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Canine heterophilic antibodies

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Cover: A dog and a mouse engaging in a potential immunization process.
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Abstract

Anamnesis, physical examination and laboratory testing are the pillars of the clinical diagnostic procedure. Alas, laboratory tests are not perfect and analytical errors happen, which can lead to misdiagnosis and detrimental consequences for patient care. Immunoassays are commonly used to measure various hormones and disease markers in patient samples. Despite decades of methodological development and technological advances, immunoassays used for clinical diagnosis are still associated with limitations and even some flaws.

This thesis focuses on a long-lived immunoassay flaw that has been poorly researched in veterinary medicine. Humans and animals both carry heterophilic antibodies, also called anti-animal antibodies, in their circulation. These antibodies can interfere with immunoassays and cause erroneous results. The mechanism of action is the same for animals as it is for humans; the heterophilic antibodies bind to animal antibodies employed by the immunoassay, usually leading to a falsely increased measurement. Due to the extensive use of mouse IgG for analyte detection in immunoassays, anti-mouse antibodies are of particular concern.

Herein, the prevalence of heterophilic antibodies against mouse IgG in a cohort of dog patients is estimated. It is demonstrated that the antibodies can have tangible consequences for patient care as they can interfere with commercial immunoassays used in veterinary laboratories. Falsely increased anti-Müllerian hormone (AMH) measurements were found, which could lead to needless surgery in dog patients. The molecular characteristics of canine heterophilic antibodies were shown to be heterogeneous. They may react with the Fc region or the Fab region of the murine IgG molecule. There is cross-reactivity with IgG from several species, and heterophilic antibodies in dogs are made up of the IgA, IgG and IgM isotypes. The prevalence of the antibodies varies between dog breeds, and the Bernese mountain dog is tentatively predisposed to heterophilic antibodies. The origin of these antibodies remains mostly unclear, but there is occasional cross-reactivity between antibodies to mouse IgG and canine autoantibodies to IgG. Canine heterophilic antibodies can persist for at least two years in serum and represent a risk factor for repeated analytical errors and misdiagnosis in patients with these antibodies.

Keywords: antibodies, anti-mouse antibodies, autoantibodies, autoimmunity, canine, ELISA, immunoassay, immunotherapy, interference, rheumatoid factors

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Heterofila antikroppar hos hund

Sammanfattning

Laboratorietest utgör tillsammans med anamnes och klinisk undersökning grunden för all sjukdomsdiagnostik. Det finns dock brister hos alla laboratorietest, och det förekommer analytiska felkällor som kan leda till att patienter får fel diagnos och behandling. Immundiagnostik används ofta till att mäta hormoner och andra biomarkörer i patientprov. Trots decennier av metodutveckling och tekniska framsteg kvarstår dock grundläggande problem hos de immundiagnostiska metoder som används inom sjukvården.

I denna avhandling undersöks en seglivad felkälla som endast har studerats sparsamt inom veterinärmedicin. Hos människor och djur förekommer anti-djurantikroppar, ofta kallade heterofila antikroppar, som kan störa immundiagnostiska metoder och orsaka felaktiga testresultat. Mekanismen är densamma oavsett djurslag; de heterofila antikropparna reagerar med testernas djurantikroppar, vilket oftast leder till falskt förhöjda mätningar. På grund av den utbredda användningen av mus-IgG i dessa test så utgör anti-musantikroppar ett särskilt stort problem inom immundiagnostik.

Resultaten beskriver för första gången förekomsten av heterofila antikroppar mot mus-IgG hos hundpatienter. Vidare demonstreras det att heterofila antikroppar hos hund kan ha påtaglig inverkan på patientsäkerheten, då de kan störa kommersiella immunanalyser som används av kliniska laboratorier. Felaktigt förhöjda mätningar av anti-müllerskt hormon (AMH) påvisades, vilket kan förorsaka onödiga kirurgiska ingrepp hos patienter. Heterofila antikroppar hos hund kan reagera med Fab- eller Fc-regionen hos mus-IgG. Det förekommer korsreaktivitet mot IgG från flera olika djurslag, och de heterofila antikropparna kan vara av IgA, IgG eller IgM-isotyp. Förekomsten av heterofila antikroppar varierar mellan hundraser, och berner sennenhundar är potentiellt predisponerade för dessa antikroppar. Antikropparnas ursprung har inte klarlagts, men det finns sporadisk förekomst av korsreaktivitet mellan autoantikroppar mot IgG och anti-musantikroppar. Heterofila antikroppar kan kvarstå i cirkulationen i åtminstone två år hos hundar, vilket är en riskfaktor för upprepade felaktiga mätningar och feldiagnostiseringar hos hundar med heterofila antikroppar.

Nyckelord: antikroppar, autoantikroppar, autoimmunitet, ELISA, hund, immundiagnostik, immunterapi, interferens, reumatoida faktorer

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List of publications

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

- I Bergman D*, Larsson A, Hansson-Hamlin H, Svensson A & Holst BS (2018) Prevalence of interfering antibodies in dogs and cats evaluated using a species-independent assay. *Veterinary Clinical Pathology* **47** (2), pp. 205–212.
- II Bergman D*, Larsson A, Hansson-Hamlin H & Ström Holst B (2019) Investigation of interference from canine anti-mouse antibodies in hormone immunoassays. *Veterinary Clinical Pathology* **48(Suppl 1)**, pp. 59–69.
- III Bergman D*, Larsson A, Hansson-Hamlin H, Åhlén E & Holst BS (2019) Characterization of canine anti-mouse antibodies highlights that multiple strategies are needed to combat immunoassay interference. *Scientific Reports* **9** (1), p. 14521.
- IV Bergman D*, Bäckström C, Hansson-Hamlin H, Larsson A & Holst BS Pre-existing canine anti-IgG antibodies: implications for immunotherapy, immunogenicity testing and immunoassay analysis. (submitted)

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The contribution of Daniel Bergman to the papers included in this thesis was as follows:

- I. Took major part in designing and planning experiments, developed the screening assay, performed the laboratory analyses, interpreted results and wrote the manuscript with input from co-authors.
- II. Took major part in designing and planning experiments, performed laboratory analyses, interpreted results and wrote the manuscript with input from co-authors.
- III. Took major part in formulating the research idea and designing and planning experiments, performed the laboratory analyses, interpreted results and wrote the manuscript with input from co-authors.
- IV. Formulated the research idea, designed the experiments, took major part in the planning of the experiments, performed the laboratory analyses, interpreted results and wrote the manuscript with input from co-authors.

Abbreviations

AIT	Autoimmune thyroiditis
AMH	Anti-Müllerian hormone
CDR	Complementarity determining region
CEA	Carcinoembryonic antigen
CH	Constant region heavy chain
CL	Constant region light chain
CRP	C-reactive protein
CV	Coefficient of variation
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen-binding
Fc	Fragment crystallizable
GluV8	Glutamyl endopeptidase
HAMA	Human anti-mouse antibodies
HNE	Human neutrophil elastase
HRP	Horseradish peroxidase
IL-13	Interleukin-13
LoD	Limit of detection
MMP	Matrix metalloproteinase
OD	Optical density
PEG	Polyethylene glycol
SD	Standard deviation
T ₃	Triiodothyronine
TgAA	Thyroglobulin autoantibodies
TSH	Thyroid-stimulating hormone
TT ₄	Total thyroxine

1 Introduction

1.1 Immunological assays

The invention of the immunoassay marked a quantum leap forward for diagnostic medicine and research in many different fields. Immunoassays can detect and quantify minuscule amounts of specific molecules in complex biological samples. In clinical practice, this ability corresponds to the need for accurate measurement of biomarkers in patient samples. Endocrinology, reproduction, oncology, cardiology and many other branches of medicine are all heavily dependent on immunoassays for the diagnostic work-up of patients. There are several different variants of immunoassays, but the principle they all share is the binding between antibody and antigen. A standard immunoassay procedure for the detection of, for example, insulin is as follows: an anti-insulin antibody pulls down sample insulin to the bottom of a microtiter well. Another anti-insulin antibody, conjugated to a label (such as an enzyme) is added, also binding the insulin. Addition of a substrate to the enzyme produces a visible signal indicating the sample concentration of insulin. This immunoassay format, with two antibodies binding the antigen, is called an immunometric assay, and its use in clinical medicine and research is widespread.

None of this would be possible if not for the remarkable specificity of affinity-matured antibodies. There are more antibody specificities in an individual than there are genes in the mammalian genomes – at least 50 million times as many according to estimates – and each one of these antibody specificities is prefabricated before the immune system is challenged with the specific antigen (Li *et al.*, 2004). However, there are nuances to this picture, because antibodies are also versatile and can bind to many targets, including molecules that do not induce an immune response. They can even adhere to inanimate objects like plastic surfaces and magnetic beads (a prerequisite for the

immunoassay technology). It is therefore not surprising that unintended antibody interactions sometimes happen, and in laboratory testing, this can lead to analytical errors and misinterpretations, ultimately to the detriment of patient care. This phenomenon is called interference. It has been defined as “the effect of a substance present in the sample that alters the correct value of the result, usually expressed as concentration or activity, for an analyte” (Kroll & Elin, 1994).

Anti-animal antibodies in humans, also known as heterophilic antibodies, have been recognized as a source of immunoassay interference for nearly 50 years, and several reports describe their impact on diagnosis and choice of treatment (Bolstad *et al.*, 2013; Tate & Ward, 2004; Kricka, 1999). However, the understanding of the nature and origin of these antibodies is incomplete, and only a few publications document their existence in animals (Solter *et al.*, 2008; Borromeo *et al.*, 2007; Kashiwazaki & Thammasart, 1998). The topic of this thesis is immunoassay interference caused by endogenous anti-animal antibodies in dogs, and the main focus is on the immunometric assay format.

1.2 A historical perspective on anti-animal antibody interference in immunoassays

The history of immunoassay interference intertwines with the history of the immunoassay technology itself. Heated argumentation from the early 1900s demonstrates that interfering serum substances were a fertile source of debate in the early days of the Wassermann test, a predecessor of the modern immunoassay used for the diagnosing of syphilis (Seelman, 1918). Years later, the specific issue of interference from anti-animal antibodies coincided with the use of animal antibodies to detect analytes in serum. The immunoassay technology was first successfully implemented in 1959 by its inventors, Solomon Berson and Rosalyn Yalow when they used it to measure insulin in the circulation of humans (Yalow & Berson, 1959). For this purpose, they used anti-insulin antibodies raised in guinea pigs. Reports that anti-animal antibodies could cause analytical errors first emerged in the early 1970s. The initial publication described anti-guinea pig-antibodies in human sera that interfered with measurements of hepatitis B antigen (Prince *et al.*, 1973). In the space between these years, several improvements on the original immunoassay principle had already been made, including the “immunometric” or “non-competitive” format, invented by Leif Wide in the late 1960s (Wide, 2005).

Immunometric assays use two antibodies in combination – one to capture the analyte and the other to detect it. In this format, the signal is directly proportional

to the binding of the analyte to the detection antibody. In other words, the signal increases in parallel with the analyte concentration. The early immunoassay format as devised by Berson and Yalow is still in use, and these tests are commonly referred to as competitive assays. This term is not universally accepted, and they are also known as reagent limited assays. At any rate, the principle for this format is that only one analyte-specific antibody is used, but it interacts with two reagents: a labelled analyte, and the unlabelled analyte present in the sample. Thus, the signal is generated by immobilized antibodies that do not bind the unlabelled sample analyte. The signal in the competitive format is therefore inversely proportional to the analyte concentration; a high signal reflects low concentrations and vice versa.

The ability of an assay to detect low concentrations of an analyte is referred to as its analytical sensitivity. In immunoassay analysis, analytical sensitivity is highly desirable as the objective often is to detect and quantify analytes present at low concentrations (Landegren *et al.*, 2012). The directly proportional relationship between signal and concentration make immunometric assays vastly superior to competitive assays in terms of analytical sensitivity. The reason is that immunometric assays measure the specific signal against a low background. Competitive assays instead measure the difference between two large signals when the analyte concentration is low. The response at low concentrations is virtually indistinguishable from the response at zero (the absence of analyte) when both signals are large. These conditions lead to an analytical insensitivity with imprecise measurements at low analyte concentrations. The immunometric format improved both the analytical sensitivity and specificity of the test. The most sensitive immunometric assays are capable of detecting trace amounts of sample protein equaling a bucketful of chemicals scattered across all oceans on earth.

The analytical specificity of an assay is its ability to distinguish a particular substance from other substances in a sample. Ultimately, the analytical specificity of an immunometric assay is contingent on the combination of antibodies used for capture and detection. If the two antibodies target two different epitopes on the same antigen, the risk for cross-reactivity is considerably lower than if only one epitope on the antigen is targeted. Immunometric assays are now by default used for measuring all analytes that are large enough to accommodate two antibodies, i.e. most proteins and peptides, but it is not a method without limitations. It soon became evident that these improvements on immunoassay performance also introduced a weakness – the susceptibility to interference from anti-animal antibodies. If a sample contains antibodies with affinity for animal antibodies, these might be capable of forming a bridge between the capture and the detection antibody, ostensibly reflecting

the presence of the analyte (Figure 1). However, this is an analytical error. Endogenous antibodies can also block the binding site on the immobilized antibody and cause negative interference, but this phenomenon has been much less investigated than positive interference. Previous immunoassay formats based on one antibody were less prone to antibody interference than immunometric assays because the specific reaction induced by the labelled analyte took precedence over the often weak, low-affinity reactions of the interfering antibodies. Unlike the competitive or reagent limited assays, the immunometric assays are reagent excess assays. In this format, the reaction is driven to completion, and even low-affinity reactions by low-concentration molecules can induce a signal. Although there are exceptions (Ghosh *et al.*, 2008), the absolute majority of reports on interference from endogenous antibodies regard immunometric two-site immunoassays (Bolstad *et al.*, 2013). The immunometric format limited or solved several rather common issues with immunoassays, but as a trade-off, it aggravated the less common issue of antibody interference.

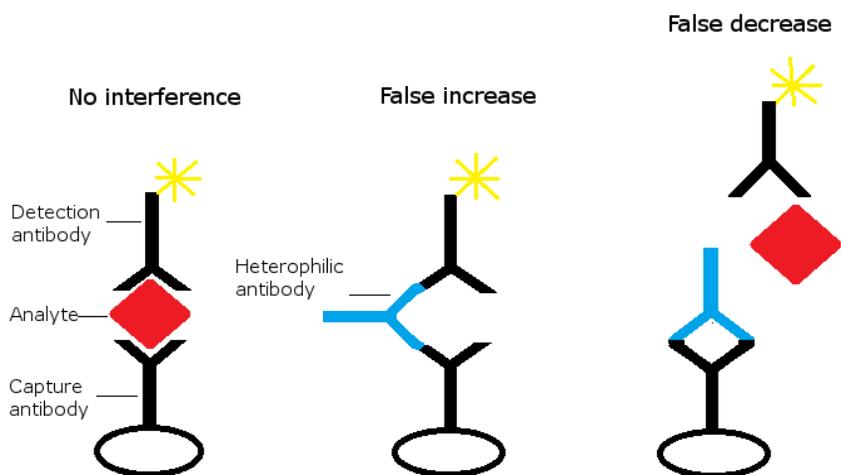


Figure 1. Illustration of interference in immunometric assays. In the scenario to the left, there is no interference, and both the capture and detection antibodies bind the analyte. In the middle scenario, a heterophilic antibody has bound to the capture and detection antibodies, causing a falsely elevated result. In the scenario to the right, a heterophilic antibody blocks the capture antibody, causing a falsely decreased result (negative interference). Illustration by Camilla Bäckström.

In the mid-1970s, the hybridoma technology was developed (Köhler & Milstein, 1975). Georges Köhler and César Milstein found that by fusing antibody-producing B cells with immortal myeloma cells, monoclonal antibodies are producible *in vitro* for an extended (in theory, indefinite) period. However, this technique requires the acquisition of B cells from a “host” – a research animal immunized with the target antigen. Köhler and Milstein used mice for this purpose, and the mouse soon became the default host for these “magic bullets” – highly specific antibodies directed at a single antigen epitope. Monoclonal mouse antibodies had an enormous impact on many scientific fields, including immunodiagnostics. Up until this point, immunoassays had used polyclonal antibodies produced in various research animals, often guinea pigs or rabbits. Since polyclonal antibodies bind to multiple epitopes on the target antigen, problems with cross-reactivity were not uncommon, and the supply was limited to the capacity of the animal host for *in vivo* antibody production. The hybridoma technology enabled improved specificity and continuous production of large antibody quantities *in vitro*, so monoclonal mouse antibodies principally came to replace polyclonal antibodies for use in immunoassays. However, the use of monoclonal antibodies does not solve the problem with interference from anti-animal antibodies, because this phenomenon is contingent on the structure of the immunoassay antibodies, and monoclonal and polyclonal antibodies are, in essence, structurally identical. Since immunoassay antibodies were now mainly derived from the mouse, the focus on interfering agents shifted to anti-mouse antibodies, and it turned out that these were very common in the human population, occurring in 40% of humans, according to an often-cited publication (Boscato & Stuart, 1986). In many cases, these antibodies appeared to be naturally occurring, or at any rate without a known source. Moreover, monoclonal mouse antibodies were also used as therapeutic drugs, so patients receiving therapeutic mouse antibodies acquired human anti-mouse antibodies (HAMAs) that interfered with immunoassays (Bolstad *et al.*, 2013).

There is now a plethora of case reports where immunoassay interference has affected the interpretation and treatment of clinical cases. Most of these reports concern anti-mouse antibodies, but there are also examples of patients with antibodies that react with rabbit, sheep or goat IgG, among others (Hennig *et al.*, 2000; Berglund & Holmberg, 1989; Hunter & Budd, 1980). Clearly, the broad spectrum of interfering agents, the variety of immunoassays available on the market and their widespread use are some of the factors that contribute to the frequent recurrence of the phenomenon, nearly 50 years after its first description.

1.3 Immunoassay interference from a veterinary perspective

Immunoassays are as indispensable to the clinical practice of veterinary medicine as they are to human medicine. Although the veterinary analytes are not as plentiful as the human analytes, their use is frequent, and they serve as important prognostic and diagnostic markers. For example, the Clinical Pathology laboratory at the University Animal Hospital in Uppsala routinely analyzes approximately 20 different analytes with immunoassays, primarily in dog, cat and horse serum. These include thyroid hormones, glucocorticoid hormones and reproductive hormones, as well as peptide markers for cardiac disease and general inflammatory markers such as C-reactive protein (CRP). There is also a wide use of immunoassays for other analytes in veterinary research and surveillance of infectious diseases, which may have the potential for zoonotic transmission and economic impact in the livestock industry.

Despite this, nearly all research that has been carried out in the field so far pertains to immunoassay interference in human samples and how it impacts the practice of human medicine. Much of the knowledge about immunoassay interference in humans could presumably apply to animals as well. Veterinary laboratories often use immunodiagnostic tests developed for use in humans to analyze animal specimens, and immunoassay kits that are species-specific include the same or similar reagents as their human counterparts. Reagent-wise, developing a kit for use in analysis of canine, feline or equine samples or samples of any other non-human source is mainly a matter of using antibodies raised against species-specific antigens. This practice has no bearing on the probability of interference since the hosts for antibody production remain the same. Mice, sheep, rabbits, goats and chickens are some of the most common hosts for immunoassay antibodies in kits developed for use in humans as well as in animals.

However, the immunoassay components only represent one side of the equation. For interference to occur, there must be interfering substances present in the analyzed samples. Do animals have antibodies against, for instance, mouse, sheep, or rabbit IgG? Accumulating evidence is now suggesting that these antibodies are not uncommon in the animal population. Anti-mouse and anti-rabbit IgG was detected in plasma from cattle (Kashiwazaki & Thammasart, 1998), and anti-IgG antibodies with fairly broad species reactivity were demonstrated in horses (Borromeo *et al.*, 2007). Later, it was found that canine plasma contained antibodies reactive with mouse IgG (Solter *et al.*, 2008). The publications in this thesis provide additional insight into the prevalence and clinical implications of interfering antibodies in dogs (Bergman *et al.*, 2019a;

Bergman *et al.*, 2019b; Bergman *et al.*, 2018). Nevertheless, these accounts are very slight in numbers compared to the body of work on immunoassay interference in people.

1.4 Interfering antibodies: definitions and nomenclature

The nomenclature on endogenous antibodies that interfere with immunoassays is often confusing because terms referring to these antibodies can have different meanings in different contexts, and the meanings are user-dependent. There have been attempts to categorize the antibodies and to standardize the nomenclature. While these attempted categorizations are likely well-intended, they all have their imperfections, and because interfering antibodies are very heterogeneous, they rarely fall squarely into one category.

Some researchers have proposed that the commonly used term “heterophilic antibodies” should be reserved for antibodies that bind IgG from two or more species (Kaplan & Levinson, 1999). However, 20 years later, this use of the term has not been adopted by the research community. It seems unrealistic to ask that investigators should evaluate each suspected sample for cross-reactivity against numerous species before the use of such a widespread term can be justified. It has also been recommended that the term “heterophilic antibodies” should indicate that the antibodies originate from an unknown exposure to antigen, in contrast to rheumatoid factors (which is debatable since rheumatoid factors are widely thought to be induced by an unknown antigen), and to HAMAs, which are acquired from monoclonal antibody therapy (Bolstad *et al.*, 2013). However, anti-mouse antibodies can also be acquired from unknown non-iatrogenic exposure to mouse antibodies, making the widely established definition of HAMAs imprecise. Furthermore, this definition of heterophilic antibodies does not include polyspecific natural antibodies that are present at birth without any prior antigen exposure. In practice, the origin of these antibodies, whether there was exposure to antigen or not is almost always unknown, and the decreased use of murine therapeutic antibodies is making the few cases where a known immunogen exists even scarcer.

The frequent use of the term “heterophilic antibodies”, in the context of immunoassay interference, indicates that a sample contains a substance that interferes, or is suspected of interfering with, a test without any further specification (Bolstad *et al.*, 2013). This broad use of the term works well in most scenarios, but if more information about the interfering substance is available, the use of a more descriptive term is motivated. Because mouse antibodies are not used for therapy in veterinary medicine, there is no principal

counterpart to HAMAs in dogs. Herein, “heterophilic antibodies” and “anti-animal antibodies” are both used as umbrella terms for immunoassay interfering antibodies, and where applicable, the term “canine anti-mouse antibodies” is used to denote that the interfering antibodies react with mouse antibodies. The species reactivity of the antibodies is their most important feature, and in the absence of further characterization, it is often one of the few facts about them that can be stated with reasonable certainty. However, the term “canine heterophilic antibodies” would also suffice to refer to these antibodies. In this thesis, “heterophilic antibodies” are not treated as a separate subset of interfering antibodies, in line with the widespread notion that all antibodies that cause immunoassay interference are heterophilic antibodies.

1.4.1 Iatrogenic anti-animal antibodies

Iatrogenic anti-animal antibodies are acquired through medical interventions. The classic example is HAMAs in people, resulting from the administration of therapeutic monoclonal antibodies.

The earliest monoclonal immunotherapies relied on mouse antibodies, but advances in chemical engineering have made it possible to modify therapeutic antibodies to minimize their immunogenicity. One of the major disadvantages of injecting a patient with a monoclonal mouse antibody, apart from the risk for immunoassay interference, is that the number of effective treatments are very few since the patient quickly develops immunity to the drug. Most therapeutic antibodies in use today are therefore chimeric or humanized. Chimeric antibodies have had their constant region replaced with human amino acid sequences, and in humanized antibodies, only the complementarity determining regions (CDRs) are of murine origin. Despite these efforts to reduce immunogenicity, all current therapeutic antibodies are immunogenic in some recipients, and therefore a potential source of immunoassay interference.

For various reasons, the development of veterinary therapeutic monoclonal antibodies has been stagnant in comparison to the avalanche of human monoclonal antibodies introduced during the 2010s. As of this writing, there are only two veterinary monoclonal antibodies approved, both in the United States, for the treatment of cancer in dogs, one for CD20 positive B cell lymphoma and one for CD52 positive T cell lymphoma (Klingemann, 2018). The first monoclonal antibody approved in the European Union for use in dogs was registered in 2017, an IL-13 antagonist for the treatment of atopic dermatitis. All of these antibodies have been speciated to limit their immunogenicity (Klingemann, 2018; Michels *et al.*, 2016), so it appears that the veterinary community is riding the wave of recent advances in antibody engineering. Once

canine immunotherapies become more widely available, a sudden outburst of immunoassay interference cases, therefore, seems quite unlikely, but some precaution is nevertheless advised.

There is another fairly common clinical treatment in veterinary medicine that could be a source of iatrogenic anti-animal antibodies. Dogs envenomated by snake bites are sometimes treated with animal antisera. Several commercial antivenom treatments for different species of snakes are available for dogs. Most of them contain polyclonal horse (Lund *et al.*, 2013) or sheep IgG (KarlsonStiber *et al.*, 1997) as whole molecules, Fab-, or F(ab')₂-fragments. Patients treated with snake antivenin develop immunity, and the iatrogenic anti-animal antibodies could cause immunoassay interference. Sheep antivenin is often advocated over horse antivenin due to higher safety, but the risk of immunoassay interference should, in theory, be higher after treatment with sheep antivenin due to the more frequent use of sheep IgG in immunoassays.

1.4.2 Non-iatrogenic anti-animal antibodies

Most commonly, the anti-animal antibodies that interfere with immunoassays are not traceable to a medical treatment or a known exposure to an immunogen. It is widely assumed that the bulk of interfering antibodies result from commonplace activities, like pet-keeping, vaccinations and food consumption (Ismail, 2009). However, causality is rarely or never proved, and correlational studies investigating risk factors are rare, although there are exceptions (Mohammadi & Bozorgi, 2019). It is also commonly assumed that these antibodies were acquired from exposure, although this is not a prerequisite. There are also natural antibodies, present before birth and exposure to antigen, which provide a rudimentary line of defence against infections by initiating weak interactions with a broad range of antigens. These antibodies are polyspecific antibodies of the IgM isotype and appear to recognize, for example, carbohydrates on the cell surface of bacteria. Antibodies with these characteristics can cause immunoassay interference (Covinsky *et al.*, 2000). There is experimental evidence showing that the binding between antibody and antigen is more flexible than implied by the traditional “lock and key”-principle for antibody-antigen interactions (James *et al.*, 2003; Kramer *et al.*, 1997) and that antibodies can change their conformation to accommodate numerous antigens. These findings could explain how polyspecific antibodies in individuals that have never been exposed to animal IgG are capable of interfering with immunoassay antibodies.

If exposure is a source of anti-animal antibodies, which seems plausible, the question is which route of exposure that most likely leads to an immune

response. Because antigen-presenting cells are abundant in subcutaneous tissues, exposed wounds would appear to be a candidate exposure route. However, in a study investigating laboratory workers that were habitually exposed to mice, the frequency of mouse bites was not a risk factor for the development of anti-mouse antibodies (Mohammadi & Bozorgi, 2019). The amount of antigen transferred through bites may be insufficient to induce antibody formation. Immunization via the gut is another possible route for the acquisition of anti-animal antibodies, and cow's milk, especially when unpasteurized, contains relatively high levels of bovine immunoglobulin (Hurley & Theil, 2011) and could be a means of immunization in people (Hunter & Budd, 1980). It is not common practice to feed dogs with milk, but in recent years, raw feeding diets for dogs have increased in popularity. These diets consist of uncooked edible bones and organs that might provide the dogs with a sufficient dose of animal immunoglobulin to form anti-animal antibodies. It is also possible that the hunting and eating of wild animals is a source of anti-animal antibodies in dogs and cats. However, this proposed immunization route is inconsistent with the concept of oral tolerance to food protein (Pabst & Mowat, 2012).

1.4.3 Rheumatoid factors

Erik Waaler described rheumatoid factors in 1940 (Waaler, 1940). Historically, they were defined as IgM autoantibodies that bind to the Fc-region of IgG (Figure 2), but numerous variants exist, including IgA/IgG to IgM, and IgG/IgM to IgA. Rheumatoid factors against its own Ig isotype are also found (IgA to IgA, IgG to IgG and IgM to IgM), but these require relatively sophisticated detection methods and are less well studied. Rheumatoid factors in people are linked to numerous conditions, including autoimmune diseases like rheumatoid arthritis and Sjögren's syndrome, as well as viral, bacterial and parasitic infections (Newkirk, 2002). Rheumatoid factors may be present in both chronic and acute conditions. In infections, the response is typically transient. It has been proposed that the rheumatoid factor acts as a "housecleaner" in infectious disease by contributing to the formation of immune complexes that are cleared from circulation by the mononuclear phagocyte system (Hogben & Devey, 1986; Van Snick *et al.*, 1978). It is also possible that B cells expressing rheumatoid factors on their surface contribute to the immune response by presenting foreign antigen to T cells (Roosnek & Lanzavecchia, 1991).

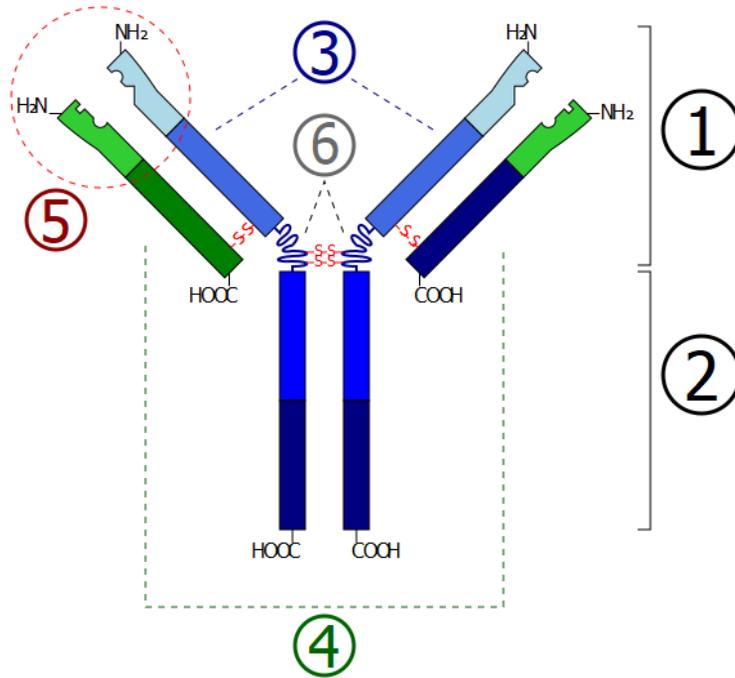


Figure 2. Schematic diagram of the IgG structure. Legend: 1. Fab region, 2. Fc region, 3. Heavy chain (from top to bottom consisting of variable heavy (VH) and constant heavy (CH₁), hinge, CH₂ and CH₃ domains), 4. Light chain (from top to bottom consisting of VL and CL domains), 5. Antigen-binding site, 6. Hinge region. By Y_tambe / CC BY-SA (https://upload.wikimedia.org/wikipedia/commons/1/19/Immunoglobulin_basic_unit.svg)

Testing for rheumatoid factors can be useful in the clinical diagnosis of rheumatoid arthritis in people. Rheumatoid factors are also of technical concern in immunological assays due to their ability to bind IgG. Knowledge about the characteristics and binding properties of rheumatoid factors is required in order to configure immunoassays in such a way that interference is limited. The most common binding site for rheumatoid factors is the CH₂-CH₃ groove or the CH₃-CH₃ groove on the Fc region of IgG (Duquerroy *et al.*, 2007; Artandi *et al.*, 1992). There are also several species-specific binding sites on the Fc region. The entire Fc region belongs to the constant region of IgG and is therefore independent of the specificity of the antibody. This region of IgG has been well conserved throughout evolution. Interspecies homology in this region enables rheumatoid factors to bind IgG from several different species and to interfere with immunoassays by cross-reacting with, e.g. mouse, rabbit, and sheep IgG.

The homology decreases as the phylogenetic distance to the target IgG increases. This principle explains why chicken IgY is a viable option for avoiding interference from rheumatoid factors in mammals – the structural difference between mammalian and avian immunoglobulin is unbridgeable for the rheumatoid factors (Larsson & Sjoquist, 1990).

Research activity on canine rheumatoid factors peaked in the late 1980s-early 1990s (Chabanne *et al.*, 1993; Carter *et al.*, 1989). It was then found that IgA and IgM rheumatoid factors are detectable in canine sera and that they occasionally may be useful in the diagnosis of rheumatoid arthritis. Most of these studies were carried out using older detection methods such as the Rose-Waaler test or the latex fixation test, which are mainly suitable for detecting rheumatoid factors of the IgM isotype. In human medicine, nephelometry is most commonly used for the detection of rheumatoid factors. The Rose-Waaler test remains the gold standard for detecting rheumatoid factors in animals, but ELISA (enzyme-linked immunosorbent assay) testing is considered an acceptable alternative, which also has the advantage of being able to detect rheumatoid factors of all isotypes (Andrysikova *et al.*, 2009). However, available tests are insensitive, and the diagnostic value is unclear. The interest in canine rheumatoid factors has declined since the 1990s, but their existence still represents a potential risk for interference in immunoassay testing for other analytes.

1.4.4 Anti-Fab/F(ab')₂-autoantibodies

Rheumatoid factors are commonly discussed in connection with immunoassay interference because they bind to conserved regions on the Fc domain of IgG. What is sometimes overlooked in this discourse is that the constant region extends well into the fragment antigen-binding (Fab) region of IgG, and thus autoantibodies against this region are capable of causing interference via the same mechanism as rheumatoid factors. These autoantibodies can be divided into different subsets depending on their exact specificity, but they are often called anti-Fab or anti-F(ab')₂-autoantibodies.

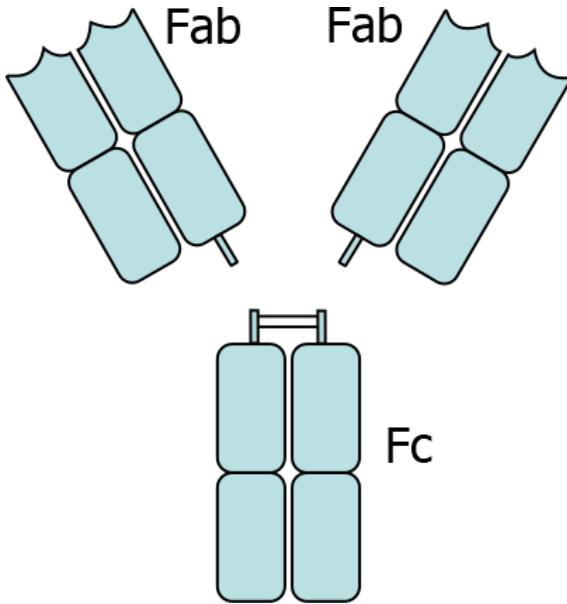


Figure 3. IgG molecule cleaved with papain to yield Fab-fragments. The Fab fragment retains the upper IgG hinge region. By Je at uwo on en.wikipedia derivative work: Vezixig / Public domain (https://commons.wikimedia.org/wiki/File:2fab_fc.svg)

In vitro, Fab-fragments are produced by cleavage of whole IgG using papain. In older literature, anti-Fab-autoantibodies were called papain agglutinators, denoting their ability to agglutinate erythrocytes covered with Fab-fragments obtained by papain cleavage (Kormeier *et al.*, 1968). Likewise, anti-F(ab')₂-autoantibodies were called pepsin agglutinators, as pepsin is used to produce F(ab')₂-fragments *in vitro* (Osterland *et al.*, 1963). If the autoantibodies bind to the constant heavy chain domain (CH₁) or the light chain domain (CL) on the Fab region of IgG, the designations anti-Fab and anti-F(ab')₂-autoantibodies are interchangeable. However, if they bind to the hinge region, these designations tend to become mutually exclusive. When Fab- and F(ab')₂-fragments are cleaved with papain or pepsin, cryptic epitopes (epitopes that are not exposed on the intact IgG molecule) become revealed in the upper or lower hinge region. Papain cleaves IgG in the upper hinge region (Figure 3). Autoantibodies targeting cryptic epitopes in the upper hinge will, therefore, recognize Fab-fragments, but not F(ab')₂-fragments, nor intact IgG. Pepsin cleaves IgG in the lower hinge region (Figure 4), so likewise, autoantibodies targeting cryptic epitopes in the lower hinge region will recognize F(ab')₂-fragments, but not Fab-fragments, nor intact IgG. Autoantibodies with these specificities are often called anti-hinge-autoantibodies to distinguish them from autoantibodies against heavy or light chain domains. Anti-hinge-autoantibodies to the lower hinge

region are more common or at least more often studied than anti-hinge-autoantibodies to the upper hinge region, although both types have been described (Elagib *et al.*, 2001). It should be emphasized that in many studies, exact characterization of the binding sites was not performed, so the designations anti-Fab or anti-F(ab')₂-autoantibodies are principally used as umbrella terms to indicate that their binding could be directed to any part of the Fab region of IgG. Technically, anti-idiotypic antibodies are also anti-Fab and anti-F(ab')₂-autoantibodies, but as such, they represent a particular subset of antibodies, and they are reviewed separately.

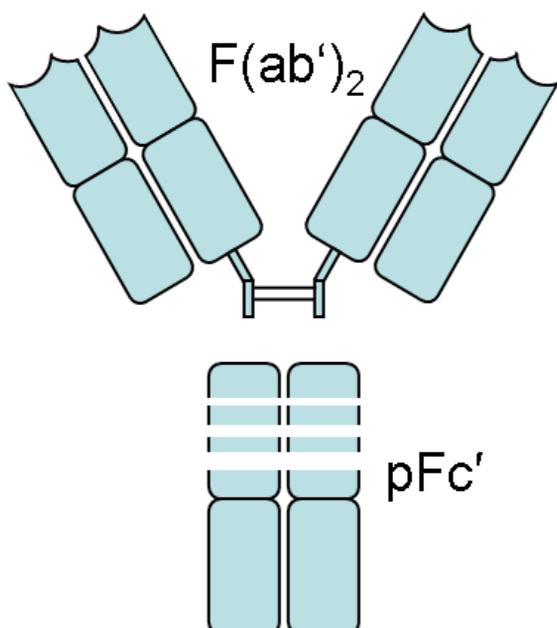


Figure 4. IgG molecule cleaved with pepsin to yield F(ab')₂-fragments. The F(ab')₂-fragment retains the entire IgG hinge region. By Je at uwo on en.wikipedia / Public domain (https://commons.wikimedia.org/wiki/File:F_ab2_pFc.png)

Initial studies revealed that anti-Fab and anti-F(ab')₂-autoantibodies were nearly ubiquitous in the human population (Waller *et al.*, 1971) and that it was not uncommon for these antibodies to recognize cryptic epitopes exposed by pepsin or papain, predominantly in the lower hinge region of IgG. However, neither pepsin nor papain is present nor biologically active under normal physiological conditions *in vivo*. Efforts were therefore made to identify other proteolytic enzymes that are capable of cleaving IgG *in vivo*. It was found that several enzymes can digest IgG *in vivo* and that many of these are disease-associated.

Notably, several tumour-associated matrix metalloproteinases (MMPs) cleave human IgG in the lower hinge region, proximal to or at the same site as pepsin (Brezski & Jordan, 2010). The bacterial enzymes glutamyl endopeptidase (GluV8) (Ryan *et al.*, 2008) and IdeS (von Pawel-Rammingen *et al.*, 2002), as well as cathepsin G, were also found to cleave IgG in this region. In addition, plasmin and human neutrophil elastase (HNE) cleave IgG in the upper hinge region (Brezski *et al.*, 2008).

Anti-F(ab')₂-autoantibodies are rarely considered as sources of immunoassay interference, despite the significant homology between CH₁ and CL domains from different species. Use of F(ab')₂-fragments instead of whole IgG molecules in immunoassays has even been endorsed as a prophylactic measure against interference (Bolstad *et al.*, 2012). This view has been challenged by other researchers claiming that most interfering antibodies target F(ab')₂-fragments (Levinson & Miller, 2002; Hennig *et al.*, 2000). In these cases, most of the discussion is focused on anti-idiotypic antibodies that bind to the variable region of animal IgG.

1.4.5 Anti-idiotypic antibodies

The distinction between “antibody” and “antigen” does not acknowledge a complicating factor: that antibodies themselves are antigenic, and can act as antigens to other antibodies. The variable region of all antibodies contain an antigen to which other antibodies can bind. This antigen is called the idiotype of the antibody, and antibodies that bind to the idiotype are called anti-idiotypic antibodies. Because the anti-idiotypic antibodies also contain an idiotype of their own, they can be bound by anti-anti-idiotypic antibodies, in a potentially endless cascade of antibody generations. Niels Jerne envisioned an “immune network” to explain this peculiar feature of humoral immunology (Jerne, 1974). Much simplified, through upregulation and downregulation of different generations of anti-idiotypic antibodies, the immune network acts to keep the immune system in a state of equilibrium when challenged with an antigen. These cascades of antibody generations might, for example, be a way for the immune system to maintain the antibody response to a specific antigen over a prolonged period.

Few immunologists subscribe to the immune network theory today, but the existence of anti-idiotypic antibodies is not in question. Although direct evidence for their role in immunoassay interference is lacking, they could account for several observations that are often made in connection to interference. First, the addition of high concentrations of non-immune IgG may be insufficient to eliminate the interference. If antibodies targeting a specific idiotype happen to be abundant in an individual sample, a serum pool of non-

immune IgG might not contain sufficient amounts of antibodies against this specific idiotypic to block the interference. Second, a sample may cause interference in one immunoassay but not in another immunoassay for another analyte, despite the assays being very similar in terms of antibody reagents and species of IgG used (Levinson & Miller, 2002). This finding is sometimes attributed to differences in the contents of the blocking buffers, but these conclusions are usually speculative at best since the contents of these buffers are rarely or never disclosed by the manufacturer. Third, the use of Fab- or F(ab')₂-fragments instead of whole IgG sometimes fails to eliminate the interferences. This observation is consistent with the presence of anti-idiotypic antibodies, although the binding could also be towards any other part of the Fab region.

1.4.6 Other interfering antibodies

Antibodies do not have to bind IgG to cause immunoassay interference; there are numerous types of antibodies with affinities for test analytes and reagents that are known to cause false-positive or false-negative test results. Although these various types of antibodies are not the main focus of this thesis, they serve as relevant differential diagnoses to anti-IgG interference in the medical conditions and detection methodologies that they are associated with, and they are therefore reviewed here.

Autoanalyte antibodies

In diseases with autoimmune etiologies, autoanalyte antibodies may be present in patient sera. Some examples are anti-thyroid hormones in Hashimoto's thyroiditis (Despres & Grant, 1998) and anti-insulin antibodies in insulin autoimmune syndrome (Casesnoves *et al.*, 1998). Autoantibodies interfering with immunoassays are also found in healthy individuals where a connection to autoimmunity has not been found (Eriksson *et al.*, 2005). Anti-insulin antibodies have been detected in healthy cats and were found to affect serum insulin measurements (Nishii *et al.*, 2010). The factors determining the nature of the interference (false increase or false decrease) are complex and depend on the detection method. Although autoanalyte antibodies potentially can affect all immunoassays, they should, in particular, be considered as sources of interference in immunoassays where anti-IgG-antibodies are less likely to affect the results, i.e. competitive assays (Miller, 2004).

In dogs, hypothyroidism often concurs with autoimmune thyroiditis (AIT), and dogs presenting with AIT may have autoantibodies against thyroglobulin (TgAA) that cross-react with triiodothyronine (T₃) and thyroxine (T₄). Unless non-immunological detection methods are used, T₃ and T₄ can only be measured

by competitive immunoassays due to their low molecular weight, and in this format, autoanalyte antibodies can impact the measurements by reacting with labelled analytes. The net result in most thyroid hormone assays is a falsely increased measurement. In a large cohort study, it was found that autoantibodies falsely increased 5.7% of free T₃ measurements and 1.7% of free T₄ measurements in canine samples (Nachreiner *et al.*, 2002). The issues of autoanalyte interference in immunological assays for free T₃ and T₄ have been considered so unacceptable that more expensive and laborious methods like equilibrium dialysis are being recommended for measurement of thyroid hormones in animals (Ferguson, 2007).

Anti-streptavidin antibodies

Immunoassays often utilize the biotin-streptavidin system for detection. Biotinylated antibodies or antigen bind to enzyme-conjugated streptavidin through one of the strongest non-covalent interactions observed in nature. One of the advantages of using enzyme-conjugated streptavidin is that it can function as a generic linker to any biotinylated antibody or antigen, eliminating the need for individual enzyme-conjugation of different antibodies and antigen. However, there are at least two mechanisms of interference specific to immunoassays employing this system. One is interference from biotin, recently described as an “emerging interferent” (Luong *et al.*, 2019) in immunoassays. In this case, the interference is mediated by high sample concentrations of biotin rather than by antibodies – biotin is a small, poorly immunogenic molecule that does not induce antibody formation. The other mechanism is interference from anti-streptavidin antibodies (Wouters *et al.*, 2019; Peltier *et al.*, 2016; Rulander *et al.*, 2013), which are receiving increased recognition as interfering agents. These antibodies might be acquired via intake of protein supplements (Harsch *et al.*, 2017). In electrochemiluminescent assays, ruthenium is used to label antibodies. Interference from anti-ruthenium antibodies has also been described (Buijs *et al.*, 2011). To date, there have not been any reports on interference from antibodies against antibody labels in animals.

2 Aims of the thesis

The overarching aim of this thesis was to investigate the prevalence, clinical impact, molecular characteristics and origin of heterophilic antibodies in dogs. The specific aims were to:

- Set up a species-independent interference assay for detecting anti-mouse antibodies, and to use it to determine the prevalence of anti-mouse antibodies in a cross-section of dog and cat patients (paper I)
- Determine if canine anti-mouse antibodies are a source of clinically relevant interference in a commercial immunoassay (paper II)
- Evaluate antibody precipitation as a method for detecting interference in a variety of hormone immunoassays used in veterinary medicine (paper II)
- Determine if there is a breed variation in the prevalence of canine anti-mouse antibodies (paper III)
- Characterize the fragment-specific binding properties, species cross-reactivity and immunoglobulin isotypes of canine anti-mouse antibodies (paper III)
- Follow up dogs with anti-mouse antibodies prospectively to determine the connection of the antibodies to disease, as well as their duration in serum over two years (manuscript IV)
- Test if autoantibodies to the Fc- and F(ab')₂-fragments of IgG cross-react with mouse IgG and if the binding is derivable to cryptic epitopes in the hinge region of IgG (manuscript IV)

3 Materials and methods

The materials and methods are presented here in a condensed version. For more detailed descriptions, see each individual paper.

3.1 Screening for anti-mouse antibodies

For paper I, we collected 369 samples from 320 dogs and 263 samples from 218 cats and screened them in an ELISA for anti-mouse antibodies. Multiple dog and cat breeds of both sexes and with varying ages were represented. The samples were collected from the routine diagnostic analysis at the University Animal Hospital, so most of the animals had some clinical diagnosis or clinical signs of disease. Except for the exclusion of samples with inadequate volumes and samples with hemolysis, bilirubinemia or lipemia, there were no selection criteria.

The screening assay was an immunometric assay using mouse IgG for capture and detection. The assay was developed in-house with commercial reagents. We used a 3-step format, with no co-incubation of sample and antibody and washed the wells between each incubation step. The detection antibody was conjugated directly to HRP (horseradish peroxidase). There was no calibration of the unknown samples, but we generated a standard curve using chicken anti-mouse IgG for evaluating the quality of each run and also as a practical means of obtaining a cut-off (the 7th point of the standard curve). Results for unknown samples were presented on a relative scale where the optical density (OD) of the sample was divided by the OD of the cut-off point. The screened patients were categorized by age, sex, breed, neutering status and clinical disease groups, and statistical analysis was performed to assess differences between patients positive and negative for anti-mouse antibodies within each of these categories. Risk

factors were assessed for both dogs and cats. The prevalence of anti-mouse antibodies was also compared between dogs and cats. Samples positive for anti-mouse antibodies were tested for cross-reactivity to chicken IgY by substituting the mouse IgG capture antibody for chicken IgY.

3.2 Interference in commercial hormone immunoassays for dogs

The sample material for the study contained in paper II consisted of serum testing positive for interference in paper I (28 sera from 27 dogs). We decided to only follow through with the positive dog samples. Serum was collected from 25 healthy dogs without anti-mouse antibodies to establish a control group. We selected four immunoassays representing a variety of formats (immunometric and competitive) and antibody reagents: mouse, sheep and rabbit IgG. The included assays were AMH, canine TSH, canine TT₄ and progesterone. They were selected and prioritized based on the importance of an individual measurement for the clinical diagnosis and the frequency of their use in veterinary patient care.

For investigating interference in the AMH assay, we matched the result against the neutering status of the animal. If a neutered dog had detectable AMH concentrations, we blocked the serum with different concentrations of mouse IgG, native and heat-treated and compared the measurements before and after treatment. We also treated the sample with 24% PEG to precipitate the immunoglobulins and evaluated the obtained result for discrepancy against the original result. This was the only sample treatment performed in the rest of the assays (canine TSH, canine TT₄ and progesterone). Measurements that differed by more than four standard deviations (SD) from the intra-assay CV were considered discrepant. By default, CVs are calculated based on one SD, which amounts to a 68% probability that two repeat measurements of a sample deviate within the confines of the assay CV. If 4 SDs instead are used to calculate the CV, the probability increases to 99.99%. Given these conditions, there is only a 0.01% probability of obtaining a discrepancy between two measurements for any other reason than the PEG treatment. The number of discrepancies for samples with anti-mouse antibodies was then compared to the number of discrepancies for samples without anti-mouse antibodies by using a T-test for two proportions.

3.3 Breed predisposition and characterization of canine anti-mouse antibodies

Serum was obtained from a mixed population of healthy dogs and dogs with clinical diagnosis, in total, 51 Bernese mountain dogs and 53 Labrador retrievers. Both excess serum from clinical cases and dogs belonging to private volunteering owners was used. The samples were screened with a method similar to the one in paper I, but here, we tested each sample for reactivity to whole molecules, Fc- fragments and F(ab')₂-fragments of mouse IgG. We then defined a positive sample for anti-mouse antibodies as one testing positive in at least one of these assays. The cut-off for each assay was set to the assay LoD (limit of detection) + 4 SD. The number of positive samples for Bernese mountain dogs and Labrador retrievers were statistically compared with a paired T-test.

Fragment-specific reactivity was obtained in the breed comparison. Samples testing positive for anti-mouse antibodies were further evaluated for cross-reactivity by substituting the capture antibody for different species of IgG (goat, rabbit, sheep and chicken IgY). Isotyping of positive samples was performed by coating the plate with mouse IgG, and detecting with goat anti-dog antibodies, isotype-specific for IgA, IgG and IgM.

3.4 Clinical significance, duration and possible origin of canine anti-IgG antibodies

A questionnaire was designed and distributed to private owners of dogs participating in the paper III study. An online survey provider hosted the questionnaire, and a link was distributed to owners that had supplied an e-mail address. Owners without e-mail contact information were surveyed via telephone. The survey was open for completion for six weeks. The questionnaire queried dog owners on any disease investigations undertaken at veterinary clinics during the years 2017 and 2019, and they were asked to specify which type or types of disease within the categories skin disease, kidney disease, gastrointestinal disease, nervous disease, tumour disease, orthopaedic disease, cardiac disease and other disease. Further questions were asked about any ongoing pharmacological treatments and observed contact with mice or other rodents. It was possible to specify the answer in free-text boxes for questions regarding clinical diagnoses or clinical signs of disease. At the end of the

questionnaire, it was possible to volunteer dogs for follow-up collection of serum samples.

Statistical testing was performed to determine if the presence of canine anti-mouse antibodies is a risk factor for the development of disease in general, as well as within specific disease categories. Follow-up samples were tested for the presence of anti-mouse antibodies with the method described in paper III. In addition, 57 serum samples collected in 2017 and 2019 were analyzed for autoantibodies to IgG (anti-Fab and anti-F(ab')₂-autoantibodies). Plates were coated with Fab- or F(ab')₂-fragments of dog IgG. Autoantibodies were detected using Fc-specific goat anti-dog IgG. The levels of autoantibodies were compared between anti-mouse positive and anti-mouse negative cohorts. Levels of anti-Fab and anti-F(ab')₂-autoantibodies were tested for statistical relationships with the health status, sex, age and breed of the dogs. Samples with anti-F(ab')₂-autoantibodies were blocked with mouse IgG and mouse F(ab')₂ to assess cross-reactivity.

4 Results

The main results from paper I-IV are summarized here. More details are found in the respective papers.

4.1 Prevalence of anti-mouse antibodies in a cross-section of dog and cat patients and interference elimination with chicken IgY as capture antibody (paper I)

In total, 9% of tested dogs (28/320) and 5% of cats (10/218) were positive for interference. The relative OD for positive samples ranged between 1.005 and 2.019 for dogs and between 1.049 and 2.081 for cats (Figure 5). The median age of interference-positive dogs (dogs with anti-mouse antibodies) was seven years. There were 11 intact males, three neutered males, ten intact females, and four spayed females of 24 different breeds. The median age of interference-positive cats was 8.5 years. There were six neutered males, one intact female, and three spayed females of 5 different breeds. Age, sex, breed, neutering status and diagnostic category comparisons between interference-positive and interference-negative dogs and cats were not statistically significant.

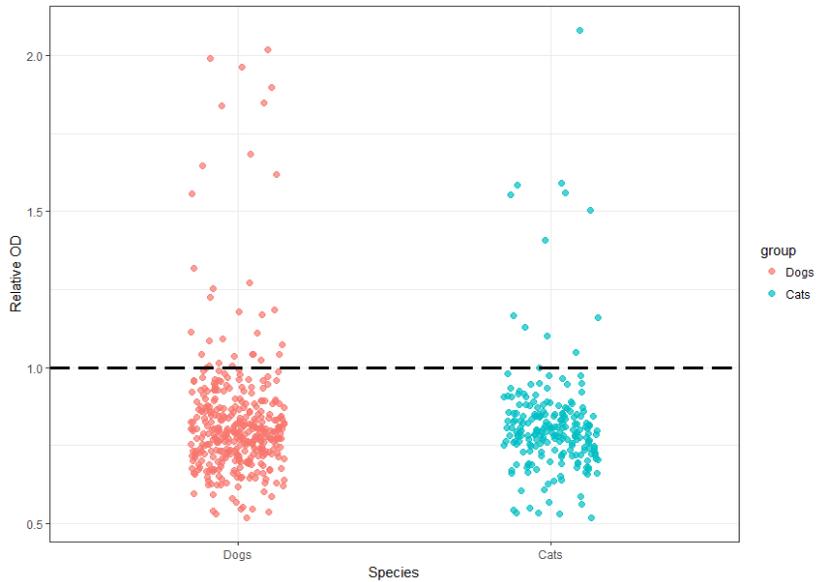


Figure 5. Dog (n = 369) and cat (n = 263) samples were screened with a 3-step immunometric ELISA using nonimmunized mouse IgG as the capture antibody. An HRP-conjugated monoclonal mouse IgG1k anti-CEA antibody was used as the detection antibody. The dashed horizontal line indicates the cutoff level. The results are presented on a relative scale and calculated according to the formula: (mean sample OD)/(mean cutoff OD). There was no significant difference in the prevalence of interference between dogs and cats (P = .06). From Paper I (Bergman et al., 2018).

Coating the microtiter wells with chicken IgY reduced the signal significantly (P < .001) as compared to wells coated with mouse IgG. All 19 assayed samples were negative when chicken IgY was used as the capture antibody. Five samples previously determined to be positive tested negative in this experiment. (Figure 6).

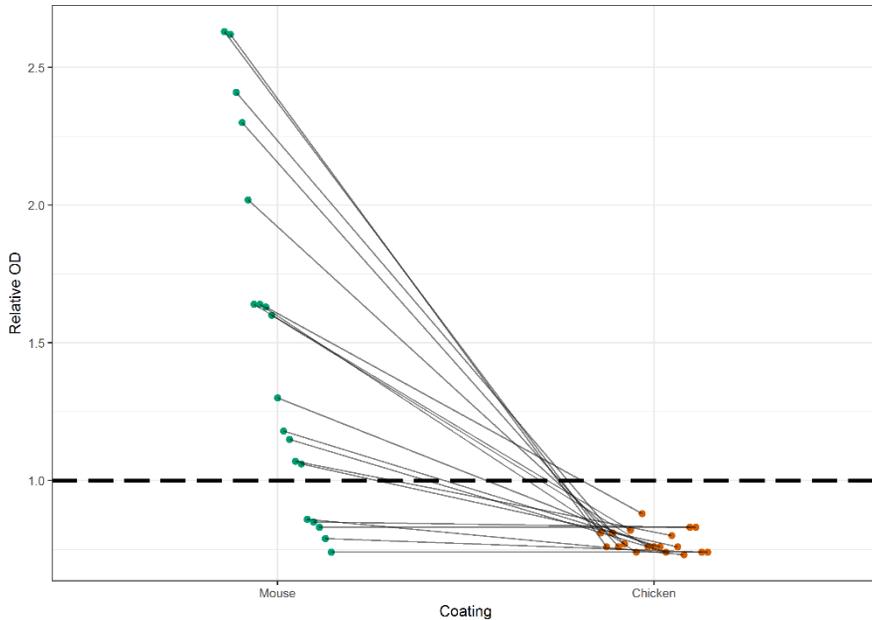


Figure 6. Reactivity against nonimmunized mouse IgG and chicken IgY using an HRP-conjugated mouse IgG1k anti-CEA antibody for detection. The dashed horizontal line indicates the positive cutoff level. The results are presented as (mean sample OD)/(mean cutoff OD)

4.2 Effects of interference elimination treatments on samples with falsely elevated AMH results (paper II)

Samples from two of seven neutered dogs with anti-mouse antibodies yielded detectable AMH concentrations. They corresponded to the strongest reactivity to anti-mouse IgG of all screened samples in study 1.

The initial AMH measurement for serum 1 was 14.49 pmol/L. Heat-aggregated MAK33 (0.5 mg/mL) yielded a decreased by 22% (to 11.28 pmol/L), and 1.0 mg/mL of heat-aggregated MAK33 yielded a decreased by 57% (to 6.28 pmol/L) (Figure 7). Combined treatment with 0.5 mg/mL heat-aggregated MAK33 + I5381 decreased the result by 62% (to 5.50 pmol/L).

The initial measurement of AMH on serum 2 was 5.71 pmol/L. The same interference testing was performed as for serum 1. Treatment with 1.0 mg/mL heat-treated MAK33 and 0.5 mg/mL MAK33 + 0.5 mg/mL I5381 both yielded undetectable AMH concentrations. The AMH concentrations for both sera were below the detection limit after treatment with PEG.

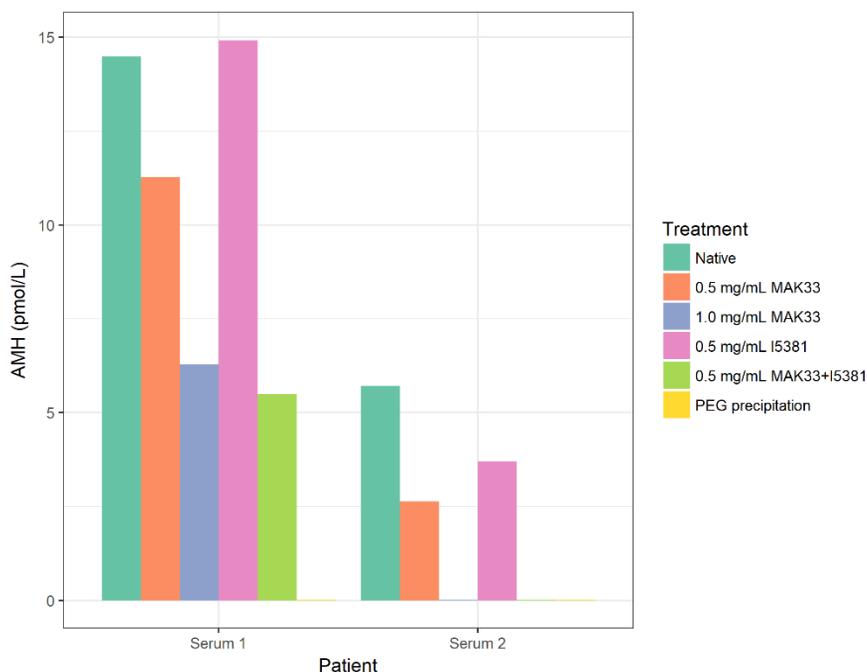


Figure 7. Effects of polyethylene glycol (PEG) treatment and antibody blocking on samples with erroneous anti-Müllerian hormone (AMH) results. For blocking, different concentrations of two mouse antibodies (MAK33 and I5381) were used. The antibodies were also used in combination with 0.5 mg/mL of each. From paper II (Bergman et al., 2019a)

4.3 Effects of antibody precipitation on hormone measurements in samples with and without anti-mouse antibodies (paper II)

The effects of the PEG treatments are summarized (Table 1). In total, 127 paired analyses were performed. Out of these analyses, 100 (79%) returned informative results. Non-informative results included results that were outside of the assay range before PEG treatment or that returned an error code after PEG treatment. When the result after PEG treatment was below the assay range, the lowest value was divided by two for statistical calculations.

There was a significant difference in the probability of getting a discrepant result post-PEG treatment depending on whether AMH, TSH, TT₄, or progesterone was analyzed. This difference was significant for samples with anti-mouse IgG, without anti-mouse IgG, as well as for all samples ($P < 0.001$ in all three cases). Presence or absence of anti-mouse antibodies did not affect

the probability of getting a discrepant result for any of the assays, except the canine TT₄ assay ($P = 0.04$).

Five of the 29 patient samples (17%) with anti-mouse antibodies did not cause discrepant results in any of the tested immunoassays. Conversely, discrepant results were found in at least one of the immunoassays for all 25 serum samples without anti-mouse antibodies.

Table 1. *A summary of the polyethylene glycol (PEG) effects. The effects on hormone measurements after PEG treatment for samples with (Anti-mouse+) and without (Anti-mouse-) anti-mouse antibodies. From paper II (Bergman et al., 2019a)*

	Number of discrepancies		Measurement decrease	
	Anti-mouse+	Anti-mouse-	Anti-mouse+	Anti-mouse-
AMH	14/14(100%)	17/17 (100%)	75%	68%
TSH	5/10 (50%)	8/10 (80%)	26%	38%
TT ₄	6/14 (43%)	15/18 (83%)	23%	38%
Progesterone	0/6	1/11 (9%)	0	0
Overall	25/44 (57%)	41/56 (73%)	29%	41%

4.4 Breed predisposition and characterization of canine anti-mouse antibodies (paper III)

4.4.1 Breed predisposition

In total, 110 samples from 104 dogs, 51 Bernese mountain dogs (two with two samples) and 53 Labrador retrievers (four with two samples) were included in the screening for breed differences. The Bernese mountain dog cohort consisted of 25 intact females, eight neutered females, 11 intact males and seven neutered males. There were fifteen Bernese mountain dogs (29%) with clinical signs of disease or disease diagnosis at the time of sampling. The median age was three years. The Labrador retriever cohort consisted of 14 intact females, eight neutered females, 28 intact males and three neutered males. Twenty-nine of the Labrador retrievers (55%) were diagnosed with disease or had clinical signs of disease. The median age was four years. The incidence of clinical disease or clinical signs of disease was increased in Labrador retrievers ($P < 0.01$) and the proportion of intact females was greater in Bernese mountain dogs ($P = 0.02$).

Fourteen samples from 12 of the 104 dogs (12%) tested positive for anti-mouse antibodies in the screening. The positive cohort consisted of ten of 51 screened Bernese mountain dogs (20%), and 2 of 53 screened Labrador

retrievers (4%). Two dogs, one Bernese mountain dog and one Labrador retriever submitted two positive samples. There was a significant difference in the prevalence of anti-mouse antibodies between the breeds ($P=0.03$). The median age of dogs positive for anti-mouse antibodies was 3.5 years. In the positive cohort, there were three each of females, males, neutered females and neutered males. There were six dogs with a diagnosed disease or clinical signs of disease at the time of sampling.

4.4.2 Characterization of anti-mouse antibodies

Out of the 14 positive samples from 12 dogs, reactivity to whole IgG and $F(ab')_2$ -fragments was found in five samples from five dogs (42% of the dogs), reactivity to whole IgG only in three samples from three dogs (25%), reactivity to whole IgG and Fc-fragments in three samples from two dogs (17%) and reactivity to Fc-fragments only in three samples from two dogs (17%). None of the samples reacted with both Fc- and $F(ab')_2$ -fragments.

Following testing for cross-reactivity to IgG from different species, reactivity to IgG from multiple species was found in two of the 14 positive samples from 12 dogs (17%). Cross-reactivity to goat IgG was found in two samples from two dogs (17%) and to sheep IgG in one sample from one dog (8%). Cross-reactivity to goat IgG and sheep IgG was found in one sample (8%).

Antibody isotyping revealed that two of the eight isotyped samples (25%) were positive for IgG only, three (37%) for IgM only, and two (25%) for IgA, IgG and IgM. One sample (12%) was negative for all isotypes. The characterization of anti-mouse antibodies is summarized (Table 2).

Table 2. Summary of the antibody characterization performed in this study. Isotyping was not performed. From paper III (Bergman et al., 2019b)

Breed	Age	Sex	Clinical signs/diagnosis	Anti-mouse reactivity	Cross-reactivity	Isotype
BMD	3	MN	-	Whole IgG, $F(ab')_2$		IgA, IgG, IgM
BMD	3	FN	-	Whole IgG		IgG
BMD	1	F	-	Whole IgG, $F(ab')_2$		IgM
BMD	1	F	-	Whole IgG, $F(ab')_2$	Goat	N/A
LR	8	M	-	Whole IgG, $F(ab')_2$	-*	N/A
BMD	4	M	-	Whole IgG		IgM
BMD	5	FN	Lipoma	Whole IgG, $F(ab')_2$		IgA, IgG, IgM

Breed	Age	Sex	Clinical signs/diagnosis	Anti-mouse reactivity	Cross-reactivity	Isotype
BMD***	8	MN	Protein-losing enteropathy	Whole IgG, Fc		IgG
BMD	10	FN	Anterior cruciate ligament injury	Whole IgG	Goat, sheep	N/A
BMD	1	F	Gastric dilatation volvulus	Whole IgG, Fc		-
BMD	6	M	Mast cell tumour	Fc		IgM
LR***	3	MN	Polyuria/polydipsia	Fc	-.**	N/A

* Not tested for reactivity to chicken IgY. **Not tested for reactivity to rabbit IgG or chicken IgY. ***Two samples from this dog were analyzed.

4.5 Clinical significance, duration and possible origin of canine anti-IgG antibodies (manuscript IV)

4.5.1 Clinical significance of anti-mouse antibodies

Out of the 107 dogs participating in the previous study in 2017, 95 dogs were followed up by contacting 73 owners via telephone or e-mail. The survey responses were inferred from available medical records for seven euthanized dogs. When all completed questionnaires and inferred responses from medical records were counted, the response rate was 73% (75/102). During the follow-up period, seventeen dogs (22.7%) had been euthanized, 11 Bernese mountain dogs (median age: eight years) and 6 Labrador retrievers (median age: nine years). One of these dogs was positive for anti-mouse antibodies in the initial testing in 2017.

In the two years between 2017 and 2019, 61% of the dogs had been investigated for disease. Out of these dogs, 24% were included in multiple disease categories. The most common disease category for Bernese mountain dogs was neoplastic disease (23%) and for Labrador retrievers orthopaedic disease (26%). The proportions of positive and negative dogs within the different disease categories are displayed (Figure 8).

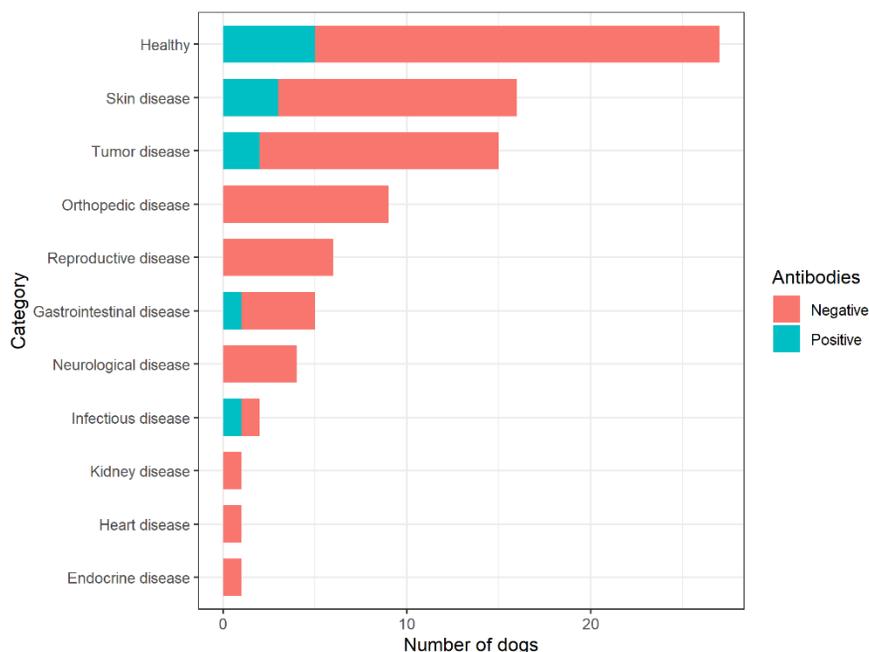


Figure 8. Allocation of diagnoses for different disease categories, including healthy dogs. Stacked bars are separated into two groups based on dogs testing positive or negative for heterophilic antibodies in 2017.

There was no overall significant difference in disease between the positive and the negative cohort ($P=0.5$); neither was there a significant difference within any of the specific disease categories.

4.5.2 Exposure to mice as a risk factor for anti-mouse antibodies

Owners reported no observed contact with mice for nine out of ten dogs that tested positive for heterophilic antibodies against mouse IgG in 2017. There was no available information on mouse contact for the tenth dog, which had been euthanized during the follow-up period. There was no observed contact with mice for three dogs testing positive for heterophilic antibodies against mouse IgG in 2019 only. Out of the dogs testing negative for anti-mouse antibodies in 2017, contact with mice was reported for 11% of the dogs (7/65).

4.5.3 Serum duration of anti-mouse antibodies

Ten out of 12 dogs that tested positive in 2017 participated in the study, and seven of them contributed new samples. Six out of these seven dogs remained positive at the follow-up sampling. Three additional dogs that were negative in 2017 were positive in 2019. The age, breed, sex, clinical signs or diagnosis and antibody reactivities for these dogs are presented in Table 3. None of the Fc-reactive samples had shifted to become F(ab')₂-reactive or vice versa.

Table 3. Breed, age, sex and clinical signs/diagnosis for dogs testing positive for heterophilic antibodies either in 2017 or 2019. Reactivity to mouse IgG in 2017 and 2019 is given.

Dog ID	Age (y)	Sex	Clinical signs/diagnosis		Antibody reactivity	
			2017	2019	2017	2019
B1	9	MN	Protein-losing enteropathy	Liver adenoma or carcinoma*	Fc	N/A
B5	8	M	Mastocytoma	Mastocytoma	Fc	Fc
B9	12	FN	Anterior cruciate ligament injury	-	Whole IgG	Whole IgG, Fc
B10	7	FN	Lipoma	Granulocytic anaplasmosis, Lyme borreliosis	Whole IgG, F(ab') ₂	Whole IgG
B11	5	FN	-	-	Whole IgG	-
B19	3	F	-	Superficial pyoderma	Whole IgG, F(ab') ₂	Whole IgG, F(ab') ₂
B24	4	F	-	Superficial pyoderma	-	F(ab') ₂
B25	6	M	-	Oral blisters, pigmentation loss and widespread redness	Whole IgG	Whole IgG, F(ab') ₂
B28	9	M	-	Gingival neoplasm	-	Fc
B42	5	MN	-	-	Whole IgG, F(ab') ₂	Whole IgG, F(ab') ₂
B44	3	F	-	Abnormal heat cycle, lameness	-	Fc
B51	3	F	-	-	Whole IgG, F(ab') ₂	N/A
L9	5	MN	Polyuria/polydipsia	-	Whole IgG	N/A

F: female; M: male; N: neutered; N/A: dog not sampled. * The dog was euthanized during the follow-up period

4.5.4 Clinical significance of anti-Fab/F(ab')₂-autoantibodies

There was no significant statistical relationship between levels of anti-F(ab')₂-autoantibodies and health status, cancer diagnosis, breed or sex. The correlation between age and levels of anti-F(ab')₂-autoantibodies was negligible.

For anti-Fab-autoantibodies, levels were higher in dogs with a diagnosis or clinical signs of disease (P=0.03), but there was no significant statistical relationship between levels of anti-Fab-autoantibodies and cancer diagnosis, breed, nor sex. The correlation between age and levels of anti-Fab-autoantibodies was negligible.

The reactivity to F(ab')₂-fragments was significantly higher than the reactivity to Fab-fragments (Figure 9, P<0.01). There was a low positive correlation between the levels of anti-Fab-autoantibodies and anti-F(ab')₂-autoantibodies ($r_s = 0.39$, P<0.01).

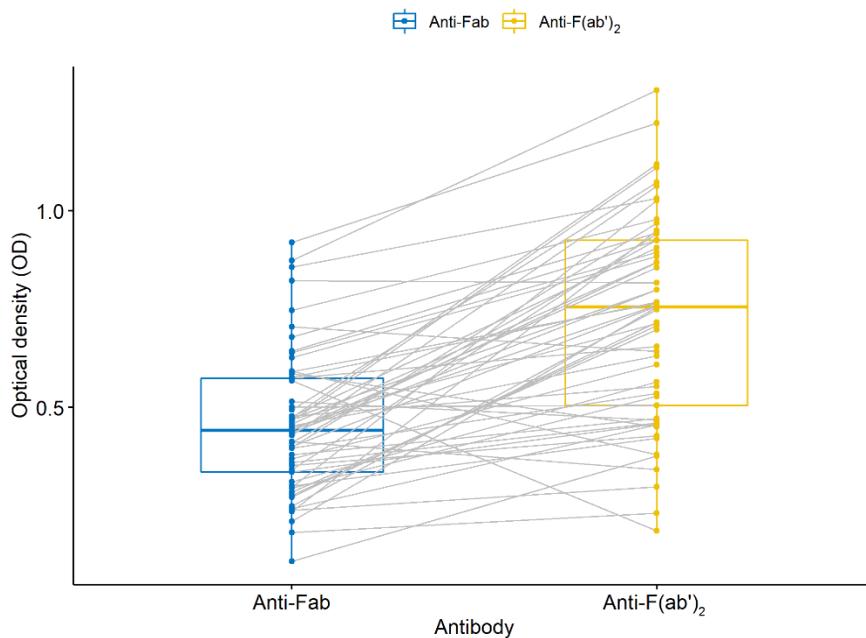


Figure 9. OD values for IgG anti-Fab and anti-F(ab')₂-autoantibodies in 57 dog samples. IgG anti-F(ab')₂-autoantibody levels were significantly higher than anti-Fab-autoantibody levels (P < 0.01).

4.5.5 Relationship between canine anti-IgG autoantibodies and anti-mouse antibodies

We blocked 35 samples analyzed for anti-F(ab')₂-autoantibodies with 0.5 mg/mL dog F(ab')₂, mouse IgG and mouse F(ab')₂ (Figure 10). Blocking with dog F(ab')₂ had an overall inhibitory effect on signal ($P < 0.01$), but mouse IgG or mouse F(ab')₂ did not have an overall effect on the signal.

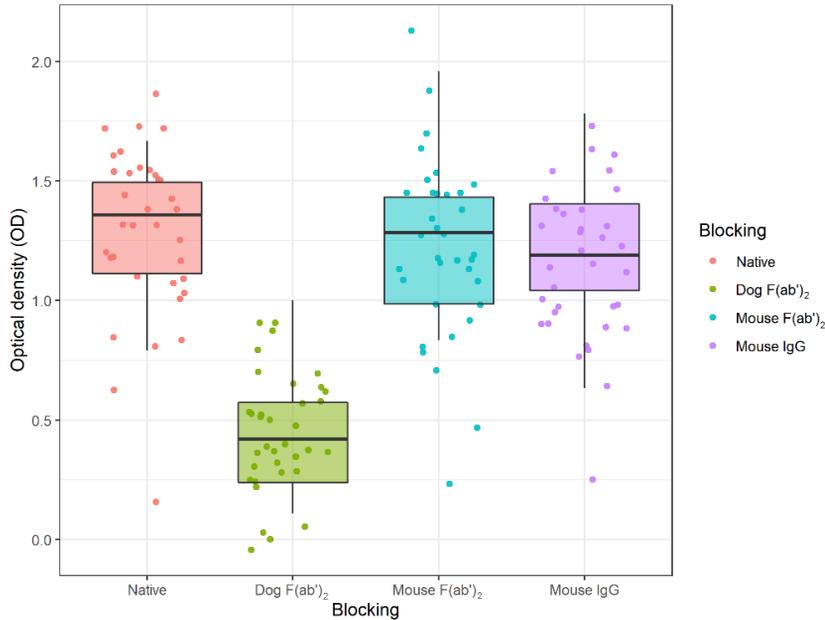


Figure 10. Thirty-five samples were assayed for IgG anti-F(ab')₂-autoantibodies native and pre-blocked with 0.5 mg/mL dog F(ab')₂, mouse F(ab')₂ and mouse IgG. There was a significant overall effect in replicates blocked with dog F(ab')₂, but not in replicates blocked with mouse IgG nor mouse F(ab')₂.

At a 99.99% prediction interval, dog F(ab')₂ decreased the signal in 35/35 samples (100%). Anti-F(ab')₂-autoantibodies cross-reacted with mouse IgG in 3/35 samples (9%) and mouse F(ab')₂ in 3/35 samples (9%). There was no cross-reactivity to mouse IgG nor to mouse F(ab')₂ in 6/9 samples (67%) with previously detected heterophilic antibodies. Three out of 26 samples (12%) without previously detected heterophilic antibodies cross-reacted with mouse IgG or mouse F(ab')₂. Anti-F(ab')₂-autoreactivity was not significantly higher in dog samples reactive with F(ab')₂-fragments of mouse IgG than in samples not reactive with F(ab')₂-fragments of mouse IgG.

5 Discussion

5.1 Screening for anti-mouse antibodies

In paper I, we focused on the bare fundamentals of heterophilic antibodies by devising a method that can be used for screening a population for their presence and estimate their prevalence. It had already been shown that heterophilic antibodies could be found in canine plasma (Solter *et al.*, 2008), but for those experiments, pooled plasma was used, preventing prevalence estimations, and the technique used only works for canine samples. There was no documentation of heterophilic antibodies in cats, another frequently occurring species in immunoassay analysis. Nearly all patients that have serum collected at an animal hospital are admitted for diagnostic work-up or follow-up. Occasionally, healthy animals are sampled at the hospital, for example in theriogenology services, or when patients are in complete remission, but the majority of the samples will come from animals with some diagnosis or clinical signs of disease. Selecting the most recent samples from a diagnostic laboratory, therefore, provides us with an appropriate study population for investigating interference, as these animals represent those who are most often subjected to immunoassay testing.

It is important to select an appropriate screening method for heterophilic antibodies. There is no gold standard method, nor any commercially available test for detecting heterophilic antibodies. In principle, there are two different ways of screening for heterophilic antibodies – either directly in each specific immunoassay by pre-treating the samples using some method that removes the interferents from the sample, or by performing parallel testing with a method that detects heterophilic antibodies, often known as an “interference assay” or “nonsense assay”. Here, we decided to first screen samples with an interference

assay, and then to screen positive samples in each specific immunoassay in a later study.

There are different ways to configure an interference assay, but the imperative is that it does not detect any specific sample analyte. This objective can be achieved by using a mismatched pair of capture and detection antibodies, such that they target epitopes that are not found on the same molecule. We used polyclonal non-immune mouse IgG for capture and a monoclonal mouse IgG1k for detection. The monoclonal antibody is specific for human carcinoembryonic antigen (CEA), a molecule not found in dog or cat serum, and at any rate, the capture antibody does not recognize human CEA, which therefore prevents detection even if CEA were present in the samples. This means that only molecules that cross-link mouse IgG, and more specifically, mouse IgG1k, will be detected in the assay. In practice, these cross-linking substances are called heterophilic antibodies or anti-mouse antibodies. The mouse IgG1k antibody was selected because it is the most frequently used antibody in commercial immunoassays. Overall, the ELISA protocol was designed to mirror a generic immunoassay procedure. However, we did not dilute or incubate the samples in any type of blocking buffer resembling a commercial immunoassay, because the contents of these are usually unknown. If this had been done, we would likely have detected less interference. On the other hand, we would probably have detected more interference if we had used a polyclonal mouse IgG for detection, and if the samples had been incubated together with the detection antibody in a so-called two-step format, which is not uncommon in commercial immunoassays.

Factors such as the selection of cut-off criteria and dilutions of reagents also affect the results, and it is well-known that estimated prevalence figures of heterophilic antibodies vary greatly depending on these and several other factors. The achieved prevalence of 9% in dogs and 5% in cats is, therefore, best seen as a rough estimate. The numbers are of less interest than the fact that we were able to demonstrate a method for screening for heterophilic antibodies that can be used in all species, and that laboratories can use without the need for special equipment or custom reagents. This study did not attempt to evaluate the usefulness of the method, as that will depend on the specific needs and its intended use. It can be used as a parallel screening tool for incoming samples at a diagnostic laboratory, for investigating interference in samples with suspected interference, or as in this case, for selecting samples of interest for a follow-up screening in specific assays. Because of the wide variety of immunoassays and configurations of immunoassays that are used in diagnostic laboratories, it is perhaps most realistically used as a tool for investigating interference in selected samples. The protocol can be modified to match the assay that the sample was

previously tested in more closely. For example, if interference is suspected in an assay using sheep IgG for capture and monoclonal mouse IgG for detection, the capture antibody would be substituted for a non-immune sheep IgG.

5.2 Interference in commercial hormone immunoassays for dogs

Investigating immunoassay interference from heterophilic antibodies is a task that is associated with several challenges. One of the major challenges is that the correct test result for a patient sample is usually unknown. In the absence of a correct result for reference, claims that a test result is incorrect are usually based on the perceived plausibility of the result. The starting point for the present investigation was a cohort of samples with anti-mouse antibodies and another cohort without anti-mouse antibodies. In clinical practice, the starting point for an interference investigation is usually a mismatch between the clinical presentation and the test result, one of the hallmarks of interference.

The retrospective nature of this study, which is presented in paper II, means that all patient information was limited to second-hand sources in the form of medical records. These may not contain the information needed to evaluate the plausibility of a particular test result. However, some test results are not intended to be interpreted together with an extensive case history and specific clinical findings. One example is the anti-Müllerian hormone (AMH) assay, which is, among other things, indicated for the determination of neutering status in dogs with unknown medical histories. There is no natural source of AMH in neutered animals, and detectable serum AMH can, therefore, be attributed to interference. The neutering status of the animal is thus all the clinical information needed to demonstrate interference in the AMH assay. We considered detectable AMH in combination with a medical record documenting the positive neutering status of the animal to be evidence for interference. Further, we attributed the source of the interference to anti-mouse antibodies if the patient also had a positive test result for anti-mouse antibodies. We then blocked interference-positive samples with different concentrations of mouse IgG and evaluated the effect of the blocking to build a strong chain of circumstantial evidence that endogenous anti-mouse antibodies altered the test result. Two samples were considered to cause interference in the AMH assay as they matched all of these criteria. The clinical implications of these findings are that dogs with falsely elevated AMH could be subjected to needless surgery if they are incorrectly determined to be unneutered. In the two specific cases found here, both dogs were male. In male dogs, needless surgery might be performed if the AMH levels falsely indicate bilateral

cryptorchidism. However, there is no indication that the sex of the dog has any bearing on the probability of interference, and a dog with a false-positive AMH result could also be a female with an unknown neutering status.

The other assays that we investigated were canine TSH, canine TT₄ and progesterone. In the measurement of these analytes, there is a myriad of factors influencing the hormone levels, and the plausibility of the test result cannot be reliably inferred from a retrospective source such as a medical record. We therefore had to use a method aimed at removing interferents from the samples to investigate them for interference, and we selected precipitation with polyethylene glycol (PEG) for this purpose. However, because this method has not previously been used for canine analytes, there were no established reference values for the expected difference in measurements after PEG treatment in interference-negative samples. Instead, we defined differences in measurements before and after PEG treatment as “discrepant” based on a 99.99% prediction interval for the expected result. These calculations are described more thoroughly in paper II and the materials and methods section of this thesis.

Based on several observations, we concluded that the PEG method is not useful as a general tool for detecting interference in the immunoassay analysis of canine serum. The method did not discriminate between samples with or without anti-mouse antibodies, it did not declare any of the tested samples free from interference, and the overall incidence of interference with interference defined as a discrepancy of more than 4 SD was very high, 73% in samples without anti-mouse antibodies. The most plausible explanation for these findings is that a fraction of the analytes co-precipitates with the antibodies. This observation was noted for all analytes except progesterone. Even if the true prevalence of interference in these assays were 73%, it would not be feasible for clinicians to re-evaluate the majority of clinical cases, and in many of these cases, the discrepancies are not likely to make a difference for the clinical interpretation as they do not push the measurement outside of the normal reference range.

5.3 Characterization of canine anti-mouse antibodies

This study was presented in paper III and consisted of two components: first, an evaluation of the prevalence of anti-mouse antibodies in two dog breeds. In immunometric assays, analytes need to bind both to the capture antibody and the detection antibody for a signal to be generated. Therefore, by substituting one of the antibodies and keeping all other variables constant, characterization can be performed. In this experiment, we put different capture antibodies on the solid

phase and kept all other steps of the ELISA procedure intact for all assays. This simple principle can be used to characterize binding to different fragments, isotypes and subclasses of immunoglobulins, cross-reactivity to immunoglobulin from different species, and other antibody properties. The limiting factor is essentially the available sample volumes at hand, so we had to be selective in deciding which experiments to perform. Here, we took a utilitarian approach and prioritized investigating the antibody properties that are most likely to impact routine immunoassay analysis.

One of the most pressing questions about interfering antibodies is which part of the target IgG molecule they bind. This knowledge can provide clues to the origin of the antibodies – for example, rheumatoid factors are expected to bind only the Fc region – but it also gives information that is immediately applicable and useful in immunoassay development. The Fc region of IgG is not needed for detection of antigen and is in immunoassays only retained for practical purposes, as removal of it would entail additional production steps and costs. However, if it can be demonstrated that interference through binding to the Fab-region is rare, it could motivate the removal of the Fc region as a prophylactic measure against interference. Determining the IgG fragment reactivity was, therefore, one of the experiments that we decided to perform.

It is also useful to know if the antibodies are capable of cross-reacting with IgG from different species. Although monoclonal mouse IgG is still the default antibody in most commercial immunoassays, it is not uncommon for immunoassays to use IgG from other species, for example, goat, sheep and rabbit, sometimes in combination with mouse IgG. Here it is important to remember that the tested samples were pre-selected for their reactivity with mouse IgG and that the detection antibody in the assay is a monoclonal mouse IgG1k. Thus, putting IgG from different species on the solid phase will only detect cross-reactive antibodies, not antibodies that are exclusively anti-goat, anti-rabbit, et cetera.

One of the experiments that cannot be performed with this general principle is immunoglobulin isotyping of the endogenous antibodies. By definition, the assay will detect anything in the sample capable of bridging two antibodies, and therefore also immunoglobulins of all isotypes. Isotyping the endogenous antibodies is the most technically challenging experiment in paper III. The most critical step is selecting appropriate detection antibodies, which must meet several criteria. The first criterion is that they are specific for different immunoglobulin isotypes. Secondly, they must not cross-react markedly with the mouse IgG that pulls down the interfering antibodies to the solid phase, or excessive background will prohibit distinction between isotypes. Moreover, the assay is only usable for samples that do not cross-react with goat IgG (all of the

detection antibodies are goat IgG), or they will react non-specifically with all the detection antibodies, again preventing distinction between isotypes. Because we had previously determined the reactivity to goat IgG, we were able to reject the samples that reacted with goat IgG for this experiment.

The results presented in paper III clearly demonstrate that canine anti-mouse antibodies are heterogeneous. They can react with IgG from different species, with different IgG regions and they can be of the IgA, IgG and IgM isotype. This heterogeneity is challenging for laboratories and manufacturers to navigate around when attempting to limit the incidence of immunoassay interference. If F(ab')₂-fragments are used to capture and detect antigen, there will still be antibodies capable of causing interference remaining. The phylogenetic distance between two species can probably be used to mitigate interference, as chicken IgY for capture and mouse IgG for detection was the combination of antibodies that resulted in the least amount of interference. However, this design may not work for all purposes, since monoclonal chicken antibodies are currently lacking. Monoclonal antibodies are only derived from mammals, and even in a small sample cohort, the possibility of cross-reactivity between different mammalian IgGs was evident. Because the endogenous antibodies consist of several isotypes, removing them from the sample is likely to be impractical. As we demonstrated in paper II, antibody precipitation with PEG is a very crude method that leads to co-precipitation of several analytes, and the mixture of different isotypes precludes the use of isotype-specific methods like Protein A and Protein G precipitation for the exclusion of IgG and gel-filtration chromatography for IgM. An adequate neutralizing incubation buffer containing non-immune IgG is probably the best protection against interference in canine samples when all factors are considered, and this is a strategy that both manufacturers and diagnostic laboratories can implement. We would also like to stress the importance of enhancing the blocking efficiency by heat-treating the IgG in this incubation buffer, as was shown in paper II. Although heat-treatment requires an extra preparation step, heat-treated incubation buffers can be produced in batches and then stored and used in the same way as untreated IgG.

Interestingly, none of the detected anti-mouse antibodies bound to both Fc- and F(ab')₂-fragments, and in some samples, there was only detectable reactivity to whole IgG. Selective binding to different IgG fragments is not expected if the source of the antibodies is exposure, which we confirmed by showing that anti-mouse IgG from chickens immunized with whole mouse IgG reacted with both Fc- and F(ab')₂-fragments. Instead, it opens up the possibility that the anti-mouse antibodies are disease-associated autoantibodies to IgG. It is counterintuitive that some samples react with whole IgG but not with either fragment, but this might be due to binding on or near the IgG hinge where the digestive enzymes

cleave the IgG molecule, possibly altering or removing epitopes in the process. These observations paved the way for the final manuscript in the thesis, where we elaborated on the clinical significance and the potential origin of the anti-mouse antibodies.

5.4 Clinical significance, duration and possible origin of canine anti-IgG antibodies

The fourth study, corresponding to manuscript IV, explored the clinical significance of heterophilic antibodies against mouse IgG from two different angles. First, it was of particular interest to investigate potential connections to autoimmune disease and cancer in heterophilic antibodies. Secondly, heterophilic antibodies can interfere with therapeutic monoclonal antibodies. Both of these scenarios postulate that the heterophilic antibodies are cross-reactive autoantibodies to IgG.

We had in paper III observed an increased frequency of heterophilic antibodies against F(ab')₂-fragments of mouse IgG in Bernese mountain dogs. This breed is not known to be predisposed to autoimmunity, but the mortality for cancer in Bernese mountain dogs is around 50% (Klopfenstein *et al.*, 2016). It was recently proposed that anti-F(ab')₂-autoantibodies can form in response to proteolytic cleavage by cancer-associated proteases (Brezski & Jordan, 2010), and previous findings did not indicate that the F(ab')₂-reactive antibodies were acquired from exposure to mice. In paper I, we did not find a relationship between the presence of heterophilic antibodies and diagnosed cancer, but this does not rule out a connection since it is quite possible that these antibodies, if they are autoantibodies, appear before disease onset, let alone before a clinical diagnosis has been made.

Two therapeutic monoclonal antibodies are currently approved by regulatory agencies for the treatment of cancer in dogs, but nearly 80 therapeutic monoclonal antibodies have been approved for use in humans (Lu *et al.*, 2020). This discrepancy is due to several factors, but one of them is an incomplete understanding of the canine immune system. In drug development, it is vital to be able to predict the immunogenicity of a candidate drug. Immunogenicity can affect the safety, efficacy and pharmacokinetics of the drug, and the development of immunogenic drugs is usually discontinued in the clinical phase, after years of basic research and development. Autoantibodies to IgG are a source of immunogenicity, and autoantibodies against the Fab region have not been described in dogs.

We investigated the clinical significance and interconnection of heterophilic antibodies and anti-Fab/F(ab')₂-autoantibodies by organizing a prospective study, where dog owners contributing samples for paper III were invited to respond to a questionnaire about any changes in their dogs' health status during the two years passed since the previous study. Follow-up samples were collected and analyzed for heterophilic antibodies to determine their serum duration. We tested samples for anti-Fab and anti-F(ab')₂-autoantibodies, and anti-Fab/F(ab')₂-reactivity was tested for statistical relationships with sex, breed, age, health status and presence of heterophilic antibodies. This information helps to understand the relationship between heterophilic antibodies and autoantibodies. The duration of heterophilic antibodies in serum can guide the clinical handling of patients presenting with antibody interference. It can also give clues into the origin of the antibodies. The persistent presence of antibodies during two years would not be expected if the antibodies are acquired from the often suggested fleeting, unknown exposures to antigen (Ismail, 2009; Kazmierczak *et al.*, 2000), and might favour the hypothesis that they are autoantibodies associated with disease. It is also of relevance to describe the presence of anti-Fab and anti-F(ab')₂-autoantibodies in dogs for the development of therapeutic monoclonal antibodies.

The results did not show any prospective relationship between the presence of heterophilic anti-mouse antibodies and overall disease, nor specific disease categories, including cancer. However, it was demonstrated that both anti-Fab and anti-F(ab')₂-reactivity is widespread in dogs. The reactivity to F(ab')₂-fragments was significantly higher than reactivity to Fab-fragments, and there was only a low positive correlation between levels of anti-Fab and anti-F(ab')₂-autoantibodies. This finding suggests that they can bind to mutually exclusive cryptic epitopes revealed by enzymatic cleaving in both the lower and the upper hinge region. These results have implications for monoclonal antibody development in dogs, as anti-Fab and anti-F(ab')₂-autoantibodies can react with therapeutic antibodies and affect their safety and efficacy. They can also interfere with immunogenicity tests that are used in preclinical and clinical drug trials (van Schie *et al.*, 2015).

There was no connection to cancer or overall disease in samples with increased anti-F(ab')₂-autoantibody levels. Thus it seems likely that the lower hinge region of canine IgG is of high general antigenicity and that many pathways lead to the formation of canine anti-F(ab')₂-autoantibodies. Dogs with a clinical disease or clinical signs of disease had significantly increased anti-Fab-autoantibody levels, which motivates future investigations of disease-associated enzymes that might cleave canine IgG in the upper hinge region *in vivo*. Increased anti-Fab-autoantibody levels in people are, for example, associated

with squamous-cell carcinomas and adenoid cystic carcinomas of the head and neck (Susal *et al.*, 1994). Autoreactivity to the F(ab')₂-region of IgG was not statistically increased in samples reactive with F(ab')₂-fragments of mouse IgG compared with samples without reactivity to F(ab')₂-fragments of mouse IgG. It appears that while all or at least nearly all dogs have some basal level of autoreactivity against the F(ab')₂-part of IgG, the increased autoreactivity is not directed towards epitopes that are shared with mouse IgG. However, there was sporadic cross-reactivity between mouse IgG or mouse F(ab')₂ and anti-F(ab')₂-autoantibodies (3/35 tested samples), indicating that heterophilic antibodies in dogs occasionally may be cross-reactive autoantibodies. There was no evidence for exposure to mice being a source of anti-mouse antibodies, as none of the dogs belonging to owners reporting observed contact with mice tested positive for anti-mouse antibodies. It is possible, though, that the owners had overlooked any hypothetical contact with mice. As yet, there is no satisfactory explanation for the origin of anti-mouse antibodies in most instances of their presence in dogs.

6 Future perspectives

The results presented in this thesis add to the basic understanding of heterophilic antibodies in veterinary medicine, which remains a largely unexplored research field. It could be argued that there are two main paths that future investigations can continue along.

One of the main paths leads to the acquisition of more epidemiological data. There is a lot of work remaining when it comes to describing the prevalence of heterophilic antibodies in animal species and species breeds. However, the presence of heterophilic antibodies does not necessarily equate to immunoassay interference. The logical next step is, therefore, to investigate the incidence of interference in specific immunoassays of relevance in veterinary medicine. This information can serve as a foundation for guidelines and recommendations on how a diagnostic laboratory should handle the hazard of antibody interference. If the problem is sufficiently common, introducing prophylactic countermeasures is a viable course of action, but if it is very sporadic, interference is perhaps better dealt with on a case-by-case basis. It would also be helpful to identify immunoassays that are especially prone to interference so that their use can be discouraged. If alternatives are lacking, it may be difficult to discontinue the use of a particular assay, but the importance of putting pressure on manufacturers to improve their products must not be neglected.

There is an ongoing trend in diagnostic medicine that veers towards using laboratory tests to identify diseases before the onset of clinical signs, to allow for early interventions and a more favourable prognosis. These promises may not be compatible with the standards of currently available diagnostic tests. Without the possibility to juxtapose test results with clinical findings, most analytical errors are initially likely to be overlooked, which by no means equates to being irrelevant. For the diagnosing of disease in apparently healthy patients to gain wide acceptance, the reliability and performance of the diagnostic tests

would likely need to be impeccable. Achieving these standards will, if at all possible, require a substantial amount of research into interference issues. Traditionally, laboratory tests have been intended to function as supplementary diagnostic tools to support the anamnestic and clinical findings. Yet, many cases of interference go unnoticed until after the patient has been misdiagnosed and administered the wrong treatment, illustrating that over-reliance on laboratory tests does occur and that it can be a harmful habit. There are many options available for investigating immunoassay interference, but the initial suspicion that a test result is incorrect will often have to come from the clinician and not from the laboratory. However, it may not always be realistic to expect clinicians to initiate interference investigations. A general lack of awareness of the problem is one reason. The interference warnings that immunoassay manufacturers frequently include in kit inserts are unlikely to be read by clinicians. It will, therefore, be motivated to research heterophilic antibodies in animals for the sole purpose of raising the awareness of immunoassay interference in veterinary medicine, thereby increasing the probability that more cases are identified. However, it must not be forgotten that medical histories and physical examinations also are subject to errors and misinterpretations, and it may be difficult to identify the missing link when clinical findings and biochemistry results are discordant. If a dubious test result is found, there are also several differential diagnoses to antibody interference that need to be considered. Here, knowledge of the magnitude of the issue with interference will help guide clinical decision making. If antibody interference is common, it would presumably be placed high on a list of potential causes for a questionable test result. If it is exceedingly rare, it may be worthwhile first to consider alternative explanations, which may include uncommon but legitimate medical causes. An example from human medicine is thyrotropinoma, a pituitary tumour with the same clinical manifestation as hyperthyroidism, but which causes raised TSH coupled with raised T₃ and T₄. This atypical combination would run a high risk of being dismissed as erroneous on analytical grounds, representing a scenario where a habit of questioning test results may instead lead to a missed or delayed diagnosis. Although knowing the incidence rate of interference is important in order to make informed clinical decisions, it is still valuable to merely demonstrate the existence of clinically relevant immunoassay interference in a species or a particular immunoassay regardless of its incidence. Even a low incidence rate could amount to a high total number of cases, and the outcome in each case is potentially devastating when patient safety is at risk.

Immunoassay interference is also an issue of relevance when veterinary care is less centred around individual animals. In the livestock sector, immunoassays are often used for active or passive surveillance of diseases with major socio-

economic impact. Avian influenza, brucellosis, leptospirosis and Q fever are some examples of zoonotic diseases that in Sweden are surveilled at least partly with immunodiagnostic methods. The rigour of the surveillance ultimately determines the potential consequences of immunoassay interference. It is well-known that immunoassays and other diagnostic methods all have their limitations and performance issues, which is why several laboratory tests often are used in combination to confirm the diagnosis of a surveilled disease. Nevertheless, a false-positive test result due to immunoassay interference is one step towards an incorrect estimate of the prevalence of the disease, which should be especially concerning when it comes to diseases that are considered to be eradicated in the surveilled area. Veterinarians and researchers also use immunoassays to analyze specimens from wild animals for a variety of purposes, including disease surveillance and monitoring of reproductive activity. Wild animals are likely to be exposed to animal species that are used to raise immunoassay antibodies, and although very little is known about autoimmunity in wild animals, it was recently shown that autoantibodies are widespread in unmanaged sheep (Graham *et al.*, 2010).

Dogs are receiving increasing attention as models for studying cancer in humans (DeWeerd, 2018), as well as recipients of novel immunotherapies (Ledford, 2016), even though a significant influx of monoclonal antibody drugs for dogs is still some distance away (Klingemann, 2018). However, if this development continues, it will open up a whole new avenue of investigation regarding canine anti-IgG antibodies. If monoclonal antibody therapies become widely available for dogs, the potential threat of immunoassay interference due to iatrogenic anti-mouse antibodies may be one of the lesser worries, as long as the therapeutic antibodies are speciated. However, autoantibodies against IgG can contribute to the immunogenicity of speciated therapeutic antibodies. Although efforts to develop novel veterinary treatments are laudable, it is disconcerting that therapeutic antibodies for dogs and cats are currently in the clinical pipeline when a basic understanding of factors that could influence the safety and efficacy of antibody therapies in these species is lacking. An inventory of pre-existing anti-IgG antibodies in dogs is much needed, as these antibodies could affect the immunogenicity of future antibody therapies. These pre-existing antibodies include rheumatoid factors, anti-idiotypic antibodies and anti-Fab/F(ab')₂-autoantibodies, most of which have been poorly studied in dogs to date. These anti-immunoglobulin antibodies could also interfere with the immunogenicity assays that are used in drug development to monitor treatment response (van Schie *et al.*, 2015). Lack of knowledge about such pre-existing antibodies is not only a concern for future immunotherapies but also one of many factors currently holding back their development. The existence of pre-existing

anti-IgG antibodies has not even been investigated for current treatments, and it is possible that canine anti-equine and anti-sheep antibodies are a source of early adverse reactions to antivenom administration following snake bites, as has been suggested in humans (Herrera *et al.*, 2005).

The second main path of research on heterophilic antibodies goes deeper into the immunological mechanisms and the molecular and biochemical interactions underlying interference. This field is relatively unexplored also in human medicine. The majority of endogenous antibodies causing interference are poorly defined. Multifaceted antibodies of a potentially wide variety of origins are lumped together, the only common denominators being that they cause interference and that their source is unknown. Because many of the publications on heterophilic antibodies are case reports, accounts of their origin are often anecdotic. Screening studies have yielded such high prevalence figures of heterophilic antibodies that there is reason to suspect that they are more or less ubiquitous in the human population, and it is not unthinkable that the same applies to other species. If that is the case, future research should focus on investigating specific sources and characteristics of these antibodies. Besides the well-known rheumatoid factors, there are other possible links between interfering antibodies and disease mechanisms. These might extend beyond the classic examples of autoimmune diseases. For example, cancers with a high mutational burden are immunogenic and could induce the formation of broadly reactive antibodies to neo-epitopes on tumour cell surfaces. These could interfere with immunoassays through a mechanism analogous to antibodies directed against surface epitopes on *E. coli* bacteria (Covinsky *et al.*, 2000). In this thesis, we investigated a potential connection between immunoassay interference, anti-F(ab')₂-autoantibodies and cancer. Although there was ultimately no evidence for a connection, it is a field of investigation that deserves further exploration. Investigating connections between severe disease and interfering antibodies makes double sense because if there is a link between a particular disease and interference, the risk for interference increases not only due to the link itself but also because more tests are presumably run on the patient for diagnostic and post-diagnostic management.

If there is a connection between heterophilic antibodies and disease, there might also be opportunities to use the antibodies as prognostic or diagnostic markers for disease. Rheumatoid factor detection in rheumatoid arthritis is not the only scenario when an interfering antibody might have diagnostic value. There is also a medical condition called cryoglobulinemia, which is associated with several causes, including hepatitis C viral infection in people. Cryoglobulins are immune complexes consisting of rheumatoid factors and polyclonal IgG that associate with the core protein of the hepatitis C virus.

Testing for cryoglobulins could be valuable for diagnosing hepatitis C, although it is rarely performed in practice (Shihabi, 2006). Hepatitis C infection is not known to occur in animals, but cryoglobulinemia is sporadically observed in dogs and cats with multiple myeloma (Giraudel *et al.*, 2002; Hickford *et al.*, 2000).

It is important to recognize that *in vitro* conditions are different from *in vivo* conditions and that knowledge about interactions taking place within organisms is not necessarily applicable to interactions in a microtiter well. Immunoassays require immobilization of antibodies or antigen on a solid phase, which is a complex reaction involving hydrophobic and electrostatic interactions. In this process, capture antibodies can be denatured and randomly oriented (Butler *et al.*, 1993), predetermining which epitopes become available for binding. When coating a microtiter well, the solid phase is overflowed with molecules, sometimes forming layers of antibodies held together by weak electrostatic interactions. It has been suggested that these stochastic events can form repetitive epitopes on the solid phase that low-affinity endogenous antibodies can initiate interactions with, even though these interactions do not happen *in vivo* (Bjerner *et al.*, 2005). Similarly, human IgG4 can bind to the Fc region of immobilized antibodies with its own Fc region (Rispen *et al.*, 2009), an interaction presumed to be exclusive to *in vitro* conditions. However, these fundamentals of immunoassay analysis remain poorly understood. It has been stated that in the current era of immunoassays, “the technological cart is before the scientific horse” (Qian *et al.*, 2000). In fact, that was the case from the beginning; as a technology utilizing animal antibodies to measure proteins and peptides, immunoassays received widespread use over a decade before it was understood how nature had been making these antibodies for millions of years to fight parasites, bacteria, viruses and other pathogens (Tonegawa *et al.*, 1974). Today, immunoassays are routinely used to measure non-immunogenic small molecules like steroid hormones, requiring laborious conjugation and modification of steroid structures. Waves of different antibody types, labels and engineering techniques have come and gone, but limitations in assay performances remain. Laboratories and clinics are using immunoassays to diagnose diseases in species for which the assay was not designed nor validated. Critics are claiming that immunoassays have been pushed past their limits and that it is time for diagnostic laboratories to phase out immunoassays, starting with steroid hormone assays (Taylor *et al.*, 2015), and move on to mass spectrometry. However, this transition would not be without complications (Auchus, 2014), and it seems likely that we will continue to use immunoassays in clinical diagnostics for the foreseeable future. Meanwhile, the search for clinically useful biomarkers is going deeper into the lower end of the plasma

proteome, requiring increasingly sensitive methods (Landegren *et al.*, 2012) for the detection of low-concentration molecules. In this process, it is crucial to keep in mind the limitations of the methods used to detect candidate biomarkers, whether immunoassays or otherwise, and to also investigate the interference issues associated with highly sensitive detection methods.

7 Conclusions

- Heterophilic antibodies reactive with mouse IgG are detectable using a species-independent assay in 9% of dogs and 5% of cats admitted to an animal hospital.
- Canine anti-mouse antibodies are a source of erroneous results in immunoassay analysis of anti-Müllerian hormone (AMH).
- Antibody precipitation with PEG is not a useful tool for detecting antibody interference in hormone immunoassays for canine samples.
- Canine anti-mouse antibodies can be of the IgA, IgG and IgM isotype, are capable of cross-reacting with IgG from several species and target both Fc- and F(ab')₂-fragments of mouse IgG.
- There are individual differences in the properties of canine anti-mouse antibodies, and the prevalence differs between dog breeds.
- Canine heterophilic antibodies against mouse IgG can persist in serum for at least two years.
- Anti-Fab and anti-F(ab')₂-autoantibodies reactive with cryptic epitopes in the hinge region of IgG are commonly found in dogs and can sporadically cross-react with mouse IgG.

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Popular science summary

Immunoassays play a crucial role in modern medicine. The technology uses target-seeking animal antibodies to detect and measure specific molecules in biological samples. For more than 60 years, this method has been used to guide the diagnosis of patients with disease. The reliance on animal antibodies comes with a drawback: they can themselves become targets for anti-animal antibodies, often called heterophilic antibodies, causing false test results. In the worst case, this leads to the patient receiving the wrong diagnosis and medical treatment.

Very little is known about this problem in veterinary medicine. In this thesis, we aimed to add some basic information about heterophilic antibodies in animals, with the main focus on dogs. The overarching aims were to investigate their prevalence, their impact on diagnostic tests, some of their molecular characteristics and their potential origins. We conceived a method for detecting these antibodies and estimated a prevalence of 9% in dog patients and 5% in cat patients. It was found that the detected antibodies can affect measurements of anti-Müllerian hormone (AMH), which could subject patients to, for example, needless surgery. There was a broad diversity in the characteristics of the antibodies, a major obstacle for eliminating their effects on diagnostic tests. The origins of the antibodies remain unknown, but they can likely be acquired from a variety of sources, and they appear to be more frequent in certain dog breeds, including the Bernese mountain dog. We found that the antibodies can persist for at least two years in dogs, which is a risk factor for repeated analytical errors and misdiagnosis in dogs with heterophilic antibodies.

Populärvetenskaplig sammanfattning

Immunanalyser spelar en nyckelroll inom dagens sjukvård. Tekniken använder sig av målsökande djurantikroppar för att detektera och mäta specifika molekyler i patientprover. I mer än 60 år har dessa metoder använts för att diagnostisera sjukdomar. Trots detta är metoderna inte felfria. Bland annat så kan de djurantikroppar som tekniken använder sig av bli måltavlor för cirkulerande anti-djurantikroppar hos patienter, även kallade heterofila antikroppar, vilket kan orsaka felaktiga provsvar. I värsta fall blir följden att patienten får fel diagnos och fel medicinsk behandling.

Inom veterinärmedicin är kunskapen om detta problem mycket begränsad. Syftet med den här avhandlingen var att undersöka grundläggande egenskaper hos heterofila antikroppar hos djur, med huvudfokus på hund. De övergripande målsättningarna var att undersöka deras förekomst, deras påverkan på diagnostiska test, några av deras molekylära egenskaper och deras tänkbara ursprung. Vi utvecklade en metod för att detektera dessa antikroppar och uppskattade förekomsten till 9 % hos hundpatienter och 5 % hos kattpatienter. Antikropparna visade sig ha förmåga att påverka mätningar av anti-müllerskt hormon (AMH), vilket kan få till följd att patienter utsätts för kirurgiska ingrepp i onödan. Det fanns en bred variation av molekylära egenskaper hos antikropparna, vilket försvårar arbetet med att förhindra deras påverkan på diagnostiska test. Antikropparnas ursprung är alltså okänt, men sannolikt finns det flera olika källor till deras uppkomst och det finns en variation i deras förekomst mellan olika hundraser. Vi visade att antikropparna kan kvarstå åtminstone två år i cirkulationen hos hundar, vilket är en riskfaktor för upprepade felaktiga testresultat och diagnoser hos hundar som har dessa antikroppar.

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As I am typing these words, it is well past midnight, and I am running out of midnight oil to burn. So I will hurry to the finish line and give a joint thank you to all of the non-name-dropped fellow PhD students and colleagues that I have met at courses, conferences, in the corridors or at start meetings with Ylva. You are simply too many to mention, but I hope to be able to celebrate with you when the COVID pandemic is over!

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Heterophilic antibodies interfere with immunoassays and cause analytical errors. The results presented herein elaborate on the prevalence, clinical impact, molecular characteristics and potential origin of heterophilic antibodies in dogs. It is demonstrated that canine heterophilic antibodies can cause incorrect measurements of anti-Müllerian hormone, with the following risk for misdiagnosis in patients. The antibodies are heterogeneous in characteristics and persistent for at least two years in circulation, making them difficult for laboratories, clinicians and test manufacturers to navigate around.

Daniel Bergman received his postgraduate education at the Department of Clinical Sciences. His veterinary degree was obtained at the Swedish University of Agricultural Sciences (SLU).

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