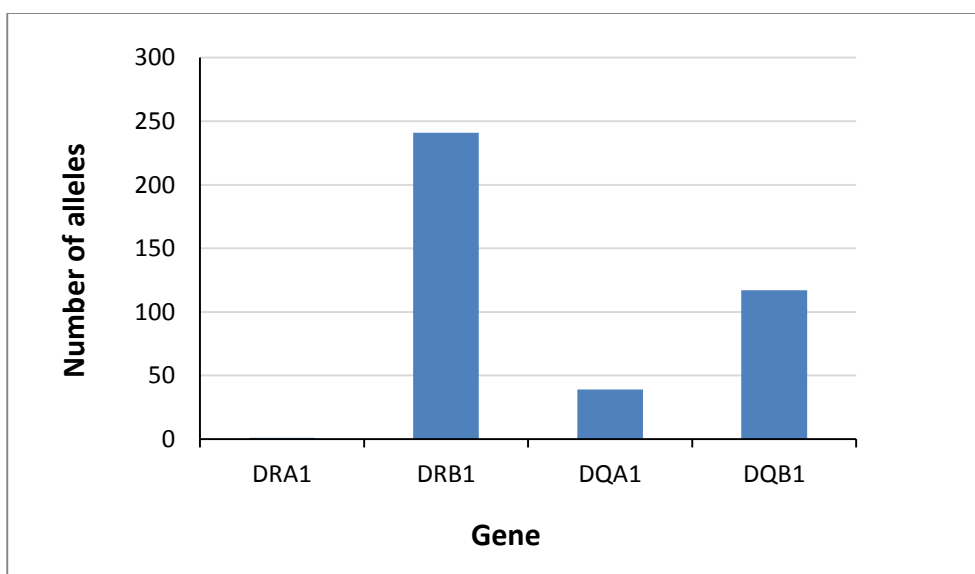


Figure 6: Number of different alleles on DR and DQ genes in dogs and other canids identified until 2012. Drawn by the author based on information from canine immunogenetics Kennedy et. al. 2012 (8).

Every dog breed has its own DLA haplotype profile. For instance, one study observed 28 different DLA haplotypes in the Saluki, whilst only two different DLA haplotypes were observed in the Rottweiler (8). Typically one dog breed has an average of seven DLA haplotypes; usually one DLA haplotype with a frequency over 20 %, two DLA haplotypes with a frequency between 10-20 %, and four DLA haplotypes with a frequency between 2-10 % (8). Usually there is extensive inter-breed but minimal intra-breed variation of DLA haplotypes (17). The GS and ES have five common DLA haplotypes, which indicates a close genetic relationship between these two breeds (18, 19).

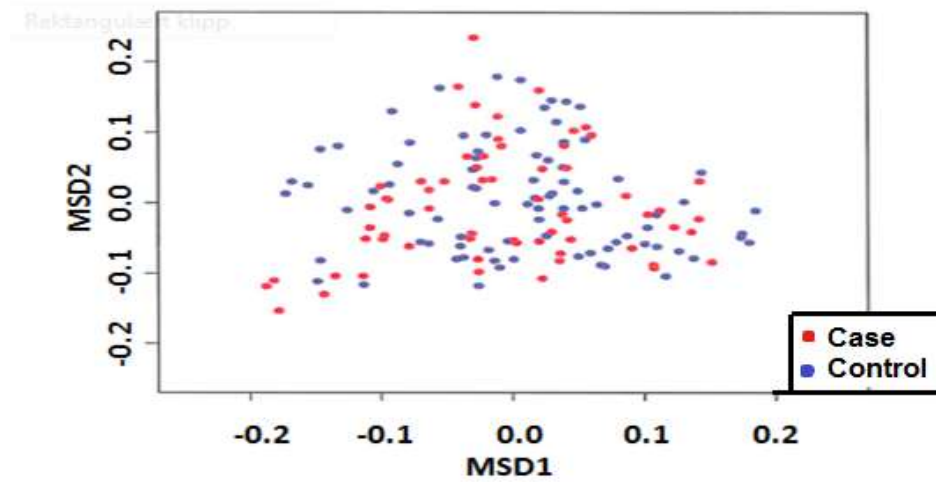
Genetic studies of complex diseases in humans and dogs

Some of the most commonly used strategies to find genes associated with disease in humans and dogs are the candidate gene approach, genome wide association studies (GWAS) and whole genome sequencing. For all methods a correct classification of the diseased phenotype is crucial for success, and a correct classification of cases and controls is therefore important. In the candidate gene approach, prior knowledge of the genes associated with the disease is the basis for the selection of genes that are subsequently compared between cases and controls (20). Genes coding for MHC (HLA/DLA) have frequently been used as candidates in studies of autoimmune diseases (8, 21). The frequencies of HLA/DLA haplotypes and alleles are compared in cases and controls and tested for any association by calculating odds ratio (OR) (8). In a true candidate gene study the whole gene is sequenced in order to find the casual mutation for the disease, but in most HLA and DLA studies only the exon 2 has been sequenced (8). Exon 2 is chosen because it is highly polymorphic and encodes the functional peptide domain which binds the antigen when MHC II presents the antigen to T cells (8, 22).

Modern population based association mapping strategies like GWAS are based on a genome wide mapping of genetic markers. The purpose is to detect genomic regions with different allele combinations in cases and controls. A collection of closely located alleles on a chromosome usually inherited together is called a haplotype (23). A haplotype harboring the disease causing mutation will have a much higher frequency in cases compared to controls. In GWAS, cases and controls are compared with genetic markers called single nucleotide polymorphism (SNP) which are distributed along the whole genome (24, 25). In 2005, the

dog genome was genotyped (24) and whole genome SNP chips used in GWAS are updated regularly (26). An associated SNP found by GWAS, could either directly affect the phenotype but in most cases it would be closely linked with genetic variants associated with the phenotype. GWAS is an efficient method to study diseases with complex inheritance (25). Canine GWAS studies usually require fewer SNP compared to humane GWAS because of the long haplotype blocks in dogs (24). Fewer cases/controls are also needed in canine GWAS than similar studies in humans (27), due to lower genetic heterogeneity for diseases within dog breeds (28). On the other hand, the long disease associated regions sometimes identified in dogs may be a challenge when fine mapping is done to try to find the mutation that causes the disease. One method to shorten the associated region found by one GWAS in one dog breed is to study the same disease in two or more dog breeds and find the overlapping region (29). GWAS is best suited to pick up frequent mutations/variants associated with the disease (25). The method does not normally reveal the actual disease mutations, but rather points to an area on the genome where important disease-associated mutations might be located (27, 28). Population stratification can lead to false associations in GWAS analyses (30). The sample population should therefore be tested, for example by creation of a multidimensional scaling (MDS) plot (Fig 7). Ideally, the cases and controls should distribute equally in the MDS plot and not form any clusters (Fig 7A). Clustering of cases can indicate population stratification which must later be considered when analyzing the data (Fig 7B) (30).

A



B

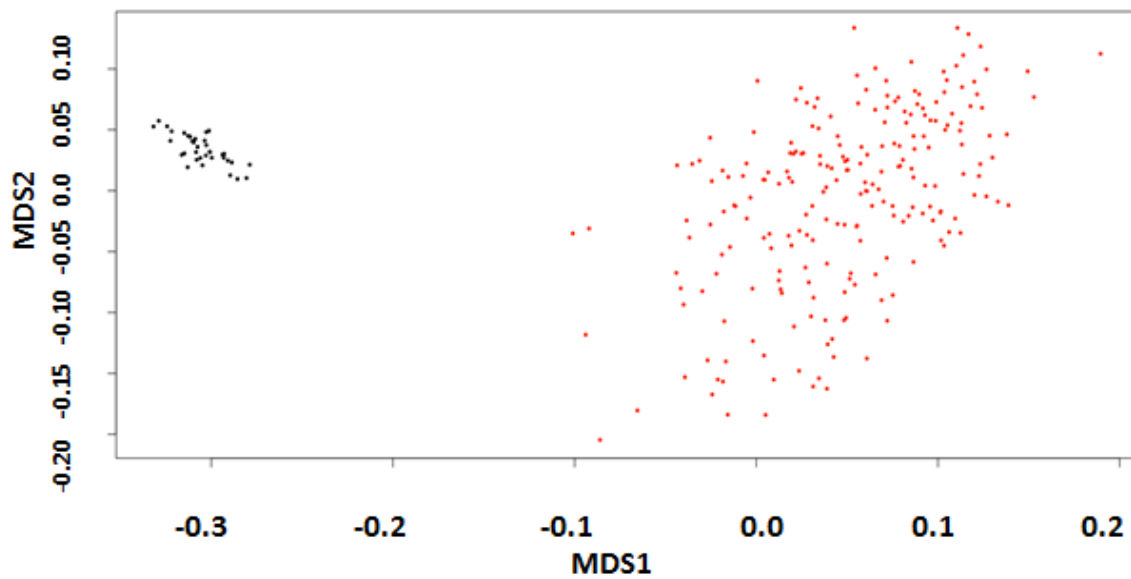


Figure 7: A: An example of equally distributed cases and controls in a multidimensional scaling plot (MDS) B: An example of population stratification seen in a MDS plot. The samples clearly separate into two clusters; ES to the left and GS to the right (paper IV).

The results from the GWAS are often displayed in a Manhattan plot (Fig 8). In the Manhattan plot each dot represents a SNP (30). The position of the SNPs are on the X-axis arranged by chromosome number and the associations of the SNP to the phenotype is displayed on the Y-axis as $-\log_{10}(\text{p-value})$. This means that the higher the value on the Y-axis, the lower the p-value. The grey horizontal line is the level of significance adjusted with the Bonferroni correction (30) (Fig 8). Bonferroni correction is defined by taking the conventional p value and dividing it by the number of tests performed. The correction is typically used to avoid false positive associations between SNPs and disease (type I error) and it assumes total independence between all SNPs in the assay. It is debated whether Bonferroni correction is too conservative to use in GWAS analysis in dogs since many SNPs are closely linked and not independent in this species (30).

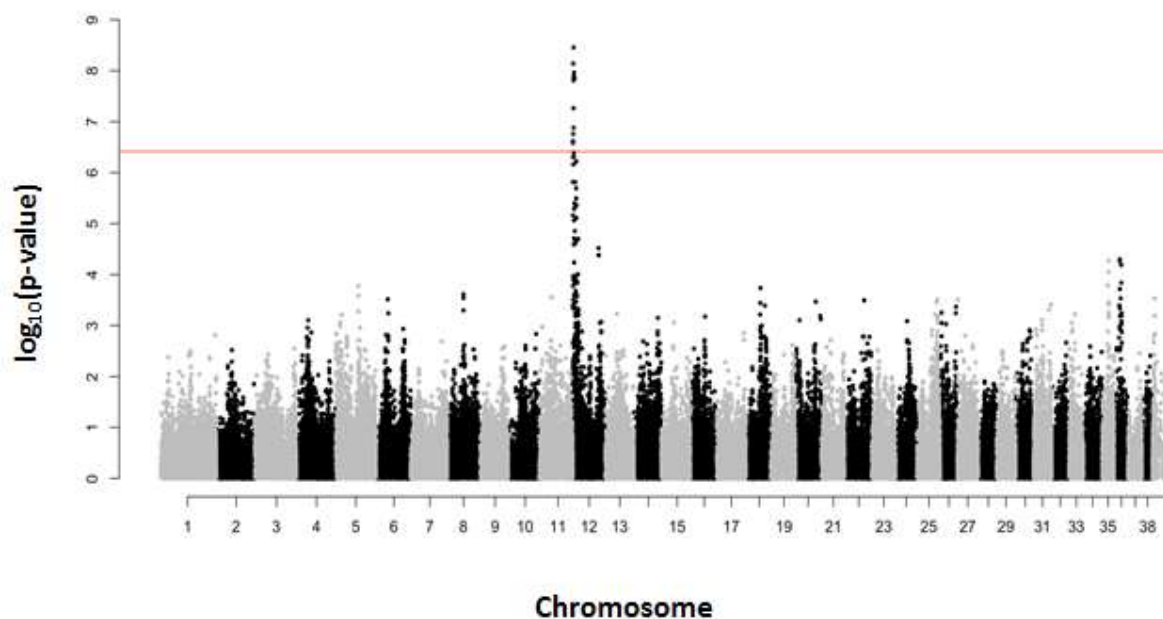


Figure 8: A Manhattan plots depicting the results of a significant hit on chromosome 12 in a GWAS in SO from GS. The grey line represents Bonferroni corrected p-value significance thresholds of 0.05. Each dot represents a single SNP. Genomic coordinates are displayed along the X-axis and the negative logarithm of the association p-value for each SNP marker is displayed on the Y-axis (paper IV).

The QQ-plot (Fig 9) shows the expected distribution of association test statistics (X-axis) across the millions of SNPs compared to the observed values (Y-axis). Any deviation from the line implies a consistent difference between cases and controls across the whole genome. A QQ plot where the results are significant should show a solid line matching X follows Y until it sharply curves at the end, representing the small number of true associations among thousands of unassociated SNPs (30).

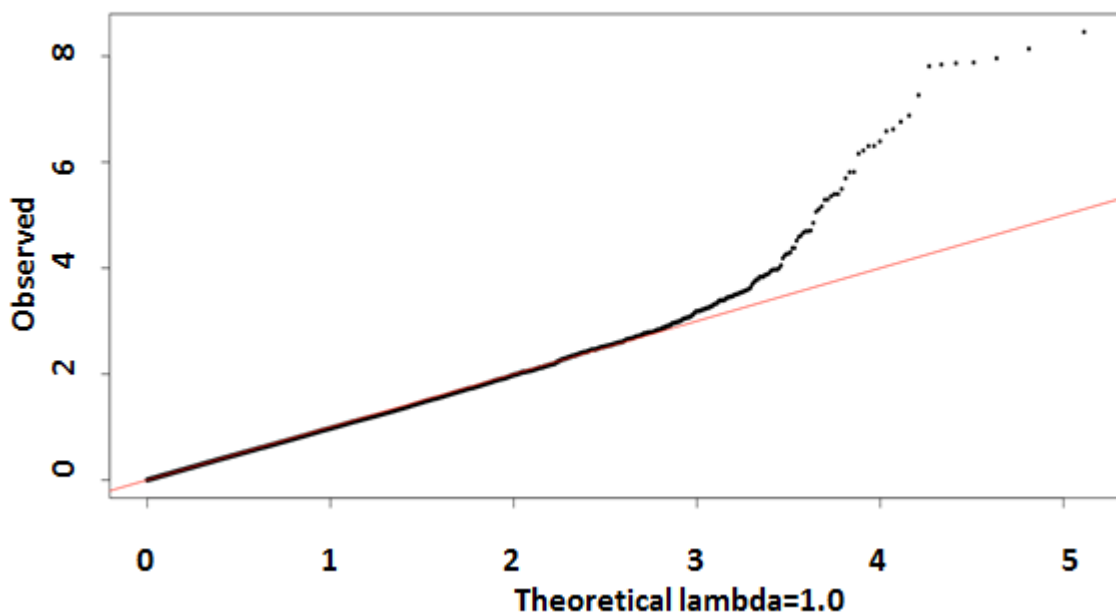


Figure 9: The QQ-plot with p -values along the X- and Y-axis (paper IV).

In a regional association plot, the region of significant hits from the Manhattan plot is magnified (Fig 10). The haploblock structure is visualized by color, between the top SNP (unfilled circle) and the associated SNPs in the associated region. Therefore every SNP with the same color has the same correlation value (r^2). If $r^2=1$, the SNPs are in perfect linkage disequilibrium and the SNPs are inherited together. An example is the three dark red SNPs that are in linkage disequilibrium with the top SNP all have r^2 -values between 0.8-1 (Fig 10). The significant SNPs from the GWAS are shown over the red significance line. Minor allele frequencies (MAF) refer to the frequency of the least common allele at a locus in a population. SNPs with MAF below 0.5 % are traditionally removed from the GWAS analysis.

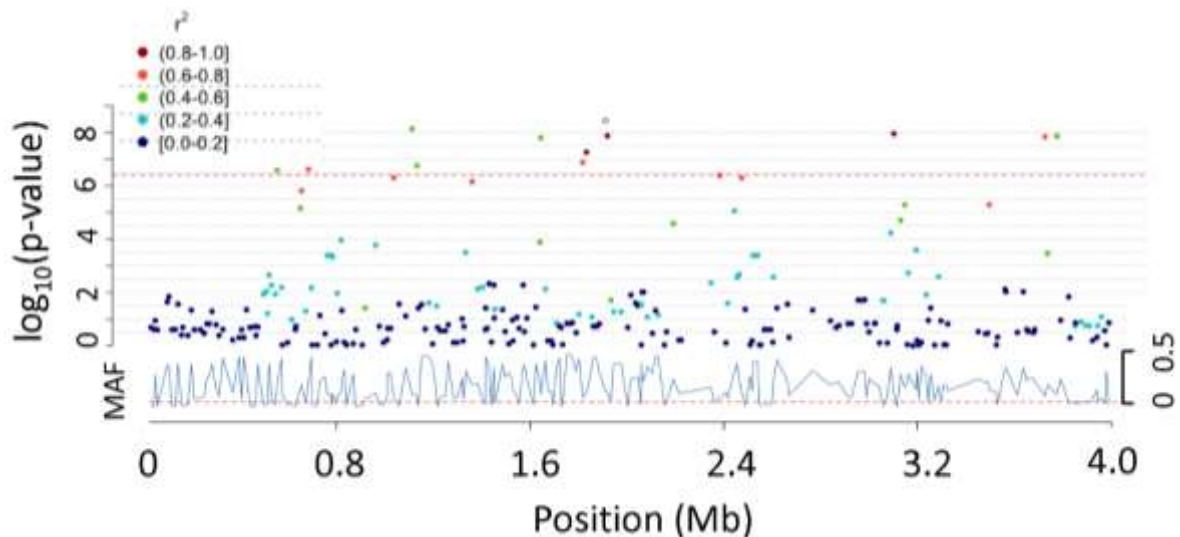


Figure 10: Regional association plot from the GWAS depicting linkage disequilibrium, measured as r^2 , between the GWAS top hit (unfilled circle) and SNPs in the associated region. Minor allele frequencies (MAF) are shown in a light blue line below. Genomic coordinates are displayed along the X-axis and the negative logarithm of the association (p-value) for each SNP marker is displayed on the Y-axis.

Autoimmune disease

Definition and pathogenesis

An autoimmune disease is defined as: “A disease that occurs when the body tissues are attacked by its own immune system” (7). There are several criteria to fulfill for a disease to be classified as autoimmune: no other underlying disease should be present, compatible organ specific or multisystemic clinical signs should be present, autoantibody or autoreactive lymphocytes should be detected in blood or tissues, and finally patients with autoimmune diseases should improve on immunosuppressive therapy (7, 15). In humans, 3-8 % develop an autoimmune disease over the course of a lifetime. Prevalence statistics for dogs are lacking, but the numbers are thought to be rising in both dogs and humans (15). In general, all lapses of regulatory control in the immune system may lead to autoimmune diseases (7, 15). In a genetically predisposed individual numerous factors can cause failure of the control mechanisms of the immune system, such as infections, vaccinations, drugs, and environmental factors. The lesions that develop in different organs systems during autoimmune diseases correspond to hypersensitivity type I-IV reactions (7).

Genetics and autoimmune diseases

Autoimmune diseases are complex diseases usually caused by many genetic and environmental factors, which poses a challenge when doing genetic studies. In humans and dogs the best known associations between genes and autoimmune diseases are associated with particular alleles of MHC II loci (15). Most of the autoimmune diseases found in humans have a canine analogue. The dog can therefore be used as a model to study genes associated with the human equivalent autoimmune disease (26). One example is multiple sclerosis in humans and necrotizing meningoencephalitis in Pugs. Both diseases are chronic autoimmune neurological diseases with high prevalence in young bitches and young women (31, 32). In humans there is a strong association between HLA-DRB1 and multiple sclerosis, and in Pugs the strongest disease association is between DLA-DQA1 and DAXX, a gene closely linked to the DLA class II genes (21, 22, 33). Another example is systemic lupus erythematosus (SLE) an autoimmune disease that affects both dogs and humans. The disease is caused by trapped immune complexes in different organs leading to an inflammation reaction. The immune complexes are formed by different self-antigens and antinuclear antibodies (ANA) (34). In dogs the disease is thought to be quite rare. Some researchers have argued that SO could be a part of SLE (35, 36). The suspicion was raised mainly because some dogs with SO had positive ANA tests (36). Also, claw biopsies from affected dogs have shown interface dermatitis similar to the dermatitis pattern found in skin biopsies from SLE patient (35, 36). To fulfill the diagnostic criteria for SLE, dogs should have a positive ANA test together with signs of systemic disease and skin disease (34). An association between SLE and HLA/DLA (MHC II) has been proven in humans and dogs (21, 37). But also genes coding for TNF and compliment (MHC III) together with other immune genes such as PTPN22, BANK1, TNFAP3, STAT4, IRF5, and ITGAM, are thought to contribute to SLE development in humans (38).

The study of the genetics of complex diseases in dogs has several advantages compared to in other species (26, 28). First, the breeding structure of modern dog breeds, with strong founder effects, genetic drifts, as well as a high degree of inbreeding, has contributed to a decreased genetic variation within some dog breeds (26). This has also reduced genetic heterogeneity for disease, meaning that fewer predisposing genes should exist for specific autoimmune diseases in each breed (26). This combination of a high frequency of disease,

with reduced genetic heterogeneity of disease-genes is an advantage in the work to identify loci associated with disease (24). Another advantage when studying autoimmune diseases in dogs is that dogs and humans are living in the same environment, and might therefore be exposed to the same triggering factors for the autoimmune disease (26, 28). Most of the autoimmune diseases are uncommon in humans, but rare diseases in humans might have a high incidence in certain dog breeds because of accumulation of risk alleles in some breeds (26).

Infections as trigger factors for autoimmune diseases

It is not totally clear how infections can trigger autoimmune diseases and several different mechanisms have been proposed (15). The infection might reveal previously “hidden” antigens and autoantibodies are subsequently produced against these new antigens. Moreover, the infections might also cause molecular alterations of the epitopes on normal endogenous proteins and autoantibodies against them are produced (7). Sometimes epitopes of the infectious agents and the autoantigens can be similar. Therefore an infectious agent with such an epitope might activate autoreactive T and B cells that continue the reaction to self-antigen after the infectious agents are cleared, which is known as molecular mimicry (7). In dogs, *Leishmania* infections are a good example of how infectious agents might trigger autoimmune responses in the body using both molecular alterations and molecular mimicry (15). Genetically susceptible dogs infected with leishmaniasis develop immune mediated polyarthritis, glomerulonephritis, vasculitis, anemia, thrombocytopenia, skin and claw disease, and hyper gammaglobulinaemia (39). Claw biopsies from *Leishmania* infected dogs have the same interface dermatitis observed in claw biopsies from dogs with SO (40). *Leishmania* infected dogs are sometimes mistaken for having SLE because the symptoms are analogous and sometimes infected dogs may also present with high ANA titers (41).

Vaccination and drugs as trigger factors for autoimmune diseases

There are also indications that vaccination might trigger autoimmune disease, although the mechanism behind this is not fully understood. The phenomenon is rare in relation to how many dogs and humans that are vaccinated annually. It has also been proposed that vaccination can induce SO (42). An association between vaccination and disease is defined as when disease onset occurs within four weeks after vaccination (43). A wide range of drugs

are known to induce autoimmune disease. One example is trimethoprim sulphamide which might induce immune mediated keratoconjunctivitis sicca, immune mediated polyarthritis and/or immune mediated hepatitis in dogs (44).

Gender and autoimmune diseases

Almost 90 % of humans with autoimmune HT are women (45). Women in general have more active humoral and cellular immune responses and this may account for the higher incidence of autoimmune diseases observed in the female population (45). Simmonds (2014) suggested that a skewed X-chromosome inactivation in early embryonic life may play a more important role in the observed sex difference seen in autoimmune diseases in humans (46). The proposed mechanism suggests an inadequate presentation of self-antigen on one X-chromosome in thymus during early development and that these antigens may induce autoimmune disease later in life. Female dominance in autoimmune diseases in dogs has been described in Pugs with necrotising meningoencephalitis (32), English Cocker Spaniels with immune mediated hemolytic anemia (47), and in Portuguese Water dogs with late onset hypoadrenocortisism (48). In dogs, there is a paucity of quality data from epidemiological studies regarding many autoimmune diseases. Day (2008) claims that early neutering of dogs may explain why we more rarely see sex differences in autoimmune diseases in dogs (15). This is not a valid argument in Scandinavian countries, where neutering of dogs is not done routinely.

Environmental factors as trigger factors for autoimmune diseases

Several environmental factors might increase the risk of various autoimmune diseases (49, 50). Smoking and stress are known to trigger several autoimmune diseases in genetically susceptible humans (51). Epigenetics, changes in gene expression without mutation in the DNA-chain, may be influenced by environmental factors as well.

Treatment of autoimmune diseases

Autoimmune diseases in dogs and cats are treated with a variety of immunosuppressive drugs with different mechanisms and strength of immunosuppression (52). In this section the main focus will be on drugs used to treat SO in dogs (53). Prednisolone is often the first choice when treating immune mediated conditions in humans and animals. Prednisolone acts by binding to receptors in the cytoplasm followed by translocation to the nucleus and

subsequent alterations in the transcription of DNA (54). Prednisolone is a potent immunomodulator because it depresses chemotaxis, margination, phagocytosis and bactericidal activity of neutrophils and macrophages. Prednisolone also depresses the antibody dependent cell mediated cytotoxicity activity of neutrophils, and depresses interleukin-1 production and antigen presentation by macrophages. The immunosuppressive effects are dose dependent and so the dose must be tapered off (52).

Omega-3 and omega-6 supplementation has been used as a treatment against SO with good effect (55, 56). It is reported that neutrophils and macrophages obtain a higher concentration of eicosapentaenoic acid and docosapentaenoic acid in cell membranes when dogs are fed fish oil which contain high amounts of omega-3 (57, 58). This might contribute to modulating the immune response so that less potent inflammatory mediators are produced. Furthermore, the expression of MHC I and II on cell surfaces decreases in animals fed a diet rich in omega-3 which leads to less antigen presentation.

Niacinamide blocks antigen induced histamine release, inhibits phosphodiesterase activity and protease release. The drug has traditionally been used together with tetracycline in the management of immune mediated dermatoses such as SO and discoid lupus in dogs (59).

Pentoxifylline is a drug used mainly in immune mediated vasculitis. The drug reduces blood viscosity and has an anti-inflammatory effect through reduction of TNF production. The drug has also been used in the treatment of SO (60).

Cyclosporine has been used to treat canine immune mediated diseases like atopic dermatitis, SO and anal furunculosis (61). Cyclosporine binds to and blocks the intracellular transmitter calcineurin when entering the cytoplasm of the cells. Subsequently the production of interleukin-2 and interferon-gamma are blocked (52). These cytokines act as important activation factors for the t helper cell 1 immune response, and without them the cell mediated immune response is blocked. Cyclosporine stimulates fibroblasts, increases the production of collagen and matrix substances as well as reduces collagen degradation. The drug also has a strong anagen effect on the hair bulb leading to increased hair growth (61).

Autoimmune thyroid disease in humans

In humans, autoimmune thyroid disease is divided into Grave's disease or Hashimoto's thyroiditis based on two completely different clinical presentations (62). Patients with Grave's disease have hyperthyroidism, because thyroid stimulating hormone (TSH) receptor autoantibodies bind to thyroid hormone receptors and cause an overproduction of thyroxin. Whilst in Hashimoto's disease, an autoimmune attack on the thyroid gland causes destruction of the gland, with a low production of thyroxin as a result (62). Autoimmune thyroid disease is one of the most common autoimmune endocrine diseases in humans (31). Contrary to dogs, the disease has a female dominance, but the symptoms of HT in humans are the same as in dogs, with weight gain, exercise intolerance, hair loss and lethargy. The diagnosis of HT in humans is based on clinical signs together with increased serum levels of TSH and the treatment consists of life long levothyroxine potassium supplementation (63).

Genetic studies of HT in humans indicate that genetic factors contribute to as much as 70-80 % of the development of autoimmune HT (64, 65). Examples of immune related genes found to be associated with autoimmune HT in humans are HLA-DR3, PTPN22, CTLA4, FOXE1, and MAGI3 (51, 66-68). Environmental factors like iodine content in drinking water are known to affect the incidence of autoimmune HT in humans (50). Supplementation of iodine in water where the iodine content is low will increase the prevalence of autoimmune HT in humans (69).

Hypothyroidism in dogs

Pathogenesis

Two types of HT have been described in dogs based on histopathological changes in the thyroid gland: lymphocytic thyroiditis and atrophic thyroiditis (70). Graham (2001) classified the lymphocytic thyroiditis as antibody-positive thyroiditis and the atrophic form as antibody-negative idiopathic thyroiditis (71). The lymphocytic thyroiditis is the most common form in ES because in one large study 84 % of the ES diagnosed with HT were positive for thyroglobuline autoantibodies (TgAA) (70). In other breeds only around 50 % of dogs with clinical and biochemical signs of HT are TgAA positive (71). It is not clear whether the lymphocytic form is the first stage of the disease and the atrophic form is the end stage

of HT, or if they are two different disease entities (71). The thyroid gland is progressively destroyed in both forms of HT and so the production of thyroid hormone gradually declines.

Epidemiology and genetic risk factors

HT is the most common autoimmune endocrine disease reported in dogs (72). The occurrence of canine HT and its similarity to Hashimoto's thyroiditis was first described in a breeding colony of Beagles in 1968 (73). The prevalence of HT varies between breeds. English Setter, Irish Setter, Cocker Spaniel, Dachshund, Doberman Pinchers, German Wirehaired Pointers, Giant Schnauzer, Golden Retrievers, Howavart, Miniature Schnauzer, Pointer, Pomeranian and Shetland Sheepdog have all been identified as high risk breeds for HT in studies from different countries (3, 74, 75). Hypothyroidism is known as a disease of middle aged dogs, but age at onset of the disease differs between breeds (71). Age at onset may also vary depending on the type of HT, and dogs with antibody-positive HT are generally younger than dogs with antibody-negative HT (70). There is no convincing data indicating a generally skewed sex distribution of HT in dogs (76), but one study concludes that neutering is a risk factor to develop HT (75). Hypothyroidism does occur in dogs with other endocrinology diseases like diabetes mellitus and/or hypercortisolism, but this is not common (77). In order to test the potential genetic background of HT, two Borzois with HT were mated and all of the offspring suffered from HT (78). Familial inheritance of HT has also been documented in a breeding colony of Beagles (79). In the Doberman Pincher, Giant Schnauzer and Rhodesian Ridgeback, several studies indicate that specific DLA haplotypes and DLA alleles are associated with HT (18, 80-82). Kennedy et al. (2006) suggested that the DQA1*00101 allele is a common risk factor for HT in these breeds (18). Wilbe et al. (2006) however, suggested that the major risk factor for HT in the Giant Schnauzer was the DRB1*01201 allele, since this DRB1 allele was only found in the risk-haplotype. The DLA haplotype (DRB1*01301/DQA1*00301/DQB1*00501) was associated with low risk for HT in the Giant Schnauzer (82). The findings of different DLA associations with HT in different breeds are consistent with previous findings of different HLA associations that have been observed between ethnic groups of humans with HT (40).

Clinical signs and diagnosis

Classical symptoms of HT in the dog include weight gain, mental dullness and lethargy. Other signs of the disease are hypothermia and poor hair quality with skin changes such as

alopecia, hyperkeratosis and seborrhea (70, 71). Diagnosing HT can be a challenge for veterinarians due to confusing blood results and slow development of clinical signs (70, 71). The clinical diagnosis should be based on the typical clinical signs together with elevated levels of TSH, subnormal total thyroxine (TT4), subnormal free thyroxine (FT4) and an elevated cholesterol concentration in patient serum. In dogs, measurement of TSH has high specificity but low sensitivity for the diagnosis of HT. Different studies have reported specificity of 0.82-1.00 and a sensitivity of 0.60- 0.87 (83-85). A high TSH value is therefore a good tool to confirm the diagnosis of HT, but it cannot be used to exclude HT, as almost 25 % of dogs with HT will have normal TSH values (83). Drugs such as prednisolone and phenobarbital can also falsely lower TT4 values, making the picture even more complicated (86). Moreover, TT4 and FT4 levels in healthy dogs of different breeds may vary, for instance sight hounds have naturally low TT4 and FT4 (87, 88). Finally, dogs that are critically ill can have low TT4 values without having HT and this is called euthyroid sickness syndrome (89). These diagnostic challenges are important to consider when performing genetic studies in dogs with HT, so that classification of cases and controls are done correctly.

Treatment and outcome

Supplementation with levothyroxine potassium is the treatment of choice for HT. Dosage is 0.02-0.05 µg twice daily and clinical response together with blood values alters the doses. The prognosis is good for long time survival (90).

Anatomy and physiology of claws

The claw is a specialised epithelial structure consisting of soft and hard keratin (Fig 11). It resembles the human nail and hair anatomically. Main claw growth originates from the germinal region (Fig 11 e) (91). Claw growth rates vary with age, housing, and diet but in general is around 0.7-2.1mm a week (60). The claw has an important function in locomotion in the normal dog. Histopathological studies of claw samples are a challenge because of presence of bone and various keratin types in the samples. Intracytoplasmic vacuoles in keratinocytes, prominent inter keratinocytes spaces and apoptotic keratinocytes in dorsal and ventral matrices are seen as normal features (92).

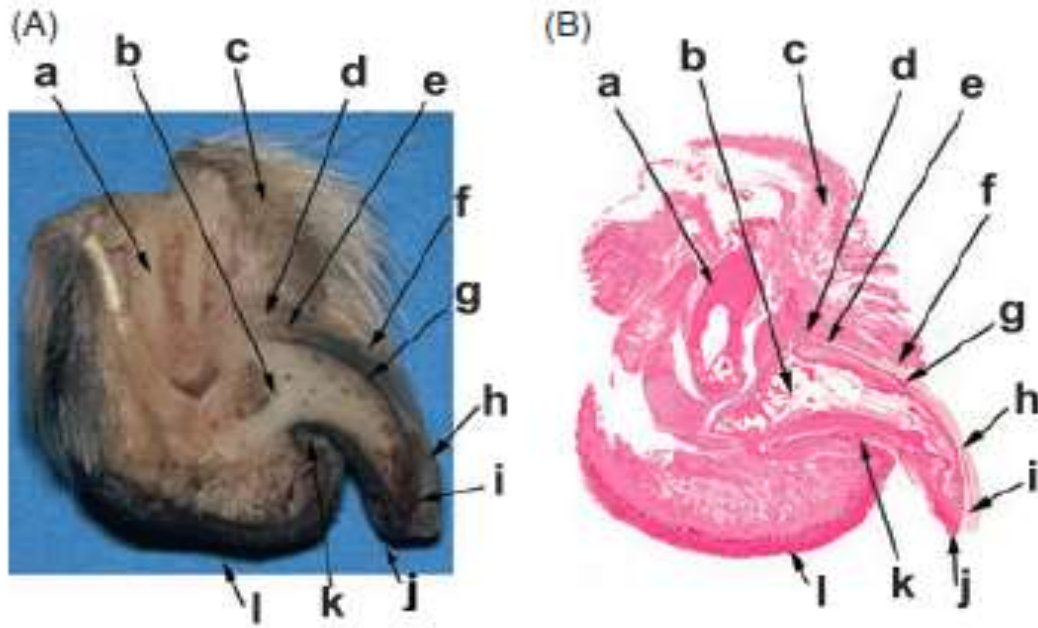


Figure 11: Macroscopic and microscopic views of the canine claw: (A): Macro photograph of a canine digit cut vertically through the center (B): Photomicrograph of a section of the same digit stained with H&E. Several regions are labelled including: a: Secondary phalanx b: primary phalanx c: hair bearing skin, d: unguis crest, e: proximal claw plate (germinative region), f: claw fold, g: inner claw plate, germinative region, h: claw plate, i: inner claw plate, j: sole, k: sole pad junction, l: digit pad. Photo from Bowden P.A ; Defining the complex epithelia that comprise the canine claw with molecular markers of differentiation. *Veterinary Dermatology*, 2009,20, 347–359. (Printed with permission from the author)

Human nail diseases

Onychomadesis occurs in humans because of trauma, infections, critical illness, drugs and autoimmune diseases (93). A few cases of dominant symmetrical familial onychomadesis have been described in the human literature (94, 95). Psoriasis, pemphigus vulgaris, alopecia areata, HT and lichen planus are all autoimmune diseases that have been associated with SO to varying degrees in humans (96).

Symmetrical onychomadesis in dogs

Pathogenesis

In 1995, Scott first described a disease named symmetrical lupoid onychodystrophy (SLO), where the dog lost all its claws within a relative short period of time without showing any other sign of skin or systemic disease (56). The name originated because of the interface

dermatitis (lupoid) found in the histopathology samples from affected claws and the regrowth of abnormal and brittle claws (onychodystrophy). Different names has been used to describe symmetrical claw disease in dogs: symmetrical onychomadesis (sloughing of claws), idiopathic onychodystrophy and idiopathic onychomadesis (60). Infections with bacteria, fungus and *Leishmania*, adverse food reactions, neoplasia, vaccination, autoimmune skin diseases like SLE, sub epidermal bullous dermatoses and Pemphigus vulgaris, are all known to be able to induce symmetrical claw disease in dogs (60). Subsequently, in a patient with claw disease, a thorough diagnostic work up must be done to rule out other diseases that possibly could affect the claws.

Epidemiology and genetic risk factors

Symmetrical onychomadesis may occur in very young and old dogs, but the mean age of onset for SO is reported to be 4.0-5.5 years (53, 97). No gender predilection has been reported for SO. Several different breeds have been reported to be affected with the disease including the Akita Inu, Bearded Collie, Boxer, Dobermann Pincher, German Shorthaired Pointer, German Shepherd, Golden Retriever, Greyhound, Cavalier King Charles Spaniel, Labrador Retriever, Miniature Poodle, Miniature Schnauzer, Rottweiler, Schipperke, Silky Terrier, Welsh Corgi and West Highland White Terrier (60). The fact that different breeds have varying risk towards developing SO implies that there could be an accumulation of genetic risk factors for SO in these breeds.

Clinical signs and diagnosis

The initial symptoms of SO include limping and that the dog suddenly starts to lick its feet. The dog's paws might be sore and the dog shows sudden onset of claw pain on several claws. The claws begin to separate from the distal attachment and this progresses proximally (onycholysis) and finally results in onychomadesis (59) (Fig 12A). The basis of the diagnosis of SO is a clinical history with descriptions of sudden onset of claw pain on several claws which results in onychomadesis. (59). Clinical examination, cytology of claw fold and biopsy are required to rule out other causes of claw diseases in addition to ensuring the disease is only limited to the claws. The biopsy is not mandatory for a correct diagnosis, but should be included when neoplastic disease, SLE, or pemphigus are suspected. Biopsy is obtained by amputation of the distal phalanx on a hind paw, or a dew claw if it is affected, or by a biopsy technique described by Mueller et al. (1999) (98).



Figure 12: *Onychomadesis in Gordon Setter. A: Claw before treatment (onychomadesis), B: Claw bed after removal of horn layer, C: Normal claw after treatment, D: Claw with onychodystrophy after treatment (paper II).*

Many dogs with onycholysis have secondary bacterial infections, necessitating bacterial cultures from the claw fold. In dogs where systemic disease is suspected as the cause of claw disease, i.e HT and hypercortisolism, analysis of blood samples for biochemistry, hormones and complete blood cells count together with urine analysis are indicated. The ANA test is only indicated in dogs with SO if an autoimmune disease is suspected to be the cause of SO (99).

Treatment and outcome

SO is a difficult disease to treat because the disease is painful for the dogs in the acute phase and improvement in claw quality progress very slowly (53, 56, 59). It takes three to four months before you can evaluate long term treatment effect in dogs with SO. Removal of damaged claw plates during anaesthesia and treatment of secondary bacterial infections are

essential in the acute phase of the disease (Fig 12B). The goal of the long term treatment is to encourage regrowth of normal quality claws and to prevent recurrence of onychomadesis (Fig 12 C and D). Most of the dogs that are kept on life-long medication will not experience recurrence of SO, but onychodystrophy is often the end result of the disease (53). Traditionally, different immune modulating drugs have been used to treat dogs with SO and complete or part remission has been achieved (53, 60). Bergvall (1998) and Scott (1995) have demonstrated positive effects of omega-3 and omega-6 supplementation (55, 56). More potent immunosuppressive drugs have also been used in treatment of SO like tetracycline/niacinamide, prednisolon, pentoxifylline and azathioprine (1, 53, 59, 100). Amputation of claws has also been used to treat SO with good results, but this might be considered an overly blunt treatment option (42). In the future, treatment outcome and success rate will hopefully improve with more insights into aetiology and/or pathogenesis of SO.

Aims of the Thesis

The main aims of this thesis were to describe the epidemiology, clinical pathology, treatment and genetic risk factors for SO and HT in the GS and ES. Further aims were to investigate whether there is a common genetic predisposition of SO and HT in both breeds.

The aims were achieved by conducting a series of studies:

1. By performing epidemiological studies in GS and ES with SO and/or HT (papers I, IV and V).
2. By collecting and evaluating the medical history, clinical signs and histopathological changes in claw biopsies from affected GS and ES (paper I, II).
3. By collecting treatment regimen (paper I) and by evaluating two different treatment regimens in GS and ES with SO (paper II).
4. By doing candidate gene DLA studies, in GS and ES with SO and/or HT (papers III, IV, V).
5. Finally, by doing a GWAS with GS and ES with SO to locate where on the genome the disease genes could be located (paper IV).

Materials and Methods

An overview of the animals and methods used in this thesis is included in the following sections. For further details please see papers I-V.

Biobank

In 2006, a biobank was established by our research group at the Norwegian School of Veterinary Science, later Norwegian University of Life Sciences (NMBU). The biobank consists of blood samples of GS and ES classified as SO and/or HT positive (cases) or negative (controls). The inclusion criteria for SO cases were breed (GS or ES), and that the diagnosis had been confirmed by a veterinarian. Inclusion criteria for the SO controls were breed (GS or ES), age > 8 years, no prior or current diagnosis of a claw problem. Inclusion criteria for HT cases were breed (GS or ES), clinical signs of HT and at least serum values of TSH ≥ 0.45 $\mu\text{g/L}$. All of the dogs included as hypothyroid did also have one or more of TT4, FT4, cholesterol, and TgAA analyses done. Inclusion criteria for HT controls were breed (GS or ES), age > 8 years, no clinical signs of HT and serum levels of TSH < 0.45 $\mu\text{g/L}$, TT4 ≥ 15 nmol/L, FT4 ≥ 6 pmol/L and cholesterol ≤ 11 mmol/L. Controls were not on any medication which could interfere with the thyroid analysis at the time the blood samples were collected. Dogs were recruited from the biobank for papers III-V.

Epidemiology studies

All owners of dogs included in paper I, IV and V received a questionnaire aimed at collecting information regarding the dog's hunting abilities, temperament, vaccination, feeding regimen and more detailed information about SO, HT or other immune mediated or skin related diseases. Questions related to SO included; season of onset of symptoms, age at onset of disease, disease progression and outcome of the disease. In paper I, two different study populations of GS were used for prevalence estimations of SO and HT (Table 1). The first study population was 380 GS more than seven years old, which were randomly picked from the NKK database. The second study population was all the 151 littermates of the affected dogs with SO. In paper V, the study population for prevalence estimation was 474 eight years old GS registered with the NKK (Table 1). In paper IV, a retrospective case/control study was performed with the GS and ES included in the GWAS for SO.

Table 1: Overview of thesis population

Paper	Aim	Source	N (cases/controls)	Breed
I	Clinical records, histopathology and treatment of SO	Clinic	18	GS
			4	ES
	Prevalence SO and HT	Siblings of SO cases NKK database	151	GS and ES
			380	GS
II	Treatment trial SO	Clinic	12	GS
			1	ES
III	Candidate gene study SO	Biobank	196 (98/98)	GS
IV	Candidate gene study SO	Biobank	215 (108/107)	GS
			34 (18/16)	ES
	GWAS SO	Biobank	225 (114/111)	GS
			34 (18/16)	ES
	Epidemiology SO	Biobank	225 (114/111)	GS
			36 (18/16)	ES
V	Prevalence HT and SO	NKK database GS born in 2002	474	GS
	Candidate gene study HT	Biobank	151 (68/93)	GS
			146 (83/53)	ES

SO = symmetrical onychomadesis. HT = hypothyroidism. GS = Gordon Setter, ES = English Setter

The same questionnaire was used for the case/control study as in the prevalence studies. In paper V, a retrospective case/control study was also performed in the GS and ES included in the DLA study for HT (Table 1). The questions regarding HT included age at onset of HT, symptoms of HT, concurrent immune mediated disease such as SO, and whether the dog received any medications that could affect serum measurements of thyroid hormones.

Clinical pathology and treatment of symmetrical onychomadesis

The diagnostic work up of the dogs participating in papers I and II included clinical examination and cytological specimens from material in the claw folds of all dogs (Table 1). Bacterial cultures were taken from the claw fold if the dog was not on antibiotics when included in the studies. Analyses of blood samples were done and included biochemistry, hematology and thyroid hormones (TT4, FT4, TSH). ANA titers were obtained in five dogs. All dogs included in paper I had claw biopsies taken according to Mueller's technique (98) or by amputation of one claw. All dogs were under general anesthesia and the biopsies were all analyzed by the same pathologist. The different treatment regimens used when treating SO in GS and ES were recorded in paper I. In paper II, 13 dogs were included in two different treatment groups. One group received cyclosporine and dietary omega-3 supplementation and one group received only omega-3 supplementation. Both groups were examined monthly for six months. All dogs were fed the same diet to assure they received the same amount of omega-3 through their food. The diet contained protein sources the dogs had not been exposed to previously. Four years after the first dogs were included in the treatment trial, a follow up examination was performed to evaluate long term outcome of SO.

Clinical pathology of hypothyroidism

Serum levels of TSH, TT4, and FT4 were analysed for all dogs on an IMMULITE® 2000 XPi Immunoassay System. The presence of TgAA was determined by an indirect enzyme immunoassay. Analysis of TgAA antibodies was not available until March 2009 and is not used routinely in Norway to diagnose HT therefore not all the included cases were analyzed for this parameter. The median TSH values at diagnosis of HT in GS and ES included in the DLA study were compared to the median TSH values of hypothyroid dogs diagnosed at the laboratory at the NMBU between 2001 and 2012. Criteria for a diagnosis of HT in the NMBU material was TSH > 0.45 µg/L, TT4 ≤ 15 nmol/L, FT4 ≤ 6 pmol/L (Table 3). Only breeds with

more than 40 hypothyroid cases were included as controls. A total of 10 breeds (with a group of mixed-breed dogs) were included in the study (Table 3).

Candidate gene studies

A candidate gene approach was used to study genetic risk factors of certain DLA class II genes in dogs diagnosed with SO and/or HT. Based on a proposed autoimmune etiology the genes DRB1, DQA1 and DQB1 were sequenced in GS and ES with SO and /or HT (Table 1). The GS sequenced in paper III were included in paper IV. The methods for gene amplification and sequencing in papers III, IV and V are described in detail in paper V.

Genome wide association study

In order to identify novel susceptibility loci for SO, a GWAS was performed in 225 GS and 36 ES (paper IV). All 261 DNA samples were subsequently genotyped with the 170K CanineHDBeadChip (Illumina, USA). The BeadChip contains more than 170 000 evenly spaced SNPs across the dog genome, derived from the CanFam 3.1 assembly. The mixed model approach was used in the GWAS for GS with SO. The GWAS of ES did not give any significant SNPs and therefore a meta- analysis of the two GWAS was performed. MetABEL was used to run a meta-analysis of the two independent datasets from the GS and the ES.

Statistical analysis

The Fischer exact test was used to evaluate the incidence of conjunctivitis, otitis externa, skin infections and HT in setters with SO and setters without SO (paper I). The Fischer exact test was also used to evaluate whether sex affected prevalence of SO (paper IV). The level of significance was set at $p < 0.05$.

Odds ratio is a statistical method used to test for associations, and is often used in case-control studies (101). The method is used in papers III-V to test for an association between different DLA genotypes, haplotypes and alleles and SO or HT. A 99 % confidence interval was used to describe the uncertainty of the estimated ORs in paper III and a 95 % confidence interval in paper IV and V.

Wilcoxon rank-sum population tests were used to compare change in the numbers of healthy claws at presentation and after six months of treatment in paper II (102). The observations from both groups were ranked as if they were from one group, then the sum of

the rank in one group was calculated. The cut-off for statistical significance was set to $p < 0.05$.

In hypothyroid dogs, the standard deviations were all greater than half of the mean for the TSH, TT4 and FT4 values. This strongly indicates that the thyroid values are skewed (102) and therefore the median values with range were reported instead of the mean with standard deviation (paper V).

Results

Epidemiology studies

The prevalence of SO was 34.3 % and HT prevalence was 4.3 % in related dogs which had at least one littermate with SO (Table 2). In the unrelated GS the prevalence of SO was 12.6 % and the HT prevalence was 5.8 % (paper I). The prevalence of SO was 8.9 % and the prevalence of HT was 2.7 % in a sample of eight years old GS (paper V). In conclusion the oldest GS had the highest HT prevalence and having a sibling with SO was a risk factor to develop SO (Table 2).

Table 2 Prevalence studies of symmetrical onychomadesis (SO) and hypothyroidism (HT)

Paper	Study design	n	Study population	Age years	SO %	HT %
I	Cross sectional	101	Unrelated dogs	9.6	12.6	5.8
	Cross sectional	104	Related dogs	6.8	34.3	4.3
IV	Case/control	94	GS SO cases	8.0	100	3.1
		89	GS SO controls	10.5	0	8.9
V	Cross sectional	234	GS birth cohort	8.0	8.9	2.7
	Case/control	57	GS HT cases	6.4	21.1	100
		90	GS HT controls	9.3	22.2	0
		53	ES HT cases	7.7	5.6	100
47		ES HT control	8.8	2.1	0	

In the case/control study performed in GS with SO, the mean age at onset of SO was 4.3 years (range 1- 9 years), with no sex differences detected in disease occurrence. In 75 % of the affected dogs the first symptoms of SO occurred between April and September (Fig 13).

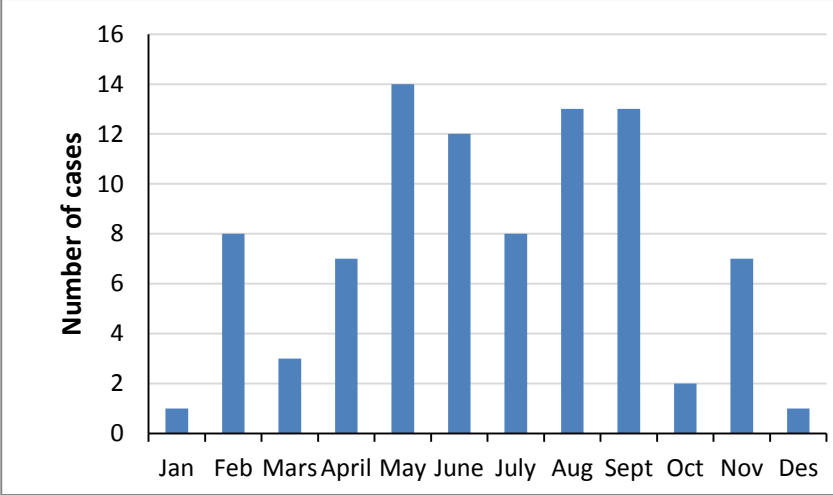


Figure 13: Symmetrical onychomadesis cases presented by month symptoms first occurred

Only 11% of the affected dogs recovered from the disease and regrew normal claws. Recurrent onychomadesis occurred in 31 % of the dogs, while chronically dystrophic claws were seen in 58 % of the affected dogs (Fig 14). All dogs were used for hunting prior to developing disease, but after the occurrence of SO, 26 % of the affected dogs were retired as hunting dogs. SO was reported as the cause of euthanasia in 12 out of 73 deaths (16 %).

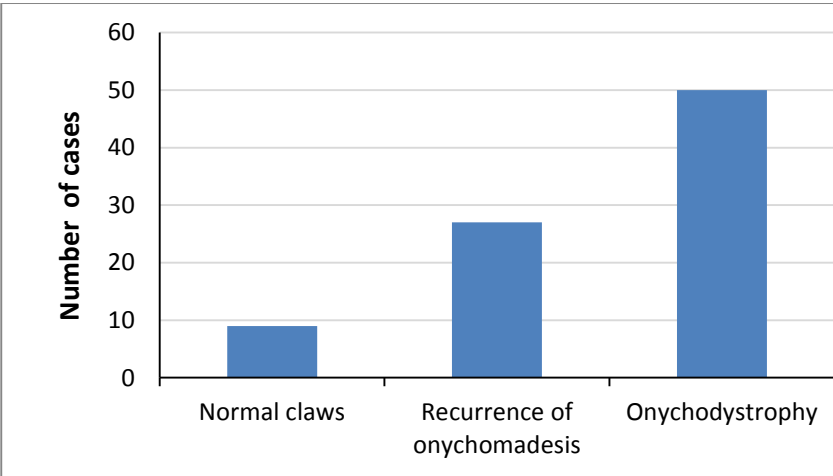


Figure 14: Outcome of cases diagnosed with symmetrical onychomadesis

In GS and ES included in the DLA study in paper V the mean age at diagnosis of HT was 6.4 years (95 % CI: 5.6-7.2) for the GS and 7.7 years (95 % CI: 7.2-8.2) for the ES (Table 4). The numbers of dogs diagnosed with HT at different ages are presented in Fig 15.

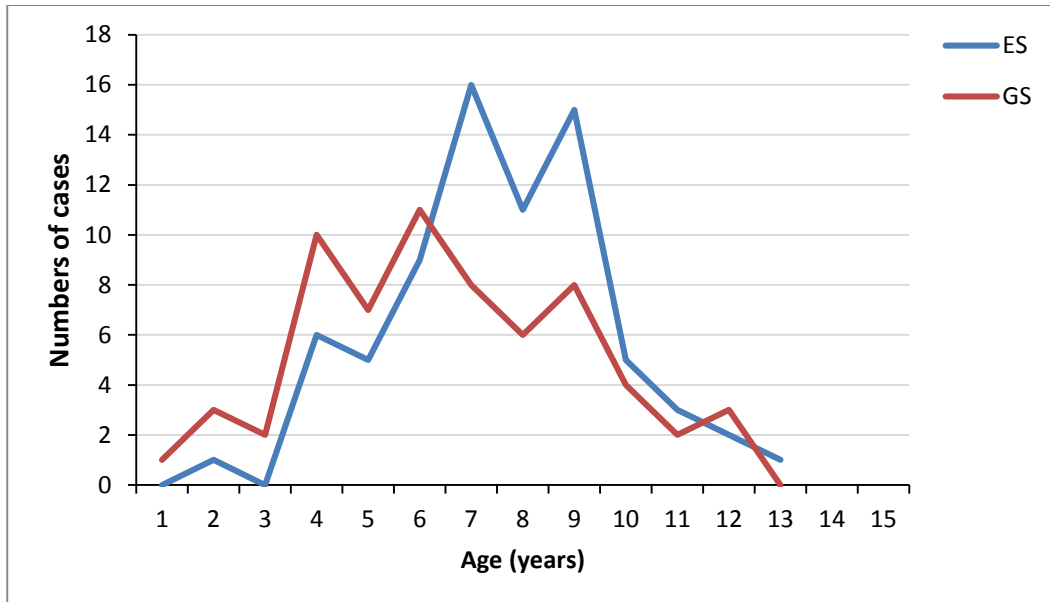


Figure 15: Age at diagnosis of hypothyroidism in the Gordon Setter (GS) and English Setter (ES).

Clinical pathology and treatment of symmetrical onychomadesis

Of the dogs participating in the clinical part of papers I and II, two dogs were diagnosed with HT and two dogs had weakly positive ANA titers. The claw biopsies analysed in paper I showed some variation between dogs, but typically included features of interface dermatitis with subepidermal cleft formation, pigment incontinence, basal cell vacuolization and necrosis, spongiosis and lymphocytic exocytosis, lymphocytic plasmacytic subepidermal inflammation and fibroplasia. In paper I, NSAIDs and antibiotics together with removal of defect claws were used in the treatment for the acute phase of SO. Prednisolone, fatty acids, niacinamide and tetracycline were used as immunosuppressive agents for long-term treatment. Two of the 22 (9.1 %) dogs included in the study showed regrowth of normal claws after six months of treatment. Eleven dogs (50.0 %) exhibited onychodystrophy characterised by soft and scaly claws, but with no recurrence of onychomadesis. Six dogs (27.3 %) had intermittent painful recurrences of onychomadesis, usually when not on some sort of therapy. Three dogs were euthanized due to SO (13.6 %). In paper II, the therapeutic effect of cyclosporine versus omega 3 supplementations was evaluated in 13 dogs with SO.

All dogs improved their claw quality during the six-month treatment trial, but no statistical differences were observed between the two groups. One dog achieved normal claws (7.6 %) and six dogs (46.1 %) suffered onychodystrophy without recurrence of SO. Six dogs had recurrence of SO, which was the cause of euthanasia in one dog (7.6 %).

Clinical pathology of hypothyroidism

In the GS and the ES from the NMBU laboratory material, the median TSH value at the time of diagnosis of HT was 3.1 µg/L in GS and 2.9 µg/L in ES (Table 3). The GS, Shetland Sheepdog and ES had the highest TSH values at time of diagnosis compared to mixed breeds dogs and the other seven breeds from the Central laboratory (Table 3). In the German Shepherd and the English Cocker spaniel there was a significant overrepresentation of females with HT. In the Shetland Sheepdog there was a significant male overrepresentation (Table 3).

Table 3: Median TSH value and female distribution in mixed breed dogs and nine other breeds at time of diagnosis of hypothyroidism (reference value for TSH: 0-0.45 µg/L)

Breed	N	Female % (95 %CI)	TSH µg/L Median	TSH µg/L Range
Gordon Setter	125	48.0 (39.2-56.8)	3.1	0.60-12.0
English Setter	200	43.5 (36.6-50.4)	2.9	0.46-12.0
American Cocker Spaniel	41	58.5 (43.5-73.6)	2.2	0.46-8.0
Boxer	44	50.0 (35.2-64.8)	1.9	0.46-4.1
English Cocker Spaniel	54	64.8* (52.1-77.5)	1.7	0.60-6.2
German Shepherd	44	72.7* (59.6-85.9)	1.8	0.46-5.4
Giant Schnauzer	75	42.7 (31.5-53.9)	1.0	0.50-9.1
Golden Retriever	68	56.7 (44.9-68.6)	1.2	0.50-9.9
Mixed breed dogs	133	50.4 (41.9-58.9)	1.6	0.50-11.5
Shetland Sheepdog	45	33.3** (19.6-47.1)	3.0	0.46-9.2
Total	655	49.0 (45.2-52.8)	2.0	0.46-12.0

**significant overrepresentation of females **significant overrepresentation of males*

Results of THS, TT4, FT4, cholesterol and TgAA analysis in hypothyroid GS and ES included in the DLA study are presented in Table 4.

Table 4: Gordon Setters (GS) and English Setters (ES) included in the dog leukocyte antigen (DLA) study for hypothyroidism (HT) (Paper V)

	Cases		Controls	
Sex and age	GS n=68	ES n=83	GS n=93	ES n=53
Male	22	42	57	33
Female	46	41	36	20
Age				
mean years (95% CI)	6.4 (5.6-7.2)	7.7 (7.2-8.2)	9.3 (9.0-9.7)	8.8 (8.3-9.3)
range years	1.8-12.9	2.7-15	8-14.4	8-16
Analysis blood samples				
TSH ug/L: median (range)	3.01 (0.46-12.0)	2.24 (0.46-12.0)	0.14 (0.03-0.45)	0.23 (0.04-0.45)
TT4 nmol/L: median (range)	7.5 (4.0-77.0)	6.0 (4.0-44.0)	28.0 (16.0-53.0)	24.0 (16.0-45)
FT4 pmol/L: median (range)	9.0 (4.0-65.0)	7.0 (4.0-77.0)	14.0 (7.0-69.0)	14.0 (7.0-47.0)
Cholesterol mmol/L: median (range)	9.1 (2.6-20.8)	11.7 (3.4-23.1)	6.1 (3.6-11.0)	5.8 (3.8-11.0)
Diagnosis of SO				
positive (<i>n</i>)	12	3	20	1
negative (<i>n</i>)	45	50	70	46
Total (<i>n</i>)	57	53	90	47

Candidate gene studies

A candidate gene approach was used to study genetic risk factors associated with SO and/or HT in the GS and the ES. The DLA genes DRB1, DQA1 and DQB1 were genotyped based on a proposed autoimmune etiology behind the two diseases. In paper III, the most common DLA haplotype (DRB1*01801/DQA1*00101/DQB1*00802) in the GS was significantly associated with SO (OR = 2.1, $p < 0.001$) (Table 5). The DLA allele DQA1*00101 had an even higher OR associated with SO in GS (OR=2.8, $p < 0.001$). When GS homozygous for the risk haplotype were compared to all dogs not carrying the haplotype, the OR was 3.96 ($p < 0.001$). The DLA haplotype (DRB1*02001/DQA1*00401/DQB1*01303) was associated with protection against SO in the GS (OR=0.02, $p < 0.001$). In the ES, 18 dogs with SO and 16 controls were genotyped (paper IV). All but two ES carried at least one copy of either DLA haplotype (DRB1*00101/DQA1*00101/DQB1*00201) or (DRB1*10201/DQA1*00101/DQB1*00201) (Table 6). The other four haplotypes ranged in frequencies between 1.5-8.8 %. The DLA haplotype DRB1*02001/DQA1*00401/DQB1*01303 appeared to be associated with decreased risk of SO in the ES as well as in GS, as it was only observed in controls.

In paper V, the most common DLA haplotype (DRB1*01801/DQA1*00101/DQB1*00802) in the GS was significantly associated with protection against HT (OR = 0.3, $p < 0.01$) (Table 5). The DLA allele DQA1*00101 was not associated with HT in the GS, but it was associated with HT in the ES (OR=2.9, $p < 0.01$). The DLA-DQB1*00201 allele was on the other hand associated with HT in the GS (OR=3.6, $p < 0.01$) and the ES (OR=1.9 $p < 0.01$). When GS homozygous for the protective haplotype were compared to all dogs not carrying the haplotype, the OR was 0.2 ($p < 0.01$). The DLA haplotype associated with HT in the GS with the highest OR was DRB1*00103/DQA1*00101/DRB1*00201 (OR=4.4, $p < 0.01$) which was present in 8.2 % of the GS population. The DLA haplotype DRB1*10102/DQA1*00101/DQB1*00201 was associated with increased risk of HT in ES (OR=2.0, $p < 0.01$). And one DLA haplotype (DRB1*00601/DQA1*005011/DQB1*00701), only present in 3.3 % of the ES population, was associated with protection of HT in ES (OR=0.2, $p < 0.01$).

Table 5: The candidate gene analysis dog leukocyte antigen (DLA) genotype, haplotypes and alleles related to symmetrical onychomadesis (SO) and hypothyroidism (HT) in the Gordon Setter (GS) and English Setter (ES)

Breed	DLA Genotype DRB1/DQA1/DQB1/ DRB1/DQA1/DQB1	OR SO (99 % C.I.) Paper III	OR HT (95 % C.I.) Paper V	Prevalence in study population (%) Paper III GS, Paper IV ES	Prevalence in study population (%) Paper V
GS	01801*00101*0080/ 01801*00101*00802	3.96 [†] (1.36-11.52)	0.18 [†] (0.05-0.53)	18.9	13.7
DLA Haplotype DRB1/DQA1/DQB1					
GS	01801*00101*00802	2.1 [†] (1.3-3.6)	0.3 [†] (0.2- 0.5)	43.4	32.0
GS	01501*00601*02301	n.s	0.2 [†] (0.1-0.8)	4.6	5.9
GS	00103*00101*00201	n.s	4.4 [†] (1.8-11.5)	8.2	8.3
GS	02001*00401*01303	0.03 [†] (0.002-0.35)	n.s	8.7	11.4
GS	04901*01001*01901	n.s	2.1 [†] (1.2-3.7)	11.2	18.9
ES	10102*00101*00201	-	2.0 (1.1-3.8)	29.4	24.6
ES	00601*005011*00701	-	0.2 (0.03-0.8)	1.5	3.3
DLA Allele					
GS	DRB1*00103	n.s	4.4 [†] (1.8-11.5)	8.2	8.4
GS	DQB1*00201	n.s	3.6 [†] (2.1.-6.4.)	25.0	21.4
ES	DQB1*00201	-	1.9 (1.05-3.3)	82.3	77.6
GS	DQA1*00101	2.8 [†] (1.48-5.23)	n.s	74.2	60.9
ES	DQA1*00101	-	2.9 [†] (1.3-6.6)	91.2	89.3

[†] denotes $p < 0.01$, n.s: non-significant. C.I: confidence interval.

Table 6: DLA haplotypes in English Setters (ES) with symmetrical onychomadesis (paper IV)

DLA haplotype (DRB1/DQA1/DQB1)	Total population % n = 68	ES cases % (n) n=36	ES controls % (n) n=32
02001/00401/01303	5.9	-	12.5 (4)
01501/00601/02301	1.5	-	3.1 (1)
00101/00101/00201	52.9	63.9 (23)	40.6 (13)
10102/00101/00201	29.4	33.3 (12)	25.0 (8)
00901/00101/008011	8.8	2.8 (1)	15.6 (5)
00601/005011/00701	1.5	-	3.1 (1)

Twelve GS had both SO and HT in paper V and nine out of these 12 GS were heterozygous for the haplotype DLA DRB1*01801/DQA1*00101/DQB1*00802. Only three ES had both SO and HT; one was homozygous for DLA- DRB1*10201/DQA1*00101/DQB1*00201, one was homozygous for DLA-DRB1*00101/DQA1*00101/DQB1*00201 and one was heterozygous with the genotype DLA- DRB1*10201/DQA1*00101/DQB1*00201/DRB1*00101/DQA1*00101/DQB1*00201.

Genome wide association study

The GWAS study of the GS identified an associated region on chromosome 12, covering 3.3 Mb pairs (CFA12: 555475 – 3775420), and with 12 significant SNPs (Fig 16). The top two SNPs (BICF2P928832 and TIGRP2P155561) from the association analysis are in high linkage disequilibrium ($r^2 > 0.8$) with their respective nearest SNPs and thus we calculated odds ratio for SO in dogs with any given SNP haplotype combination at these two short linkage disequilibrium-blocks. The first haplotype contained three SNPs with an OR =13.0, 95 % CI: 5.1- 33.2, $p < 0.01$. The second haplotype contained four SNPs with an OR =9.7, 95 % CI: 4.5- 20.9, $p < 0.01$. The GWA analysis of 36 ES revealed no significant association to SO (Fig.17). However, meta-analysis across ES and GS revealed that $p_{corrected}$ improved by between 1-2 orders of magnitude for many of the SNPs in the SO-associated region revealed in GS alone. The meta- analysis top SNP was the same top SNP as in the GWAS analysis of GS alone.

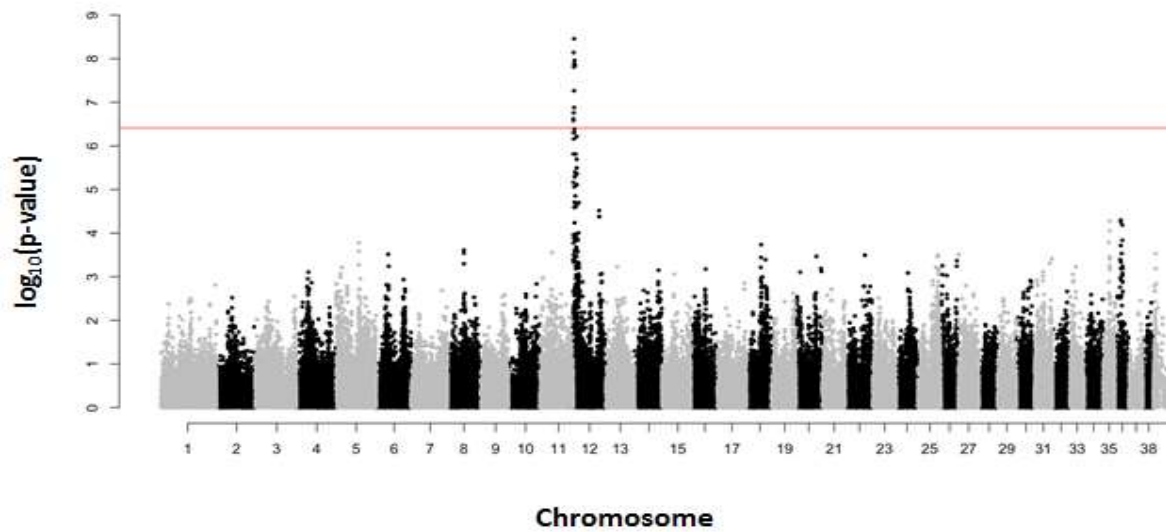


Figure 16: Manhattan plot reveals a strongly associated region on chromosome 12 of the GWAS from Gordon Setters (paper IV). The red line represents the Bonferroni corrected p-value significance threshold of 0.05. Each dot represents a single SNP. Genomic coordinates are displayed along the X-axis and the negative logarithm of the association p-value for each SNP marker is displayed on the Y-axis.

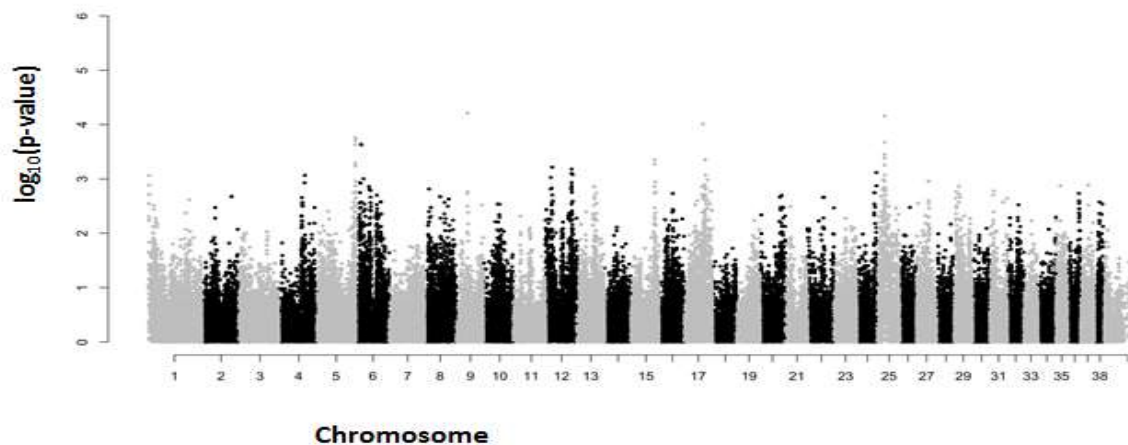


Figure 17: Manhattan plot does not reveal any strongly associated region on a chromosome of the GWAS from English Setters (paper IV)

General Discussion

Symmetrical onychomadesis was confirmed to be a severe claw disease affecting the GS and ES in this thesis. The dogs lost all claws during a period of a few months, and only 11 % of the affected dogs regrew normal claws (papers I and IV). The prevalence of SO in unrelated GS was found to be between 8.9 % (paper V) and 12.6 % (paper I). Symmetrical onychomadesis has previously been reported in several other dog breeds, but no specific breed prevalence studies of SO have been performed (60). In the studies presented in this thesis the GS was documented to be a high risk breed for SO (papers I, V). The low numbers of ES with SO participating in papers I, II, IV and V reflected the difficulties in finding ES with SO. This could indicate that SO is a more rare disease in the ES compared to the GS, but a prevalence study is needed to confirm this assumption.

Ideally, a population as old as possible should be sampled for HT prevalences studies, since the risk of HT increases with age in both dogs (70, 103) and humans (31). This was confirmed in the GS where the estimated prevalence of HT in GS with a mean age of 9.6 years was 5.8 % (paper I) and 2.7 % in the eight years old GS (paper V). There are few published reports on breed prevalence of HT, but an increased risk of HT has previously been reported in the Giant Schnauzer and the Howavart in Sweden (74). Different diagnostic criteria used for HT, differences in age and breed variations as well as misclassification may influence prevalence estimates of HT (70, 104). Misclassification bias is a relevant issue in prevalence studies of HT due to difficulties in diagnosing the disease (104), but this kind of bias is less likely to occur with SO because the dramatic appearance of the disease for the dogs and their owners. To deal with classification bias regarding HT, the questionnaire used in the prevalence studies was designed with two questions confirming the HT diagnosis (105). First the owners were asked whether their dogs were diagnosed with HT, and in the next question whether they administered levothyroxine. Only dogs with owners that confirmed both questions were included as HT positive dogs, but in both studies, the HT prevalence was based on dogs that were examined by a veterinarian because of their owners' suspicion of HT. The HT prevalence may therefore be underestimated because some owners misinterpret the signs of HT as normal signs of aging in their dogs and hence do not seek medical care (103). Selection bias may occur if the sampled population is not representative for the general

population, and can result in an incorrect prevalence in the study population (106). The dogs participating in the prevalence studies in this thesis were all registered in the NKK database. In Norway, it is very uncommon not to register GS or ES with the NKK, therefore this database can be considered to represent the general GS and ES population. The results confirmed that the GS has a higher prevalence of HT the older the study population gets. Unfortunately, the prevalence of HT in the ES could not be investigated in this study, but this breed has previously been described as a high risk breed for HT in several countries at different continents (3, 18, 71).

In humans multiple autoimmune diseases may occur in one single patient, indicating that these diseases may share some susceptibility genes (31). Although rare in dogs, multiple autoimmune diseases in individual patients have been reported (77, 107). One of the aims of this thesis was to investigate whether there is indication of a common genetic predisposition for SO and HT in GS. If such a common predisposition exists, the prevalence of HT in GS with SO should be higher than the prevalence of HT in the general GS population at the same age. Conversely, the prevalence of SO should be higher in GS with HT than in the general GS population. The prevalence of HT was similar in two GS population at eight years of age; 3.1 % in the GS population with SO and 2.7 % in the GS population without SO. Also, the prevalence of SO was similar in GS with HT (21.1 %) compared to GS without HT (22.2 %) included in paper V, further supporting the conclusion that there is no indications of a common genetic predisposition between SO and HT.

Symmetrical onychomadesis in dogs has been thought to have clinical similarities with SLE, another autoimmune disease (35). This conclusion was drawn because some of the dogs with SO had positive ANA titers, they responded well to immunosuppressive treatment, and biopsies from affected claws showed the same histopathological features found in skin biopsies from dogs with SLE (35, 36, 56). In patients with SLE a lupus band on a skin biopsy is a diagnostic test used to confirm presence of IgG, Ig M, Ig A and/or complement deposits in the dermoepidermal junction (108). Direct immunofluorescence testing did not reveal a lupus band on histopathology samples from cases with SO (109). The interface dermatitis found on histopathology samples from claw biopsies of affected GS and ES (paper I) were similar to what has been described in previous studies of SO in other breeds (60). Recent studies have shown that claw biopsies from dogs with *Leishmaniosis* and bacterial infections

have the same interface dermatitis as seen in SO (40, 59). Thus, the lupoid reaction seen histopathologically in cases of SO may represent a reaction pattern of the claw rather than the etiology of the disease (97). In dogs with SLE, concurrent systemic signs of disease are important clinical features together with claw symptoms. This is in contrast to our observations in GS and ES with SO, where the symptoms were only localized to their claws (Paper I, II, IV). Dogs diagnosed with SLE should also have positive ANA test with high titers, and in our material only a few of the GS and ES had positive ANA tests but with low titers (Paper I). A positive ANA test with low titer might be found in a variety of chronic inflammatory, infectious or neoplastic diseases (99). The conclusion is therefore that SO in the GS and ES is not a part of SLE in dogs (Paper I, II, IV), but it might still be an immune mediated disease. In humans, onychomadesis has been associated with a number of other autoimmune diseases, but in all of them the patients do also have other concomitant skin diseases together with the onychomadesis (96). Concurrent skin diseases have not been observed in setters with SO, however the results from the genetic studies in this thesis indicated an immunological background of SO (paper III and IV). The improvement of SO on immunosuppressive treatment further supported the conclusion that the disease might be linked to immunoregulatory mechanisms (paper I, II). Symmetrical onychomadesis is a difficult disease to treat. The re-growth of claws and improvement takes time and is hard to monitor accurately. Removal of damaged claw plates during anaesthesia and treatment of secondary bacterial infections are essential in the acute phase of the disease (53, 59, 100). From a dermatological point of view, a dog is cured of the disease if it develops new claws of normal quality with no recurrence of onychomadesis during the dog's lifetime. However, a significant proportion of owners will report the dog as "cured" as long as the dog does not have painful relapses of onychomadesis, even though it still has onychodystrophy. Bergvall (1998) demonstrated positive effects of omega-3 and omega-6 supplementation (55). Other treatment plans have included the use of tetracycline/niacinamide, prednisolone, pentoxifylline and azathioprine (1, 55, 60) . In paper II, the therapeutic effect of cyclosporine versus omega 3 supplementations was evaluated in 13 dogs with SO. Cyclosporine was chosen as a therapeutic agent for comparison with omega 3 in this study because it showed good response in a pilot study, and has previously been used to treat canine immune mediated diseases such as atopic dermatitis and anal furunculosis (110, 111). Cyclosporine is a potent immunosuppressive agent that inhibits the activation of many of the cells involved

in cell-mediated immunity (112). The hypothesis was that cyclosporine would give a better treatment outcome compared to omega-3, but after thirteen dogs, no significant difference between the two groups was observed and inclusion of new dogs were stopped. All dogs improved their claw quality during the six-month treatment trial. One dog achieved normal claws and six dogs suffered onychodystrophy without recurrence of SO. Six dogs had recurrence of SO, which was the cause of euthanasia in one dog. The extent of the treatment effects on the outcome is unknown, because a negative control group was not included in the study design. Potential selection bias was avoided through the random allocation of dogs to treatment groups. The fact that a binominal scale was chosen to measure treatment effect (number of affected claws before and after treatment) instead of a graduated scale or subjective measurements of improvement should decrease the risk for measurement error introduced by the lack of blinding.

Autoimmune diseases can be triggered following exposure to several external factors, like infections, vaccinations and environmental factors. (35). Symmetrical onychomadesis and hypothyroidism are suspected to be autoimmune diseases (56, 71). Bacterial samples were taken from all dogs (except animals that received antibiotic treatment) at the time of inclusion in papers I and II, and no associations between specific bacterial agents and SO were identified. *Leishmania* infection has previously been described as a cause of claw disease in dogs (40). Since *Leishmania* is only found on imported dogs in Norway or Sweden (113), this could not be the cause of SO in the dogs included in this thesis. No association was found between vaccination and SO (paper I). Allergies, adverse food reactions and other immune mediated skin diseases have also been associated with SO in dogs in other studies (59, 97). The dogs that participated in the treatment study were investigated for adverse food reactions and none reported recurrence of SO after they returned to their normal diet after the treatment trial was finished (paper II). In this thesis no associations were identified between SO and other skin diseases (papers I, II, IV). Time of year did have an influence on the incidence of SO in the GS and ES. In 75 % of the dogs the first symptoms of SO occurred between April and September (papers I, IV). The summer through autumn is the most active period for the dogs, resulting in increased mechanical stress on the claws during training on hard surfaces. These environmental factors may contribute to development of

onychomadesis as mechanical stress has previously been reported as a cause of symmetrical onychomadesis in humans (114).

Sex predilection is common in human autoimmune disease, usually with more women than men being affected (115). Similar sex-association has only been documented in a few autoimmune disease in dogs (32, 47, 48) and has not yet been described in SO or autoimmune HT. No sex predilections were detected in the occurrence of SO in the GS or ES in the present thesis (papers I and IV). However, in the material from the NMBU laboratory the German Shepherd and the English Cocker Spaniel did have a significantly higher proportion of females with HT compared to males with HT, whereas the Shetland Sheepdog had a significantly higher proportion of males with HT (Table 3). Sex predilections in HT in dogs might have been overlooked in the past, since previous studies have mainly analyzed data with a combination of breeds rather than investigating the disease in individual breeds (76). Further studies are needed to confirm whether there exists a potential skewed sex distribution of autoimmune HT in different dog breeds. Breed specific TSH levels at time of diagnosis of HT have not been reported previously. However, the levels of both TT4 and FT4 in healthy dogs of different breeds are known to vary, for example are sight hounds known to have naturally low TT4 and FT4 values compared to most other breeds (87, 88). TSH secretion is pulsatile in both normal and hypothyroid dogs, but the peak level of TSH increases in dogs that develop hypothyroidism (83, 116, 117). The median serum TSH value from the NMBU laboratory material at the time of diagnosis of HT was among the highest in GS with 3.1 µg/L and ES with 2.9 µg/L. The significance of breed differences in TSH values at time of diagnosis of HT need further studies to be explored. The GS and ES were middle aged when they were diagnosed with HT and age at onset of HT is known to vary among breeds (70). Different age of diagnosis and TSH values in the same phenotypic disease might indicate that different genes or mutations affect disease development in different breeds (118-120).

A hereditary predisposition to SO in the GS and ES is likely, based on the high prevalence among GS and ES and an even higher prevalence of SO among dogs with siblings with SO (paper I). In one litter of GS, six of eleven dogs were affected with the disease and in another litter of GS four out of eight dogs developed the disease during their lifetime (paper I). A candidate gene approach was used to study genetic risk factors associated with SO and/or

HT in the GS and the ES. Mueller et al. (2003) suggested that SO and HT may share genetic factors encoding a vulnerability to the diseases due to high incidence of HT in breeds known to be predisposed to SO (53, 59). The DLA genes DRB1, DQA1 and DQB1 were genotyped based on a proposed autoimmune etiology behind the two diseases (18). The DLA haplotype DRB1*01801/DQA1*00101/DQB1*00802 was the most common haplotype in the GS and was significantly associated with SO in GS, when GS homozygous for the risk haplotype were compared to all dogs not carrying the haplotype the highest OR for SO was found (paper III). The DLA allele DQA1*00101 in the GS population was significantly associated with SO and 73.7 % of the GS had this allele (paper III). The DLA haplotype DRB1*02001/DQA1*00401/DQB1*01303 was associated with protection against SO in GS but only 8.1 % of the GS population had this haplotype (paper III).

The haplotype with highest OR associated with HT in GS was the DLA haplotype DRB1*00103/DQA1*00101/DQB1*00201 and this haplotype was present in 8.3 % of the GS (paper V). Notably, the DLA- DQB1*00201 was associated with HT in both GS and ES (Table 5). The allele DLA-DQA1*00101 was found to be associated with HT in the ES but not in the GS. This DLA- DQA1 allele was found in 89.3 % of the ES included in paper V. In the GS in paper V, this DLA- DQA1 allele was part of the most common and also protective haplotype DLA-DRB1*01801/DQA1*00101/DQB1*00802. Homozygosity for this DLA haplotype (DLA-DRB1*01801/DQA1*00101/DQB1*00802) was the strongest protective factor against HT in the GS. Nine out of 12 GS with SO and HT in paper V were heterozygous for the DLA haplotype DLA (DB1*01801/DQA1*00101/DQB1*00802). In summary, the same DLA haplotype in the GS was associated with both increased risk for one presumed autoimmune disease (SO) and decreased risk for another autoimmune disease (HT). This indicates a genetic complexity of this group of diseases. DLA genes have previously been associated with several different immune mediated diseases in dogs (121-123). Traditionally, only a portion of exon 2 from DRB1, DQA1 and DQB1 has been sequenced because most of the variation has been found in these regions (8). Kennedy et al. (2006), suggested that the DLA-DQA1*00101 allele was associated with HT in many breeds (18). The findings in our study support this for the ES but not for the GS. The findings of different DLA associations with HT in different breeds are consistent with previous findings of different HLA associations with HT observed both within and between ethnic groups in humans (21). Safra et al. (2011) have

questioned some of the published associations between autoimmune diseases and different DLA class II genes because the low numbers of different DLA haplotypes in some breeds can lead to spurious associations (22). In our study the SO results from the GWAS in the GS supported a linked association between DLA haplotypes and SO. In the ES the association was not confirmed with GWAS, probably due to few individuals and maybe too little variation in the ES in the actual region on the genome.

The GWAS in the GS revealed 12 significant SNPs, covering 3.3Mb pairs of chromosome 12 (paper IV). This region spans most of the MHC class I, II and III region and furthermore, including 16 genes downstream the MHC region (124). The most associated SNP was localized at 1911694 (BICF2P928832). No associated SNPs were revealed within the DLA-DRB1/DQA1/DQB1 genes and the likelihood for SO was higher for dogs carrying the major allele at each of the 12 SNPs compared to the other recorded DLA-DRB1/DQA1/DQB1 haplotypes. One haplotype from the GWAS contained three SNPs with an OR=13.0, $p < 0.01$ (paper IV). Another haplotype contained four SNPs with an OR=9.7, $p < 0.01$. These OR numbers are much higher than the OR found based on the DLA associations studies (Table 5). The significant SNPs in the GWAS can be used to work out a genetic risk index for SO in GS, but it is crucial to account for potential unfavorable correlations to other diseases. A genetic risk index could be helpful to assist selection of breeding animals, and contribute to a reduced incidence of SO in the GS and the ES. There are 129 genes in the SO associated region identified by the GWAS and, because of high linkage disequilibrium between the SNPs in this region, exact identification of disease causing variants has proven difficult. Whole genome sequencing of the associated area in cases and controls can reveal the disease causing genes. The CDSN gene could be of interest for elucidating the disease progression of SO (125). CDSN encodes a protein found in corneodesmosomes, which are localized in the epidermis where they serve as intracellular junctions with cell adhesion as a major function (125). Two other genes involved in non-immunological pathways could potentially be relevant for the development of SO, namely ITPR3 that has been associated with regulation of the hair cycle (126), and LEMD2 that encodes proteins involved in providing mechanical stability to the nucleus of a cell (127). Theoretically, LEMD2 mutations could contribute to disease if mechanical properties in the nuclear structure become altered and thereby render tissues that undergo harsh mechanical stress more prone to cell death (127). LEMD2 has

been linked to human diseases called laminopathies, which have a wide range of clinical symptoms (127). Although LEMD2 has been shown to have important myogenic function, it is expressed in virtually all differentiated cells and could possibly play a role in the development of SO because claws of dogs undergo harsh mechanical stress, particularly during intensive training.

Although rare in dogs, multiple autoimmune diseases in individual human patients are not uncommon, indicating that autoimmune diseases may share some susceptibility genes (31, 77, 107). Our results do not support any common genetic factors that contribute to the development of SO and HT in GS and ES. On the contrary, the same DLA haplotype in the GS was associated with decreased risk of HT and increased risk of SO. The fact that the same DLA haplotype is associated with increased risk of one autoimmune disease and decreased risk of another presumed autoimmune disease, suggests that specific combinations of DLA alleles do not necessarily predispose dogs for autoimmune disease in general. It may also be an indication that the functional effect, leading to the observed associations to disease, may be due to closely linked genes.

Conclusions

In conclusion, SO is a disease in GS with high prevalence, which decreases the quality of life for affected dogs and have a high euthanasia rate. The ES does not have the same high prevalence of SO. Symmetrical onychomadesis is most likely immune mediated based on association to a region on chromosome 12 rich in genes involved in the immune response and the response of SO to immunosuppressive treatment.

The GS, as with the ES, is confirmed to be a high risk breed for HT in this study. Even if some DLA alleles are associated with increased risk of HT and/or SO in several breeds, the breed specific allele associations of some DLA haplotypes indicate that the functional effect may be associated to closely linked genes instead of the DLA loci itself. There were not found any common genetic risk factors for SO and HT in this thesis. The identified associated SNPs with SO could be valuable in marker assisted selection that might help to lower the incidence of SO in the GS and possibly also the ES.

Future prospects

In recent years there has been an increased focus on how to decrease the high incidence of diseases and conformational problems in pure-bred dogs (128). This has resulted in an increased focus on health issues in breeding by the American and European kennel clubs. A large number of genetic tests for simple Mendelian-inherited diseases have been developed over the years. Such tests can be implemented in the selection of healthy breeding animals without the risk of losing too much genetic variance in one dog breed (20, 129). The picture is more challenging when it comes to complex diseases like autoimmune diseases and cancers where there is not just a single gene that causes the disease. Instead, several genes in combination with environmental factors influence disease development. Fortunately, new genetic tools are being developed to assist breeders in breeding healthier dogs even in relation to complex diseases (27). For instance has one genetic index test been calculated based on GWAS results from Bernese Mountain dogs with histiocytic sarcoma (130, 131). Further genetic index tests based on GWAS results are also developed for hip and elbow dysplasia in the same breed (132). One example of a genetic test for an autoimmune disease is necrotizing meningoencephalitis in the Pug, where an association between DLA-genes and the disease is offered as a genetic test to breeders (33). The associated SNPs found in the GWAS from GS with SO can be used in a similar manner as a new genetic index test to reduce disease frequency. To further explore any associations between SO and HT a GWAS in hypothyroid GS and ES should be performed and the results compared with the GWAS of SO. To find the genes which cause SO, sequencing of the candidate region found on chromosome 12 in the GWAS should be performed in cases and controls. The studies in this thesis have shown that it has been possible to identify genetic risk factors for two important complex diseases in the GS and ES. Hopefully this may contribute to a reduced disease incidence and an improved welfare of these wonderful hunting breeds in the future.

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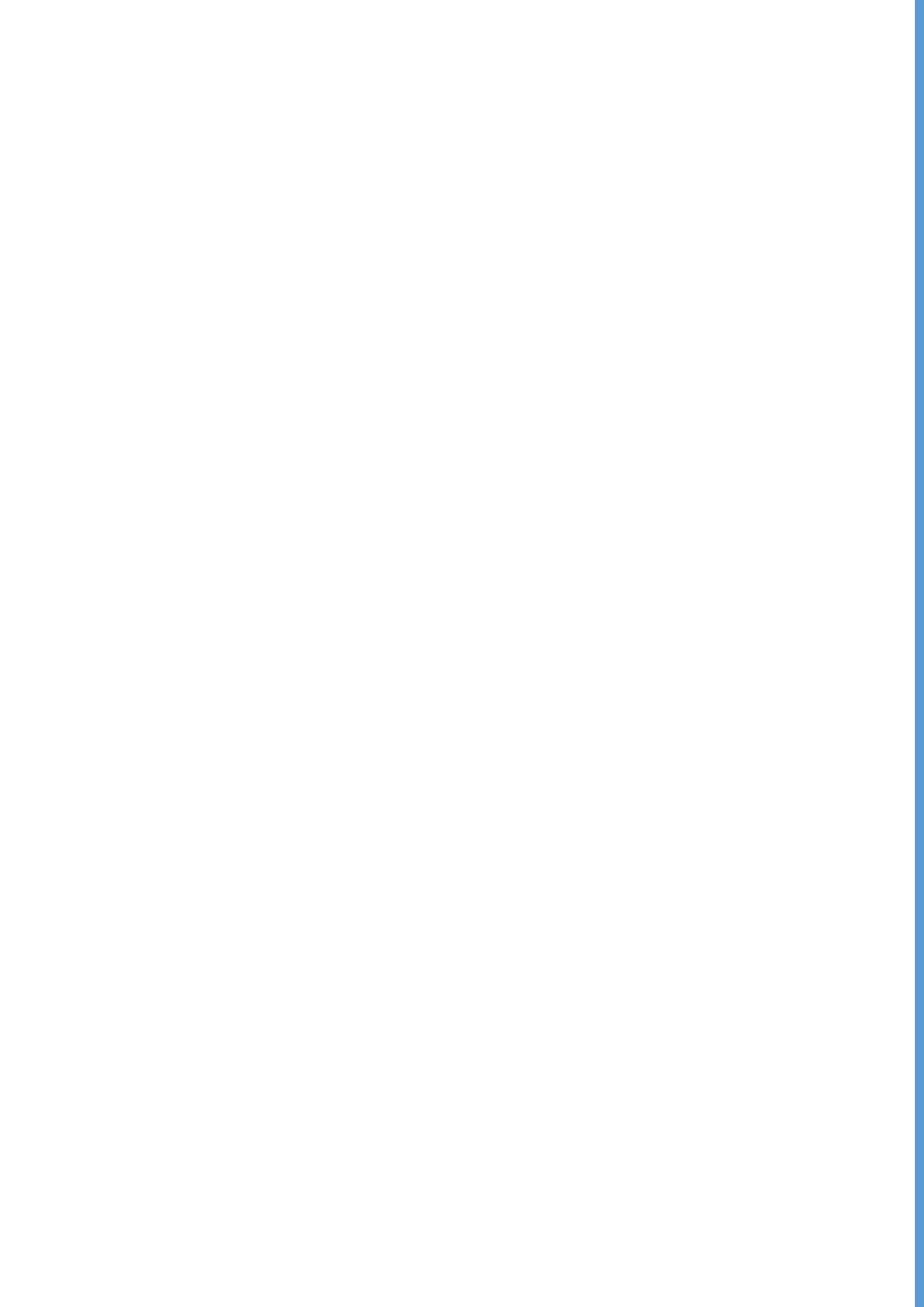
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Papers I-V

Paper I



Symmetrical onychomadesis in Norwegian Gordon and English setters

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Due to the close relationship of the affected dogs, pedigree map analysis was not possible. Three dogs were euthanized because of the disease and two had regrowth of normal claws. Seventeen dogs had persistent onychodystrophy that typically was nonpainful during therapy which in most dogs consisted of fatty acid supplementation or prednisolone.

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What is known about the topic of this paper

- Symmetrical onychomadesis is a painful and frustrating disease for dogs and their owners.
- Clinically the disease is characterized by an acute onset with loss of one or several claw plates, followed rapidly by shedding of most or all other claw plates. The dogs are otherwise healthy. Histopathologically the disease is characterized by hydropic and lichenoid interface dermatitis.
- Treatment may consist of fatty acid supplementation, tetracyclines and niacinamide or prednisolone.

What this paper adds to the field of veterinary dermatology

- Norwegian Gordon and English setters are prone to symmetrical onychomadesis.
- The disease seems clinically and histologically similar to what is reported in other breeds.
- Treatment leads to improvement and nonpainful but not completely normal claws.

Abstract

This study reports the condition onychomadesis affecting multiple claws in Norwegian Gordon and English setters. Medical records of and claw biopsies from 18 Gordon and four English setters with onychomadesis of multiple claws were obtained from July 2005 to January 2007. Only dogs with symmetrical onychomadesis and no signs of concurrent disease were included. Histopathological features varied between dogs, but typically included interface dermatitis with subepidermal cleft formation, pigment incontinence, basal cell vacuolization and necrosis, spongiosis and lymphocytic exocytosis, a lymphocytic, plasmacytic subepidermal inflammation, and fibroplasia. In two dogs, histopathological signs of a superficial infection were present. The age of onset of disease varied between 2 and 7 years with a mean of 3.9 years, and was not correlated with vaccination time. Six of the affected dogs also had siblings with the disease.

This study was presented at the 22nd North American Veterinary Dermatology Forum in Kauai, Hawaii, USA, 2007. Published in *Veterinary Dermatology* 2007; 18: 195.

Introduction

Onychology (the study of nails) is an area of veterinary dermatology that has only recently become the focus of more detailed study.¹⁻⁷ One of the more common claw diseases in dogs has been previously named idiopathic onychomadesis⁴ or lupoid onychodystrophy.⁷ This was first reported by Scott *et al.*⁷ Affected dogs showed a symmetrical sudden onset of onychomadesis and no other clinical signs. All these patients underwent an extensive evaluation including haemograms, urinalysis, serum biochemistry panels and skin sampling. A lichenoid interface dermatitis was reported histopathologically; other test results were nonconclusive. The authors proposed the name 'symmetrical lupoid onychodystrophy' for this claw disorder. German shepherd dogs seemed to be predisposed.⁷ Harvey and Markwell⁴ examined the mineral composition of claws in 21 dogs with idiopathic onychomadesis and compared it with that of 32 normal dogs. The concentration of a number of minerals was increased in the dogs with claw disease including calcium and phosphorus; thus, a lack of calcium or phosphorus was considered an unlikely cause. There was a significant difference in mineral content between affected German shepherd dogs and other affected breeds, suggesting a different pathogenesis of onychomadesis in German shepherds. In a later prospective study, Mueller *et al.*⁵ evaluated 24 dogs with exclusive claw disease with an extensive diagnostic work-up. Again, 6 of 24 dogs were German shepherds: a significant overrepresentation. A genetic predisposition for German shepherds seems likely based on the published literature. The pathogenesis so far has not been completely elucidated, but allergic, infectious and immune-mediated diseases have all been associated with symmetrical onychomadesis.⁵ Thus, the lupoid reaction seen histopathologically and the clinical signs of onychomadesis may represent a reaction pattern of the claw, rather than an aetiological disease.⁵

A syndrome clinically resembling idiopathic onychomadesis has been recognized for years among breeders and owners of Norwegian Gordon and English setters (both

breeds are among the five most popular breeds in Norway, with 900–1100 puppies born annually), and the prevalence is thought to be high in this population. The disease is devastating for the dogs and their owners, as they are frequently used as hunting dogs in Scandinavia.

The aim of this study was to evaluate the history, clinical signs, histopathological changes, treatment and prognosis of symmetrical onychomadesis in Norwegian Gordon and English setters.

Materials and methods

In July 2005, a request for assistance in identifying setters with symmetrical onychomadesis was sent out to 250 small animal practices in Norway. In this letter, veterinarians were asked to contact one of the authors (M.L.Z.) if Gordon or English setters with claw disease affecting multiple claws and no other clinical signs were presented. A further inclusion criterion to participate in the study was a claw specimen. Medical records from dogs matching the inclusion criteria were obtained from the referring veterinarian with owner permission. All dogs underwent a thorough clinical and dermatological examination either by the local veterinarian or by the primary investigator (M.L.Z.). Claw specimens were obtained by biopsy under general anaesthesia using one of two techniques. In some dogs, one or two affected claws were amputated; in others, claw samples were obtained by onychobiopsy without onychectomy.⁸ Specimens were fixed in formalin, paraffin-embedded and stained with haematoxylin and eosin. A periodic acid Schiff reaction was also performed on the histopathological sections, which were evaluated by two of the authors (S.B. and R.M.). Serum biochemistry and haematology analyses, blood thyroid profiles [total thyroxine (TT4), free T4 (FT4), and thyroid-stimulating hormone (TSH)], cytological specimens from material in the claw fold, bacterial and/or fungal cultures and antinuclear antibody titres were obtained where possible.

Subsequently, a questionnaire-based telephone interview with owners of affected dogs was conducted by the primary investigator (M.L.Z.). Detailed information was obtained about the type, frequency and dates of vaccinations, feeding regimens, previous or concurrent diseases, and clinical signs of coat abnormalities (especially hypotrichosis, scales and pyoderma). Furthermore, age and time of year of disease onset, disease duration, treatment type(s), doses and response and disease outcome were recorded. The same questionnaire was mailed to 151 owners of littermates and where possible followed up with a telephone interview. Three hundred and eighty additional Gordon setter owners were randomly selected from the Norwegian Kennel Club register and asked to complete the same questionnaire by telephone interview or email. The dogs whose owners responded to that questionnaire formed the control group.

Using Fisher's exact test, the incidence of conjunctivitis, otitis externa, skin infections and hypothyroidism was compared between the control group and the affected group of setters; a *P*-value of < 0.05 was considered significant.

Results

Study participants

Twenty-two dogs were enrolled in the study: 8 male and 10 female Gordon setters and 2 male and 2 female English setters. All of the English setters were black and white and both black and white nails were affected. Eighteen of the dogs were used as hunting dogs every September.

All of the dogs were fed commercial dog food and table scraps. One dog underwent a commercial elimination diet with Hill's Canine z/d™ Low Allergen (Kruuse As, Drøbak, Norway) diet for 6 weeks and subsequent rechallenge with normal food without any influence on the onychomadesis.



Figure 1. Onychomadesis involving all digits on the front foot of a Gordon setter.

The age of the dogs at the time of sampling varied between 2 and 11 years. The age of onset of disease varied between 2 and 7 years with a mean and a median of 4 years and there was no sex difference (*P* = 0.077).

Seven of the owners reported that they had first noticed onychomadesis during the hunting season in August–September. The others did not recognize any relationship between stress, oestrous, hunting or other illness and the outbreak of onychomadesis. In 15 of the dogs, disease onset was between May and September (Table 1).

One dog had onychomadesis on all claws of one front paw only. Three showed no involvement of dew claws but involvement of all the other claws. In the rest of the dogs, onychomadesis started with one claw and then spread to all claws within a few weeks (Fig. 1).

Six dogs had a clinical history of recurrent otitis externa, 10 dogs recurrent conjunctivitis and three dogs previously had superficial pyoderma (Table 1). Dog number 14 had recurrent otitis externa, conjunctivitis and pyoderma. Owners recognized neither a seasonal pattern nor a correlation of these symptoms to the onset of onychomadesis.

Clinicopathological changes

In eight of the diseased dogs, routine blood biochemistry and haematology analyses were performed. In two dogs, albumin and globulin were slightly decreased and, in another two, alanine transferase and alkaline phosphatase concentrations were increased, although the values were less than doubled. No other significant abnormalities were found. A thyroid profile (TT4, FT4 and TSH) was obtained in seven of these and one additional diseased dog. Five were within reference values and one was previously diagnosed with hypothyroidism that was well controlled on T4 supplementation. Two had values of FT4 just below the reference range associated with normal TSH values. A euthyroid sick syndrome due to the onychomadesis was suspected. The dog with hypothyroidism had been diagnosed 3 years prior to developing the claw

Table 1. Age and month of disease onset, vaccination times and concurrent diseases or clinical signs in setters with onychitis/onychomadesis

Dog number	Age at disease onset	Month of disease onset	Time of annual vaccination	Previous otitis externa	Previous conjunctivitis	Previous pyoderma
1	3	May 2005	September	No	Yes, 4–6 times	No
2	3	May 2002	March	No	No	Yes, 2 times
3	2	August 2005	June	No	Yes, 1–3 times	No
4	5	September 2005	June	No	No	No
5	5	October 2005	January	No	Yes, 4–6 times	No
6	3	September 2005	Not known	No	No	No
7	3	November 2005	April	No	Yes, 1–3 times	No
8	4	February 2006	October	No	No	No
9	5	June 2006	February	No	No	No
10	2	September 2006	September	Yes, 4–6 times	No	Yes, 1–3 times
11	4	June 2004	January	Yes, 4–6 times	Yes, 1–3 times	No
12	7	Not known	January	No	No	No
13	3	May 2004	June	Yes, 1–3 times	No	No
14	3	August 2005	July	Yes, 1–3 times	Yes, 1–3 times	Yes, 1–3 times
15	5	June 2005	August	No	No	No
16	6	April 2004	Not vaccinated	No	Yes, 1–3 times	No
17	3	September 2005	April	No	Yes, 1–3 times	No
18	4	August 2006	June	No	Yes, 1–3 times	No
19	4	July 2006	September	No	Yes, 1–3 times	No
20	4	June 2006	March	Yes, 1–3 times	No	No
21	4	January 2007	June	Yes, 1–3 times	No	No
22	6	October 2006	May	No	No	No

problems. An antinuclear antibody titre was determined in five of these dogs; in two the titres were weakly positive.

All dogs had paronychia associated with onychomadesis, but swabs of the claw folds for bacterial culture and sensitivity were only obtained in seven. The results showed a mixed infection of Gram-positive and -negative organisms in three dogs, *Enterococcus* spp. in one and *Staphylococcus intermedius* in three. Fungal cultures obtained from three dogs were negative

Six of the dogs were sampled by biopsy during a recurrence of their previously clinically diagnosed disease. Specimens from 16 dogs were obtained during the first onset of disease. None of the dogs had been treated with prednisolone or other immunosuppressive drugs prior to sampling. Two dogs had received fatty acid supplementation and/or antibiotics for 7 days prior to biopsy. In three dogs, one or two affected claws were amputated. In the other dogs, claw biopsies were obtained by onychobiopsy without onychectomy.⁸

Histopathological features varied from dog to dog, but typically included features of interface dermatitis with subepidermal cleft formation, pigment incontinence, basal cell vacuolization and necrosis, spongiosis and lymphocytic exocytosis, a lymphocytic, plasmacytic subepidermal inflammation and fibroplasia. In two dogs, histopathological signs of infection (including cocci in the stratum corneum and intracorneal neutrophilic pustules) were present. In general the samples obtained by claw amputation were easier to interpret, largely because there was more material available, but in some of the cases biopsied without onychectomy, the technical orientation of the sample in the block was also not optimal.

One dog was never and two were only rarely vaccinated. Seventeen were vaccinated with live attenuated canine distemper virus, parvovirus type 2, and adenovirus type 2, parainfluenza virus and attenuated *Bordetella bronchiseptica* every other year. Two dogs were vaccinated annually against

rabies and leptospirosis. The time between vaccination and onset of disease varied from 1 to 12 months and no relationship between time of vaccination and time of disease onset was apparent (Table 1).

Treatment and disease outcome

All dogs except one received fatty acid supplementation for various durations, most received a commercial fatty acid supplementation containing 150 mg mL⁻¹ eicosapentaenoic acid, 94 mg mL⁻¹ docosahexaenoic acid and 1.0 mg mL⁻¹ vitamin E (Dr Baddaky fish oil®, Dr Baddaky, Skotterud, Norway). Approximately half of the dogs initially also received short-term oral tetracycline and niacinamide. Prednisolone, carprofen and/or antimicrobial therapy were also used; treatment durations and doses are listed in Table 2.

Two dogs responded well after treatment for 6 months; regrowth of normal claws ensued. These two dogs were treated with prednisolone, fatty acid supplementation, niacinamide and tetracyclines and with intensive nail cutting and trimming. Eleven dogs exhibited onychodystrophy characterized by soft and scaly claws but no recurrence of onychomadesis. Six had intermittent painful recurrences of onychomadesis, usually when not on some sort of therapy. Three were euthanized due to the disease (Table 2).

Further information available from littermates and randomly selected dogs

Of the 151 owners of littermates of included dogs, 101 sent back the questionnaire and were subsequently interviewed via telephone. Of the 380 randomly selected owners, 104 responded. The overall prevalence of symmetrical onychomadesis was 12.6% (13/104) in the randomly selected Gordon setter owners and 34.3% (35/101) in the affected dogs included in the study and their littermates. The latter group consisted of the 22 dogs included in the

Table 2. Summary of treatment of setters with onychitis/onychomadesis

Dog number	Initial treatment	Maintenance treatment	Outcome
1	Cefalexin (20 mg kg ⁻¹ q 12 h)	Niacinamide (500 mg q 24 h), fatty acids, prednisolone (20 mg q 24 h)	Onychodystrophy, not painful when on prednisolone (20 mg q 24 h)
2	Trimethoprim/sulfonamid, prednisolone (20 mg q 24 h), fatty acids	Fatty acids	Onychodystrophy not painful
3	Fatty acids, niacinamide (500 mg q 12 h), tetracycline (500 mg q 8 h), prednisolone (0.5 mg kg ⁻¹ q 48 h)	Fatty acids, prednisolone (0.5 mg kg ⁻¹ q 48 h), niacinamide (500 mg q 12 h)	Normal claws
4	Cefalexin (25 mg kg ⁻¹ q 12 h), fatty acids, prednisolone (1.5 mg kg ⁻¹ q 24 h)	Fatty acids	Normal claws
5	Clavulanic acid/amoxicillin (10 mg kg ⁻¹ q 12 h), fatty acids, niacinamide (500 mg q 12 h × 60 days), tetracycline (500 mg q 8 h × 60 days)	Fatty acids	Nonpainful onychodystrophy, relapse in November 2006 on four claws with pain and exudate
6	Prednisolone (20 mg q 24 h)		Euthanized 2 months after diagnosis
7	Fatty acids, niacinamide (500 mg q 12 h × 60 days), tetracycline (500 mg q 8 h × 60 days)	Fatty acids	Nonpainful onychodystrophy
8	Niacinamide (500 mg q 12 h × 60 days), tetracycline (500 mg q 8 h × 60 days), fatty acids, Hills Z/D, prednisolone (20 mg q 24 h × 14 days)	Fatty acids on and off	Nonpainful onychodystrophy with relapse in May
9	Prednisolone (20 mg daily), fatty acids, niacinamide (500 mg q 12 h), tetracycline (500 mg q 8 h)	Fatty acids, niacinamide (500 mg q 12 h), tetracycline (500 mg q 8 h)	Euthanized due to relapse of disease, 3 months after diagnosed
10	Prednisolone (20 mg 24 q), fatty acids, niacinamide (500 mg q 12 h × 50 days), tetracycline (500 mg q 8 h × 60 days)	Fatty acids	Claws still dystrophic after 1 month, then lost to follow-up. Euthanized by another veterinarian
11	Prednisolone (10 mg q 24 h), fatty acids, niacinamide (500 mg q 12 h × 60 days), tetracycline (500 mg q 8 h × 60 days), clavulanic acid/amoxicillin (200 mg q 12 h × 14 days)	Prednisolone (10 mg q 24 h), fatty acids	Nonpainful onychodystrophy, when on prednisolone
12	Fatty acids, clavulanic acid/amoxicillin (200 mg q 12 h × 14 days)	Thyroxine (500 g q 24 h, fatty acids)	Normal claws in between relapses. The owner believes relapses follow cessation of fatty acid supplementation
13	Fatty acids, enrofloxacin (75 mg q 24 h × 30 days), prednisolone (20 mg q 24 h × 10 days, 10 mg q 24 h × 10 days, 10 mg q 48 h × 30 days)	Fatty acids	Onychodystrophy
14	Clavulanic acid/amoxicillin (200 mg q 12 h × 14 days), prednisolone (2 mg kg ⁻¹ q 24 h), fatty acids, vitamin B	Fatty acids	Onychodystrophy
15	Cefalexin (500 mg q 12 h × 20 days), clavulanic acid/amoxicillin (200 mg 12 q for 14 days), tetracycline (500 mg q 8 h × 60 days), vitamin C and fatty acids	Fatty acids, vitamin C	Onychodystrophy
16	None	None	Onychodystrophy
17	Prednisolone and tetracycline (500 mg q 8 h × 30 days)	Fatty acids	Onychodystrophy with recurrent episode of pain
18	Tetracycline (500 mg q 8 h × 60 days), fatty acids 1 month	None	Onychodystrophy
19	Carprofen (4 mg kg ⁻¹ q 12 h × 5 days), niacinamide (500 mg q 12 h × 90 days), tetracycline (500 mg q 12 h × 60 days), prednisolone (20 mg q 24 h × 14 days, 10 mg q 24 h × 30 days)	Prednisolone (10 mg q 48 h), niacinamide (500 mg q 12 h), fatty acids	Onychodystrophy
20	Carprofen (4 mg kg ⁻¹ q 12 h × 5 days), fatty acids, tetracycline (500 mg q 8 h × 60 days)	None	Nonpainful onychodystrophy
21	Carprofen (4 mg kg ⁻¹ q 12 h q × 5 days), fatty acids, clavulanic acid/amoxicillin (200 mg q 12 h × 14 days)	Fatty acids	Onychodystrophy
22	Carprofen (4 mg kg ⁻¹ q 12 h × 5 days), niacinamide (500 mg q 12 h × 90 days), tetracycline (500 mg q 12 h × 60 days), fatty acids	Fatty acids	Onychodystrophy

Fatty acids: Dr Baddaky fish oil® (Dr Baddaky, Skotterud, Norway).

study and 13 more affected littermates. In one litter, 6 of 11 Gordon setters developed symmetrical onychomadesis and in another litter 4 of 8 had the disease. There was no significant difference in the frequency of hypothyroidism, skin infections, conjunctivitis or otitis externa between affected dogs and the control group. However, a trend to significance was observed in dogs with conjunctivitis ($P = 0.053$).

Discussion

This study evaluated symmetrical onychitis and onychomadesis in Norwegian Gordon and English setters. The overall prevalence of symmetrical onychomadesis was 12.6% in the control group and 34.7% in the litters of the included dogs. Although the true prevalence of the disease in Gordon setters is unknown (as only 104 of 380 owners replied to the questionnaire), the occurrence of claw disease in this breed based on our study is high enough to postulate a distinct breed predisposition. Based on previous reports, German shepherd dogs were most likely predisposed.^{4,5,7} The findings of this study emphasize the differences in breed predispositions in different locations.

Mueller *et al.*⁵ identified reproducible onychomadesis associated with an adverse food reaction in one dog and resolution of clinical signs following an elimination diet in three others, and suggested the lupoid changes to be a reaction pattern of the canine claw from a number of possible causes including allergic skin disease. Due to the nature of this study (multiple practitioners and/or long distances with follow-up predominantly via telephone), there was no attempt made to rule out adverse food reactions in the setters, although owners were questioned about feeding regimens. However, the authors firmly believe that in dogs with symmetrical onychomadesis an appropriately formulated elimination diet for at least 12 weeks should be recommended to rule out an adverse food reaction.

Conjunctivitis, otitis externa and recurrent skin infections are known clinical signs or complications of allergic skin disease.⁹ The prevalence of conjunctivitis, skin infections, and otitis externa in setters with claw disease was compared with that of a randomly chosen control group of setters. There was no significant difference between the two groups, although there was an observed trend for setters with claw disease to develop conjunctivitis more frequently. These findings do not support an allergic aetiology resulting in concurrent clinical signs consistent with atopic dermatitis in the dogs with claw disease.

Most dogs developed the claw disease during summer when they are trained more intensively for hunting in September. It is possible that mechanical stress on the claws contributes to onychomadesis. The role of increased exercise on hard surfaces and resultant increased stress on the claws is unknown and needs to be further explored. Repeated mechanical stress is a reported cause of multiple nail onychomadesis in humans.¹⁰ Whether the frequent disease onset in summer in this group of Norwegian Gordon and English setters was due to such an increased mechanical stress, an increased allergenic load, or other causes is not clear.

It has been proposed that symmetrical onychomadesis may be caused by vaccination,³ but other authors failed to find a correlation between onset of disease and time of vaccination.⁵ In this study, the time periods between vaccination and onset of disease varied widely, a number of vaccination types were used with rabies vaccination administered only in a minority of dogs, one of the dogs was never vaccinated. One of the cutaneous vaccine-induced reactions is the induction of a vasculitis. Evidence of a vasculitis was not seen histologically, although not all of the samples contained a large dermal component. It thus seems unlikely that vaccination is a cause of disease. However, as subtle early signs of the disease may be missed by owners, vaccine involvement cannot be completely ruled out.

In this study, dogs were examined and diagnostic testing performed by a number of clinicians in different practices. In seven dogs, bacterial infection was suspected based on clinical presentation and cultures. In two other studies, secondary bacterial infections were also common.^{5,7} Such infections are not surprising and most likely due to the inflammation and trauma associated with onychomadesis and/or with the subsequent licking. Cytological evaluation of the claw fold was not performed in any of the dogs. However, it may confirm a clinically relevant bacterial infection and provide rapid clues on organisms involved. Thus, cytological evaluation from swabs of the claw fold and bacterial cultures are an important part of the diagnostic work-up.

Claw samples without onychectomy have been recommended to evaluate changes of the claw matrix.⁸ Two of the authors (R.M. and S.B.) have extensive experience with this technique and specimens obtained with it provide a clear view of pathologic changes in the claw matrix. However, possible pitfalls include a lack of the clinician's experience with the technique and of the laboratory technician's experience with orienting such specimens in the paraffin blocks. Despite some poor specimens in this study, a diagnosis was not prevented in any of the cases.

Skin specimens obtained from all dogs were evaluated by two of the authors (S.B. and R.M.) and showed an interface dermatitis with plasmacytic and lymphocytic superficial dermal infiltrate. Some authors term this syndrome 'symmetric lupoid onychodystrophy'.^{1,7} The authors chose *not* to refer to these cases as symmetric lupoid onychodystrophy even though some of the samples were compatible with and supportive of such a finding. They feel such 'supportive' changes are a site-related reaction pattern and the term 'lupoid' implies an immunological aetiopathogenesis. Symmetrical onychitis, onychomadesis and onychodystrophy are more appropriate clinical terms.^{2,4}

Most dogs were treated with fatty acid supplementation for extended time periods as essential fatty acids have been reported as effective in the majority of dogs with lupoid onychodystrophy or symmetrical onychomadesis.^{2,7} However, other studies found therapy with fatty acids less efficacious.^{1,6} The total supplemented amounts of fatty acids per kilogram body weight in all four of these studies was comparable. In the present study, all dogs were supplemented, but only very few achieved remission. The role of fatty acids in the treatment of symmetrical onychomadesis remains unclear. However, based on the previous

reports and a good clinical response in some dogs in the present study, it must be regarded as a possible therapeutic trial agent. A combination of tetracyclines and niacinamide has been shown to be one of the treatments of choice in symmetrical onychomadesis in a recent study.⁶ That study did not report complete regrowth of normal claws. In the present study, most dogs did not show complete resolution on therapy. Two dogs showed complete resolution: one after therapy with prednisolone, essential fatty acids, and niacinamide and one with essential fatty acids combined with repeated trimming of the claws. Repeated trimming of the claws should help regrowth where mechanical trauma is involved by initiating or perpetuating pathogenesis. Whether remission was due to therapy is not known, as spontaneous remission has been reported.⁶

In summary, onychomadesis of multiple claws is a syndrome seen in a number of breeds but particularly prevalent in Norwegian Gordon and English setters. It is characterized by an interface onychitis, often associated with secondary bacterial infection and is partially responsive to symptomatic therapy, although complete remission is rare. The exact pathogenesis and possible underlying diseases still need to be elucidated.

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Résumé Un syndrome caractérisé par une onychomadèse de plusieurs griffes est décrit chez les Setters Gordon et anglais. Les dossiers médicaux de 18 Setter Gordon et 4 Setter anglais avec onychomadèse de plusieurs griffes ont été répertoriés entre juillet 2005 et janvier 2007. Seuls les chiens à onychomadèse symétrique et sans signe de maladie de système ont été inclus. Les lésions histopathologiques étaient variables, mais typiquement une dermatite d'interface avec fissuration subépidermique, incontinence pigmentaire, vacuolisation des cellules basales, nécrose, spongiose, infiltrat subépidermique lymphoplasmocytaire et fibroplasie étaient observés. Pour deux chiens, des signes d'infection étaient associés. L'âge à l'apparition des signes cliniques variait entre deux et sept ans, avec une moyenne de 3.9 ans, sans corrélation avec la vaccination. Six des chiens avaient des frères et sœurs avec la maladie. Cependant, une analyse de pedigree n'a pas été possible. Trois chiens ont été euthanasiés à cause de leur maladie, et deux ont présenté une repousse normale de leurs griffes. Dix sept chiens ont présenté une onychodystrophie récurrente, qui n'était pas douloureuse pendant le traitement, à base d'acides gras essentiels ou de prednisolone.

Resumen Se describe un síndrome de onicomadesis afectando varias garras en Gordon Setters Noruegos y Setter Ingleses. Se compilaron los historiales clínicos y las biopsias de 18 Gordon Setters y cuatro Setter Ingleses con onicomadesis en varias garras desde Julio 2005 a Enero 2007. Sólo se incluyeron perros con onicomadesis simétrica y sin signos de otra enfermedad. Las características histopatológicas variaron entre los perros, pero generalmente incluían dermatitis en interfase con formación de hendiduras subepidermales, incontinencia pigmentaria, vacuolización de las células basales y necrosis, espongiosis y exocitosis de linfocitos, inflamación linfocítica y plasmacítica subepidérmica y fibroplasia. En dos perros se observaron signos histopatológicos de infección superficial. La edad de aparición de la enfermedad fue entre 2 y 7 años con una media de 3.9 años y no se observó correlación con la administración de vacunas. Seis de los perros afectados también tenían hermanos con la enfermedad. Debido a la relación tan cercana de los perros afectados no fue posible realizar un análisis del mapa de pedigree. Tres perros se eutanasiaron debido a la enfermedad y dos presentaron crecimiento normal de garras. Diecisiete perros tuvieron onicodistrofia persistente típicamente sin dolor durante el tratamiento, que en la mayoría de los perros consistió en suplementación de ácidos grasos y prednisolona.

Zusammenfassung Ein Syndrom der Onychomadese mehrerer Krallen wird in diesem Artikel bei norwegischen Gordon und English Settern beschrieben. Die Krankengeschichten und die Krallenbiopsien von 18 Gordon und vier English Settern mit Onychomadese multipler Krallen wurden von Juli 2005 bis Jänner 2007 gesammelt. Es wurden nur Hunde mit symmetrischer Onychomadese und ohne Anzeichen sonstiger Krankheit inkludiert. Das histopathologische Bild variierte zwischen den einzelnen Hunden, beinhaltete aber typischerweise eine Interface-Dermatitis mit subepidermaler Spaltenbildung, Pigmentinkontinenz, Vakuolisierung der Basalzellen und Nekrose, Spongiose und lymphozytäre Exozytose, eine lymphozytär-plasmazytäre subepidermale Entzündung und Fibroplasie. Bei zwei Hunden bestanden histopathologische Hinweise auf eine oberflächliche Infektion. Das Lebensalter am Beginn der Erkrankung lag zwischen zwei und sieben Jahren mit einem arithmetischen Mittel von 3,9 Jahren, der Beginn der Erkrankung korrelierte nicht mit dem Zeitpunkt einer Impfung. Sechs der betroffenen Hunde hatten auch Geschwister mit derselben Erkrankung. Aufgrund der engen Verwandtschaft der betroffenen Hunde konnte eine Pedigree-Analyse nicht durchgeführt werden. Drei Hunde wurden wegen der Krankheit euthanasiert und bei zwei Hunden wuchsen normale Krallen nach. Siebzehn Hunde hatten eine persistierende Onychodystrophie, die während der Therapie, die bei den meisten Hunden aus der Supplementierung von Fettsäuren oder Prednisolon bestand, typischerweise nicht schmerzhaft war.

Paper II

RESEARCH

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A treatment study of canine symmetrical onychomadesis (symmetrical lupoid onychodystrophy) comparing fish oil and cyclosporine supplementation in addition to a diet rich in omega-3 fatty acids

Martine L Ziener^{1,2*} and Ane Nødtvedt²

Abstract

Background: Treatment of symmetrical onychomadesis (symmetrical lupoid onychodystrophy) is a challenging task for dermatologists. The acute phase is characterized by sloughing of claw plates and loose claws have to be removed and secondary infections treated. The goal of long-term treatment is to allow claws to re-grow with normal quality and to achieve life-long lack of recurrence. The aim of this randomized treatment trial was to see if adding fish oil or cyclosporine to a diet rich in omega-3 could improve the treatment outcome of symmetrical onychomadesis in Gordon and English setters. All dogs were fed Eukanuba Veterinary Diets Dermatitis[®] exclusively during the six month treatment trial. The treatment outcome was measured as the change in number of healthy claws during treatment, as well as the long-term effect on hunting ability and recurrence of onychomadesis. The hypothesis was that cyclosporine provides a stronger and different immune modulating property than fish oil and therefore would give a better treatment outcome in dogs with symmetrical onychomadesis eating a diet rich in omega-3 fatty acids.

Results: Six Gordon setters and one English setter were treated with 5 mg/kg cyclosporine once daily for six months and seven Gordon setters were treated with 10 ml Dr Baddaky fish oil[®] once daily for six months. All dogs were evaluated every month and the numbers of healthy claws were recorded. There was a statistically significant improvement in the number of healthy claws after six months of treatment with a median increase of 13.5 claws for both groups. However, there was no statistically significant difference between the two treatment groups regarding the improvement in number of healthy claws, as assessed using the Wilcoxon rank-sum test ($P = 0.15$). Dogs in the cyclosporine group had a median increase of 10 healthy claws after six months of treatment while the median for the fish oil group was 14. Long-term cure was not achieved with either treatment.

Conclusion: Cyclosporine and fish oil appeared to be equally effective in treating symmetrical onychomadesis when the dog is fed a diet high in omega-3.

Keywords: Randomized treatment trial, Symmetrical onychomadesis, Symmetrical lupoid onychodystrophy (SLO), Cyclosporine, Fish oil

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Background

Dogs often damage their claws during hunting, but with symmetrical onychomadesis all claws slough off within three months and when the claws grow back, they are brittle and misshapen (onychodystrophy). The dogs experience pain in the acute phase of the disease. Symmetrical onychomadesis also diminishes the dogs' hunting abilities and welfare through the lack of functional claws [1]. Previously, symmetrical onychomadesis was named canine symmetrical lupoid onychodystrophy (SLO) and was first described by Scott *et al.* [2]. The disease was reported to affect different breeds of dogs and mainly dogs between three and eight years of age. According to Scott *et al.* [2], affected dogs shed their claws (onychomadesis) without any signs of systemic disease and the claws are dystrophic when they grow back. Histopathological examinations of claw biopsies showed band-like subepidermal infiltrates predominantly along the dorsal aspect of the claw [2]. The disease is thought to be quite rare in most breeds, but Ziener *et al.* [1] described a high prevalence among Gordon and English setters in Norway.

Symmetrical onychomadesis is a difficult disease to treat for a veterinary dermatologist. The re-growth of claws and improvement takes time and is hard to monitor accurately. Removal of damaged claw plates during anaesthesia and treatment of secondary bacterial infections are essential in the acute phase of the disease. The goal of the long-term treatment is to encourage re-growth of normal quality claws and to prevent recurrence of onychomadesis. From a dermatological point of view, a dog is cured of the disease if it develops new claws of normal quality with no recurrence of onychomadesis during that dog's lifetime. However, a significant proportion of owners will report the dog as "cured" as long as the dog does not have painful relapses of onychomadesis, even though it still has onychodystrophy.

Traditionally, different immune-modulating drugs have been used to treat dogs diagnosed with symmetrical onychomadesis and complete or partial remission has been achieved. Bergvall [3] demonstrated positive effects of omega-3 and omega-6 supplementation and other treatment plans have included the use of tetracycline/niacinamide, prednisolone, pentoxifylline and azathioprine [1-5]. Amputation of claws has also been used to treat symmetrical onychomadesis with good results, but this might be considered an overly aggressive treatment option [6]. In addition, a pilot study using cyclosporine in three dogs was conducted prior to initiating the current treatment trial. All dogs had re-growth of normal claws after treatment with cyclosporine for six months (unpublished data).

The aim of this randomized treatment trial was to compare fish oil supplementation and cyclosporine for

the treatment of symmetrical onychomadesis in Gordon and English setters fed a diet rich in omega-3. The effect of treatment was measured by comparing the number of healthy claws in each dog before and after six months of treatment and a follow-up survey was done four years after the first dog was included in the study. The hypothesis was that dogs fed a diet rich in omega-3 would have a better treatment outcome by adding cyclosporine than fish oil, given the more pronounced and different immune modulating properties of cyclosporine compared to fish oil. To our knowledge this is the first randomized treatment trial comparing different treatment regimens in symmetrical onychomadesis.

Materials and methods

Study population and design

Twelve Gordon setters and one English setter diagnosed with symmetrical onychomadesis were recruited for a treatment trial at the first author's clinic in Norway from 2008 until 2010. Participation was based on informed owner consent. The execution of the project was in compliance with ethical regulations at the Norwegian University of Life Sciences. All dogs were fed Eukanuba Veterinary Diets (EVD) Dermatitis[®] exclusively from the time of initiation of treatment and for the following six months. In addition, the owners were allowed to give their dogs' potatoes and low fat white fish, as these were ingredients in the diet, but nothing else. EVD Dermatitis[®] was fed as an elimination diet during the study because neither of the owners had previously given their dog catfish or herring, which are the protein sources in EVD Dermatitis[®].

Allocation to treatment groups was based on a systematic random procedure. This means that the first dog was allocated to treatment group based on the toss of a coin and subsequently every other dog entering the study received either fish oil (Dr Baddaky fish oil[®], Dr. Baddaky AS, Skotterud, Norway) or cyclosporine (Atopica[®], Novartis, Oslo, Norway) to produce groups of equal size. In both groups, the treatment was administered per os by the owners. For practical reasons, the treatment allocation was not blinded to the owner or to the responsible veterinarian.

A thorough clinical and dermatological examination of all dogs included in the study was performed. A medical history was obtained with special emphasis on ruling out previous skin diseases as a possible cause for the symmetrical onychomadesis. The number of claws without visible signs of disease was recorded.

All dogs were placed under short time general anaesthesia to remove affected claw plates and inspect claw beds at initial presentation. Samples for bacterial culture and antimicrobial sensitivity were obtained from the

dogs that were not on antibiotics when included in the study. Bacterial culture and sensitivity testing are recommended as a part of the diagnostic workup for dogs with symmetrical onychomadesis [7]. All dogs had routine serum biochemistry and complete blood counts performed. A thyroid gland profile including thyroid stimulating hormone (TSH), total thyroxin (TT4), free thyroxin (FT4) and cholesterol was also obtained from all dogs.

The dogs received carprofen 2 mg/kg BID for five days and amoxicillin clavulanic acid 10 mg/kg BID for fourteen days following the removal of claw plates. Additionally, the owners were instructed to use socks on the dog's paws as long as the claw beds were sore and to wash the paws with antibacterial shampoo daily if exudate was present.

The diagnosis was based on clinical signs of symmetrical onychomadesis on several claws (Figure 1A and B) and by exclusion of other dermatological diseases as the cause of onychomadesis. Only English and Gordon setters with symmetrical onychomadesis on several claws were included in the study. All dogs which had previously received any form of immune suppressive therapy and all dogs showing only single digit onychomadesis were excluded from this study.

Follow up

All dogs included in this study were presented for clinical evaluation once monthly for six months. During these examinations, all claw beds were inspected and the absence or presence of re-growth of claw plates was recorded. Photo documentation of re-grown claws was performed. Each claw was either characterized as normal (Figure 1C) or as having signs of onychodystrophy (Figure 1D). For every visit the number of claws that had become normal was recorded. The outcome of the treatment trial was the difference between the number of normal claws for each individual at presentation and after six months of treatment. After six months the medication was discontinued and feeding of the dog's original diet resumed. In 2012, four years after the first dog was included in the study, a follow-up questionnaire was mailed to the owners. The owners were asked to describe their dog's claws as normal, misshapen (dystrophic) or with recurrence of onychomadesis. The dog's hunting abilities after onychomadesis was recorded. Furthermore, if the dog was not alive, the reason for death/euthanasia was recorded.

Statistical methods

The change in number of healthy claws at presentation and after six months of treatment was compared using



Figure 1 Onychomadesis in Gordon setter. A) Claw before treatment (onychomadesis); B) Claw bed after removal of horn layer; C) Normal claw after treatment; D) Onychodystrophy after treatment. Figures A and B: dog no. 3 in the cyclosporine group; Figure C: dog no. 5 in cyclosporine group; Figure D: dog no.7 in the fish oil group.

the Wilcoxon rank-sum equality of populations test. Furthermore, the effect of treatment in the two groups was compared and the same test was also used to evaluate if the sex of the dog influenced the treatment outcome. The software package Stata/SE version 11 (Statacorp, College Station, Texas) was used for the analysis. The cut-off for statistical significance was set to $P < 0.05$. A post-hoc power calculation was performed using JavaStat (<http://statpages.org/postpowr.html>).

Results

Twelve Gordon and one English setter diagnosed with symmetrical onychomadesis were included in the treatment trial. The dogs were aged between three and seven years when entering the study and this was their first appearance of onychomadesis. The sex, age and weight distributions, as well as the long-term outcome for dogs in the two treatment groups are presented in Tables 1 and 2.

Dog no. 3 in the cyclosporine group is the same dog as dog no. 7 in the fish oil group. This dog had normal claws for six months after finishing treatment with cyclosporine, before it relapsed with symmetrical onychomadesis on all claws. The dog then entered the fish oil supplementation group following random allocation.

A skin biopsy was performed in dog no. 6 from the fish oil group because of mild alopecia when included in the study. The histopathology report showed mild atrophic dermatopathy and mild superficial perivascular dermatitis. In general, the dogs showed only minor changes in albumin, globulin, alanine transferase (ALT), alkaline phosphatase (ALKP) and glucose. These were considered clinically irrelevant.

Dog no. 7 in the cyclosporine group was diagnosed with hypothyroidism when it was included in the study, based on low serum TT4, FT4, high TSH and cholesterol values. In this dog, a euthyroid state was maintained adequately by

supplementation of levothyroxine (Levaxin®, Nycomed Pharma, Oslo, Norway). Dog no. 4 in the cyclosporine group had values of TT4 of 52 nmmol/l (16–46 nmmol/l) and tested thyroglobulin autoantibodies (TgAA) negative. Dog no. 6 in the fish oil group had TT4 of 47 nmmol/l (16–46) and she was not tested for TgAA.

All dogs had paronychia associated with onychomadesis. Bacterial culture and antimicrobial sensitivity analysis was obtained in nine dogs; the remaining dogs were already on antibacterial treatment upon entering the study. The results showed three samples with beta toxin producing *Staphylococcus* sp. and one with *S. pseudintermedius* from the dogs in the cyclosporine group. From dogs in the fish oil group, there were two samples with a mixed culture of Gram positive and Gram negative bacteria and one sample with *Streptococcus canis*.

Adverse effects of cyclosporine and fish oil

None of the owners of dogs in the fish oil group reported adverse effects of the treatment. In the cyclosporine group, three owners reported that their dogs were vomiting during the initial week of treatment, but this resolved within the following four to five days, during which a small amount of food was given with the drug.

Outcome

All dogs, except one in the cyclosporine group, showed an improvement in the number of normal claws during the course of the study period. The median number of normal claws when entering the study was 5/18 for the cyclosporine group and 0/18 for the fish oil group. After six months, the median numbers of normal claws were 15/18 in cyclosporine group and 14/18 in fish oil group (Tables 1 and 2). There was a statistically significant improvement in the number of healthy claws after six months of treatment with a median increase of 13.5 claws for both groups. However, there was no statistically

Table 1 Demographic data from seven dogs diagnosed with onychomadesis allocated to cyclosporine treatment

Dog	Sex	Age of onset	Weight	Proportion of normal nails when entering study	Proportion of normal nails after six months of treatment	Long term outcome; Claw outcome; 1 = Still episodes of onychomadesis 2 = Onychodystrophy with out onychomadesis 3 = Normal claws	Time of follow up in years	Long term outcome: Is the dog used as a hunting dog?
1	Male	3	23	6/18	18/18	Euthanized after six months	4	
2	Female	6	20	0/18	18/18	2	4	No
3	Female	4	18	5/18	15/18	1	3	Yes
4 [#]	Female	6	24	10/16	7/16	2	4	No
5	Female	3	19	0/18	6/18	3	3	Yes
6*	Female	5	19	0/18	15/18	2	3	Yes
7	Male	7	23	10/18	17/18	2	3	Yes
Average		5	21	5/18 (median)	15/18 (median)	2 (median)	3.4 (3median)	

[#]Dog number four had two digits amputated.

*Dog number six was an English setter.

Table 2 Demographic data from seven Gordon setters diagnosed with onychomadesis allocated to fish oil treatment

Dog	Sex	Age of onset	Weight	Proportion of normal nails when entering study	Proportion of normal nails after six months of treatment	Long term outcome; Claw outcome; 1 = Still episodes of onychomadesis 2 = Onychodystrophy without onychomadesis	Time of follow up in years	Long term outcome: Is the dog used as a hunting dog?
1	Female	6	21	0/18	14/18	1	4	Yes
2	Male	6	22	0/18	16/18	1	4	Yes
3	Female	4	17	0/18	13/18	1	4	Yes
4	Male	7	19	4/18	18/16	1	4	Yes
5	Male	3	20	0/18	12/18	1	3	Yes
6	Female	7	15	0/18	14/18	2	3	No
7	Female	7	19	10/18	14/18	2	2	No
Average		6	19	0/18 (median)	14/18 (median)	1 (median)	3.4 (median4)	

significant difference between the two groups regarding the differences in number of healthy claws after six months of treatment as assessed using the Wilcoxon rank-sum test ($P = 0.15$). Furthermore, the change in number of healthy claws over time did not differ by sex ($P = 0.89$). A post-hoc power calculation showed that the power of this study to detect a difference in number of healthy claws of four between the fish-oil and cyclosporine groups was 30%. By doubling the sample size the observed difference could be detected with a power of 80% and a P of 0.05.

None of the owners reported any recurrence of symmetrical onychomadesis in the first month after their dog discontinued treatment and where fed their original diet.

Four years after the first dog was included in the study a questionnaire was mailed to the owners. In the cyclosporine group; one dog had been euthanized due to onychomadesis and aggressive behavior, one dog had normal claws, one still had recurrence of onychomadesis and four dogs had onychodystrophy. Four of the seven dogs were used for hunting (Table 1).

In the fish oil group; two dogs had onychodystrophy and five dogs had recurrence of onychomadesis. Five of the seven dogs were used for hunting (Table 2).

None of the dogs, in either group, had received any further treatment for their claw disease after completing the trial.

Discussion

The primary aim of this randomized treatment trial was to evaluate if adding cyclosporine to a diet rich in omega-3 could improve treatment outcome regarding canine symmetrical onychomadesis compared to adding fish oil to the same diet. A second aim was to observe the course of the disease on a long term basis after withdrawal of treatment.

Evaluating the outcome in a prospective treatment study of symmetrical onychomadesis is challenging,

because within the same dog, claws will show different stages of disease at any given time. If the inclusion criteria of this treatment trial had been absence of normal claws, the groups would have been more comparable at the start and hence the results easier to interpret.

Therefore, in order to minimize the possible effect of any difference between groups at inclusion into this study, the change in number of normal claws was used to assess treatment success. No difference in the change of number of normal claws was detected between the two groups. This could be due to low effect of adding cyclosporine to a diet high in omega-3 when treating symmetrical onychomadesis.

It was considered necessary to have a treatment period of six months for studying a disease with such a long recovery phase. The results could potentially have been different if the treatment period had been longer or shorter than six months, but earlier studies have reported efficacy of treatment within a time period of three to four months [4].

To assess the natural course of the disease without treatment, a follow up was done four years after the first dog was included in the study. Owners reported that even though none of the dogs were on any treatment, it seemed that the dogs with recurrence of onychomadesis or onychodystrophy showed less sign of pain and lameness compared to what was seen during the initial acute phase of the disease. In previous studies, effect of treatment has been reported as excellent, good, partial or poor, based on owner interviews [4]. Many owners and their dogs will consider a good response to treatment to be absence of painful relapses of onychomadesis, despite the claws showing signs of onychodystrophy. In medical terms a dog with symmetrical onychomadesis should be classified as "cured" if it has no recurrence of sloughing of claws for the remainder of its life, as well as complete re-growth of normal claws. In this study only one dog in the cyclosporine group was still "cured" after four years.

The number of “cured” dogs could probably have been higher if the dogs were kept on life-long treatment, but the question is if the dogs need this, if they only have onychodystrophy without pain and lameness. Perhaps a diet change to a diet rich in omega-3 might be enough as a long term treatment in the majority of cases, as long as the affected claws are removed in the acute phase of the disease. The results could also have been different in heavier breeds known to be predisposed to symmetrical onychomadesis such as Rhodesian ridgeback and giant schnauzers. These breeds might possibly need life-long treatment because they have a higher body weight than setters and therefore could show more pain upon recurrence.

Because Mueller *et al.* [5,7] showed that symmetrical onychomadesis could be due to food allergy in two cases, all the dogs in this trial were fed the same diet. To rule out food responsive symmetrical onychomadesis all the dogs were fed their original diet after completion of the trial, and none of the owners reported any recurrence of symmetrical onychomadesis within the first month. It was also important to assure that all dogs participating in the study got the same amount of eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA) from the diet. A dose of EPA/DHA at 50–250 mg/kg body weight appears to have an anti-inflammatory effect [8]. The mean weight of the dogs participating in the study was 20 kg and a total dose of 1–5 g EPA/DHA daily would therefore be sufficient to anticipate a treatment effect. In this study each dog had a daily intake of 3.3 g EPA/DHA from the diet. Every dog ate an average of 300 g food and in EVD Dermatitis[®] the EPA level is 0.9% per gram and the DPA level is 0.2% per gram.

In the fish oil group each dog had a daily intake of 2.7 g EPA/DHA from the fish oil supplementation. Dr Baddaky fish oil[®] contains 165 mg EPA/ml, 106 mg DHA/ml and 1.0 mg vitamin E/ml and each dog in the fish oil group received 10 ml daily for six months.

Omega-3 fatty acids, in the form of fish oil, have previously been shown to have a good effect against symmetrical onychomadesis and are relatively inexpensive with few side effects [5]. It is reported that neutrophils and macrophages obtain a higher concentration of EPA and DHA in cell membranes when dogs are fed fish oil. This might contribute to modulating the immune response so that less potent inflammatory mediators are produced. Furthermore, the expression of MHC class I and II on cell surfaces decreases in animals fed a diet rich in omega-3 fatty acids [8-11].

Cyclosporine was chosen as a therapeutic agent for comparison in this study because it had shown good response in the pilot study and has previously been used to treat canine immune mediated diseases such as atopic dermatitis and anal furunculosis [12,13]. Cyclosporine

is a potent immune suppressive agent that has been shown to inhibit the activation of most cells involved in cell-mediated immunity. The compound does not have major impact on humoral immune response in the short term.

Two dogs in the cyclosporine group and one dog in the fish oil group had complete re-growth of normal claws after six months of treatment. Twelve of the thirteen dogs had a higher number of normal claws at the end of the treatment period. To which extent the omega-3 from the EVD[®] diet contributed to the outcome of this treatment trial is unknown, because a negative control group that did not get the diet was not included in the study design.

Potential selection bias of this study was avoided through the random allocation of dogs to treatment groups. There was no blinding of the treatment for the veterinarian that performed the evaluation of the outcome and this is a limitation of the study. Blinding was not considered practically achievable because of the different formulation of the two drugs. The fact that a binominal scale was chosen to measure treatment effect (number of affected claws before and after treatment) instead of a graduated scale or subjective measurements of improvement should decrease the risk for measurement error introduced by the lack of blinding. The hypothesis was that cyclosporine together with the diet would give a better treatment outcome compared to fish oil with diet, but after thirteen dogs, no significant difference between the two groups was observed. The post-hoc power calculation showed that the power to detect a difference in improvement in the magnitude of four claws between the two treatment groups was 30%, and that a doubling of the sample-size could have resulted in a power of 80% for this difference and significance level. However, because a difference of this size was considered unlikely to represent a biologically relevant effect the inclusion of additional patients was stopped.

Conclusions

Cyclosporine and fish oil appeared to be equally effective in treating symmetrical onychomadesis when the dog is fed a diet high in omega-3. No statistically significant differences in the change in number of healthy claws were observed between dogs in the two treatment groups after six months of treatment. Symmetrical onychomadesis has been described in several other breeds and it is likely that the treatment regimens used in this trial could be used for dogs from other breeds as well. With long term follow-up, eleven of thirteen dogs continued to have onychomadesis or onychodystrophy after discontinuation of treatment and diet. However, the dogs appeared to live well with their disease after discontinuing treatment, although three dogs were not used for hunting because

of discomfort. A diet rich in omega-3 may play an important role in suppressing the inflammatory reaction that takes place in the claw fold. Because of the high costs and lack of effect of cyclosporine compared to fish oil, we are inclined to suggest that omega-3 might be the first choice of treatment for canine symmetrical onychomadesis incorporated in the diet or as a supplement. However, the current study included a limited number of dogs and further investigation is warranted before treatment guidelines for the disease can be proposed.

Abbreviations

BID: Bis in die- twice a day; DHA: Docosahexaenoic acid; DPA: Docosapentaenoic acid; EPA: Eicosapentaenoic acid; EVD: Eukanuba veterinary diets; FT4: Free thyroxin; TgAA: Thyroglobulin autoantibodies; TSH: Thyroid stimulating hormone; TT4: Total thyroxin; SLO: Symmetrical lupoid onychodystrophy.

Competing interests

The authors declare no competing interests. Dr. Baddaky supported all the dogs in the fish oil group with fish oil without cost for the owner. Nova Artis supported all the dogs in the cyclosporine group with cyclosporine without cost for the owner. Eukanuba veterinary diets gave all the dogs EVD Dermatitis for six months, as long as they participated in the study. Neither of the companies provided salary or other financial contributions to the project, nor did company representatives take part in data-analysis or writing of the manuscript.

Authors' contributions

MLZ planned the study, did all the clinical work and was responsible for drafting the manuscript. AN contributed with statistical analyses as well as writing the manuscript. Both authors read and approved the final manuscript.

Authors' information

MLZ is a veterinarian working at Fredrikstad Animal Hospital and PhD student at Norwegian School of Veterinarian Science. The working title of the thesis is "Hypothyroidism and symmetrical onychomadesis in Norwegian Gordon and English setter". MLZ is also a member of the breeding council in the Norwegian Gordon setter club. AN is a veterinary epidemiologist and professor of population medicine at the Norwegian School of Veterinary Science.

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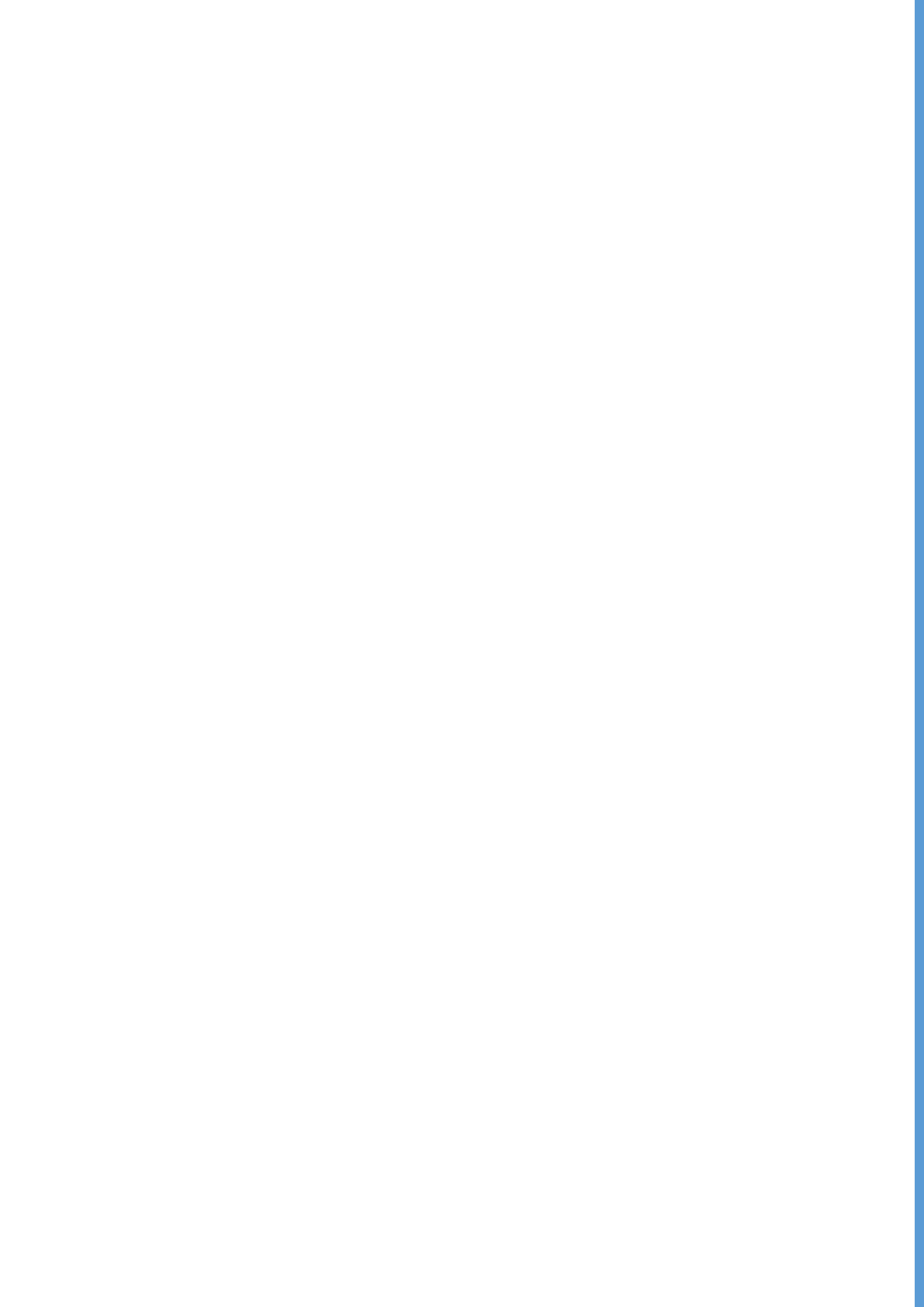
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Paper III



DLA Class II Alleles Are Associated with Risk for Canine Symmetrical Lupoid Onychodystrophy (SLO)

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Abstract

Symmetrical lupoid onychodystrophy (SLO) is an immune-mediated disease in dogs affecting the claws with a suggested autoimmune aetiology. Sequence-based genotyping of the polymorphic exon 2 from DLA-DRB1, -DQA1, and -DQB1 class II loci were performed in a total of 98 SLO Gordon setter cases and 98 healthy controls. A risk haplotype (DRB1*01801/DQA1*00101/DQB1*00802) was present in 53% of cases and 34% of controls and conferred an elevated risk of developing SLO with an odds ratio (OR) of 2.1. When dogs homozygous for the risk haplotype were compared to all dogs not carrying the haplotype the OR was 5.4. However, a stronger protective haplotype (DRB1*02001/DQA1*00401/DQB1*01303, OR=0.03, 1/OR=33) was present in 16.8% of controls, but only in a single case (0.5%). The effect of the protective haplotype was clearly stronger than the risk haplotype, since 11.2% of the controls were heterozygous for the risk and protective haplotypes, whereas this combination was absent from cases. When the dogs with the protective haplotype were excluded, an OR of 2.5 was obtained when dogs homozygous for the risk haplotype were compared to those heterozygous for the risk haplotype, suggesting a co-dominant effect of the risk haplotype. In smaller sample sizes of the bearded collie and giant schnauzer breeds we found the same or similar haplotypes, sharing the same DQA1 allele, over-represented among the cases suggesting that the risk is associated primarily with DLA-DQ. We obtained conclusive results that DLA class II is significantly associated with risk of developing SLO in Gordon setters, thus supporting that SLO is an immune-mediated disease. Further studies of SLO in dogs may provide important insight into immune privilege of the nail apparatus and also knowledge about a number of inflammatory disorders of the nail apparatus like lichen planus, psoriasis, alopecia areata and onycholysis.

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Introduction

Canine symmetrical lupoid onychodystrophy (SLO) is described as separation and sloughing of several claws from claw beds and ultimately affecting all claws. The pathogenesis of SLO is incompletely elucidated, but allergic, infectious and immune-mediated diseases have all been associated with symmetrical onychomadesis [1]. Thus, the lupoid reaction observed histopathologically and the clinical signs of onychomadesis represent an immune-mediated disease of the claw, rather than an actual triggering event of the disease. Initially, separation of claw from claw bed and subsequent sloughing is noted on one or more claws, but within two to three months all claws might be affected. Regrowth results in dystrophic claws, brittle, crumbling and misshapen claws [2]. A thorough clinical investigation is important for a correct diagnosis [3]. Histopathology of affected claws present infiltrates of mononuclear cells, apoptosis of the epidermal

basal cells and hydropic degeneration of the epidermal basal cells. Typically the inflammatory infiltrate forms a parallel band to the basement membrane called lichenoid pattern. Pigmentary incontinence is also often observed in the dermis [4]. Treatment with immunosuppressive drugs, such as glucocorticoids, has been reported to be successful [5] as well as fatty acid supplementation [6] suggesting an autoimmune aetiology of the disease.

The genomic structure in the dog is unique, with long haplotype blocks and extensive linkage disequilibrium. Dog and human share a similar set of orthologous genes, lives in the same environment and are affected by diseases of similar aetiology. Therefore, the dog is an excellent model for studies on genetic diseases [7–9].

Autoimmune diseases in humans have complex patterns of inheritance [10]. In humans, several keratin disorders exist with similarities to SLO [11]. Inflammatory diseases of the nail are common. This includes the group of non-infectious inflammatory disorders of the nail apparatus like psoriasis, lichen planus and other

lichenoid diseases, alopecia areata, pemphigus vulgaris, as well as erythema multiforme [12]. Nail changes in lupus erythematosus are not common, but in acute lupus erythematosus, onycholysis is the most common symptom. Nail loss is uncommon but has been observed. Lichen planus is affecting the nail more often than lupus erythematosus and may lead to complete nail loss, either insidiously or relatively rapidly in case of ulcerating lichen planus [12]. In dogs, SLO is a significant health concern and appears to be the most common immune-mediated claw disease [4]. Particularly high disease prevalence has been reported in certain breeds such as Gordon setters (12.6%) [13], [2]. English setters, giant schnauzers (10%) and also bearded collies are reported to have a high incidence of SLO (unpublished observation). The increased risk in specific breeds suggests a significant genetic component, influencing the power of mapping the genetic risk factors underlying SLO. The inheritance of the disease has not been reported but is suggested to be complex. Studies on pedigrees, indicate large differences in incidence between breeding lines and an increased risk in relatives of affected dogs (unpublished observation) supporting a high heritability for the diseases.

In the dog, the major histocompatibility complex (MHC) class II is called DLA (dog leukocyte antigen) class II and consists of three highly polymorphic genes known as DLA-DRB1, DLA-DQA1 and DLA-DQB1 and one monomorphic gene, DLA-DRA [14], [15]. Due to the high linkage disequilibrium in the region and the extensive polymorphism in exon 2 (which encodes the antigen-binding domain), genetic typing of this exon is likely to detect most of the variation in the locus. There are currently 106 DLA-DRB1, 26 DLA-DQA1 and 62 DLA-DQB1 alleles identified in the dog [15]. The DLA class II genes are in high linkage disequilibrium (LD). However, the extension of LD within this region remains unknown. Many previous studies have identified DLA class II as a genetic risk or protective factor for various autoimmune or immune-mediated diseases in dogs [16-22]. In humans the association to MHC class II is also well established in autoimmune diseases [16].

The aim of this study was to elucidate the potential associations between DLA class II and risk of developing SLO.

Results

Haplotypes, alleles and genotypes

In 196 Gordon setters, a total of 10 different DLA-DRB1/DQA1/DQB1 haplotypes were identified. The majority of the

dogs (43.4%) carried haplotype DRB1*01801/DQA1*00101/DQB1*00802. The other nine haplotypes ranged in frequencies between 0.3–12.8% (Table 1). Also, 10 DRB1 alleles, six DQA1 alleles and eight DQB1 alleles were found in the population (Table 2). DRB1*01801 was the most common allele with a frequency of 43.4% and the other nine DRB1 alleles had frequencies between 0.3–12.8%. 74.2% of Gordon setters had allele DQA1*00101 and the other alleles ranged in frequency between 0.3–11.2%. Eight DQB1 alleles were found with DQB1*00802 being the most common (43.4%) and the others in frequencies between 0.3–25.0%. We also identified 34 different genotypes (haplotype combinations) with frequencies between 0.5–18.9% (Table S1).

Five haplotypes were found in the 10 bearded collies. The two most common, DRB1*01801/DQA1*00101/DQB1*00802 and DRB1*01801/DQA1*00101/DQB1*00201 had frequencies of 50% and 30%, respectively. The other haplotypes ranged in frequencies between 5–10% (Table S2). There were three DRB1 alleles (*01801, *00201 and *01501) and three DQA1 alleles (*00101, *00901 and *00601). Five DQB1 alleles were identified. Two were common (30–50%) and three were rare (5–10%) (Table S3). A total of four different genotypes were found (Table S1).

In the 110 giant schnauzers, we identified 10 haplotypes. Four were common (13.6–23.6%) and six were rare (0.5–9.%) (Table S4). We also identified eight DRB1 alleles, four DQA1 alleles and six DQB1 alleles (Table S5). We found 32 genotypes (frequencies of 0.9–10.0%) (Table S1).

Disease association

In Gordon setters, two haplotypes differed markedly in frequency between cases and controls. The haplotype DRB1*01801/DQA1*00101/DQB1*00802 occurred in 52.6% of the cases compared to 34.2% of the controls (OR = 2.1, $p < 0.001$) and was defined as a risk-haplotype. This haplotype was also the most common haplotype in the Gordon setter population (43.4%). The allele frequencies of each of the genes involved in the risk haplotype were higher in cases compared to controls and showed a significant disease association (DRB1*01801: 52.6% vs. 34.2%, OR = 2.1, $p < 0.001$; DQA1*00101: 83.7% vs. 64.8%, OR = 2.8, $p \leq 0.001$ and DQB1*00802: 52.6% vs. 34.2% OR = 2.1, $p \leq 0.001$). All of these alleles are the most common alleles in the population with frequencies of 43.4%, 74.2% and 43.3% (DRB1/DQA1/DQB1, respectively).

Table 1. Haplotype frequencies in Gordon setter.

Number	Haplotype DRB1/DQA1/DQB1	Total population % (392)	Cases % (196)	Controls % (196)
1	01801/00101/00802	43.4 (170)	52.6 (103)	34.2 (67)
2	01501/00601/02301	4.6 (18)	2.6 (5)	6.6 (13)
3	1800103/00101/00201	8.2 (32)	8.2 (16)	8.2 (16)
4	00101/00101/00201	12.8 (50)	15.3 (30)	10.2 (20)
5	02001/00401/01303	8.7 (34)	0.5 (1)	16.8 (33)
6	04901/01001/01901	11.2 (44)	12.8 (25)	9.7 (19)
7	00901/00101/00801	5.9 (23)	1.5 (3)	10.2 (20)
8	00601/00501/00701	0.3 (1)	0.0 (0)	0.5 (1)
9	00501/00301/00501	1.0 (4)	0.5 (1)	1.5 (3)
10	10102/00101/00201	4.1 (16)	6.1 (12)	2.0 (4)

A total of 10 different haplotypes were found. DRB1*01801/DQA1*00101/DQB1*00802 had an increased frequency in cases and DRB1*02001/DQA1*00401/DQB1*01303 was significantly more frequent in controls, both numbers shown in bold.

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Table 2. DLA DRB1/DQA1/DQB1 allele frequencies in Gordon setter.

Allele	Total population % (392)	Cases % (196)	Controls % (196)
DRB1			
01801	43.4 (170)	52.6 (103)	34.2 (67)
01501	4.6 (18)	2.6 (5)	6.6 (13)
1800103	8.2 (32)	8.2 (16)	8.2 (16)
00101	12.8 (50)	15.3 (30)	10.2 (20)
02001	8.7 (34)	0.5 (1)	16.8 (33)
04901	11.2 (44)	12.8 (25)	9.7 (19)
00901	5.9 (23)	1.5 (3)	10.2 (20)
00601	0.3 (1)	0.0 (0)	0.5 (1)
00501	1.0 (4)	0.5 (1)	1.5 (3)
10102	4.1 (16)	6.1 (12)	2.0 (4)
DQA1			
00101	74.2 (291)	83.7 (164)	64.8 (127)
00601	4.6 (18)	2.6 (5)	6.6 (13)
00401	8.7 (34)	0.5 (1)	16.8 (33)
01001	11.2 (44)	12.8 (25)	9.7 (19)
005011	0.3 (1)	0.0 (0)	0.5 (1)
00301	1.0 (4)	0.5 (1)	1.5 (3)
DQB1			
00802	43.4 (170)	52.6 (103)	34.2 (67)
02301	4.6 (18)	2.6 (5)	6.6 (13)
00201	25.0 (98)	29.6 (58)	20.4 (40)
01303	8.7 (34)	0.5 (1)	16.8 (33)
01901	11.2 (44)	12.8 (25)	9.7 (19)
008011	5.9 (23)	1.5 (3)	10.2 (20)
00701	0.3 (1)	0.0 (0)	0.5 (1)
00501	1.0 (4)	0.5 (1)	1.5 (3)

Altogether, 10 DRB1 alleles, six DQA1 alleles and eight DQB1 alleles were found in the population. The alleles DRB1*01801, DQA1*00101 and DQB1*00802 was observed in a higher frequency in cases while the alleles DRB1*02001, DQA1*00401 and DQB1*01303 was more frequent in controls. Numbers in bold indicate significant different between cases and controls.
doi:10.1371/journal.pone.0012332.t002

A lower frequency of SLO was found in Gordon setter dogs carrying haplotype DRB1*02001/DQA1*00401/DQB1*01303. This haplotype is rare in the population (8.7%) and was found in 16.8% of the controls compared to 0.5% of the cases (OR = 0.03, $p \leq 0.0001$) and thus is significantly associated with protection against disease development. Calculation of the allele frequencies at each of the three loci separately showed the same results, as these alleles were unique to this haplotype.

Gordon setter dogs homozygous for the risk haplotype, DRB1*01801/DQA1*00101/DQB1*00802 had even higher odds for developing disease. In fact, 18.9% in the population was homozygous for this risk haplotype, which was found in 28.6% of cases compared to 9.2% of controls (OR = 4.0, $p = 0.001$). None of the 11 dogs heterozygous for the risk and the protective haplotypes had SLO, emphasizing the strength of the protective allele (Table 3).

We also compared the dogs homozygous for risk haplotype vs. those not carrying this haplotype (OR = 5.41, $p = 0.0004$) and vs. heterozygous for risk haplotype (OR = 3.24, $p = 0.01$) and finally heterozygous for risk haplotype vs. not carrying risk haplotype (OR = 1.67, $p = 0.17$) (Table 4). However, when the protective haplotype was removed from the analysis, the odds ratio for homozygosity for the risk haplotype vs. all without risk alleles was 2.97 (Table 4), for homozygotes vs. heterozygotes the OR was 2.52 and for heterozygotes vs. no risk the OR was 1.18 although none of these results were significant based on the smaller sample size.

Comparative analysis between breeds

Bearded collies and giant schnauzers were analyzed for comparative purposes, despite small sample numbers. Interestingly, the risk haplotype found in Gordon setter (DRB1*01801/DQA1*00101/DQB1*00802) was also found in increased frequency among the bearded collie cases (cases 40% vs. controls 20%). A similar haplotype (DRB1*01801/DQA1*00101/DQB1*00202), where only the DQB1 allele differs from the risk haplotype was also identified (cases 60% vs. controls 40%). These two haplotypes are the only ones occurring in cases, whereas in controls, three more haplotypes exist. When evaluating the alleles it appears that all bearded collie cases carry DRB1*01801 and DQB1*00101, further supporting the findings in Gordon setter.

In giant schnauzer we observed a higher number of possible cases carrying haplotype DRB1*00101/DQA1*00101/DQB1*00201 compared to controls (26.9% vs. 15.0%). This risk haplotype of DRB1 and DQB1 differs from Gordon setter and

Table 3. Haplotypes, alleles and genotypes in Gordon setter cases and controls.

Haplotype, allele or genotype	Total population % (392)	Cases % (196)	Controls % (196)	OR	P-value	99% CI
01801/00101/00802	43.4 (170)	52.6 (103)	34.2 (67)	2.1	0.0004	1.3–3.6
02001/00401/01303	8.7 (34)	0.5 (1)	16.8 (33)	0.03	0.0001	0.002–0.35
DRB1*01801	43.4 (170)	52.6 (103)	34.2 (67)	2.1	0.0004	1.25–3.64
DRB1*02001	8.7 (34)	0.5 (1)	16.8 (33)	0.03	<.0001	0.002–0.35
DQA1*00101	74.2 (291)	83.7 (164)	64.8 (127)	2.8	<.0001	1.48–5.23
DQA1*00401	8.7 (34)	0.5 (1)	16.8 (33)	0.03	<.0001	0.002–0.35
DQB1*00802	43.4 (170)	52.6 (103)	34.2 (67)	2.1	0.0004	1.25–3.64
DQB1*01303	8.7 (34)	0.5 (1)	16.8 (33)	0.03	<.0001	0.002–0.35
Genotype 1	18.9 (37)	28.6 (28)	9.2 (9)	3.96	0.001	1.36–11.52
Genotype 5	5.6 (11)	0 (0)	11.2 (11)	0	0.002	0

Odds ratio (OR) and Yates p-values were calculated for differences greater than 10% between cases and controls. A 99% confidence interval (CI) was used.
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Table 4. Comparison of OR for SLO depending on the number of risk haplotypes.

Haplotype	Cases (n)	Controls (n)	OR	Yates P-value	99% CI
Homozygous risk	28	9	5.41	0.0003	1.64–17.88
No risk	23	40			
Homozygous risk	28	9	2.97	0.04	0.84–10.49
No risk - no protection	22	21			
Homozygous risk	28	9	3.24	0.01	1.06–9.93
Heterozygous risk	47	49			
Homozygous risk	28	9	2.52	0.05	0.81–7.83
Heterozygous risk - no protection	47	38			
Heterozygous risk	47	49	1.67	0.17	0.71–3.92
No risk	23	40			
Heterozygous risk - no protection	47	38	1.18	0.79	0.045–3.10
No risk - no protection	22	21			
Homozygous risk	28	9	93.3	<. 0001	5.68–1532
Protection	1	30			

The difference in OR according to the number of risk haplotypes (homozygous- heterozygous-none). Risk haplotype is DRB1*01801/DQA1*00101/DQB1*00802 and protective haplotype is DRB1*02001/DQA1*00401/DQB1*01303.
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bearded collie, but has the same risk DQA1 allele as the risk haplotype found in Gordon setter and bearded collie further supporting the findings in these breeds. When DQA1*00101 was analyzed separately in giant schnauzer it was not significantly associated with an increased risk. We also identified a protective haplotype, DRB1*01301/DQA1*00301/DQB1*00501 in giant schnauzer.

Discussion

In the present study we have evaluated the association of MHC class II haplotypes and alleles with symmetrical lupoid onychodystrophy in a large cohort of Gordon setter and smaller numbers from two additional breeds of dogs, the giant schnauzer and the bearded collie. The analysis revealed that DLA class II is significantly associated with SLO and provides support for the hypothesis of SLO being an immune-mediated disease. Genetic association between DLA and SLO and similar claw conditions has been established in all the breeds analyzed here even if the number of dogs studied and the level of significance varied between breeds. We found that dogs homozygous for the risk haplotype had elevated risk of 2.97 of developing SLO, but that a rare protective haplotype had a stronger 33-fold protective effect. The protective haplotype had an uneven distribution between cases and controls and only three (10%) of the controls were homozygous. Because 90% of the controls were heterozygotes, including 11 (37%) heterozygotes for the combination of risk and protective haplotypes, the results indicate a dominant effect of the protective haplotype. It is worth emphasizing the very high OR of 93.3 when comparing dogs homozygous for the risk haplotype vs. dogs with at least one protective haplotype, even if the observed number of the protective haplotype is low in cases (Table 4).

The relatively high frequency of dogs homozygous for DLA class II haplotypes, provides a unique opportunity to estimate an unbiased risk effect of the most common haplotypes, and to compare homozygote vs. heterozygote dogs. In humans, homozygosity for HLA haplotypes is extremely rare, preventing analyses of genetic risk association between MHC class II and immune-

mediated disease in homozygotes. The comparison of OR depending on the presence of two (homozygous) or one (heterozygous) risk haplotype should be carefully interpreted (Table 4). Comparison of the group of dogs being homozygous for the risk haplotype to those with no risk haplotype (OR = 5.41), to the heterozygotes (OR = 3.24) and the heterozygotes versus those with no risk (OR = 1.67) could indicate a co-dominant effect of the risk haplotype.

When we compared the SLO risk haplotype in the Gordon setter, with those of the bearded collie and the giant schnauzer, we observed that the DRB1-DQA1-DQB1 haplotypes were relatively similar although the sample number was small. Even if there were some differences, all the major risk haplotypes in the three breeds contained the same DQA1 allele. Strikingly, the DQA1*00101 molecule associated with SLO has an Arg at position 55 instead of an Asp as well as (DQA1*00601, *00901, *01001, *01301, *01401 and *01501). Kennedy et al suggested that this amino acid is likely to be important for peptide binding to the canine DQ class II molecule [23], but this remains to be verified. One might speculate that some antigenic peptides bound by the Arg containing DQ molecules leads to increased risk of aberrant immune responses ultimately leading to de-regulated immunity and disease. The exact type of immune-mediated mechanism involved in development of SLO remains to be established. In several human autoimmune diseases, MHC class II-mediated autoantigen presentation leads to broken tolerance and similar mechanisms are likely to operate during SLO progression.

MHC class II-mediated autoantigen presentation leading to broken tolerance is likely to operate during SLO progression. The autoantigen/s involved in SLO remains to be defined. Identification of such antigens could lead to increased understanding of similar diseases in human.

The human hair follicles and the nail apparatus is reported to harbour an unusual site of immune reactions called immune privilege [24], originally described as sites providing a relative protection against rejection of transplants [25]. There are strong indications that parts of the hair follicle and nail epithelium is

characterized by an absence of or very low expression of some MHC antigens. These mechanisms remain to be fully understood, but there are reasons to believe that a disturbance of these mechanisms may play a key role in the pathogenesis of one of the most common organ-specific autoimmune diseases, alopecia areata, which shows frequent nail involvement [12], [24], [26].

In summary, we have identified both a risk haplotype for developing SLO in Gordon setter and also a haplotype that may protect dogs from developing SLO. The risk is even higher in dogs homozygous for the specific risk haplotype. In contrast, dogs heterozygous for the risk and protective haplotypes are over-represented among controls suggesting that the protective effect is phenotypically stronger. To be able to distinguish a potential functional effect of DRB1*1801 from the closely linked loci in strong LD, it will be important to type more dogs/breeds to identify dogs with DRB1*1801. However, other variants of linked loci, but our findings may provide an impetus for future breeding practices in an effort to increase the frequency of the protective haplotype to reduce the incidence of SLO in Gordon setters. Moreover, canine SLO could provide new information that could benefit a number of related non-infectious inflammatory disorders of the nail apparatus in humans including lichen planus, psoriasis, alopecia areata, pemphigus vulgaris, and onycholysis and provide new insight in immune privilege of the nail apparatus.

Materials and Methods

Study population

The main study population was based on Gordon setters. A total of 196 dogs were included, among these, 98 dogs were classified as SLO cases and 98 as healthy controls. Most of the cases were unrelated at the grand-parental level. The average age of cases were 5.6 years of age while the control dogs had an average age of 10 years (Table S6).

We also used 10 bearded collies and 110 giant schnauzers for comparative purpose. Five bearded collies were defined as SLO cases and five as healthy controls. In giant schnauzer, we used 30 healthy controls and 80 dogs classified with different claw abnormalities. A total of seven SLO cases were identified among the giant schnauzer population. The age of onset for SLO in both giant schnauzers and Gordon setters is between two and seven years of age, with an average of about 4.5 years of age. All the control dogs used for comparative purpose in this study were older than seven years of age and unrelated (Table S6).

Clinical examination

The inclusion criteria used for cases were veterinary-verified diagnosis, where dogs two to seven years of age lost all claws on all four paws within a short time span. Inclusion criteria for all the control dogs were that they had never experienced claw disorders and were more than eight years of age for the Gordon setters and more than seven years of age for all the other breeds. The giant schnauzers were classified according to owner's questionnaire and grouped according to Table S7.

Isolation of genomic DNA

Genomic DNA from the Gordon setters was extracted using 250 μ l EDTA blood using the E.Z.N.A. Blood DNA Kit (Omega bio-tek, Norcross, GA).

Genomic DNA from bearded collies and giant schnauzers was extracted from 200 μ l EDTA blood by using a standard salt extraction protocol, the Qiagen QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA).

PCR amplification, DNA Sequencing and data analysis

The methods for amplification, sequencing and data analysis of DLA-DRB1, -DQA1 and -DQB1 were performed as previously described [21]. Primer pairs used for amplification of DLA-DRB1, -DQA1, and -DQB1 was DRB1 (forward): gatcccccgctccacag, DRB1 (reverse): cgcccgctgcgtca, DQA1 (forward): taaggttctttctccctc, DQA1 (reverse): ggacagattcagtgaagaga, DQB1 (forward): ctctactggcccgctgtctc and DQB1 (reverse): cacctcgccgctgcaactg [14]. A T7 tail (taatcagctactatag) was used to label the PCR products for sequencing. The purified PCR products were sequenced using capillary-electrophoresis on an Applied Biosystems 3730. BigDye[®] Terminator v3.1 (Applied Biosystems, Foster City, CA). Finally, the nucleotide sequences for DLA-DRB1, -DQA1, and -DQB1 were analyzed using MatchTools and MatchToolsNavigator (Applied Biosystems) also used for assigning alleles, haplotypes and genotypes. The most frequently occurring haplotypes could easily be identified in homozygous dogs. Due to the high frequency of these haplotypes, the probability that they are present also in heterozygous dogs is high. After thorough manual checking of each diploid sequence, additional haplotypes not found in homozygous individuals could be identified by "subtracting" the haplotypes initially identified in homozygotes. All alleles identified in this study are available in the IPD - MHC Database (<http://www.ebi.ac.uk/ipd/mhc/dla/index.html>).

Statistical analyses

Statistical analyses were performed with the statistical program VassarStats (<http://faculty.vassar.edu/lowry/VassarStats.html>). 2x2 Contingency tables were used for calculations of Odds ratio and Yates p-value, which is corrected for continuity. The number of SLO-cases and controls having each specific allele, haplotype or genotype compared to the overall number of cases and controls in dogs not carrying it, was the basis for the OR-estimates. We also evaluated dogs homozygous for the risk haplotype vs. dogs heterozygous for the risk haplotype, dogs homozygous for the risk haplotype vs. the other haplotypes and dogs heterozygous for the risk haplotype vs. all other haplotypes. A 99% confidence interval (CI) was used for all tests. Statistical analysis was only performed for Gordon setters, due to an insufficient number of dogs in bearded collies and uncertain disease classification in giant schnauzers.

Ethics Statement

Ethical approval for performing this study has been granted by the Ethical board for experimental animals in Uppsala, Sweden (Dnr C138/6).

Supporting Information

Table S1 Genotype frequencies in Gordon setter, bearded collie and giant schnauzer. Genotype 1 in Gordon setter (being homozygous for DRB1*01801/DQA1*00101/DQB1*00802) gave an even higher risk for developing SLO and genotype 5 (DRB1*01801/DQA1*00101/DQB1*00802 and DRB1*02001/DQA1*00401/DQB1*01303) was protective.

Found at: doi:10.1371/journal.pone.0012332.s001 (0.10 MB DOC)

Table S2 Haplotype frequencies in bearded collie. Only two different haplotypes were found in cases whereas five were found in control dogs. Haplotype 1 and 2 occur in higher frequency in cases compared to controls (not significant).

Found at: doi:10.1371/journal.pone.0012332.s002 (0.03 MB DOC)

Table S3 DLA DRB1/DQA1/DQB1 allele frequencies in bearded collies. Altogether, three DRB1, three DQA1 and five DQB1 alleles were found in the population. Only one DRB1, one DQA1 and two DQB1 alleles were identified in the cases compared to three DRB1, three DQA1 and five DQB1 alleles in control dogs.

Found at: doi:10.1371/journal.pone.0012332.s003 (0.03 MB DOC)

Table S4 Haplotype frequencies in giant schnauzer. 10 different haplotypes were identified in the total population. Haplotype DRB1*00101/DQA1*00101/DQB1*00201, was more common in cases compared to control dogs. Haplotype DRB1*01301/DQA1*00301/DQB1*00501 was more frequently occurring in controls compared to cases.

Found at: doi:10.1371/journal.pone.0012332.s004 (0.04 MB DOC)

Table S5 DLA DRB1/DQA1/DQB1 allele frequencies in giant schnauzer. Altogether, eight DRB1 alleles, four DQA1 alleles and six DQB1 alleles were found in the population. The allele DRB1*00101 was increased in cases compared to controls. A protective effect was found for dogs carrying allele DRB1*01301. Found at: doi:10.1371/journal.pone.0012332.s005 (0.04 MB DOC)

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Table S6 Diagnostic information and DLA-DRB1, -DQA1 and -DQB1 alleles for all dogs included in the study (Gordon Setter (GSet), bearded collie (BC) and giant schnauzer (GSch)). Found at: doi:10.1371/journal.pone.0012332.s006 (0.51 MB DOC)

Table S7 The inclusion criteria used for giant schnauzers. Found at: doi:10.1371/journal.pone.0012332.s007 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: MW GA FL. Performed the experiments: MW CH. Analyzed the data: MW CH LA KLT GA FL. Wrote the paper: MW KLT GA FL. Phenotypic characterization of dogs and sample collection: MW ML AA KS EN AH FL.

Paper IV

A genome-wide association study identifies a region strongly associated with symmetrical onychomadesis on chromosome 12 in dogs

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Abstract

Symmetrical onychomadesis causes periodic loss of claws in otherwise healthy dogs, and an autoimmune aetiology is presumed. Genome-wide association analysis in 225 Gordon setters identified a single region associated with symmetrical onychomadesis on chromosome 12. Meta-analysis across a small number of English setters and the Gordon setters indicated that this genomic region also predispose for symmetrical onychomadesis in English setters. The associated region spans most of the major histocompatibility complex and nearly 1 mega base pairs downstream. Like many other autoimmune diseases, associations of symmetrical onychomadesis with DLA class II alleles have been reported. In this study no associated markers were revealed within any of the *DLA-DRB1*, *-DQA1*, or *-DQB1* genes and the odds for symmetrical onychomadesis were higher for Gordon setters carrying the major allele at each of the significant single nucleotide polymorphisms compared to the odds of any of the recorded *DLA-DRB1/DQA1/DQB1* haplotypes. We noticed that specific DLA-haplotypes, some which were different in the English setters and the Gordon setters, were more common in cases or controls. Interestingly, associated SNP chip markers showed a more consistent pattern of allelic variants related to cases or controls regardless of breed. In conclusion, we suggest that any *DLA-DRB1/DQA1/DQB1* association with symmetrical onychomadesis could be caused by linkage disequilibrium to nearby genes with stronger functional effect, and most important, the associated genetic markers identified in this study hold the potential to aid in selection of breeding animals to reduce the frequency of symmetrical onychomadesis in the dog.

Keywords: dog, DLA, English setter, Gordon setter, GWAS, symmetrical lupoid onychodystrophy, symmetrical onychomadesis

Introduction

Symmetrical onychomadesis (SO) causes severe claw problems in otherwise healthy dogs. The disease is characterised by an acute onset of claw loss (onychomadesis), and within a few months all claws may be lost. Replacement claws are typically brittle and misshapen and may be lost repeatedly during new episodes of onychomadesis.

A relatively high frequency of SO compared with other breeds has been reported in German shepherds (Scott *et al.* 1995; Mueller *et al.* 2000), English setters (ES) and Gordon setters (GS) (Ziener *et al.* 2008; Wilbe *et al.* 2010b), indicating a likely genetic background to SO. Moreover, Ziener *et al.* (2008) observed higher prevalence of SO among siblings of dogs with SO compared to unrelated dogs. Histopathological changes reported in affected claws with SO and examples of successful treatment of affected dogs with immunosuppressive drugs and fatty acid supplementation suggest an autoimmune aetiology (Scott *et al.* 1995; Bergvall 1998; Ziener *et al.* 2008). Similar to other autoimmune diseases the inheritance of SO is most likely complex and caused by a combination of numerous genetic and environmental factors. Non-genetic risk factors for SO are unknown, but adverse food reactions, vaccination, infections, and repeated mechanical stress on the claws have been suggested (Mueller *et al.* 2000; Ziener *et al.* 2008).

Like many other autoimmune diseases in humans and in dogs, associations of SO to major histocompatibility complex (MHC) class II alleles (=dog leukocyte antigen (DLA) in dogs) have been revealed (Wilbe *et al.* 2010b). However, previous studies of other autoimmune diseases indicate that some of the reported DLA associations may be spurious and not causative (Safra *et al.* 2011; Pedersen *et al.* 2012).

Since 2005, following the release of a high quality draft of the dog genome sequence together with a dense map of single nucleotide polymorphisms (SNPs) across

dog breeds (Lindblad-Toh *et al.* 2005), genome-wide association studies (GWAS) have identified several loci associated with complex diseases in the dog (Wood *et al.* 2009; Wilbe *et al.* 2010a; Madsen *et al.* 2011; Mogensen *et al.* 2011; Philipp *et al.* 2012; Roque *et al.* 2012; Ahonen *et al.* 2013; Tengvall *et al.* 2013).

The aim of this study was to use GWAS, to confirm DLA class II associations with SO and to identify genetic variants across the whole genome associated with the disease.

Material and Methods

Blood sampling

EDTA blood samples were collected between 2006 and 2011 from dogs in Norway and Sweden. The sampled dogs comprised 114 GS cases (68 females and 46 males) and 113 GS controls (65 females and 48 males), and 18 ES cases (10 females and 8 males) and 18 ES controls (14 females and 4 males). Controls were defined as dogs eight years or older that never had experienced onychomadesis and cases were dogs with onychomadesis. In 75% of cases the first symptoms of SO occurred in the months between April and September.

Certified veterinarians collected the blood samples in agreement with the provisions enforced by the Norwegian Animal Research Authority. Ethical approval for collecting blood samples is not required in Norway (Regulation on Animal Experimentation of January 15, 1996, in accordance with the Animal Welfare Act of June 19, 2009). For each of the dogs that participated in the study, the consent of its owner was obtained prior to inclusion.

GWAS and meta-analysis

Genomic DNA was extracted using E.Z.N.A® blood DNA kit (Omega Bio-Tek), and the samples were subsequently genotyped with the 170K CanineHDBeadChip (Illumina). The SNP locations are given according to the CanFam3.1 assembly.

The genotyped SNPs were analysed using GenABEL and MetABEL (Aulchenko *et al.* 2007), libraries implemented in the R statistical software (Ihaka & Gentleman 1996). GenABEL was used for quality control of the SNP genotype data, to analyse population structure, to determine possible gender association with SO, to run association analyses, and to estimate LD between associated SNPs. SNPs were filtered to satisfy a call rate of > 95%, Hardy-Weinberg equilibrium (HWE) test $p > 10^{-8}$, and minor allele frequency (MAF) < 0.5%. Dogs with > 5% missing genotypes were removed. To determine the population structure a genomic kinship matrix was computed after pruning of dogs, using genotype information from the autosomal chromosomes. This kinship matrix was also used in the association analyses. K-means clustering was used to determine the optimal number of subpopulations in downstream analyses. The mixed model approach was used in all analyses and the polygenic_hgml function (Rönnegård *et al.* 2010) was used to fit the model. Genome wide significance was ascertained by permutation testing ($n = 100000$).

An r^2 value between significant SNPs was calculated to evaluate LD across the region using the r2fast function (Hao *et al.* 2007), and we ran a conditional GWAS on the top SNP in order to determine the number of genetic signals that could explain SO.

MetABEL was used to run a meta-analysis of the two independent datasets from the GS and the ES.

Haplotype analysis

SNP genotypes of the associated region were phased into haplotypes using fastPHASE (Scheet & Stephens 2006). Cases and controls were phased separately in order to avoid prediction of false haplotypes. We estimated the length of SNP haplotypes spanning the DLA class II genes by grouping SNP haplotypes according to *DLA-DRB1/DQA1/DQB1* risk-haplotype and then identified SNPs that indicated the haplotype borders.

DLA class II genotyping

The aim was to obtain *DLA-DRB1*, *DQA1*, and *DQB1* exon 2 DNA sequences from all dogs; thus, 40 GS and 34 ES were genotyped in the present study, whereas 175 of the GS had been genotyped previously (Wilbe *et al.* 2010b). Methods for genotyping and classification of DNA sequences followed previously described methods (Wilbe *et al.* 2010b), with the exceptions that DNA was extracted using E.Z.N.A® blood DNA kit (Omega Bio-Tek), and the PCR products were sequenced on a 3500xL Genetic Analyzer (Applied Biosystems).

Statistical analyses

Odds ratios (OR) were calculated using the web-based statistical calculator in OpenEpi 3.01 (Dean *et al.*). Two by two contingency tables were used to evaluate associations. In calculations with a 0 cell the value 0.5 was added to each cell. One-tail Mid-p exact value was calculated in calculations having one expected value (row total x column total/grand total) <5. For all other OR computations, Yates p-value was calculated. A 95% confidence level was used for all tests.

Results

Multidimensional scaling (MDS) analysis of all dogs ($N = 263$) revealed two distinct breed populations (Figure 1a), thus separate GWA analysis were run in the GS and the ES, followed by a meta-analysis across the two breeds.

A region on chromosome 12 is associated with SO

Initial association analysis of the GS revealed a genomic inflation factor $\lambda = 1.45$. As visualised by an MDS plot, the GS appeared to form three overlapping subpopulations (Figure 1b), also supported by K-means clustering. Cases and controls were evenly distributed in the three subpopulations, and the Norwegian GS were distributed evenly across all clusters, whereas the majority of Swedish GS were allocated to two of the three clusters (Figure 1b). No other recorded phenotypic trait could explain the grouping of dogs into subpopulations. No gender predisposition for SO was detected as revealed by Fisher's exact test ($p = 0.79$).

The final dataset for the GWAS in GS comprised 225 dogs (114 cases and 111 controls) and 129407 SNPs. Population substructure effects and other cryptic confounding factors in the dataset were most efficiently accounted for by the mixed model approach using three subpopulations as a vector ($\lambda = 1.00$). The GWAS revealed significant associations of SO to markers on chromosome 12 distributed between 555475 and 3775420 base pair (bp) (Figure 2a). Twelve markers in this region reached genome wide significance ($p_{\text{genome}} < 0.05$) after permutation testing (Table 1). P-values after correction by the mixed model, but before permutation testing, $p_{\text{corrected}}$, ranged between 2.4×10^{-7} - 3.5×10^{-9} (Table 1). Pair-wise LD calculations of the 12 significant SNPs revealed that strong linkage ($r^2 > 0.8$) occurred between many of the markers across the associated region (Table 2). All significant associations disappeared after conditional

GWAS using marker BICF2P928832 as a covariate. Based on allele frequency in the GS, we will refer to the two alternative alleles at each significant SNP as the *major allele* and the *minor allele*. A higher frequency of the major allele was revealed in cases compared to controls at each of the significant SNPs (Table 1). Calculations of odds ratio for SO in dogs with certain allele combinations at each of the 12 GWAS hits revealed the following: OR for homozygous major allele vs. heterozygous or homozygous minor allele ranged between 5.7-52.0 ($p < 0.01$); OR for homozygous major allele or heterozygous vs. homozygous minor allele ranged between 4.9-15.4 ($p \leq 0.03$); and OR for homozygous major allele vs. heterozygous ranged between 5.3-46.1 ($p < 0.01$) (Table 1). The top two SNPs (BICF2P928832 and TIGRP2P155561) from the association analysis are in high LD ($r^2 > 0.8$) with their respective nearest SNPs and thus we calculated odds ratio for SO in dogs with any given SNP haplotype combination at these two short LD-blocks (Table 3). The first LD-block contains three SNPs (TIGRP2P155561, TIGRP2P155568, and BICF2S23023653), and OR for the most common haplotype GCG vs. ATA, ACA, GCA, and ATG was 13.0 ($p < 0.01$). The second LD-block contains four SNPs (BICF2S23519405, BICF2P928832, BICF2P543796, and BICF2S23416139), and OR for the most common haplotype GGAT vs. AAGG, GAGG, AAAG, and AAGT was 9.7 ($p < 0.01$).

GWA analysis of 36 ES revealed no significant association to SO. However, meta-analysis across ES and GS ($\lambda = 0.97$) revealed that $p_{\text{corrected}}$ improved by between 1-2 orders of magnitude for many of the SNPs in the SO-associated region revealed in GS alone (Figure 2b and Supplementary Table 1). The meta-analysis top SNP was BICF2P928832 ($p_{\text{corrected}} = 1.4 \times 10^{-10}$), the same as in the association analysis of the GS alone. Examination in the ES of SNP alleles at 11 of the 12 SO-associated SNPs identified in the GS, revealed that all ES cases except two (94.4%) carried the major allele at 9 of these SNPs located between CFA12: 1112342-3726208, compared to 24 out of 36

controls (66.6%). (BICF2P1056154 at position 3775420 bp was excluded from this comparison since it appeared to be fixed for the major allele in the ES).

DLA class II associations

The *DLA-DRB1*, *-DQA1*, and *-DQB1* genes are located between CFA12: 2151409-2307711.

The nearest associated SNPs in the GWA-analyses were located at CFA12: 1919031 (significant in GWAS in GS and in meta-analysis across ES and GS) and CFA12: 2383201 (significant in meta-analysis across ES and GS).

Altogether, nine *DLA-DRB1/DQA1/DQB1* haplotypes were identified among the GS and six haplotypes were identified in the ES population (Table 4). Haplotype *DRB1*02001/DQA1*00401/DQB1*01303* has been associated with decreased risk of SO in GS and the haplotype *DRB1*01801/DQA1*00101/DQB1*00802* has been associated with increased risk of developing SO (Wilbe *et al.* 2010b). These results were confirmed in the GS in the present study (Table 4). Haplotype *DRB1*02001/DQA1*00401/DQB1*01303* appeared to be associated with decreased risk of SO in the ES as well, as it was only observed in controls. Haplotype *DRB1*01801/DQA1*00101/DQB1*00802* was not present among the genotyped ES. In this breed, all but two ES carried at least one copy of either haplotype *DRB1*00101/DQA1*00101/DQB1*00201* or *DRB1*10102/DQA1*00101/DQB1*00201*, and with the exception of one case, they were the only haplotypes observed among cases. Interestingly, most DLA-haplotypes overrepresented in ES- and GS cases carried the major allele at all 12 SO-associated SNPs from the GWAS in GS, whereas most DLA-haplotypes overrepresented in ES- and GS controls carried the minor allele at a varying number at these 12 SNPs (Table 4).

Haplotype analysis in the GS indicated that both haplotypes *DRB1*02001/DQA1*00401/DQB1*01303* and *DRB1*01801/DQA1*00101/DQB1*00802* span SNP chip markers located between CFA12: 3991 (the first marker on CFA12 on the SNP chip) -3541928, thus including 10 out of the 12 SO-associated GWAS hits. A comparison between GS and ES of SNP haplotypes carrying the shared haplotype *DRB1*02001/DQA1*00401/DQB1*01303* revealed common blocks spanning SNP chip markers located between CFA: 961974-2757946, thus covering 7 out of the 12 SO-associated GWAS hits.

Discussion

We have identified a strongly associated region affecting SO in GS on chromosome 12 using GWAS. No markers on other chromosomes were near reaching genome-wide significance. GWAS of a small number of ES did not identify associations to SO, possibly due to the fact that we had too few cases to provide the necessary statistical power in the analysis. Nevertheless, meta-analysis across GS and ES suggested that the identified region on chromosome 12 is associated with SO in ES as well.

The identified SO-associated region spans most of the MHC region, with the exception of a few DLA class I genes. However, haplotype analysis revealed the presence of long haplotypes in the associated region, spanning all SNP markers upstream this region on chromosome 12, and hence including all DLA class I genes as well.

Additionally, the associated region encompasses a closely linked region downstream of the MHC. Many of the associated SNPs are closely linked and they are also spread across the entire associated region, making it difficult to assess the exact location of specific disease-causing variants. The abundance of genes in the MHC region, many of which are involved in the same function i.e., the immune response, also impede predictions about

which gene(s) is (are) involved in the pathogenesis of SO. The SO-associated region outside the MHC encompassed 16 protein-coding genes. One significant SNP was situated within one of these genes, namely *PASCIN1*, which also has an important immune function by regulating the interferon response (Esashi *et al.* 2012). Within this confined region outside the MHC region there are two genes involved in non-immunological pathways that could potentially be relevant for the development of SO, namely *ITPR3* that has been associated with regulation of the hair cycle (Sato-Miyaoka *et al.* 2012), and *LEMD2* that encodes proteins involved in providing mechanical stability to the nucleus of a cell (Brachner *et al.* 2005; Huber *et al.* 2009). Theoretically, *LEMD2* mutations could contribute to disease if the mechanical properties in the nuclear structure become altered and thereby renders tissues that undergo harsh mechanical stress more prone to cell death (Huber *et al.* 2009). *LEMD2* is expressed in virtually all differentiated cells (Brachner *et al.* 2005) and could possibly play a role in the development of SO as claws of dogs undergo harsh mechanical stress, particularly during intensive training. Notably, in our study, the majority of SO-cases showed first symptoms of disease in the months between April and September, the time of the year when the dogs are trained on hard surface.

In addition to protein coding genes, heritable changes in gene expression and cellular function not involving alterations in the DNA could also play a role in the development of SO. Hence, epigenetics modifications, including the non-coding microRNAs (a few present in the identified SO-associated region) as well as cytosine methylation and histone modification, play important roles in the pathogenesis of autoimmune diseases in humans (Lu 2013).

Previous studies have implied association of SO with DLA class II genes (Wilbe *et al.* 2010b). In the present GWAS, no significant associations were found between dogs

with SO and markers on the SNP chip located within the *DLA-DRB1* or the *DLA-DQA1* gene. Markers for the *DLA-DQB1* gene were absent on the SNP chip used for genome-wide genotyping, however, *DLA-DRB1/DQA1/DQB1* exon 2 typing is assumed to detect most of the variation at this locus (Kennedy 2007). Even though our GWAS revealed association between SO and the MHC region, the role of the class II genes *DRB1*, *DQA1*, and *DQB1* in disease development is uncertain. Notably, the odds of SO were higher given carrying the major allele at any of the 12 significant GWAS hits or given carrying haplotype blocks containing SO-associated SNPs in high LD, compared to carrying any of the recorded *DLA-DRB1/DQA1/DQB1* haplotypes. Hence these results may suggest that LD to other primary disease-predisposing gene(s) caused the *DLA-DRB1/DQA1/DQB1* associations. An important conclusion of this study is that although different or the same DLA-haplotypes may be associated with increased or decreased risk of SO in different dog breeds, we have identified SNP markers with a more consistent pattern of allelic variants in cases and controls regardless of breed. Thus, these findings holds promising potential for the development of a genetic tool that based on a limited number of SNPs, can be used to aid in the selection of breeding animals and thereby reduce the frequency of SO in the dog.

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Legends to Figures

Figure 1: Multidimensional scaling plots

(a) The Gordon setters and the English setters form distinct genetic populations. (b) Illustrates the division of the Gordon setters into three overlapping populations, and the distribution of Swedish- and Norwegian cases and controls.

Figure 2: A region on chromosome 12 is associated with symmetrical onychomadesis

Manhattan plots depicting the results of (a) the genome wide association study of SNP markers to symmetrical onychomadesis in Gordon setters, and (b) the meta-analysis across Gordon setters and English setters. The red line represents Bonferroni corrected p-value significance thresholds of 0.05. Each dot represents a single SNP. Genomic coordinates are displayed along the X-axis and the negative logarithm of the association p-value for each SNP marker is displayed on the Y-axis.

Table 1 Top 12 SNP alleles from the association analysis in Gordon setters

Marker name and position on chromosome 12 (alleles, major one capitalised)	F_A / F_U major allele%	$P_{\text{corrected}}$	OR (95% CI) for homoz. major allele vs. heteroz. or homoz. for minor allele	OR (95% CI) for homoz. major allele or heteroz. vs. homoz. for minor allele	OR (95% CI) for homoz. major allele vs. heteroz.
BICF2P301485 555475 (T/g)	89.5/ 65.8	2.6×10^{-7}	5.8 (3.2-10.6) ($p < 0.001$)	4.9 (1.4-17.7) ($p < 0.001$)	5.4 (2.8-10.2) ($p < 0.001$)
BICF2S23222412 684307 (T/g)	88.2/ 64.0	2.4×10^{-7}	5.7 (3.2-10.3) ($p < 0.001$)	4.9 (1.4-17.3) ($p < 0.001$)	5.3 (2.8-9.8) ($p < 0.001$)
TIGRP2P155561 1112342 (G/a)	97.8/ 77.9	7.2×10^{-9}	18.1 (6.2-52.5) ($p < 0.001$)	5.3 (0.6-46.4) ($p = 0.06$)*	21.3 (6.3-71.8) ($p < 0.001$)
TIGRP2P155568 1132974 (C/t)	98.2/ 79.7	1.7×10^{-7}	20.9 (6.2-69.9) ($p < 0.001$)	5.3 (0.6-46.4) ($p = 0.06$)*	27.4 (6.4-117.3) ($p < 0.01$)*
BICF2S23023653 1643804 (G/a)	98.2/ 77.9	1.5×10^{-8}	17.4 (6.0-50.6) ($p < 0.001$)	6.5 (0.8-54.5) ($p = 0.03$)*	20.0 (5.9-67.2) ($p < 0.001$)
BICF2P827116 1817494 (T/c)	99.2/ 82.4	1.3×10^{-7}	52.0 (7.0-387.9) ($p < 0.001$)	8.5 (0.4-163.1) ($p = 0.06$)*	46.1 (6.2-344.8) ($p < 0.001$)
BICF2S23519405 1832582 (G/a)	96.9/ 75.7	5.4×10^{-8}	11.2 (4.8-26.3) ($p < 0.001$)	15.4 (0.9-273.7) ($p < 0.001$)*	9.6 (4.0-22.6) ($p < 0.001$)
BICF2P928832 1911694 (G/a)	96.5/ 73.9	3.5×10^{-9}	11.3 (5.0-25.3) ($p < 0.001$)	15.4 (0.9-273.7) ($p < 0.001$)*	9.6 (4.0-22.6) ($p < 0.001$)
BICF2P543796 1919031 (A/g)	96.5/ 75.2	1.3×10^{-8}	10.2 (4.5-22.7) ($p < 0.001$)	15.4 (0.9-273.7) ($p < 0.001$)*	8.6 (3.8-19.6) ($p < 0.001$)
BICF2S23416139 3101865 (T/g)	96.5/ 75.7	1.1×10^{-8}	9.7 (4.3-21.9) ($p < 0.001$)	15.4 (0.9-273.7) ($p < 0.001$)*	8.2 (3.6-18.8) ($p < 0.001$)
BICF2P568857 3726208 (C/t)	98.7/ 80.2	1.4×10^{-8}	20.0 (6.0-67.3) ($p < 0.001$)	10.8 (0.6-199.2) ($p = 0.03$)*	17.5 (5.2-59.0) ($p < 0.001$)
BICF2P1056154 3775420 (A/g)	97.8/ 78.4	1.4×10^{-8}	13.3 (5.0-35.2) ($p < 0.001$)	6.5 (0.8-54.5) ($p = 0.03$)*	14.2 (4.8-41.7) ($p < 0.001$)

F_A , allele frequency in cases; F_U , allele frequency in controls; $P_{\text{corrected}}$, p-value corrected

for population substructure; CI, confidence interval; homoz., homozygosity; heteroz.,

heterozygosity.*One-tail Mid-p exact value was calculated if one expected value (row total x column total/grand total) < 5. For all other OR computations, Yates p-value was calculated.

Table 2 Linkage disequilibrium measured by r^2 values between the top 12 SNP alleles from the association analysis in Gordon setters

Position on CFA 12	555475	684307	1112342	1132974	1643804	1817494	1832582	1911694	1919031	3101865	3726208
684307	0.9										
1112342	0.5	0.5									
1132974	0.4	0.5	0.9								
1643804	0.5	0.5	1.0	0.9							
1817494	0.4	0.4	0.7	0.8	0.7						
1832582	0.5	0.6	0.5	0.6	0.5	0.7					
1911694	0.5	0.7	0.6	0.5	0.6	0.7	1.0				
1919031	0.5	0.6	0.6	0.5	0.6	0.6	0.9	1.0			
3101865	0.6	0.6	0.5	0.4	0.5	0.6	0.9	1.0	0.9		
3726208	0.4	0.5	0.8	0.7	0.8	0.8	0.7	0.8	0.8	0.7	
3775420	0.6	0.5	0.9	0.8	0.9	0.6	0.5	0.6	0.6	0.6	0.8

Table 3 Haplotypes in Gordon setters containing symmetrical onychomadesis associated SNPs in high linkage disequilibrium ($r^2 > 0.8$)

Marker name (position on CFA12)	Haplo- type	Frequency total population GS % (n = 450)	Frequency GS cases % (n = 228)	Frequency GS controls % (n = 220)	OR (95% CI) for haplotype vs. the other haplotypes (Yates p < 0.001)
TIGRP2P155561 (1112342), TIGRP2P155568 (1132974), BICF2S23023653 (1643804)	GCG	87.8	97.8	77.5	13.0 (5.1- 33.2)
	ATA	10.7	1.8	19.8	0.1 (0.0-0.2)
	ACA	1.1	0.4	1.8	-.1
	GCA	0.2	0.0	0.5	-.1
BICF2S23519405 (1832582), BICF2P928832 (1911694), BICF2P543796 (1919031), BICF2S23416139 (3101865)	ATG	0.2	0.0	0.5	-.1
	GGAT	85.3	96.5	73.9	9.7 (4.5-20.9)
	AAGG	12.0	3.1	21.2	0.12 (0.1-0.3)
	GAGG	1.1	0.4	1.8	-.1
	AAAG	0.7	0.0	1.4	-.1
	AAGT	0.9	0.0	1.8	-.1

ES, English setters; GS, Gordon setters. ¹OR was not calculated due to the low number of dogs carrying this haplotype.

Table 4 DLA haplotypes, and alleles at the top 12 SNPs from the association analysis in Gordon setters. (ES, English setters; GS, Gordon setters)

Haplotype (DRB1/DQA1/DQB1)	GS cases % (n)	GS controls % (n)	ES cases % (n)	ES controls % (n)	Alleles at each of the 12 top SNPs, major one capitalised
<i>01801/00101/00802</i>	52.3 (113)	36.0 (77)	-	-	TTGCGTGGATCA
<i>04901/01001/01901</i>	13.4 (29)	12.6 (27)	-	-	TTGCGTGGATCA
<i>00103/00101/00201</i>	8.3 (18)	6.1 (13)	-	-	TTGCGTGGATCA
<i>00501/00301/00501</i>	-	1.4 (3)	-	-	TTGCGTGGATCA
<i>02001/00401/01303</i>	0.5 (1)	15.9 (34)	-	12.5 (4)	TgatacaagTtA (ES) ggatacaaggtg (GS)
<i>01501/00601/02301</i>	2.8 (6)	6.1 (13)	-	3.1 (1)	TTGCGTGGATCA (ES) GgataTGGATC ^A / _g (GS)
<i>00101/00101/00201</i>	18.1 (39)	11.2 (24)	63.9 (23)	40.6 (13)	^T / _g ^T / _g GCGTGGATCA (ES) TTGCGTGGATCA
<i>10102/00101/00201</i>	2.8 (6)	1.9 (4)	33.3 (12)	25.0 (8)	^T / _g ^T / _g GCGTGGATCA (ES) TTGCGTGGATCA (GS)
<i>00901/00101/008011</i>	1.9 (4)	8.9 (19)	2.8 (1)	15.6 (5)	ggGCGTaaggCA (ES) ggGCGTaaggC ^A / _g (GS)
<i>00601/005011/00701</i>	-	-	-	3.1 (1)	ggGCGcaaATCA

Supplementary Table 1

P-values of top 20 SNPs from the association analysis in Gordon setters and a comparison with p-values from the meta-analysis

Marker name	Position on CFA 12	$P_{\text{corrected GS alone}}$	$P_{\text{corrected meta-analysis across GS and ES}}$
BICF2P928832	1911694	3.5×10^{-9}	1.4×10^{-10}
TIGRP2P155561	1112342	7.2×10^{-9}	NA
BICF2S23416139	3101865	1.1×10^{-8}	4.6×10^{-10}
BICF2P543796	1919031	1.3×10^{-8}	4.6×10^{-10}
BICF2P1056154	3775420	1.4×10^{-8}	NA
BICF2P568857	3726208	1.4×10^{-8}	3.9×10^{-10}
BICF2S23023653	1643804	1.5×10^{-8}	NA
BICF2S23519405	1832582	5.4×10^{-8}	2.3×10^{-9}
BICF2P827116	1817494	1.3×10^{-7}	2.6×10^{-8}
TIGRP2P155568	1132974	1.7×10^{-7}	8.1×10^{-9}
BICF2S23222412	684307	2.4×10^{-7}	3.7×10^{-7}
BICF2P301485	555475	2.6×10^{-7}	NA
BICF2S23219052	2383201	4.1×10^{-7}	1.4×10^{-8}
BICF2S23661198	2473964	5.0×10^{-7}	9.0×10^{-9}
BICF2P855985	1037055	5.0×10^{-7}	2.7×10^{-8}
BICF2P814186	8076110	6.1×10^{-7}	1.0×10^{-7}
BICF2P680923	1359971	6.9×10^{-7}	7.0×10^{-7}
BICF2S23032363	655802	1.5×10^{-6}	4.6×10^{-6}
BICF2P1322975	5497565	1.5×10^{-6}	NA
BICF2P63611	7755067	2.0×10^{-6}	7.0×10^{-4}

NA, SNP removed during quality control of genotype data of English setters; ES, English setters; GS, Gordon setters

Figure 1a

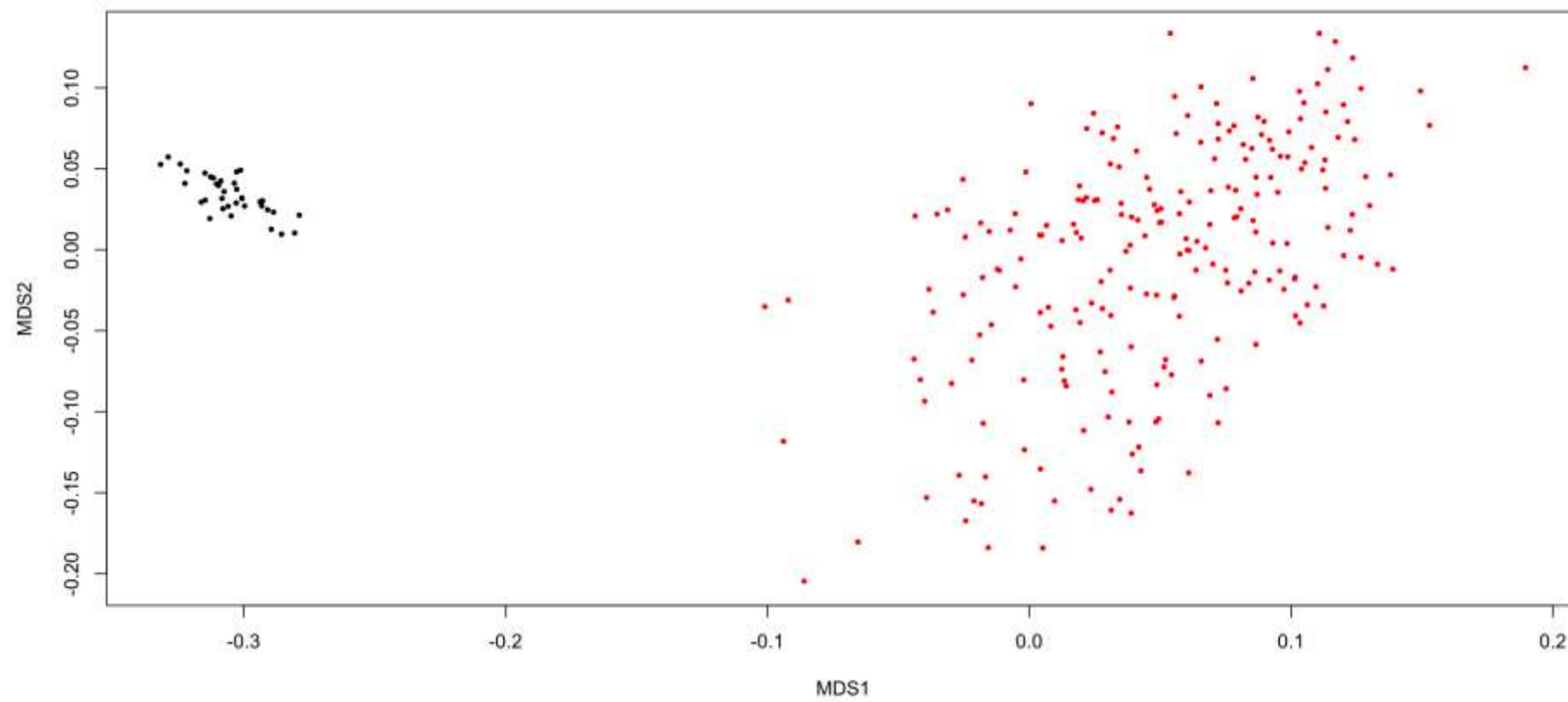


Figure 1b

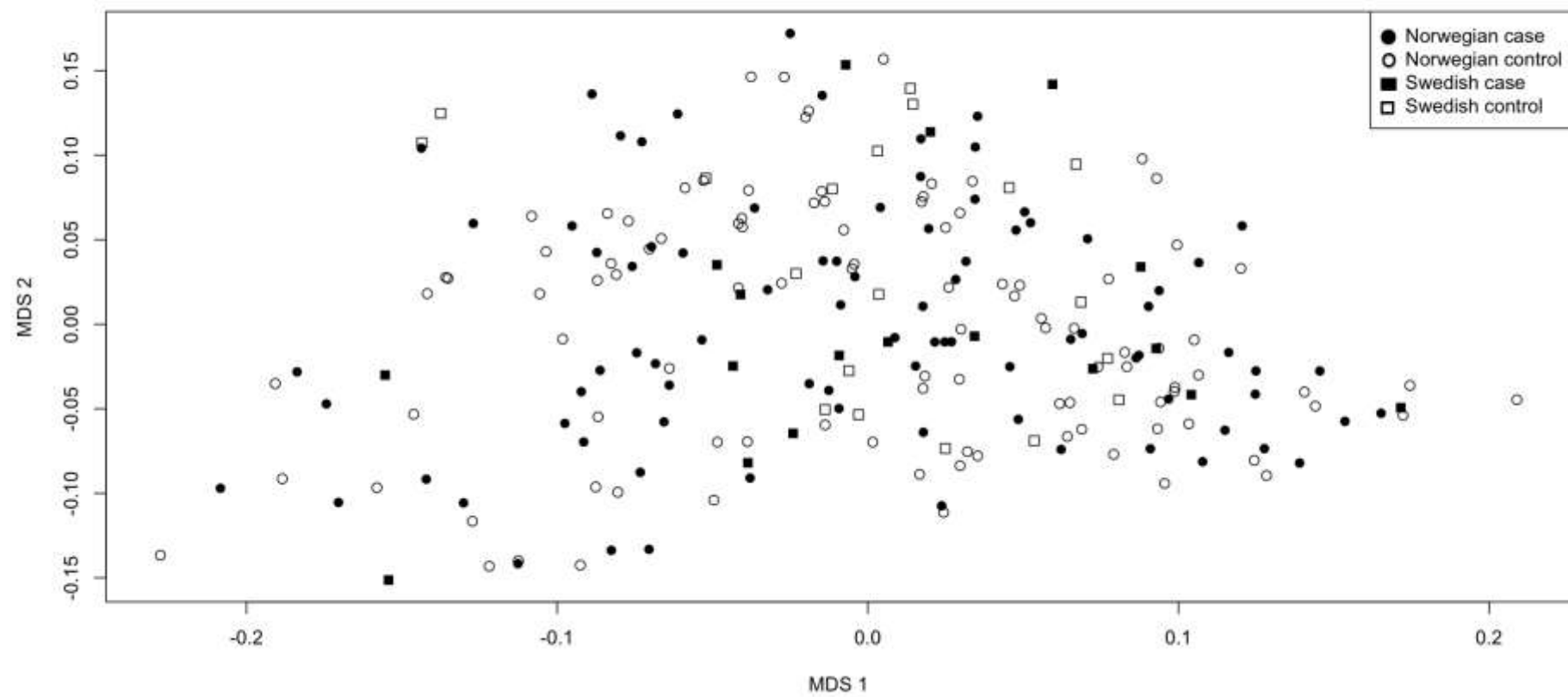


Figure 2a

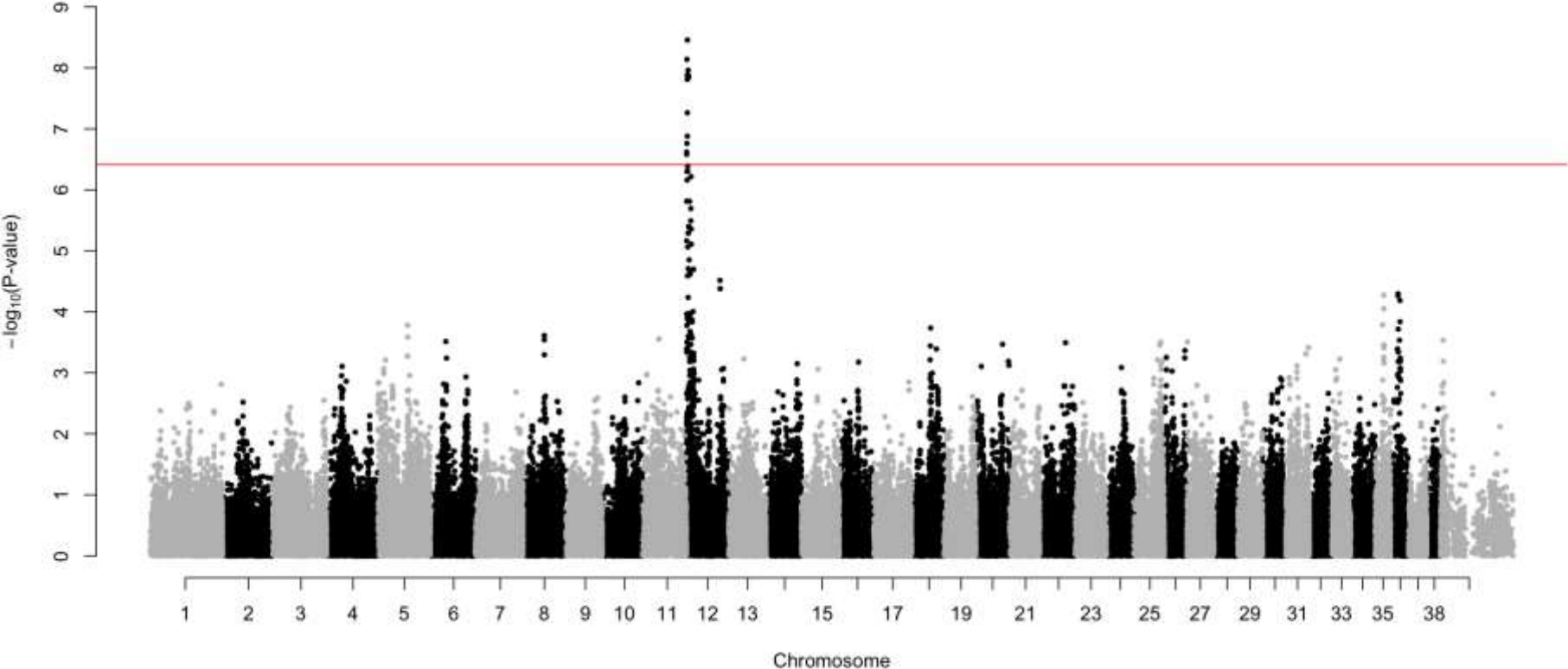
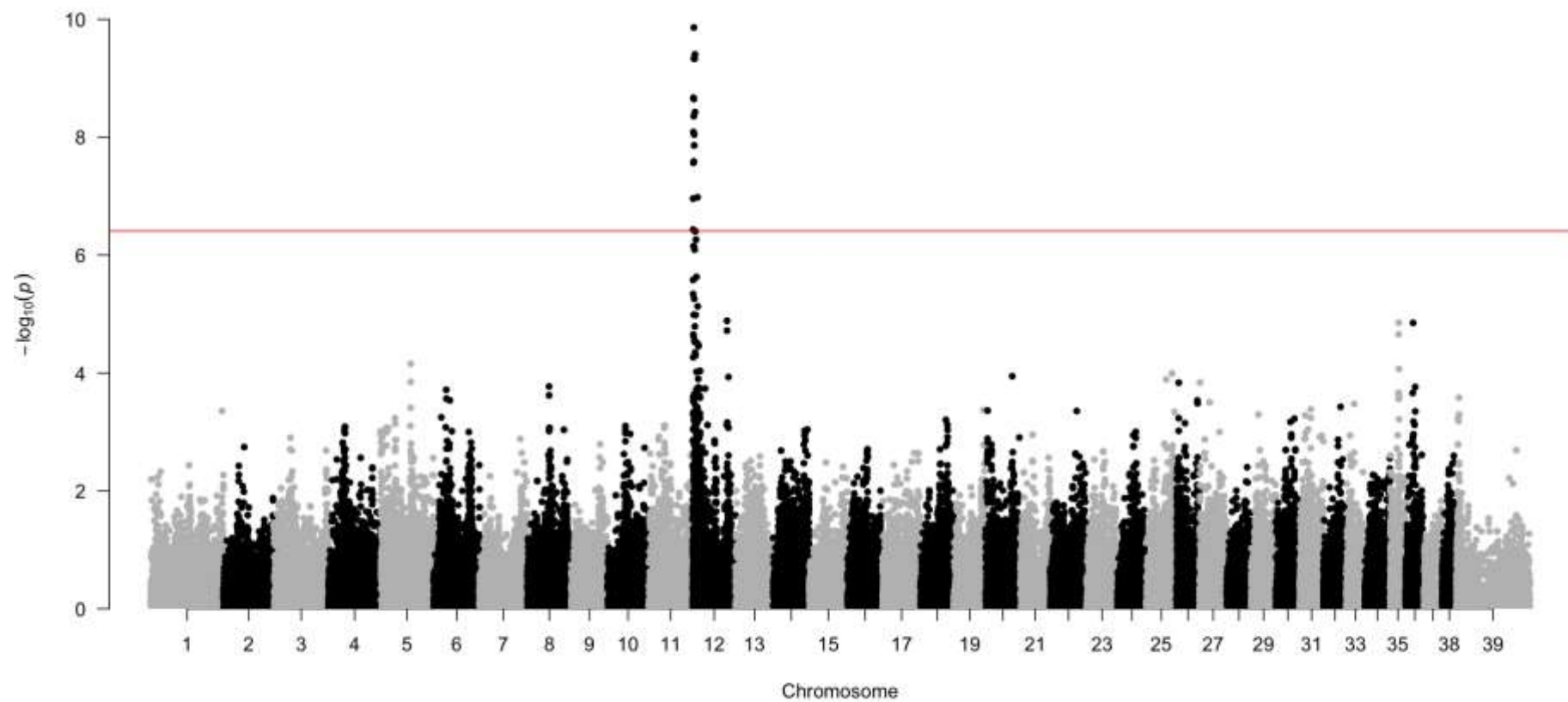


Figure 2b



Paper V

Genetics and epidemiology of hypothyroidism and symmetrical onychomadesis in the Gordon setter and the English setter

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Abstract

Background: Hypothyroidism is one of the most common endocrine disorders, whereas symmetrical onychomadesis is a rare claw disease in the general dog population. The aims of this study were to estimate the prevalence of hypothyroidism and symmetrical onychomadesis in a birth cohort of 291 Gordon setters at eight years of age. Further, to describe the age at diagnosis of hypothyroidism in the 68 Gordon setters and 51 English setters included in the DLA study. Finally, to elucidate potential associations between dog leukocyte antigen (DLA) class II and hypothyroidism and/or symmetrical onychomadesis in the Gordon setter and the English setter.

Results: In the birth cohort of eight years old Gordon setters, 2.7 % had hypothyroidism and 8.9 % had symmetrical onychomadesis, but only one out of these 291 dogs (0.3 %) had both diseases. Mean age at diagnosis of hypothyroidism for dogs included in the DLA study was 6.4 years (95 % CI: 5.6-7.2 years) in the Gordon setters and 7.7 years (95 % CI: 7.2-8.2 years) in the English setters. The DLA alleles most associated with hypothyroidism in the Gordon setter and English setter were DLA-DQB1*00201 (OR=3.6, 95 % CI: 2.1-6.4, $p<0.001$) and DLA-DQA1*00101 (OR=2.9, 95% CI: 1.3-6.6, $p<0.001$), respectively.

In the Gordon setter, the haplotype DLA-DRB1*01801/DQA1*00101/DQB1*00802 was significantly associated with both symmetrical onychomadesis (OR=2.9, 95% CI: 1.7-5.2, $p<0.001$) and with protection against hypothyroidism (OR=0.3, 95% CI: 0.2-0.5, $p<0.001$).

Conclusion: Hypothyroidism is a complex disease where DLA genes together with other genes may be involved in the pathogenesis of the disease. In the Gordon setter, one DLA haplotype that was associated with protection against hypothyroidism was also associated with symmetrical onychomadesis. These findings indicate that closely linked genes instead of the DLA genes themselves may be associated with hypothyroidism and symmetrical onychomadesis. In a breed where several autoimmune diseases are prevalent all possible associations between DLA genes and actual diseases need to be investigated before DLA is considered used as a tool for marker-assisted selection.

Lay summary

An autoimmune disease occurs when the body's immune system attacks normal cells of the body. For instance an autoimmune attack on the thyroid gland may cause hypothyroidism in both dogs and humans. Thyroid hormones are involved in several energy demanding processes in the body and subnormal hormone levels in dogs may cause a variety of clinical signs such as dullness, weight gain, lethargy, and skin changes. Dogs with compatible signs of hypothyroidism are commonly diagnosed based on subnormal levels of thyroid hormones and elevated levels of thyroid stimulating hormone (TSH) in serum.

Symmetrical onychomadesis is an acute claw disease where the dogs lose all claws during 3-4 months. The disease is known to occur in Gordon setters and many other dog breeds.

Hypothyroidism and symmetrical onychomadesis appear to be common in some dog breeds like Rhodesian Ridgeback and Giant Schnauser which could indicate that genetic factors contribute to disease development. In a birth cohort of Norwegian Gordon setters at eight years, the prevalence of hypothyroidism was 2.7 % and the prevalence of symmetrical onychomadesis was 8.9 % (n=291). The Gordon setters and the English setters were middle aged when they were diagnosed with hypothyroidism.

Dog leukocyte antigen (DLA)-genes play an essential role in the immune response and they have been associated with different autoimmune diseases in dogs. In this study, different DLA haplotypes and alleles were associated with hypothyroidism in the Gordon setter and the English setter. In the Gordon setter, one DLA haplotype that may protect against hypothyroidism may concurrently predispose dogs to symmetrical onychomadesis. All possible immune mediated diseases in one breed needs to be investigated for associations to DLA before information about DLA is considered used as a tool for marker-assisted selection to reduce disease prevalence.

Background

Hypothyroidism is one of the most common endocrine diseases reported in dogs [1]. In most cases, the disease is considered to be the result of an autoimmune attack of the thyroid gland [2].

Subclinical hypothyroidism is characterised by the presence of autoantibodies against thyroglobulin (TgAA) in serum and elevated serum TSH values [2-4], and at this stage the thyroid gland shows lymphocytic infiltrates [4]. In dogs with overt hypothyroidism, more than 75 % of the functional gland is destroyed and blood chemistry is characterised by the presence of TgAA, elevated TSH, and in most cases decreased free thyroxin/total thyroxin (FT4/TT4) levels [2-4]. End stage hypothyroidism is characterised by absence of TgAA in serum, normal or elevated TSH, and decreased FT4/TT4 levels [2, 3]. The gland now has a non-inflammatory and an atrophic appearance on histopathology [4]. It is still debated whether the lymphocytic form is the first stage and the atrophic form is the end stage of the disease, or if they are two different disease entities [2-4]. Hypothyroidism is considered to be a disease of middle aged dogs, but age at diagnosis has been reported to differ between breeds [4]. The age at diagnosis may also depend on the stage of hypothyroidism hence dogs with TgAA-positive hypothyroidism are in general younger than dogs with TgAA-negative hypothyroidism [2, 3].

Diagnosing hypothyroidism in dogs can be a challenge due to confusing blood results and vague clinical signs [2]. TSH, as a diagnostic test for hypothyroidism, has high specificity but low sensitivity in dogs [5]. A high TSH value is therefore well suited to confirm a diagnosis of hypothyroidism, but it cannot be used to exclude hypothyroidism [5, 6]. Autoantibodies may also falsely increase TT4 and FT4 levels in serum when TT4 and FT4 are measured by chemiluminescence [7]. Moreover, dogs suffering from other critical diseases or severe chronic illnesses may have low TT4 values without having hypothyroidism (euthyroid sick syndrome) [8, 9]. These diagnostic aspects are important to consider when collecting cases and controls for genetic studies of hypothyroidism.

Autoimmune hypothyroidism is a multifactorial disease in which many genetic and environmental factors are assumed to affect disease development and disease outcome [2]. Comparably higher

prevalences of hypothyroidism in the English setter, the Giant Schnauzer, and the Hovawart indicate an accumulation of genetic variants in these breeds [10, 11]. Research on autoimmune hypothyroidism in human patients indicates that genetic factors contribute as much as 70-80 % to the risk of developing the disease [12, 13]. The major histocompatibility complex (MHC) class II has been associated with various autoimmune diseases in both humans and dogs [14, 15]. In dogs, genes coding for this complex are named DLA. The DLA class II genes comprise three highly polymorphic and closely linked genes, DLA-DRB1, DLA-DQA1, and DLA-DQB1 [16]. In the Doberman Pinscher, the Rhodesian Ridgeback and the Giant Schnauzer associations between specific DLA class II haplotypes/alleles and hypothyroidism have been described [17-20]. In the Gordon setter and the Giant Schnauzer associations between specific DLA class II haplotypes/alleles and symmetrical onychomadesis have been reported [21].

Common clinical signs of hypothyroidism in dogs include weight gain, mental dullness, and lethargy [3]. Other signs of the disease are hypothermia and poor hair coat quality with skin changes, such as alopecia, hyperkeratosis, and seborrhea [3, 4]. In humans, detached nails from the nail bed (onycholysis) has been described in hypothyroid patients [22]. Claw changes in dogs with hypothyroidism are uncommon [23]. Symmetrical onychomadesis is a claw disease where the dogs lose all their claws within a few months [24]. Both symmetrical onychomadesis and autoimmune hypothyroidism occur with high prevalence in Giant Schnauzers, German Shepherds and Rhodesian Ridgebacks [21, 23]. Mueller et. al. (2003) suggested that these diseases may share genetic risk factors since they both occur with high prevalence in the same breeds, although not necessarily in the same dog [24, 25].

The aims of this study were to: 1) Investigate the prevalence of hypothyroidism and symmetrical onychomadesis in a large birth cohort of Gordon setters; 2) Describe the age at diagnosis of hypothyroidism in the Gordon setter and English setter; 3) To investigate potential associations

between DLA class II haplotypes/alleles and hypothyroidism and/or symmetrical onychomadesis in the Gordon setter and the English setter.

Results

Epidemiology of hypothyroidism and symmetrical onychomadesis

The prevalence of hypothyroidism in eight years old Gordon setters was 2.7 % (95 % CI: 1.3-5.1 %, n=291). The prevalence of symmetrical onychomadesis in the same study population was 8.9 % (95 % CI: 6.0-12.6 %). Only one of these 291 dogs had both hypothyroidism and symmetrical onychomadesis.

The Gordon setters from the DLA study comprised 68 cases and 93 controls and the English setters comprised 83 cases and 53 controls. The age at diagnosis, median TSH, TT4, FT4, cholesterol values at time of diagnosis in cases are presented in table 1. The mean age of the control dogs together with their median TSH, TT4, FT4 and cholesterol values are also presented in table 1. TgAA analysis was not performed in all cases because it was not available at the time of samples collection. Thus only, 31 out of 151 hypothyroid dogs were tested for TgAA and 114 out of 146 controls were tested for TgAA (Table 1). Five of the controls had positive TgAA titers and these dogs were still included as controls because they were followed until death, and none of them developed clinical or biochemical signs of hypothyroidism.

Medical records of symmetrical onychomadesis were obtained in retrospect from 83.2 % of the Gordon setters and English setters participating in the DLA study (n=297). Among the hypothyroid Gordon setters 21.1 % of the dogs had symmetrical onychomadesis (n=57) and 5.6 % of the hypothyroid English setters had symmetrical onychomadesis (n=53). In Gordon setters without hypothyroidism, 22.2 % of the dogs had symmetrical onychomadesis (n=91) and 2.1 % of the English setters without hypothyroidism had symmetrical onychomadesis (n=47) (Table 1).

DLA haplotypes and alleles associated with hypothyroidism in the Gordon setter and the English setter

Ten DLA haplotypes were present in the Gordon setter population (n=161), and seven DLA haplotypes were present in the English setter population (n=136). Five DLA haplotypes occurred in both breeds (Table 4 and 5). A complete overview of DLA allele frequencies in the Gordon setters and the English setters are given in supplementary tables S1 and S2. The haplotype, DLA-DRB1*00103/DQA1*00101/DQB1*00201, was present in 8.3 % of the Gordon setters and found to be associated with hypothyroidism in this breed (OR=4.4, 95 % CI: 1.8-11.5, p<0.001) (Table 2). Only five Gordon setters were homozygous for this haplotype, three cases and two controls. The more common haplotype among the Gordon setters, DLA-DRB1*04901/DQA1*01001/DQB1*01901 was also associated with hypothyroidism (OR=2.1, 95 % CI: 1.2-3.7, p=0.008) (Table 2).

In the English setter the haplotype DLA-DRB1*10201/DQA1*00101/DQB1*00201 was associated with hypothyroidism (OR=2.0, 95% CI: 1.1-3.8, p=0.01) (Table 3). Seven cases were homozygous, whereas none of the controls were homozygous for this haplotype. The allele DLA-DQA1*00101 was present in 89.3% of the English setters and was found to be associated with hypothyroidism (OR=2.9, 95% CI: 1.3-6.6, p=0.002) (Table 3).

This DLA- DQA1 allele was also the most common allele in the Gordon setter, however, it was not associated with hypothyroidism in this breed. In the Gordon setter this allele was instead part of the most common and also protective haplotype DLA-DRB1*01801/DQA1*00101/DQB1*00802 (OR=0.3, 95% CI: 0.2-0.5, p<0.001) (Table 2). Notably, DLA- DQB1*00201 was associated with hypothyroidism in both the Gordon setter (OR=3.6, 95% CI: 2.1-6.4, p<0.001) and the English setter (OR=1.9, 95% CI: 1.1-3.3, p=0.03). It is also worth noting that 77.6% of the English setters and 21.4% of the Gordon setters carried the allele DLA- DQB1*00201 (Table 2 and 3).

Three Gordon setter cases and 19 controls were homozygous for the most common DLA haplotype in this breed (DLA-DRB1*01801/DQA1*00101/DQB1*00802). Thus, homozygosity for this DLA haplotype was associated with the strongest protection for hypothyroidism in the Gordon setter (OR=0.2, 95% CI: 0.1-0.6, p=0.004). Eighteen cases and 41 controls were heterozygous for this haplotype (DLA-DRB1*01801/DQA1*00101/DQB1*00802) in the Gordon setter (OR=0.51, 95% CI: 0.29-0.98, p=0.04).

Haplotype DLA-DRB1*01801/DQA1*00101/DQB1*00802 was absent in the English setter. The only DLA haplotype associated with protection for hypothyroidism in the English setter was DLA-DRB1*00601/DQA1*005011/DQB1*00701 (OR=0.22, 95% CI: 0.03-0.81, p=0.02). Only nine English setters (3.3%) and none of the Gordon setters had this haplotype. All alleles in the haplotype DLA-DRB1*00601/DQA1*005011/DQB1*00701 were unique to that haplotype.

DLA haplotypes associated with symmetrical onychomadesis in the Gordon setter and English setter

The haplotype DLA-DRB1*01801/DQA1*00101/DQB1*00802 has previously been associated with symmetrical onychomadesis in the Gordon setter [21] which was confirmed in the present study (Supplementary S 5). Nine out of 12 Gordon setters with symmetrical onychomadesis and hypothyroidism were also heterozygous for this haplotype (DLA-DRB1*01801/DQA1*00101/DQB1*00802). Only three English setters had both symmetrical onychomadesis and hypothyroidism; one was homozygous for DLA-DRB1*10201/DQA1*00101/DQB1*00201, one was homozygous for DLA-DRB1*00101/DQA1*00101/DQB1*00201 and one was heterozygous with the preceding haplotypes.

Discussion

Gordon setters and English setters are frequently used as hunting dogs and both hypothyroidism and symmetrical onychomadesis reduce their hunting abilities besides welfare concerns [26]. The true breed prevalence of hypothyroidism is challenging to determine. In this study, a large sample from a birth cohort of Gordon setters was investigated at eight years of age. The prevalence of hypothyroidism was 2.7 %. This estimate was based on dogs that were examined by a veterinarian because of their owners' suspicion of hypothyroidism. The prevalence may therefore be underestimated because some owners misinterpret the signs of hypothyroidism as normal signs of getting old and hence do not seek medical care. The estimated prevalence of 8.9 % of Gordon setters affected with symmetrical onychomadesis was in accordance with previous published results [26]. The prevalence for hypothyroidism and symmetrical onychomadesis in English setter could not be estimated in the present study, but this breed has been described as a high risk breed for hypothyroidism in previous studies [3, 4]. Only four of the English setters in this study had symmetrical onychomadesis. The prevalence of symmetrical onychomadesis in the English setter is thought to be lower than that observed in the Gordon setter [26].

Overall, 50 % of hypothyroid dogs have TgAA positive hypothyroidism, but the frequencies of TgAA positive hypothyroidism vary among breeds [3]. This could suggest that different genetic factors affect disease development in various breeds [2, 3]. Graham et. al. (2007) discovered that 61 out of 73 English setters (84%) had TgAA positive hypothyroidism, while for instance only 13 out of 81 Dachshunds (16 %) were TgAA positive [3]. Graham et. al. (2007) also discovered that dogs diagnosed with TgAA positive hypothyroidism in general were younger than dogs diagnosed with TgAA negative hypothyroidism [3]. In our study, English setters were relatively old when they were diagnosed with hypothyroidism, but since neither TgAA measurements nor biopsies of the thyroid gland were available for all the dogs in this present study, it can not be determined if they had the atrophic form of hypothyroidism or a late form of TgAA-positive hypothyroidism.

DLA has been associated with several different immune mediated diseases in dogs [27-29]. The most common DLA haplotype in the Gordon setter (DLA-DRB1*01801/DQA1*00101/DQB1*00802) in this study was associated with protection for hypothyroidism, and interestingly, this DLA haplotype was also reported associated with symmetrical onychomadesis in that same breed. The fact that the same DLA haplotype is associated with one autoimmune disease and also associated with protection for another presumed autoimmune disease, clearly suggests that specific combinations of DLA alleles do not necessarily predispose dogs for autoimmune diseases in general. It may also indicate that potential functional effects of autoimmune disease may be associated to closely linked loci to DLA-haplotypes. The allele DLA-DQA1*00101 was both associated with hypothyroidism in the English setter and part of a haplotype associated with protection of hypothyroidism in the Gordon setter. Several hypothyroidism protective DLA haplotypes occurred at a high frequency in the Gordon setter, whereas such protective DLA haplotypes were rare in the English setter. Another DLA haplotype associated with protection of hypothyroidism (DLA-DRB1*01301/DQA1*00301/DQB1*00501), has been reported from the Giant Schnauzer [20], but this haplotype was not observed among our Gordon setters or English setters. One previously reported DLA haplotype associated with hypothyroidism in the Giant Schnauzer and the Doberman (DLA-DRB1*01201/DQA1*00101/DQB1*00201) [18, 20], contained two of the most common alleles associated with hypothyroidism in English setter (DLA-DQA1*00101, DQB1*00201). The DLA-DQB1*00201 was also associated with hypothyroidism in the Gordon setter. This allele together with DLA-DQA1*00101 has also previously been reported associated with symmetrical onychomadesis in the Gordon setter [21]. Kennedy et al. (2006), suggested that the DLA-DQA1*00101 allele is associated with hypothyroidism in many breeds [17]. The findings of different DLA associations with hypothyroidism in different breeds are consistent with previous findings of different HLA associations with hypothyroidism observed both within and between ethnic groups in humans [15].

Conclusions

The Gordon setters had a prevalence of 2.7 % of hypothyroidism and 8.9 % of symmetrical onychomadesis. Both the Gordon setters and the English setters were middle aged when they were diagnosed with hypothyroidism. One allele (DLA- DQB1*00201) was associated with hypothyroidism in both the Gordon setter and the English setter. Another allele (DLA- DQA1*00101) was associated with hypothyroidism in the English setter, but not in Gordon setter. The same haplotype (DLA- DRB1*01801/DQA1*00101/DQB1*00802) was associated both with symmetrical onychomadesis and with protection against hypothyroidism in the Gordon setter. At the moment the functional genes in hypothyroidism and symmetrical onychomadesis are not known. The opposite effects of some alleles/haplotypes on two different autoimmune diseases may indicate that closely linked genes to DLA genes are involved. Further studies are needed to investigate how DLA genes or other closely linked genes participate in the pathogenesis of hypothyroidism and symmetrical onychomadesis. It is also important to state that selection of breeding animals supported by DLA haplotypes/alleles could only be used if taking into account also potential associations to other autoimmune disorders in a dog breed as well as how it influence the genetic variation in that breed.

Material and methods

Epidemiology of hypothyroidism and symmetrical onychomadesis

Gordon setters eight years of age received a health survey in 2012 (n=474). Non-responders were followed up by telephone. The response rate was 61% (n=291). Dogs with reported hypothyroidism and/or symmetrical onychomadesis were confirmed by an evaluation of veterinary records. All dogs with hypothyroidism (n=8) fulfilled the diagnostic criteria: TSH >0.45 µg/L, TT4 ≤16 nmol/L, and FT4 ≤8 pmol/L. Symmetrical onychomadesis cases were dogs with one or multiple episodes of onychomadesis during their lifetime (n=26).

The cases and controls for the DLA study were collected between 2006 and 2012. Cases were analysed for serum values of TSH, TT4, FT4, and cholesterol (Table 1). Cases were dogs with clinical signs of hypothyroidism with serum TSH > 0.45 µg/L and at least one of the following diagnostic criteria; TT4<16 nmol/L and/or TT4>46 nmol/L, FT4<7 pmol/ and/ or FT4>44 pmol/L and/or cholesterol> 10 mmol/L and/or positive TgAA analysis. Controls were dogs eight years or older without signs of hypothyroidism and the following serum levels: TSH ≤ 0.45µg/L, TT4 > 15 nmol/L, FT4 > 6 pmol/L, and cholesterol < 11 mmol/L (Table 1). The reference values at the Central laboratory was; TSH = 0-0.45 µg/L, TT4 = 16-46 nmol/L, FT4 = 7-44 pmol/L, cholesterol: 3.4-10.0 mmol/L. All dogs were screened for relatedness and showed no indication of a closer relationship than the general population in these breeds.

Blood analysis

Serum levels of TSH, TT4, and FT4 were analysed on an IMMULITE® 2000 XPi Immunoassay System (Siemens Healthcare Diagnostics, Siemens AG, Germany). In the present study, two chemiluminescent assays for measuring free T4 in canine serum were used. Initially, an assay primarily evaluated for analyzing free T4 in human sera was used (IMMULITE® 2000 Free T4). However, this assay has been evaluated for canine samples [30]. When IMMULITE® 2000 Veterinary Free T4 became available this assay was preferred and calibrated to use identical reference intervals as related to the previous method for healthy and hypothyroid dogs. Most of the serum samples were analysed within five days, but a few samples had been kept frozen at -20 °C before they were analysed. Storage of serum samples at -20 °C should not interfere with serum TT4 and FT4 levels [31]. Analysis of TgAA antibodies was not available at the Central Laboratory until March, 2009. The presence of TgAA was determined by an indirect enzyme immunoassay using microplates containing both thyroglobulin coated (Tg+) and non-specific binding (Tg-) strips (Oxford Laboratories, Inc., Oxford, MI, USA) and an microplate absorbance reader (Tecan Sunrise™, Tecan Group Ltd., Switzerland) reading the absorbance at 450 nm for each well. The values for the non-specific binding

wells were subtracted from the corresponding positive and negative sera. Then the average of the non-specific binding wells was subtracted from the average of each sample. Each sample was divided by the corrected positive reference serum and multiplied by 100. Samples > 25 % of the positive reference serum are positive for TgAA, samples below 10 % are negative and samples from 10-25 % are inconclusive (“grey zone”) for TgAA.

DLA class II genotyping

DLA-DRB1, DLA-DQA1, and DLA-DQB1 exon 2 DNA sequences were obtained from 161 Gordon Setters and 136 English Setters. DNA was extracted using E.Z.N.A.[®] blood DNA kit and then PCR amplified. One PCR reaction mixture contained 1.5 µl aliquots of the DNA solution, 0.05 µl HotStarTaq Master Mix (Qiagen GmbH), 0.5 µl of each forward and reverse DLA-specific primer (20 pmol; Eurofins MWG Operon) (see supplementary S 4), 1.5 µl dNTP (2.5 mM; Amplicon), 1.5 µl PCR Buffer (Qiagen), 1 µl Q-Solution (Qiagen), and 8.45 µl distilled water. PCR reactions were carried out in a Veriti[®] Thermal Cycler Applied Biosystems using the following PCR protocol: initial Hot Start at 95 °C for 15 min; 14 touch down cycles of 95 °C for 30 seconds, followed by 1 minute annealing, starting at 62 °C (DLA-DRB1), 54 °C (DLA-DQA1), 73 °C (DLA-DQB1) and reducing by 0.5 °C each cycle, and 72°C for 1 minute; then 20 cycles of 95 °C for 30 seconds, 55°C (DLA-DRB1), 47°C (DLA-DQA1) 66°C (DLA-DQB1) for 1 minute, 72°C for 1 minute; and final extension at 72 °C for 10 minutes. PCR products were separated on 1% agarose gel and visualized under UV light after staining with ethidium bromide to check for appropriately sized products, and subsequently purified using the Illustra[™] Exostar[™] clean-up kit (GE Healthcare). Sequencing reaction mixtures were prepared using BigDye[®] Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and the samples were then sequenced on a 3500xL Genetic Analyzer (Applied Biosystems). PCR products were initially sequenced in one direction, but if the DLA allele could not be completely determined the opposite direction was sequenced as well. All DNA sequences were finally analysed and alleles were designated for each sequence using MatchTools and MatchToolsNavigator (Applied Biosystems). Alleles not present in the built-in allele library were identified by a BLAST search [32].

Statistical analyses

Standard deviations and confidence intervals for means were calculated using Microsoft Excel 2011. In hypothyroid dogs, the standard deviations were all greater than half of the mean for the TSH, TT4 and FT4 values. This strongly indicated that the thyroid values were skewed and therefore the median values with range were reported instead of the mean with standard deviation [33]. Two by two contingency tables were used to evaluate association between DLA-DRB1/DQA1/DQB1 haplotypes and individual DLA alleles. Odd ratio (OR) were calculated using the web-based statistical calculator in OpenEpi 3.01. Chi-square test was used and two-tail mid-p exact values were calculated. A 95% confidence level was used for all tests.

Ethics statement

Certified veterinarians collected the blood samples during routine clinical consultations, and in agreement with the provisions enforced by the Norwegian Animal Research Authority. Ethical approval for collecting blood samples is not required in Norway (Regulation on Animal Experimentation of January 15, 1996, in accordance with the Animal Welfare Act of June 19, 2009). For each of the dogs that participated in the study, the consent of its owner was obtained prior to inclusion.

List of abbreviations

DLA- Dog leukocyte antigen , FT4- Free thyroxin, HLA- Human leukocyte antigen, LD- Linkage disequilibrium, MHC-Major histocompatibility complex, OR- Odds ratio, TgAA- Thyroid globulin autoantibodies, TSH- Thyroid stimulating hormone, TT4- Total thyroxin.

Competing interests

The authors declare that they have no competing interests.

Authors' information

MLZ is a PhD student at Norwegian University of Life Sciences (NMBU) and works as a clinician at Fredrikstad Animal Hospital in Norway. She is also a member of the breeding council for the Norwegian Gordon Setter club. SD is post doc in medical genetics at NMBU. ST is a diplomat and professor in clinical pathology at the Central Laboratory at NMBU, and FL is a professor in medical genetics and leader of the Genetics department at the Veterinary faculty, NMBU.

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Authors' contributions

MLZ: Study design, sample collection, phenotypic diagnosis, prevalence study, statistical analysis and drafting of the manuscript. SD: Study design, DLA genotyping, and critical evaluation of the manuscript, ST: sample collection, thyroid hormone analysis of the blood, and critical evaluation of the manuscript. FL: Study design, sample collection, statistical analysis and critical evaluation of the manuscript. All authors read and approved the final manuscript.

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Tables:

Table 1 Gordon setters and English setters in the DLA study

	Cases		Controls	
	GS (n=68)	ES (n=83)	GS (n=93)	ES (n=53)
Sex and age				
Male	22	42	57	33
Female	46	41	36	20
Age (y): mean (95% CI)	6.4 (5.6-7.2)	7.7 (7.2-8.2)	9.3 (9.0-9.7)	8.8 (8.3-9.3)
Range age, years	1.8-12.9	2.7-15	8-14.4	8-16
Analysis blood samples				
TSH ug/L: median (range)	3.01 (0.46-12.0)	2.24 (0.46-12.0)	0.14 (0.03-0.45)	0.23 (0.04-0.45)
TT4 nmol/L: median (range)	7.5 (4.0-77.0)	6.0 (4.0-44.0)	28.0 (16.0-53.0)	24.0 (16.0-45)
FT4 pmol/L: median (range)	9.0 (4.0-65.0)	7.0 (4.0-77.0)	14.0 (7.0-69.0)	14.0 (7.0-47.0)
Cholesterol mmol/L: median (range)	9.1 (2.6-20.8)	11.7 (3.4-23.1)	6.1 (3.6-11.0)	5.8 (3.8-11.0)
TgAA positive (n)	14	6	4	1
TgAA negative (n)	2	8	59	45
TgAA inconclusive (n)	0	1	0	5
Diagnosis of SO				
positive (n)	12	3	20	1
negative (n)	45	50	70	46

TSH = thyroid stimulating hormone (reference values: 0-0.45 ug/L). TT4 = total thyroxin (Reference values: 16-46 nmol/L), FT4 = free thyroxine (reference values: 7-44 pmol/L). cholesterol: (reference values: 3.4-10.0 mmol/L). TgAA = thyroglobulin autoantibodies.

Table 2: DLA-haplotypes and alleles associated with hypothyroidism or protection of hypothyroidism in the Gordon setter

DLA-haplotype or allele	Total		Cases		Controls		Cases/controls		
	%	n=322	%	n=136	%	n=186	OR	95 % CI	p-value
DRB1*00103/DQA1*00101/DQB1*00201	8.3	27	14.7	20	3.7	7	4.4	1.8 - 11.5	<0.0001
DLA-DQB1*00201	21.4	69	33.8	46	12.4	23	3.6	2.1 - 6.4	<0.0001
DRB1*04901/DQA1*01001/DQB1*01901	18.9	61	25.7	35	13.9	26	2.1	1.2 - 3.7	0.008
DRB1*00101/DQA1*00101/DQB1*00201	11.1	36	15.4	21	8.0	15	2.1	1.0 - 4.3	0.04
DRB1*01801/DQA1*00101/DQB1*00802	31.9	103	18.3	25	41.9	78	0.3	0.2 - 0.5	<0.0001
DRB1*01501/DQA1*00601/DQB1*02301	5.9	19	2.2	3	8.6	16	0.2	0.1 - 0.8	0.001

Table 3: DLA-haplotypes and alleles significantly associated with hypothyroidism or protection against hypothyroidism in the English setter

DLA-haplotype or allele	Total		Cases		Controls		Cases / Controls		
	%	n=272	%	n=166	%	n=106	OR	CI 95 %	p-value
DRB1*10201/DQA1*00101/DQB1*00201	24.6	67	29.5	49	17.0	18	2.0	1.1-3.8	=0.01
DLA-DQA1*00101	89.3	243	93.4	155	83.0	88	2.9	1.3-6.6	=0.002
DLA-DQB1*00201	77.6	211	81.9	136	70.8	75	1.9	1.1-3.3	=0.03
DRB1*00601/DQA1*005011/DQB1*00701	3.3	9	1.2	2	6.6	7	0.2	0.03-0.8	=0.02

Table 4: DLA haplotypes in Gordon setter (GS)

DLA-haplotype	All GS		GS cases		GS controls		GS cases/ GS controls		
	%	n=322	%	n=136	%	n=186	OR	CI 95 %	p-value
DRB1*01801/DQA1*00101/DQB1*00802	31.9	103	18.3	25	41.9	78	0.3	0.2-0.5	<0.0001
DRB1*01501/DQA1*00601/DQB1*02301	5.9	19	2.2	3	8.6	16	0.2	0.1-0.8	=0.001
DRB1*00103/DQA1*00101/DQB1*00201	8.3	27	14.7	20	3.7	7	4.4	1.8-11.5	<0.0001
DRB1*00101/DQA1*00101/DQB1*00201	11.1	36	15.4	21	8.0	15	2.1	1.03-4.28	=0.04
DRB1*02001/DQA1*00401/DQB1*01303	11.4	37	11.0	15	11.8	22	n.s		
DRB1*04901/DQA1*01001/DQB1*01901	18.9	61	25.7	35	13.9	26	2.1	1.2-3.7	=0.008
DRB1*00901/DQA1*00101/DQB1*008011	7.4	24	6.6	9	8.0	15	n.s		
DRB1*00501/DQA1*00301/DQB1*00501	0.9	3	1.4	2	0.5	1	n.s		
DRB1*10201/DQA1*00101/DQB1*00201	1.8	6	3.6	5	0.5	1	n.s		
DRB1*01501/DQA1*00601/DQB1*00301	1.8	6	0.7	1	2.6	5	n.s		

Cases; Gordon setter with hypothyroidism, Controls; Gordon setters without hypothyroidism, n.s; non significant, OR; Odd ratio, CI; confidence interval

Table 5: DLA haplotypes in English setter (ES)

DLA-haplotype	All ES		ES cases		ES controls		ES cases/ ES controls		
	%	n=272	%	n=166	%	n=106	OR	CI 95 %	p-value
DRB1*01501/DQA1*00601/DQB1*02301	2.1	5	0.6	1	3.8	4	n.s		
DRB1*00101/DQA1*00101/DQB1*00201	52.9	144	52.4	87	53.8	57	n.s		
DRB1*02001/DQA1*00401/DQB1*01303	4.0	11	3.6	6	4.7	5	n.s		
DRB1*00901/DQA1*00101/DQB1*008011	11.7	32	11.4	19	12.3	13	n.s		
DRB1*00601/DQA1*005011/DQB1*00701	3.3	9	1.2	2	6.6	7	0.2	0.03-0.8	=0.02
DRB1*10201/DQA1*00101/DQB1*00201	24.6	67	29.5	49	17.0	18	2.0	1.1-3.8	=0.01
DRB1*03802/DQA1*00901/DQB1*00101	1.4	4	1.3	2	1.8	2	n.s		

Cases; English setters with hypothyroidism, Controls; English setters without hypothyroidism, n.s; non significant, OR; Odd ratio, CI; confidence interval

S1 DLA alleles in the Gordon setter (GS)

Allele	All GS %	All GS n=322	GS cases %	GS cases n=136	GS controls %	GS controls n=186	GS cases/GS controls OR	CI 95%	p-value
DLA-DRB1									
01801	32.0	103	18.4	25	41.9	78	0.3	0.2-0.5	<0.0001
01501	7.8	25	2.9	4	11.3	21	0.2	0.1-0.8	=0.001
00103	8.4	27	14.0	20	3.8	7	4.4	1.8-11.5	<0.0001
00101	11.2	36	15.4	21	8.1	15	2.0	1.03-4.28	=0.04
02001	11.5	37	11.0	15	11.8	22	n.s		
04901	18.9	61	25.7	35	14.0	26	2.1	1.2-3.7	=0.004
00901	7.5	24	6.6	9	8.1	15	n.s		
00501	0.9	3	1.5	2	0.5	1	n.s		
10201	1.9	6	3.7	5	0.5	1	n.s		
DLA-DQA1									
00101	60.9	196	58.8	80	62.4	116	n.s		
00601	7.8	25	2.9	4	11.3	21	0.2	0.1-0.8	=0.001
00401	11.5	37	11.0	15	11.8	22	n.s		
01001	18.9	61	25.7	35	14.0	26	2.1	1.2-3.7	=0.004
00301	0.9	3	1.5	2	0.5	1	n.s		
DLA-DQB1									
00802	32.0	103	18.4	25	41.9	78	0.3	0.2-0.5	<0.0001
02301	5.9	19	2.2	3	8.6	16	0.2	0.1-0.8	=0.001
00201	21.4	69	33.8	46	12.4	23	3.6	2.1-6.4	<0.0001
01303	11.5	37	11.0	15	11.8	22	n.s		
01901	18.9	61	25.7	35	14.0	26	2.1	1.2-3.7	=0.004
008011	7.5	24	6.6	9	8.1	15	n.s		
00501	0.9	3	1.5	2	0.5	1	n.s		
00301	1.9	6	0.7	1	2.7	5	n.s		

S2 DLA alleles in the English setter (ES)

Allele	All ES	All ES	ES cases	ES cases	ES controls	ES controls	ES cases/ ES controls		
DLA-DRB1	%	n=27	%	n=166	%	n=106	OR	CI 95%	P value
01501	1.8	5	0.6	1	3.8	4	n.s		
00101	53.1	144	52.4	87	53.8	57	n.s		
02001	4.1	11	3.6	5	4.7	6	n.s		
00901	11.8	32	11.4	19	12.3	13	n.s		
00601	3.3		1.2	2	6.6	7	0.2	0.03-0.8	=0.02
10102	24.7	67	29.5	49	17.0	18	2.0	1.1-3.8	=0.01
03802	1.5	4	1.2	2	1.9	2	n.s		
DLA-DQA1									
00601	1.8	5	0.6	1	3.8	4	n.s		
00101	89.3	243	93.4	155	83.0	88	2.9	1.3-6.6	=0.002
00401	4.0	11	3.6	6	4.7	5	n.s		
005011	3.3	9	1.2	2	6.6	7	0.2	0.03-0.8	=0.02
00901	1.5	4	1.2	2	1.9	2	n.s		
DLA-DQB1									
02301	1.8	5	0.6	1	3.8	4	n.s		
00201	77.6	211	81.9	136	70.8	75	1.9	1.05-3.3	=0.03
01303	4.0	11	3.6	6	4.7	5	n.s		
008011	11.8	32	11.4	19	12.3	13	n.s		
00701	3.3	9	1.2	2	6.6	7	0.2	0.03-0.8	=0.02
00101	1.5	4	1.2	2	1.9	2	n.s		

Cases; English setter with hypothyroidism, Controls; English setter without hypothyroidism, CI; confidence interval, OR; odds ratio, n.s; non significant

S3 DLA haplotypes associated with symmetrical onychomadesis and protection for symmetrical onychomadesis in the Gordon setter

DLA-haplotype	GS total		GS cases		GS controls		GS cases/ GS controls		
	%	n=294	%	n=64	%	n=230	OR	95 % CI	p-value
DRB1*01801/DQA1*00101/DQB1*00802	32,0	94	51,6	33	26,5	61	2.9	1.7-5.2	=0.0002
DRB1*01501/DQA1*00601/DQB1*02301	2,0	6	0,0	0	2,6	6	n.s		
DRB1*00103/DQA1*00101/DQB1*00201	8,2	24	7,8	5	8,3	19	n.s		
DRB1*00101/DQA1*00101/DQB1*00201	10,5	31	12,5	8	10,0	23	n.s		
DRB1*02001/DQA1*00401/DQB1*01303	11,6	34	7,8	5	12,6	29	n.s		
DRB1*04901/DQA1*01001/DQB1*01901	18,7	55	12,5	8	20,4	47	n.s		
DRB1*00901/DQA1*00101/DQB1*008011	7,8	23	3,1	2	9,1	21	n.s		
DRB1*00501/DQA1*00301/DQB1*00501	1,0	3	0,0	0	1,3	3	n.s		
DRB1*10201/DQA1*00101/DQB1*00201	1,7	5	1,6	1	1,7	4	n.s		
DRB1*01501/DQA1*00601/DQB1*00301	6,5	19	3,1	2	7,4	17	n.s		

Cases; Gordon setter with symmetrical onychomadesis, Controls; Gordon setter without symmetrical onychomadesis, CI; confidence interval, OR; odds ratio, n.s; non significant

S4 Oligonucleotide primers used in the study

Primer	Primer sequence
DLA-DRB1 (forward)	5'-CCGTCCCCACAGCACATTTTC-3'
DLA-DRB1 (reverse) ¹	5'-TAATACGACTCACTATAGGGTGTGCTACACACCTCAGCACCA-3'
DLA-DQA1 (forward)	5'-CTCTCACGGAGCATACACGA-3'
DLA-DQA1 (reverse)	5'-ATGCTAGGGAGGAAGGGAAA-3'
DLA-DQB1 (forward) ¹	5'-TAATACGACTCACTATAGGGCTCACTGGCCCCGGCTGTCTC-3'
DLA-DQB1 (reverse)	5'-CACCTCGCCGCTGCAACGTG-3'
T7	5'-TAATACGACTCACTATAGGG-3'

¹Primer tailed with T7 promoter at the 5' end.