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CANINE DISEASE MODELS FOR IGA DEFICIENCY

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Canine disease models for IgA deficiency

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Der Wille ist die treibende Kraft jeder Veränderung in der Welt.

-Arthur Schopenhauer

To the people I love

ABSTRACT

Selective IgA deficiency (IgAD) is the most common primary immunodeficiency disorder in Caucasians and is defined as serum IgA concentrations below or equal to 0.07 g/l, with normal serum concentrations of IgM and IgG, in individuals 4 years of age or older. The prevalence of IgAD is approximately 1:600 in the general population and recent results have shown that patients with IgAD have significantly poorer physical health and an increased risk of early death.

The domestic dog is more than just a companion and working animal. The almost 400 distinct modern dog breeds represent great phenotypical diversity and there are more than 350 naturally occurring genetic diseases in dogs which clinically resemble the corresponding human diseases. As a result of domestication, the dog's genome is characterised by long haplotype blocks and linkage disequilibrium (LD) making the dog an appealing model for genetic studies of human diseases.

Low serum IgA concentrations in dogs have been reported to clinically resemble human IgAD. However, even though the literature on IgA concentrations in dogs is extensive, the normal range of serum IgA as well as a generally accepted cut-off value for IgAD deficiency has not yet been established.

We performed an extensive screen of serum IgA concentrations in more than 1,500 dogs from 22 breeds. Dog breed-specific differences in the prevalence of IgAD indicate the involvement of genetic factors in the development of the disease. Furthermore, certain dog breeds were found to stand out as high-risk breeds.

Genome-wide association studies (GWAS) in selected high-risk breeds show that IgAD is associated with genes involved in B cell development and haematopoiesis. Additionally, serum IgA concentrations were found to play an important role in the aetiology of canine atopic dermatitis (CAD), an autoimmune disease in dogs.

Molecular identity allowed the quantification of IgA in serum samples from Canadian and Scandinavian wolves with the same antibodies as those used in dogs. Interestingly, wolves from Scandinavia show significantly lower IgA concentrations as compared to Canadian wolves. Due to its size, the Scandinavian wolf population is prone to inbreeding and therefore, it is known to suffer from a decrease in genetic variation. Further analyses are needed to investigate whether Scandinavian wolves and dogs with a high-risk profile for IgAD share certain genetic factors.

LIST OF SCIENTIFIC PAPERS

I. Olsson M, **Frankowiack M**, Tengvall K, Roosje P, Fall T, Ivansson E *et al*. The dog as a genetic model for immunoglobulin A (IgA) deficiency: Identification of several breeds with low serum IgA concentrations. Vet Immunol Immunopathol. 2014; 255–259.

II. Tengvall K, Kierczak M, Bergvall K, Olsson M, **Frankowiack M**, Farias FHG *et al*. Genome-wide analysis in German shepherd dogs reveals association of a locus on CFA 27 with atopic dermatitis. PLoS Genet. 2013; 9: e1003475.

III. Olsson M, Tengvall K, **Frankowiack M**, Kierczak M, Bergvall K, Axelsson E *et al*. Genome-wide Analyses Suggest Mechanisms Involving Early B-cell Development in Canine IgA Deficiency. PLOS Genetics. Currently under review

IV. **Frankowiack M**, Hellman L, Zhao Y, Arnemo JM, Lin M, Tengvall K *et al*. IgA deficiency in wolves. Dev Comp Immunol. 2013; 40: 180–184.

V. **Frankowiack M**, Olsson M, Cluff HD, Evans AL, Hellman L, Månsson J *et al*. IgA deficiency in wolves from Canada and Scandinavia. Dev Comp Immunol. 2015; 50: 26-28.

LIST OF RELATED SCIENTIFIC PAPERS

A. **Frankowiack M**, Kovanen RM, Repasky GA, Lim CK, Song C *et al*. The Higher Frequency of IgA Deficiency among Swedish Twins is not Explained by HLA Haplotypes. Genes Immun. 2015; doi:10.1038/gene.2014.78

B. Viktorin A, **Frankowiack M**, Padyukov L, Chang Z, Melén E, Sääf A *et al.* IgA measurements in over 12 000 Swedish twins reveal sex differential heritability and regulatory locus near CD30L. Hum Mol Genet. 2014; 23: 4177–4184.

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LIST OF ABBREVIATIONS

APRIL	Tumour necrosis factor superfamily
BSD	Belgian shepherd dog
CAD	Canine atopic dermatitis
CFA	Canine chromosome
$C_H\alpha$	Gene loci encoding the α heavy-chain
CI	Confidence interval
CLEC16A	C-type lectin domain family 16
CNV	Copy number variation
CVID	Common variable immunodeficiency
DLA	Dog leucocyte antigen
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GPR149	G protein-coupled receptor 149
GR	Golden retriever
GSC	Goosecoid homeobox
GSD	German shepherd dog
GWAS	Genome-wide association study
HLA	Human leucocyte antigen
HWD	Hovawart dog
IFIH1	Interferon induced with helicase C domain 1
Ig	Immunoglobulin
IgAD	Selective immunoglobulin A deficiency
IGHA	IgA heavy chain constant region gene
IL	Interleukin
kb	Kilo base pairs
KIRREL3	Kin of IRRE-like protein 3
LD	Linkage disequilibrium
LR	Labrador retriever
MAF	Minor allele frequency
Mb	Mega base pairs
MHC	Major histocompatibility complex
NED	Norwegian elkhound dog
NSDTR	Nova Scotia duck tolling retriever
PBST	Phosphate-buffered saline with Tween
PCR	Polymerase chain reaction
PKP2	Plakophilin 2
PNPP	Para-Nitrophenylphosphate
PPP4R4	Protein phosphatase 4
RETNLB	Resistin like beta
SERPINA	Serpin peptidase inhibitor family
SLE	Systemic lupus erythematosus
SLIT1	Slit homolog 1
SNP	Single nucleotide polymorphism
SP	Shar Pei
T1D	Type 1 diabetes
TACI	Transmembrane activator and CAML interactor
TBST	Tris-buffered saline and Tween
TNF	Tumour necrosis factor

1 INTRODUCTION

1.1 HUMAN IGA

Immunoglobulin (Ig) A accounts for at least 70 % of all Igs produced in the human body. It is the predominant Ig class at the mucosal sites (secretory IgA) and the second most prevalent antibody class, after IgG, in serum [1].

Humans have two functional gene loci encoding the α-heavy chain ($C_H\alpha$) domain of the antibody molecule, resulting in two IgA subclasses (IgA1 and IgA2). The predominant difference between IgA1 and IgA2 is the length of the hinge region [1, 2].

Whilst serum IgA is predominantly monomeric IgA1, the secretory IgA is polymeric, mainly dimeric, with an increased proportion of IgA2 [2]. Serum IgA is produced in the bone marrow while secretory IgA is produced locally at the mucosal surfaces, in direct contact with the environment [2]. In the vast majority of individuals, there is a high correlation between serum IgA concentrations and mucosal IgA concentrations [3].

As the basis of the secretory immune system, IgA protects the lining of the gastrointestinal, respiratory and genitourinary tracts from invading pathogens by neutralising antigens and preventing the adherence of bacteria [2, 4, 5]. The function of serum IgA is less clear, however, there is evidence that serum IgA triggers effector functions [2, 6].

Genome-wide association studies (GWAS) found the regulation of normal serum IgA concentrations in humans to be associated with members of the tumour necrosis factor (TNF) ligand superfamily. Interestingly, both candidate loci are involved in the regulation of B cell proliferation [7, 8].

1.2 SELECTIVE IGA DEFICIENCY IN HUMANS

Selective IgA deficiency (IgAD) is defined as serum IgA concentrations equal to or less than 0.07 g/l, with normal concentrations of IgG and IgM, in individuals four years of age or older [9]. IgAD is the most common primary immunodeficiency in humans and its frequency ranges from 1:143 in Saudi Arabia [10] to 1:18,500 in Japan [11]. In Sweden, the frequency is around 1:600 [12].

IgAD can appear or disappear spontaneously [2]. Furthermore, a transient form of the deficiency can be induced by captopril, penicillamine and other drugs [13]. Until recently, it was believed that only one third of the patients with IgAD have an increased risk of recurrent infections at mucosal sites and an increased predisposition for autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus (SLE), allergy and gastrointestinal infections [14–16]. Recent studies, however, show that IgA-deficient individuals have significantly poorer physical health [17] and an increased risk of early death [18].

The genetic background of IgAD is complex. It is assumed that regulatory mechanisms in the production or secretion of IgA are the cause of the deficiency, since both $C_H\alpha$ genes are expressed and functional in individuals with IgAD and no mutations have been identified within the coding regions of these genes [19].

There is a strong association of IgAD with the major histocompatibility complex (MHC) region, in particular the human leucocyte antigen (HLA) [20]. Up to 45 % of IgAD patients harbour at least one copy of the risk-conveying *HLA-B*08, DRB1*03, DQB1*02* haplotype [21, 22] in comparison to 16 % in the general population [23]. Other haplotypes, like the *HLA-DRB1*07, DQB1*02* and *DRB1*01, DQB1*05* are also associated with IgAD [24]. The *HLA-DRB1*15, DQB1*06* haplotype has been shown to confer almost complete protection against the disorder [24–26].

Furthermore, non-MHC genes (interferon induced with helicase C domain 1 [*IFIH1*] and C-type lectin domain family 16 [*CLEC16A*]) and to a lesser extent, several autoimmunity risk alleles have been found to be associated with IgAD [27]. Lastly, missense mutations in the TNF receptor family member TACI, mediating isotype switching in B cells, were reported in 1:16 individuals with IgAD [28].

1.3 MURINE DISEASE MODELS FOR IGAD

The foremost model for genetic studies in mammals has been the mouse, but with significant limitations. Spontaneously occurring diseases in humans must be induced in mice, thus limiting the possibility to study complex, polygenic human diseases.

Until today, there is no animal model that fully resembles the manifestation of IgAD in humans. In mice, the phenotype is usually genetically or experimentally induced and therefore, resembles the human disease only to a limited extent [13].

Several knockout mice have been generated by targeted deletion of either the joining chain [29], which links two monomeric IgA molecules, or the IgA switch region and/or parts of the antibody's constant region. Although the secretion of IgA was successfully impaired, the concentrations of IgM and (subclasses of) IgG in serum and/or secretion increased significantly [30–34].

Another strategy to artificially create IgA-deficient mouse models is the disruption or deletion of cytokine or cytokine receptor genes crucial for B cell development, such as lymphotoxin α [35, 36], tumour necrosis factor superfamily (APRIL) [37] and the interleukin (IL)-5 receptor [38]. With the exception of IL-5 receptor, a significant decrease in serum IgA concentration has been observed in these models. However, the observed side effects have been substantial. They range from disrupted development of secondary lymphoid organs [35] to an increased number of effector and memory B and T cells [37].

More or less successful, the murine disease models are used to investigate the protection from infection, such as influenza [32] and rotavirus [31, 39], through secretory IgA.

1.4 DOMESTICATION OF THE DOG

The domestic dog (*Canis lupus familiaris*) emerged from its wild ancestor, the gray wolf (*Canis lupus*), through domestication; a process that started around 15,000 years ago [40]. The domestication is characterised by two narrow genetic bottlenecks where only a limited number of wild ancestors contributed to the generation of a very restricted gene pool (Figure 1) [40, 41]. These genetic

restrictions are the reason for considerably short haplotype blocks on a species level and long breed-specific haplotypes [40].

The haplotype blocks and the linkage disequilibrium (LD) in the whole domesticated dog population are about 10 kilo base pairs (kb) and thus, shorter than in humans [40, 41]. LD breaks down over time [42]; as the first genetic bottleneck occurred around 15,000 years ago, there was enough time for breakdown of the haplotype blocks in the whole dog population [41].

There are nearly 400 distinct dog breeds showing great phenotypic diversity for morphology, physiology and behaviour. The phenotypic variety was generated based on few founder animals and stringent breeding strategies. The overall genetic drift led to a loss of genetic diversity within breeds and a greater divergence among them [43]. Long breed-specific haplotype blocks of 0.1 to 1 mega base pairs (Mb) and a LD that is 40 to 100*x* more extensive than in humans are the result [41 ,44, 45]. The generation of modern dog breeds started around 200 years ago [41], not leaving enough time for the haplotypes and the LD to break down. As a consequence, the dog genome displays unique features making it an excellent model for genetic studies.

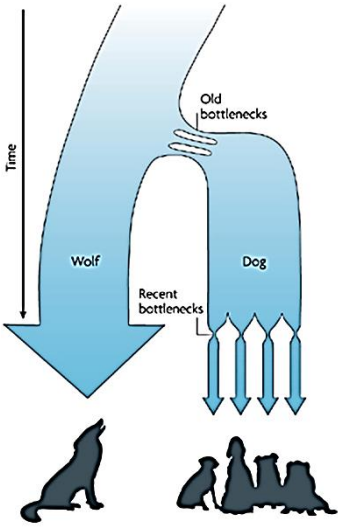

Figure 1. The domestication of modern dog breeds is characterised by two genetic bottlenecks. The first one occurred around 15,000 years ago when the domestic dog diverged from the gray wolves. At the time of the creation of modern dog breeds, around 200 years ago, a very restricted number of founder animals contributed to the genetic pool. As a consequence, our modern dog breeds display long haplotypes and long LD in a dog breed-specific manner. This figure was adapted from Karlsson and Lindblad-Toh, Nature Reviews. Genetics. 2008 Sep; 9(9): 713-725, with permission from Nature Publishing Group.

1.5 THE CANINE LEUCOCYTE ANTIGEN

The dog leucocyte antigen (DLA) is the canine counterpart of the HLA. Like in all vertebrates, the genes inside the DLA are essential for the presentation of foreign and self-peptides to the immune system [46, 47]. The DLA is thought to be under balancing selection to maintain the required diversity. The proposed selection mechanisms are based on providing resistance for diseases and parasites, maternal-foetal interactions and mate choice to avoid inbreeding [48–50].

Low levels of genetic diversity have been suggested to increase vulnerability to infectious diseases [51–53]. Furthermore, a higher level of heterozygosity in the HLA region in humans is thought to provide an advantage in fighting viral diseases [54–57]. Similar results were found for some wildlife species [58], but not all [49, 59–61].

The structure of the DLA was identified through large-scale mapping studies [62]. In general, it is divided into class I, II and III regions [63, 64]. DLA class I and II genes, involved in the processing and presenting of antigens [65], are characterised by low mutation rates and high levels of polymorphism [66–68]. In addition, these genes display a significant sequence homology with the corresponding HLA regions in humans [69–71].

The DLA class II complex is composed of a tightly linked cluster of three functional genes (*DRB1*, *DQA1* and *DQB1*) and one pseudogene (*DRB2*) on canine chromosome (CFA) 12. The complex shows high inter-breed and low intra-breed variation of alleles and haplotypes resulting in a characteristic distribution of DLA alleles for individual breeds (Table 1) [67, 72, 73]. Theoretically, there is a great capacity for allelic variation within the DLA class II region. However, this potential is limited, due to the natural LD and the breeding restrictions [74].

Table 1. Distribution of selected DLA alleles in dogs. This table was adapted from Kennedy et al., Tissue Antigens. 2002 Mar; 59(3): 194-204, with permission from John Wiley and Sons.

DLA allele	Beagle	German shepherd	Labrador retriever	Golden retriever
DRB1*n				
001	40	16	23	6
002	13	7	-	-
008	13	-	3	-
011	2	32	1	-
012	1	2	31	54
015	17	38	22	14
DQA*n				
00101	44	13	25	13
00201	2	15	2	1
00301	15	-	6	4
00401	1	10	32	64
00601	1	37	20	1
00701	1	16	2	1
00901	31	-	-	-
DQB1*n				
101	13	-	-	-
201	50	-	10	-
401	3	-	10	-
701	13	-	15	-
1303	3	-	10	-
1701	-	-	35	-
2301	-	-	15	-

1.6 CANINE DISEASE MODEL

After humans, the dog is the most intensively studied animal in medical practice with detailed records of its family history [41, 75]. There are more than 350 naturally occurring genetic diseases described in dogs that clinically resemble the corresponding human disease [75, 76]. The strong selection of certain traits, popular sires and inbreeding during the domestication of the dog led to an enrichment of disease mutations in different breeds. Often these diseases occur exclusively in a limited number of breeds, due to the enrichment of risk alleles suggesting a heritable component [77].

Due to the similar genomic content and genetic predisposition, the dog is an excellent model for mapping disease-causing genes of common and even complex diseases [41, 78]. Gene mapping was successfully performed for narcolepsy [79], copper toxicosis [80, 81], ichthyosis [82], haemophilia, retinal degeneration, and muscular dystrophy [83] amongst others.

The dog's many advantages, beside its particular genetics, make it a superior model system for translational medicine. In 2005, the first high-quality draft sequence of the euchromatic genome of a dog, a female boxer named Tasha, was published [41]. Additional analysis of the canine haplotype structure and a dense single nucleotide polymorphism (SNP) map enabled the study of even more complex disease backgrounds, including human aging and Alzheimer's disease [84], cancer [85], SLE [86], as well as the environmental contributions to diseases, aging, and disease heredity [83].

There are further advantages the canine disease models have to offer. The dog is a companion animal with which we share our environment and it receives good care, including medical care. The lifespan of a dog is approximately seven times shorter than that of humans, making disease development and progression shorter. Reduced regulatory guidelines and increasing acceptance by regulatory bodies make clinical trials in dogs even more attractive [83, 85].

The enormous genetic diversity between different dog breeds is the cause for some limitations of canine disease models. Breed-specific differences in physiology and metabolism make a generalisation of results difficult. This is particularly the case in pharmacodynamics and pharmacokinetics [87]. Compared to rodent models, their generation time is longer, they are more expensive to house, due to their size, and use for research purposes is more strictly regulated [83, 84].

1.7 IGA AND IGAD IN DOGS

In contrast to humans, only a single $C_H\alpha$ gene has been described in dogs [88]. It occurs in four different allelic variants which differ mainly in the length of the hinge region [89]. The polymeric, predominantly dimeric, mucosal IgA is produced by plasma cells at the mucosal sites of the intestine [90, 91]. Interestingly, mucosal IgA represents the main source of serum IgA in dogs [92].

Also, in dogs there is a correlation between IgA concentration and age. While the concentrations are low [93] or even undetectable [94] in young animals, they later increase with ageing [95, 96].

The quantification of immunoglobulins is commonly used in dogs to assess immune function. Still, the normal range of serum IgA and a generally accepted, physiologically proven cut-off value for IgAD have not been established yet. This is partially due to the strong variation of IgA concentrations among different dog breeds [97].

Previously suggested cut-offs in different dog breeds range from 0.15 g/l [98, 99] to 0.3 g/l [100]. Selected dog breeds, including the Chinese Shar Pei (SP) [98, 100] and selected Beagle dogs [95, 101], show innately low IgA concentrations or even overt deficiency. For German shepherd dogs (GSD), conflicting results have been published. Some studies report no difference in serum IgA concentrations [102] and faecal samples [103] as compared to other breeds. Other studies find lower IgA concentrations in serum [104–107], tears [108], faeces [109] and secretions of duodenal explants [110].

The majority of animals displays no clinical symptoms [107]. In terms of recurrent infection, especially of the skin and the upper respiratory tract [95, 100, 105] and the increased susceptibility to immune-mediated diseases [95], low IgA concentrations in dogs clinically resemble human IgAD. Furthermore, intestinal bacterial overgrowth has been found in dogs with IgAD [105, 106].

1.8 IGA CONCENTRATIONS IN WOLVES

Despite all the work on IgA concentrations in dogs, very little is known on Ig concentrations in wolves. This is remarkable, especially since the grey wolf is considered to be the ancestor of the dog [111].

One study on serum IgE in wolves found concentrations approximately twice as high as the ones in dogs [112].

1.9 CANINE ATOPIC DERMATITIS

Canine atopic dermatitis (CAD) is a complex disease involving immune dysregulation, allergic sensitisation, skin barrier defects, microbial colonisation and environmental factors [113]. It is an inflammatory and pruritic allergic skin disease with clinical manifestations similar to human atopic dermatitis. CAD develops in young dogs between six months and three years of age [114]. It affects 10-15 % of all dogs [113–116].

There is a genetic component with a strong breed predisposition (e.g. for Labrador retriever [LR]) involved in CAD [117, 115]. Environmental factors have been found to be crucial for the development of CAD and this might explain its seasonal manifestations [118].

Most dogs with CAD produce IgE antibodies directed towards environmental allergens [119, 120]. However, IgE concentrations are not correlated to the disease course [121]. Moreover, total IgE concentrations in normal and atopic dogs have not been found to differ significantly [122].

Allergic symptoms manifest in the muzzle, on the ears, the paws, the extremities, the ventrum and flex zones in the form of pruritus and erythema [123].

2 AIMS

2.1 GENERAL AIM

The aim of this thesis is to evaluate the potential of a canine disease model for the study of genetic risk factors associated with IgAD in humans.

2.2 SPECIFIC AIMS

The specific aims of this thesis are to investigate the prevalence of IgAD in different dog breeds, identify dog breeds with a high-risk profile for IgAD and to analyse genomic loci associated with IgAD in selected high-risk breeds using GWAS.

Furthermore, the breed-specific differences in the prevalence of IgAD raised the question as to whether canine IgAD is a breeding-enriched phenomenon. To address this question, serum IgA concentrations in wolves from North America and Scandinavia have been analysed.

3 MATERIALS AND METHODS

3.1 SAMPLE COHORTS

3.1.1 Dog samples

Blood samples (ethylenediaminetetraacetic acid [EDTA] blood for the extraction of deoxyribonucleic acid [DNA] and serum for IgA quantification) were collected from 1,623 privately owned, purebred dogs aged between 0.5 and 17 years in collaboration with veterinaries in the United States, Sweden and Switzerland (Table 2). The samples were obtained all year round between 2007 and 2013. Owner consent was collected for each dog.

Serum was isolated from blood samples after centrifugation and stored at -20 °C/ -80 °C until use. The sampling was conformed to the approval of the Swedish Animal Ethical Committee, the Swedish Animal Welfare Agency, the Broad Institute (Cambridge, MA, USA) and the canton of Bern (Switzerland).

Table 2. Number of dogs from different dog breeds included in the studies. This table was adapted from Olsson et al., Vet. Immunol. Immunopathol. 2014 Aug; 160(3-4): 255-259, with permission from Elsevier.

Breed	Abbreviation	Study 1	Study 2	Study 3	Total
American Staffordshire bullterrier	-	13	-	-	13
Bearded collie	-	49	-	-	49
Belgian shepherd	BSD	10	-	-	10
Berner sennen	-	21	-	-	21
Border collie	-	20	-	-	20
Boxer	-	20	-	-	20
Bullterrier	-	14	-	-	14
German shepherd	GSD	319	207	516	516
Giant schnauzer	-	20	-	-	20
Golden retriever	GR	168	-	187	187
Hovawart	HWD	19	-	-	19
Jämthund	-	19	-	-	19
Labrador retriever	LR	141	-	302	302
Leonberger	-	20	-	-	20
Norwegian elkhund	NED	14	-	-	14
Nova Scotia duck tolling retriever	NSDTR	11	-	-	11
Rottweiler	-	20	-	-	20
Samoyed	-	19	-	-	18
Shar Pei	SP	157	-	96	157
Staffordshire bullterrier	-	20	-	-	20
Standard Poodle	-	137	-	-	137
Tibetan spaniel	-	16	-	-	16
Total population		1247	207	1101	1623

3.1.2 Wolf samples

Blood samples were collected from 150 free-ranging Scandinavian wolves in the winters (December–March) from 1998 through 2014 primarily from the central region of Sweden and Norway (59–61°N, 11–15°E, [Table 3]). Ten individuals were sampled in northern Sweden, partially at the border to Finland. These 150 wolves correspond to approximately 75 % of the entire free-ranging wolf population in Sweden and Norway. The individuals were chemically immobilised for radio collaring, health assessment, and sampling of biological materials. Blood was collected in tubes with clot activating factor (Venoject IIsystem, Terumo Europe N.V., Leuven, Belgium). The tubes were kept at room temperature for 1–2 h to ensure complete coagulation. Serum was then separated by centrifugation and stored at -20 °C until use. Thirteen samples were collected from wolves living in captivity. The samples were collected from chemically immobilised individuals during routine check-ups. Blood was collected in tubes with gel and clot activating factor (Venoject® IIsystem, Terumo Europe N.V., Leuven, Belgium) and treated as described above.

Blood samples from 33 free-ranging Canadian wolves were collected in Nunavut and the rest of the North-West Territories in Canada in March 2012 or June 2013 (Table 3). The individuals were chemically immobilised for radio collaring, health assessment, and sampling of biological materials. Blood was collected in red top vacutainer tubes with clot activating factor (Becton, Dickson and company, Franklin Lakes, NJ, USA) and treated as described above.

Capture of free-ranging wolves was approved by the Ethical Committee on Animal Experiments in Uppsala, Sweden, by the National Animal Research Authority in Oslo, Norway and the Northwest Territories Wildlife Care Committee in Yellowknife, Canada.

Table 3. Number of Canadian and Scandinavian wolves

		Study 4	Study 5	Total
Scandinavian wolves	Captive	13	-	13
	Free-ranging	58	92	150
	Total	71	92	163
Canadian wolves		-	33	-

3.2 DNA EXTRACTION

Genomic DNA was extracted from EDTA blood samples using Qiagen mini- and/or midiprep extraction kits (Qiagen, Hilden, Germany) or from whole blood using the phenol-chloroform or the salting out method.

3.3 SERUM IMMUNOGLOBULIN CONCENTRATIONS

Total serum IgA concentrations were determined by enzyme-linked immunosorbent assay (ELISA) at room temperature using non-commercial canine reference samples with known IgA concentrations, as described previously [124, 125]. All samples were quantified at least two times. Samples showing

strong variation between the individual measurements were run again and potential outliers were subsequently excluded.

For studies 1, 3, 4 and 5, polyclonal goat anti-dog IgA antibodies (AbD Serotec, Oxford, UK) and alkaline phosphatase-conjugated polyclonal goat anti-dog IgA antibodies (Bethyl Laboratories, Montgomery, TX, USA) were diluted 1:2,000 with carbonate-bicarbonate buffer (0.05 M, pH 9.6) or Tris-buffered saline and Tween (TBST) respectively. Samples were diluted 1:25,000, 1:50,000 and 1:100,000 with phosphate-buffered saline with Tween (PBST). Para-Nitrophenylphosphate (PNPP) was used as substrate.

For studies 2 and 4, polyclonal goat anti-dog IgA antibodies (AbD Serotec, Oxford, UK), monoclonal mouse anti-dog IgA antibodies (AbD Serotec, Oxford, UK) and alkaline phosphatase-conjugated polyclonal goat anti-dog IgA antibodies (Jackson Immunoresearch, West Grove, PA, USA) were diluted 1:2,000 in PBST. All samples were diluted three times as described above and PNPP was used as a substrate.

3.4 STATISTICS

All descriptive statistics were calculated using routine procedures and software such as GraphPad (La Jolla, CA, USA), IBM SPSS (Armonk, NY, USA) and R (www.r-project.org). Normal distribution of IgA concentrations was tested by D'Agostino normality test. The number of animals in different groups was assayed for significance by Fisher's exact test (study 2). The effect of age, sex, subpopulations and/or CAD on IgA concentration was assessed either by Spearman correlation test (studies 1 and 4), Pearson's correlation coefficient (study 2) or Kendall's rank correlation test (study 5). Mann-Whitney test (studies 1 and 5) or Welch two-sample t-test (study 2) was used to compare the IgA concentrations between different groups of animals. Differences were considered significant if $p<0.05$.

3.5 GENOME-WIDE ASSOCIATION STUDY

Genome-wide scans were performed for 1,101 dogs (516 GSD, 187 GR, 302 LR and 96 SP) with previously determined IgA concentrations using the Illumina 170k Canine HD Bead-Chip (Illumina, San Diego, CA, USA). A further 179 GSD were successfully genotyped, of which 91 were CAD cases and 88 were healthy controls.

Quality control was conducted; non-informative markers, markers with a low call rate (≤ 0.95) and markers not in Hardy-Weinberg equilibrium were removed to minimise potential bias and error in GWAS results.

In study 3, an association analysis of CAD was performed in GSD with IgA concentrations (as scale measure) and age at sampling as covariates.

In study 4, an association of IgA concentrations with genetic markers was analysed. Age at sampling was used as covariate. A different GWAS was performed in each of the four breeds separately. The IgA concentrations were divided into groups of percentile intervals, hence the analysis was run in a close-to-continuous manner. A subpopulation assignment was included in the analysis as an additional

covariate only for GSD; cases and controls were included for this analysis. All individuals with IgA concentrations lower than the 25th percentile were considered cases. Controls were all individuals with IgA concentrations higher than the 75th percentile.

To control for cryptic relatedness and population sub-structure a mixed model approach was applied in both studies. Genome-wide significance was defined as p-values below 0.05 after 100,000 permutations or by using an empirical threshold (97.5 % confidence interval [CI]) calculated from the distribution of p-values and after permutations.

3.6 SEQUENCING OF THE *IGHA* GENE LOCUS

The IgA heavy chain constant region gene (*IGHA*), encoding the heavy chain constant region (C_H1 to C_H3) was sequenced in genomic DNA from the blood of two wolves. The gene was amplified by polymerase chain reaction (PCR) using primers targeting the *IGHA* gene in dogs:

5’ CATGAAGACCTGTGCATTTC TCA 3’,
5’ AGGGACTCAATTGTGAGGAGGAA 3’.

The resulting products were cloned into a pMD19-T vector and sequenced by Sanger sequencing.

3.7 IMMUNODIFFUSION

Serum samples from dog and wolf were analysed for identity by double immunodiffusion, as described previously [126], using polyclonal goat anti-dog IgA antibodies (AbD Serotec, Oxford, UK) and monoclonal mouse anti-dog IgA antibodies (AbD Serotec, Oxford, UK). Antisera and serum samples were added to holes with a defined distance in an agarose matrix. The samples were allowed to diffuse in the matrix.

4 RESULTS

4.1 SERUM IGA CONCENTRATIONS AND PREVALENCE FOR IGAD IN DOGS

4.1.1 IgA concentrations in different dog breeds

The measured IgA concentrations range from 0.01 g/l to 3.0 g/l (Figure 2). The concentrations vary widely among the 22 different purebred dog breeds analysed. The SP stands out as the most high-risk breed for low median serum IgA concentrations followed by HWD, NED, NSDTR, BSD, American Staffordshire terrier, Bullterrier, GSD, GR, LR and Staffordshire bullterrier.

The difference in IgA concentrations between males and females as well as castrated and non-castrated dogs was found to be insignificant. A positive correlation between IgA concentrations and age was found.

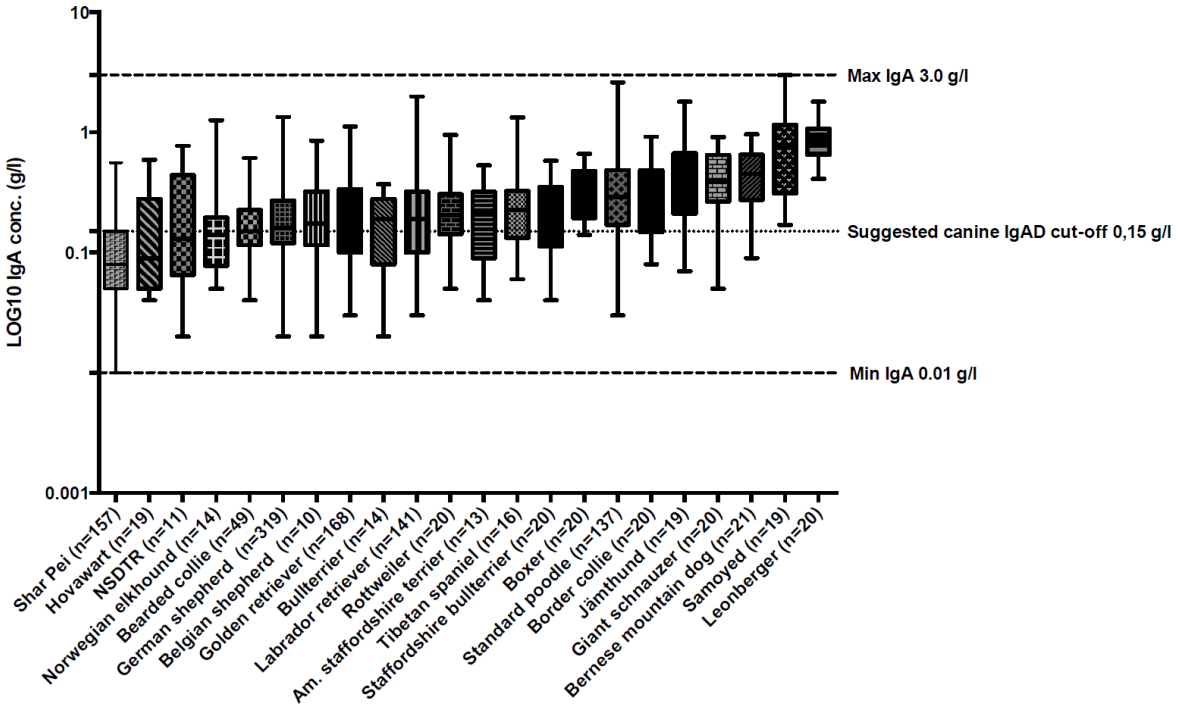

Figure 2. IgA concentrations measured for 22 different dog breeds. This figure was adapted from Olsson et al., Vet. Immunol. Immunopathol. 2014 Aug; 160(3-4): 255-259, with permission from Elsevier.

4.1.2 Defining an IgAD cut-off

In accordance with previously suggested cut-offs for IgAD [95, 98, 99, 102], we calculated a 95 % CI of the mean resulting in a cut-off of 0.26 g/l for the total population of 1,623 dogs. Due to the strong variation in IgA concentrations among different breeds, a breed-specific cut-off might be preferred.

4.1.3 Prevalence of IgAD in different dog breeds

Low serum IgA concentrations or even overt deficiency have previously been found in the SP [98, 100], the GSD [104, 105, 107, 109] and Beagle dogs from selected kennels [95, 101].

Our studies suggest an average prevalence of IgAD in the investigated dog breeds of 15 % (Figure 3). Furthermore, the following five dog breeds show an increased prevalence for IgAD compared to the average: SP, HWD, NED, NSDTR and BSD.

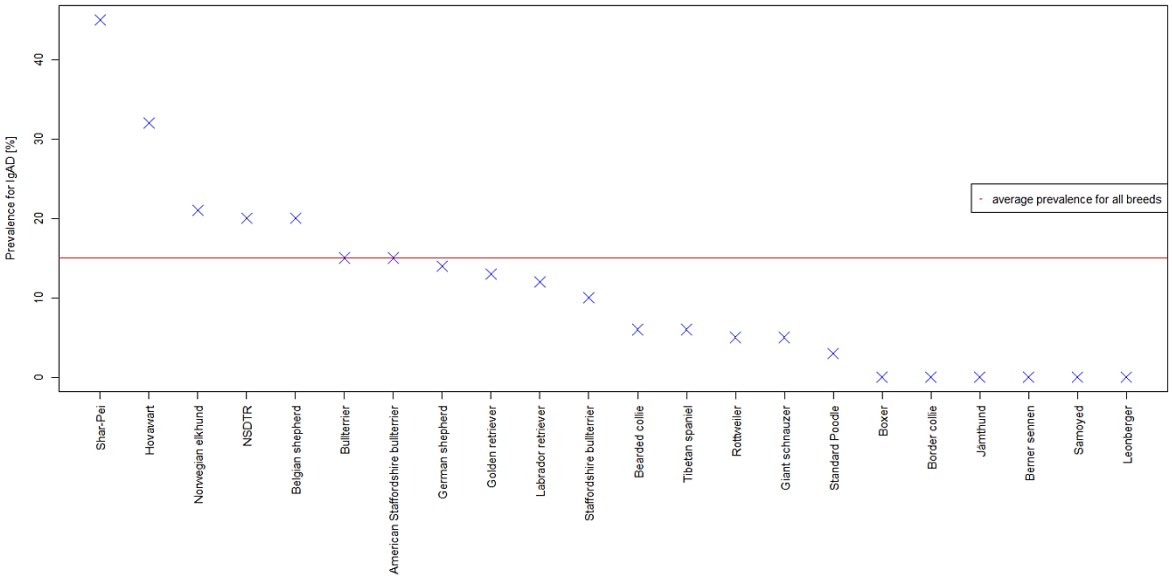

Figure 3. Prevalence of IgAD in different dog breeds.

4.2 SERUM IGA CONCENTRATIONS AND PREVALENCE OF IGAD IN WOLVES

4.2.1 Molecular identity of dog and wolf IgA

The sequenced *IGHA* gene in wolves has shown complete sequence homology to one of the four known gene variants [89] in dogs. Moreover, the fused precipitation lines indicate that the applied detection antibodies bind to the same epitope in the IgA molecule in dog and wolf (Figure 4) [124].

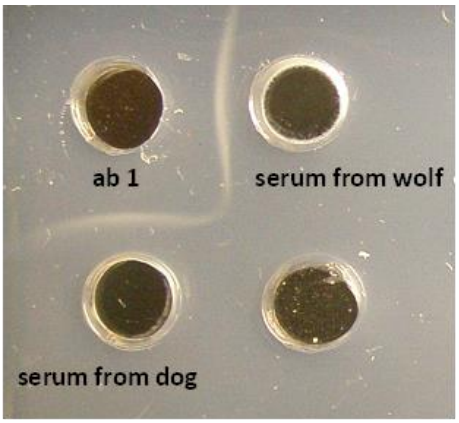

Figure 4. Double immunodiffusion showing binding of one of the detection antibodies to IgA in serum samples from dogs and wolves. This figure was adapted from Frankowiack et al., Dev. Comp. Immunol. 2013 Jun; 40(2): 180-184, with permission from Elsevier.

4.2.2 IgA concentrations in wolves

The IgA concentrations in wolves range from 0.01 g/l to 0.23 g/l. No sex- or age-dependency has been found. Furthermore, no difference has been found between free-ranging and captive wolves.

Scandinavian and Canadian wolves differ significantly in their IgA concentrations. The median IgA concentration for Scandinavian wolves is 0.05 g/l and 0.18 g/l for Canadian wolves.

Applying an arbitrary cut-off at 0.1 g/l, the average prevalence for IgAD in Scandinavian wolves is 80 %. The prevalence of IgAD in wolves from North America is only 14 %.

4.3 GENETIC ASSOCIATION OF IGAD AND CAD

4.3.1 Genetic association of CAD and IgAD

Low serum IgA concentrations correlate strongly to the CAD phenotype in GSD. In total, 19 SNPs on CFA27 show a significant association to CAD. Furthermore, these 19 SNPs have been found to form a 1.5 Mb-long associated haplotype with an 86 % correlation with the case and control haplotype pattern. The associated haplotype includes eight genes, with the top two SNPs surrounding the gene plakophilin 2 (*PKP2)*.

4.3.2 Genetic association of IgAD

In the association analysis performed in four dog breeds prone to low IgA concentrations, 35 genomic loci were found to be suggestively associated. However, there were no overlapping association signals in the different breeds.

In GSD, three loci on CFA5, CFA8 and CFA23 showed a significant association with IgA concentrations. The first locus on CFA5 is around 1 Mb long, consisting of 14 SNPs. Based on the SNPs in LD with the top SNP, a region around 1.7 Mb long was identified. This region consists of 18 SNPs and it resulted in 12 different haplotypes. Two of the haplotypes were defined as risk haplotypes, one as an IgA concentration-independent haplotype and nine as rare haplotypes. Only the gene kin of IRRE-like protein 3 (*KIRREL3*) is harboured by these haplotypes, despite its size.

Two single SNPs on CFA8 and CFA23 showed significant association. The CFA8 top SNP is in high LD with three SNPs spanning a region of about 0.5 Mb. Eleven haplotypes could be defined from this region. None of these haplotypes were found to convey either a risk for or a protection from low IgA concentrations. However, the region harbours 13 genes, such as goosecoid homeobox (*GSC*), resistin-like beta (*RETNLB),* protein phosphatase 4 (*PPP4R4*), and 10 members (member 1, 2, 3-6, 9-13) in the Serpin peptidase inhibitor family (*SERPINA*). The CFA23 top SNP is located in the intron of the gene G protein-coupled receptor 149 (*GPR149*) and is not in LD with any other SNP.

In SP, a significant association to a region on CFA28 was found. The region is part of a ~20 kb-long haplotype. In total, two rare haplotypes, two common haplotypes, four risk haplotypes, and one protective haplotype were defined. A significant difference in IgA concentrations between the risk and the protective haplotype was found in homozygous dogs. Furthermore, both the dogs homozygous for the risk haplotype and the dogs heterozygous for the risk haplotype show a significant difference in IgA concentration. The haplotype spanned the first intron of the gene slit homolog 1 (*SLIT1).*

Pathway analysis unveils significant connectivity between genes in the IgA associated regions across three breeds (GSD, GR and SP). The results indicate that around 45 % of the enriched genes are involved in inflammation. Gene set enrichment analysis indicates the involvement of, amongst others, transcriptional activity, haematopoiesis and regulation of cell growth.

5 DISCUSSION

5.1 IGA CONCENTRATIONS AND IGAD IN DIFFERENT DOG BREEDS

The proportion of dogs with a low IgA concentration or even an overt IgAD, as well as the range of IgA concentrations, has been found to vary widely among different breeds. The inter-breed variability makes it difficult to establish a generally accepted, physiologically proven cut-off for IgAD. Previously suggested cut-offs in different dog breeds include 0.3 g/l [100], 0.22 g/l [102], 0.18 g/l [95], and 0.15 g/l [98, 99], these being higher than the cut-offs we chose. In agreement with some studies, we suggest the lower limit of the 95 % CI for the mean in the studied population to be used as a cut-off [95, 98, 99].

Most purebred dogs have low levels of genetic variation caused by founder effects, strict breeding practices and reproductive isolation. As a consequence, these breeds show a very high susceptibility to one or more genetic diseases [40, 127]. IgAD appears to be one of the diseases shared among multiple breeds.

The SP is known to be a high-risk dog breed for IgAD [98, 100] and it stands out as the breed with the highest prevalence of IgAD. Amongst the other dog breeds with a considerably increased prevalence are the HWD, the NED and the GSD; at least for the latter, inconsistent results have previously been published. Normal faecal IgA concentrations [103] and a larger variance in serum IgA concentrations as compared to other breeds [102] have been reported, as well as low IgA concentrations or IgAD in faeces [109] and serum [105–107]. Our results suggest that the GSD is a high-risk breed for IgAD [97].

5.2 IGAD-ASSOCIATED DISEASES IN DOGS

In humans, IgAD is associated with autoimmune diseases such as SLE and celiac disease (CD) [16]. In dogs, IgAD is known to increase susceptibility to immune-mediated diseases [95].

The GSD has an exceptionally high susceptibility to immunological diseases or immune-related disorders including skin as well as gastrointestinal problems, such as CAD [128, 129]. We found a significant difference in IgA concentrations in CAD cases as compared to CAD controls that indicates a functional role for IgA in the aetiology of CAD [125]. Furthermore, we identified the gene *PKP2* as a target gene. The protein encoded by *PKP2* is involved in linking cadherins to intermediate filaments in the cytoskeleton [130]. Degradation processes in the surface of the skin are known to be common in skin diseases including atopic dermatitis [131], underlining that *PKP2* is a good candidate gene for CAD. Furthermore, altered mRNA expression of *PKP2* has been detected between atopic and healthy skin and even correlated with clinical severity in atopic skin [132].

5.3 GENETIC ASSOCIATIONS WITH IGAD IN DOGS

Our results suggest that canine IgAD is a polygenetic, multifactorial disease. Results from our GWAS performed in four breeds prone to low IgA concentrations identified risk haplotypes and target genes like *KIRREL3*, *SERPINA9* and *SLIT1*. These genes are known to play a role in naïve B cell

maintenance and the regulation of haematopoiesis in the bone marrow [133–139]. Even though the molecular basis of IgAD is unknown, defects were suggested at several steps, including the stem cell level [140]. Furthermore, most patients with IgAD were found to have a lower percentage of class-switched memory B cells [141], or both memory B cells and class-switched memory B cells, as compared to the healthy controls [142]. Interestingly, a reconstitution of IgA production could be demonstrated in IgAD subjects by in-vitro induction of class-switch recombination (CSR) through CD40 together with IL-4 or IL-10, or IL-21 exclusively [143–148]. This suggests that a lack of one or more essential signals following class-switching may result in a selective elimination of IgA^+ B cells.

In humans, a mechanistic and genetic difference between low concentrations of serum IgA and IgAD has been suggested [149]; contrary to humans, there is no clear distinction between low concentrations and IgAD in dogs. In conclusion, the background IgAD in dogs might be equivalent to low serum IgA concentrations in humans and thus explain why, at this stage, we were not able to prove homology to human IgAD-associated genes for our candidate genes.

5.4 DLA AND AUTOIMMUNE DISEASES

In both humans and dogs the class II region of the HLA and DLA respectively, is one of the most important genetic risk factors for autoimmune disease [74, 150]. As a consequence of a shrinking population size, geographic isolation of populations or selective breeding for specific traits, the genetic diversity of the DLA can be limited. What follows are strong inter-breed, low intra-breed polymorphisms and high levels of homozygosity in 35 % [72] to 40 % [67] of the DLA class II region in purebred dogs [67].

Consequently, the breed-specific distribution of DLA haplotypes [67, 72, 151] and the decrease in genetic diversity might cause the breed-specific increases in disease predisposition of immunodeficiencies such as IgAD [97], autoimmune diseases like canine immune arthritis [152], diabetes mellitus [153–155], SLE [86], and cancer [67]. Studies on diabetes mellitus have found similar risk-associated haplotypes and genotypes in different susceptible breeds [153] confirming this notion.

Similarly to the human HLA, the DLA might not explain the complete heritability of complex diseases. It might indicate a link to other genetic markers outside the DLA or to genetic variations in the form of polymorphisms or gene copy number variation (CNV) elsewhere in the genome. Such a link has been found for SLE in humans [156]. Consequently, alleles inside the DLA might not only represent a potential genetic cause for a disease, but might also be an indicator for disease susceptibility.

Although studies in humans have found associations between IgAD and the HLA [20, 27], no association has yet been found with the DLA. The lack of a physiologically proven cut-off makes a distinction between cases and controls challenging. Strong, but not significant, day-to-day variations in IgA concentrations have also been found in saliva, tears and faeces [93, 103]. Since mucosal IgA is the main source of serum IgA in dogs [92], one could assume similar variations of serum IgA. Unfortunately, due to the complexity involved in sample drawing, a repeated sampling of the dogs, albeit highly desirable, might not be possible to achieve.

Despite breed-specific differences in the DLA, different dog breeds susceptible to diabetes mellitus show similar risk haplotypes and alleles [153]. Assuming an analogous underlying disease cause for IgAD, genotyping could help to increase the power of a GWAS due to further population stratification.

Although the applied chip allows for genome-wide genotyping with a uniform coverage, including the DLA, some of the SNPs failed due to technical reasons or quality control. Even though the majority of SNPs that failed quality control has been excluded due to a low minor allele frequency (MAF), actual mutations or variants might have been missed.

5.5 IGAD IN WOLVES

The nuclear coding-DNA sequence from the domestic dog differs only by 0.04 % from the gray wolf [41, 77]. As a result of this close relationship within wolf-like canids, genetic tools developed for dogs are usually applicable to wolves as well [77, 157]. SNPs identified in the domestic dog can therefore be used to characterise variations in wild wolves [158].

We could prove complete sequence homology of the *IGHA* gene in wolves to one of the four known gene variants in dogs [89, 124]. Moreover, the antibodies applied for the quantification of serum IgA concentrations in dogs and wolves have been found highly reliable, because of the strong correlation between the results obtained using the two different sets of antibodies and a unique line of precipitation in immunodiffusion [124].

High levels of inbreeding in the Scandinavian wolf population might be the cause for the difference in serum IgA concentrations displayed by Canadian and Scandinavian wolves [159, 160]. Despite the observation that reasonably high levels of DLA diversity can be maintained in small populations [161], the Scandinavian wolf population is an exception as it was established by only three animals [162, 163]. The breeding restriction-dependent reduction in genetic variation could have led to an increased proportion of disease-associated DLA alleles or haplotypes in comparison to the Canadian wolves. Therefore, a potential enrichment of the same genetic risk factors (supposedly in the DLA region) in certain high-risk dog breeds as well as the Scandinavian wolf population might be the cause for the increased prevalence of IgAD. These risk factors might have been enriched during the domestication and passed on to certain dog breeds. This would explain the breed-specific variation in IgA concentrations, the prominent differences in disease prevalence as well as the similarly low IgA concentrations in Scandinavian wolves and SP dogs.

Parts of the DLA class II genes in gray wolves from North America were found to be identical in structure to the DLA of domestic dogs with similar polymorphisms at each of the DLA class II sites [164]. Thus, the combination of DLA class II alleles might be under strong maintaining pressure. Even though wolves from North America had a few dog DLA alleles, Canadian wolves in particular showed high levels of variation in the DLA class II region [164]. A reduced susceptibility to IgAD might thus be the result of a larger gene pool in the North American wolf population.

Interestingly, data on the DLA class II alleles in wolves from Scandinavia [162] and North America [164] indicated only a minor overlay. However, this data has to be interpreted with caution, since it is based on a limited number of animals.

5.6 TWINS AS DISEASE MODELS FOR IGAD

Twins, in particular monozygotic twins discordant for IgAD, provide a unique possibility to study the complex interplay of genes and environment in propensity to disease. So far, there are seven references on serum IgA concentrations in twins [165–171]. Three of them are case reports [167–169] and two studies suggest either a genetic influence on serum IgA concentrations [165] or a combination of genetic and environmental influences [166].

More recent studies suggest non-HLA or even non-genetic predisposing risk factors for the development of IgAD [170, 172]. One study on the prevalence of IgAD among patients with type 1 diabetes (T1D), which is known to be higher compared with the general population [16, 173], suggests an incomplete penetrance for certain HLA-determined Ig deficiencies [170]. The other study finds an increase in disease prevalence among twins compared to the healthy population which is not explained by a genetic association with the HLA. Furthermore, the heritability for IgAD has been found to be 35 % [172].

The problem of missing heritability is shared by most common diseases [174–176] and mapping of complex human diseases finds many mutations outside known genes that are likely to alter gene regulation rather than the coding sequence [177]. The findings described in here stress the importance of non-HLA genetic differences or environmental factors and support the conclusions drawn from the studies in dogs and wolves.

6 FUTURE PERSPECTIVES

6.1 ESTABLISHMENT OF A PHYSIOLOGICALLY PROVEN CUT-OFF FOR IGAD

A physiologically proven cut-off is crucial to distinguish between low concentrations of IgA and IgAD. Unfortunately, there is no generally accepted cut-off in dogs as yet. Even if the majority of animals displays no clinical symptoms [107], low IgA concentrations resemble human IgAD in a proportion of dogs. Hence, an additional screening of medical records might help identify dogs with recurrent infections, especially in the skin and the upper respiratory tract [95, 100, 105] as well as an increased susceptibility to immune-mediated diseases [95]. Their IgA concentrations could consequently be compared to a control group in a breed-specific manner.

6.2 IGAD AND AUTOIMMUNE DISEASES

An in-depth analysis of medical records would also allow for the investigation of an association between IgAD and immune-mediated diseases including autoimmune diseases and allergies. There is a well-established connection between human IgAD and autoimmune diseases [16]. Furthermore, we found indications for IgA to play an important role in the aetiology of CAD [125].

Despite the complexity involved in drawing multiple samples from the same animal, it would allow for analysis of the variation in IgA concentrations in dogs. This is of particular interest since seasonal fluctuation of IgA concentrations are known in humans [178]. Furthermore, IgAD in humans can

appear and disappear without a known cause [2]. Multiple samples could help confirm the permanent status of the deficiency in the animals.

In summary, not only could a retrospective analysis of the medical records help to establish a physiologically proven cut-off for IgAD, it could also further substantiate the similarities in disease manifestation in humans and dogs.

6.3 DLA HAPLOTYPE ANALYSIS

Breed specificity in the prevalence of IgAD [97] and the haplotype distribution [67, 72, 151] indicate a possible association between IgAD and the DLA. The genotype data from the GWAS could be used to impute the DLA haplotypes in the study cohorts and to investigate the presence of risk-conveying DLA alleles or haplotypes similar to ones known in humans (see 1.2). Ultimately, GWAS could be run on the proportion of cases harboring a respective risk haplotype. This way, the power of the analysis would be increased due to the exclusion of genetic variance in the DLA.

The genotype array used allows for genome-wide genotyping of more than 170,000 SNPs, which uniformly cover the whole genome, including the DLA. Should further genotype information be needed, despite the strong LD, sequencing of parts of the DLA class II region might be required. In this case, sequencing of only one gene of the DLA class II region might be sufficient, due to the small number of possible alleles at each locus in purebred dogs [74].

Similarly to dogs, wolves from both Canada and Scandinavia could be genotyped; their DLA haplotypes could be imputed and compared. It can be assumed that the Scandinavian wolves display a much lower diversity of the DLA region, since they represent a much smaller population which is also prone to inbreeding (see 5.5).

It is known that wolves from North America and domestic dogs share similarities in their DLA class II genes [164]. One should investigate if this accounts also for potential IgAD risk-haplotypes. Furthermore, a comparison of the DLA haplotypes from wolves and dogs with IgAD might support the idea that IgAD is a breeding-enriched phenomenon that has been passed on to some purebred dogs from the gray wolf. Finally, it should be analysed if there are greater similarities between the DLA haplotypes of the Scandinavian wolves and Canadian wolves or one of the high-risk dog breeds. A limitation to running GWAS on these wolves is the size of the available sample cohort. Even for a simple multiplicative risk model, 100 cases and 100 controls are needed, according to power calculations, to detect a fivefold risk allele in 98 % of data sets [40].

6.4 WHOLE-GENOME SEQUENCING

In common variable immunodeficiency (CVID), another primary immunodeficiency, disease-associated CNVs have been found [179]. However, array-based genotyping methods do not allow for the detection of structural variants, such as CNV, prediction of functionality, non-coding mutations and genes located in non-conserved elements or in complex regions with low sequence coverage. To aggravate this situation, structural variants in dogs are poorly understood and until today

there are only five large studies on structural variants in dogs using comparative genomic hybridisation (CGH) [180].

One approach to target structural variants, genetic differences outside the DLA and outside SNPs included on the array is whole genome sequencing. DNA sequences from Canadian and Scandinavian wolves could thus be compared in an attempt to identify differences that might account for different disease prevalence for IgAD.

6.5 WOLF SAMPLES FROM DIFFERENT POPULATIONS

More serum samples from wolves, ideally from different populations could be used to investigate whether low serum IgA concentrations are unique for the Scandinavian wolves. Additionally, an assessment on whether low IgA concentrations have a negative effect on the health and fitness of these wolves might be informative. For this purpose, it might be feasible to focus on captive wolves as regular health checkups are quite common for most of them.

7 CONCLUSIONS

The dog displays an appealing disease model for IgAD. Not only do dogs with low serum IgA concentrations resemble human IgAD from a clinical point of view but there is also a genetic background present in canine IgAD. Serum IgA concentrations were found to differ between different dog breeds. Moreover, several additional dog breeds were identified as high-risk breeds.

GWAS have identified new, highly interesting target genes associated with low concentrations of serum IgA using a novel percentile groups-approach that allowed for the establishment of breed-specific cut-offs and the performance of analysis in a close to continuous manner. The results suggest that genetic markers involved in B cell development and haematopoiesis play an important role in canine IgAD. Furthermore, our results suggest that canine IgAD is a polygenic, multifactorial disease.

IgA concentrations were found to be correlated with the immune-related disease CAD and a novel target gene was found to be associated with CAD. Furthermore, our results indicate that Scandinavian wolves might represent another high-risk population for IgAD possibly sharing a similar genetic background with high-risk dog breeds.

Our results suggest additional studies, especially on a physiologically relevant cut-off and genotyping of the DLA in different dog breeds and wolves from Canada and Scandinavia which will allow for investigation of genetic association inside and outside the DLA.

8 POPULAR SCIENCE SUMMARY

The dog, our furry companion, believe it or not, has an incredible impact on human health; an impact that goes beyond animal-assisted therapy to increase social behaviour or the detection of cancer and diabetes by disease-characteristic smells.

The story of the dog began around 15,000 years ago when it emerged from its ancestor, the gray wolf. Since then, humans have selected dogs with desirable traits and thereby strictly reduced the size of the available breeding pool. Over time, the dog's genetic material started to change resulting in strong variations between different dog breeds, but very limited variation within individual breeds.

Today, the dog's genes are arranged in long blocks that are characteristic to individual dog breeds. These blocks help researchers to perform genetic studies in dogs; however they also increase the dog's susceptibility to diseases.

IgA deficiency (IgAD) is one such disease and individuals suffering from it lack the ability to produce IgA antibodies. Since IgA protects the body from invading pathogens by covering the mucosal surfaces of the gastrointestinal and respiratory tracts, individuals with IgAD are likely to experience recurrent infections at these sites. Interestingly, the clinical manifestations of IgAD are similar in humans and dogs.

The results presented herein suggest that the dog is a suitable model to study the background of human IgAD. More specifically, we found IgA concentrations and the number of dogs with IgAD to be breed-specific, suggesting the involvement of genes in the development of the disease.

By selecting dog breeds with a high susceptibility to IgAD and analysing their genes, we identified genetic markers involved in formation of blood cells to play an important role in the development of the disease.

We also found very low IgA concentrations in Scandinavian, but not in Canadian, wolves so further genetic studies are currently planned to help identify common genetic denominators in dog breeds with a high prevalence of IgAD and wolves from Scandinavia. These studies will also investigate the genetic differences in Canadian and Scandinavian wolves, which could account for the observed difference in IgA concentrations.

In conclusion, this study portrays dogs and wolves as appealing disease models to study the background of IgAD.

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