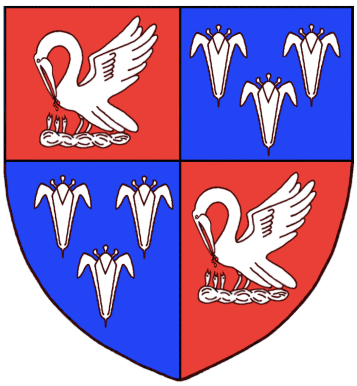
UNIVERSITY OF CAMBRIDGE

A study of a multi-systemic immune mediated disease in the English Cocker Spaniel

María Francisca Coddou



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Summary

Chronic pancreatitis (CP) is a common disease in the English Cocker Spaniel (ECS) and shows a distinctive clinical and histopathological appearance. It is characterised by duct destruction, interlobular fibrosis, dense periductular and perivenous lymphocytic aggregates, the presence of multi-organ manifestation, and increased numbers of IgG4+ plasma cells in the pancreas, and other affected organs. These features are also seen in human autoimmune pancreatitis (AIP) type 1, which is part of a multi-organ syndrome, recently recognised as IgG4-Related Disease (IgG4-RD). Human IgG4-RD may affect one or several organs, pancreatitis being the most common manifestation. It is often seen that serum IgG4 levels and $IgG4^+$ plasma cell numbers are increased in the affected tissues.

The aims of this PhD were to further investigate CP in ECS dogs with a focus on its immunology and genetics to increase our understanding of its diagnosis and pathogenesis and its similarities and differences from human IgG4-RD. Thus, 104 ECS affected by CP and 44 age-matched ECS controls were recruited for this study. Clinical features of affected dogs were characterised and clinical similarities between CP in ECS and human IgG4-RD, including a high prevalence of multi-organ manifestation were seen. Affected ECS presented with a high prevalence of keratoconjunctivitis sicca, proteinuria, anal gland disease, suspected xerostomia, atopy and other immune-mediated diseases. A strong association between CP and ECS presenting blue roan coat colour was seen, suggesting a link between coat colour and immune-mediated conditions in this breed. Moreover, the development of an enzyme-linked immunosorbent assay (ELISA) capable of quantifying serum IgG4 was attempted without success. Therefore, serum IgG4 concentrations were assessed using a commercial ELISA kit. It was demonstrated that affected ECS had significantly higher IgG4 serum concentrations compared with controls. Cases presenting with clinical inflammatory manifestations in more than one organ had significantly higher concentrations of serum IgG4 than those with only pancreatic manifestation. Total serum IgG, IgE concentrations and IgG4/IgG ratios were also measured in cases and controls. No significant difference was observed regarding concentrations of total IgG and IgE in serum**.** Serum IgG4/IgG ratio was significantly higher in cases than controls. Finally, a genetic study trying to identify risk factors in affected dogs was performed. Potential genetic association previously found in a pilot study between CP in the ECS and the SNP rs852916308 located on chromosome 16, which overlies a region containing a group of T-cell receptor beta variable loci and the anionic trypsinogen gene, was rejected. The SNP rs852916308 was not polymorphic in ECS. Results found on the SNP rs22196038 located on chromosome 12, which is downstream of the branched-chain alpha-keto acid dehydrogenase BCKDHB gene, demonstrated some interesting potential genotype associations that need to be confirmed including a greater number of healthy ECS.

A greater understanding of the manifestation of the disease in ECS was obtained. Further studies are required to assess the efficacy of immunosuppressive therapy, and identify the pathogenesis and genetic basis of the disease. This will contribute to improve diagnosis and treatment resulting in the improvement of the quality of life in affected dogs.

Preface

The study work described in this dissertation was performed at the Department of Veterinary Medicine, University of Cambridge, between February 2016 and July 2020 under the supervision of Dr Penny Watson and Dr Barbara Blacklaws.

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically indicated in the text.

The dissertation does not exceed the word limit (39,144 words) for the respective Degree Committee.

The contents have not previously been submitted for any degree or any other qualification at the University of Cambridge or any other institution.

María Francisca Coddou

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Firstly, I would like to express my sincere gratitude to my supervisor Dr Penny Watson and my co-supervisor Dr Barbara Blacklaws for the continuous support of my PhD study and related research, for their patience, motivation, and immense knowledge. Their guidance helped me in all the time of research and writing of this thesis.

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Last but not least, I would like to thank my husband Sergio, my daughter Martina, and my son Clemente for their love and support.

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Abbreviations

AIP	Autoimmune Pancreatitis
AS	Anal Sacculitis
ASAC	Anal Sac Adenocarcinoma
CP	Chronic Pancreatitis
cPLI	Canine pancreatic lipase immunoreactivity
CSS	Chronic Sclerosing Sialadenitis
CT	Computed Tomography
DCM	Dilated cardiomyopathy
DGGR	1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester
DLA	Dog Leukocyte Antigen
DM	Diabetes Mellitus
ECS	English Cocker Spaniel
EHBO	Extra Hepatic Biliary Tract Obstruction
ELISA	Enzyme-linked immunosorbent assay
EPI	Exocrine Pancreatic Insufficiency
FN	Neutered Female
GN	Glomerulonephritis
GWAS	Genome-Wide Association Study
H&E	Haematoxylin and eosin
HPF	High Power Field
HRP	Horseradish Peroxidase
IBD	Inflammatory Bowel Disease
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgG4-RD	IgG4-Related Disease
IgG4-RKD	IgG4-Related Kidney Disease
IgG+	Immunoglobulin G positive
IHC	Immunohistochemistry
IL	interleukin
IMHA	Immune-Mediated Haemolytic Anaemia
IMPA	Immune-Mediated Polyarthritis
IMTP	Immune-Mediated Thrombocytopenia

IVDD	Intervertebral Disc Disease
KCS	Keratoconjunctivitis sicca
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
mAb	Monoclonal Antibody
MN	Neutered Male
MVD	Mitral Valve Disease
NR	Not Recorded
OR	Odds Ratio
PBS	Phosphate-Buffered Saline
PLE	Protein-Losing Enteropathy
RBC	Red blood cell
RTX	Rituximab
SD	Standard deviation
SE	Standard error
SNP	Single nucleotide polymorphism
STT	Schirmer Tear Test
TBST	Tris-buffered saline, 0.1% Tween 20
TCC	Transitional Cell Carcinoma
UPC	Urine Protein:Creatinine
USMI	Urethral Sphincter Mechanism Incompetence

CHAPTER 1: GENERAL INTRODUCTION

1.1. Motivation

English cocker spaniels (ECS) are predisposed to immune-mediated diseases with a significant negative effect on their welfare. Chronic pancreatitis (CP) is a common disease in ECS and it has distinctive clinical and histological appearances that differ from CP in other breeds. Histologically, it is characterised by duct destruction, interlobular fibrosis, dense periductular and perivenous lymphocytic aggregates, and increased numbers of $IgG4^+$ plasma cells. Clinically affected ECSs commonly present with associated concurrent autoimmune diseases, keratoconjunctivitis sicca (KCS) and glomerulonephritis (GN). These features are also found in human autoimmune pancreatitis (AIP) type 1, which is part of a steroid-responsive, multi-organ syndrome, newly recognised as IgG4-Related Disease (IgG4-RD). Human IgG4-RD affects one or several organs, often showing a predominance of $IgG4^+$ plasma cells histologically, with an $IgG4^+$/total IgG^+ plasma cell ratio > 40 %.

The aims of this PhD were to investigate CP in ECS dogs with a focus on its immunology and genetics to increase our understanding of its diagnosis and pathogenesis and its similarities and differences from human IgG4-related disease.

1.2. Thesis Outline

Chapter 1, *Introduction*, examines the field of immune-mediated diseases in English cocker spaniels and human IgG4 related-disease, providing an overview of previously published work.

Chapter 2, *Case and control recruitment*, describes the process of finding English cocker spaniels affected by chronic pancreatitis and the clinical definition of cases and controls and characterises the clinical features of CP in this particular breed.

Chapter 3, *Development of a canine serum IgG4 test*, outlines the materials produced and the techniques employed in order to develop an ELISA test for the quantitative determination of canine IgG4 in serum.

Chapter 4, Concentration of total serum IgG, IgG4 and IgE in English cocker spaniels affected by chronic pancreatitis*,* describes the assessment of serum Ig concentrations and IgG4/IgG ratios in affected and controls ECSs and determines its correlation with disease.

Chapter 5*, Genotypic evaluation of English cocker spaniels affected by chronic pancreatitis,* presents a fine map study of the genetic loci already identified as potentially linked to CP in a previous preliminary genome-wide association study (GWAS)

Chapter 6*, Conclusions and future work,* presents the main findings of this work and outlines future suggestions for experimental work.

1.3. Literature Review

1.3.1. Immune-mediated diseases in English Cocker Spaniels

English cocker spaniel (ECS) dogs are greatly predisposed to immune-mediated diseases with a significant negative effect on their welfare (Day, 1996). Haematological conditions, such as immune-mediated haemolytic anaemia (IMHA) and immune-mediated thrombocytopenia (IMTP), are the most common autoimmune diseases recognised in ECS (Carr *et al*., 2002; Miller *et al*., 2004; Kennedy *et al*., 2006). IMHA and IMTP are conditions predominantly mediated by a type II hypersensitivity reaction in which red blood cells or platelets are destroyed as a consequence of breakdown of immune self-tolerance and the production of anti-erythrocyte or anti-platelet antibodies, respectively. Sixty to 70 % of IMHA and IMTP cases in dogs are considered idiopathic. In other words, no underlying cause is found in these cases (Jackson and Kruth, 1985; Piek *et al*., 2008). Clinical signs of IMHA may include lethargy, inappetence, pale mucous membranes, exercise intolerance, tachycardia, tachypnoea, and fever (Reimer *et al*., 1999). Hypercoagulability is a characteristic feature of canine IMHA that is believed to increase the risk of thrombosis, being an important cause of morbidity and mortality (Hamzianpour and Chan, 2016). The diagnosis of IMHA is based on the identification of antibodies targeting RBC through a positive direct Coombs' test and presence of anaemia with signs of haemolysis (Carr *et al*., 2002). IMTP is associated with spontaneous bleeding that can occur in any organ or tissue, even when no obvious trauma has occurred. This can be manifested as petechial haemorrhages, haematuria, haematemesis, melaena or haematochezia. The diagnosis of IMTP is made based on a combination of bone marrow examination and exclusion of other known causes of thrombocytopenia or underlying diseases (Putsche and Kohn, 2008). Anti-platelet antibody testing has been used with some success to confirm the diagnosis but the assay is not commercially available (Kristensen *et a*l., 1994; Bachman *et a*l., 2015). Corticosteroids are the current treatment of choice for both disorders, along with a variety of other immunosuppressive agents such as ciclosporin and azathioprine. The spleen is responsible for removing damaged platelets and red blood cells and has an integral role in antigen presentation and autoantibody production (Weledji, 2014). Therefore, on some occasions, when there is not a response to immunosuppressive therapy or there are adverse effects associated with drug therapy, splenectomy and blood transfusions are performed in order to keep more platelets and/or red blood cells circulating in the body. Although these

treatments have been beneficial for many patients, it is not the case for many others (Jackson and Kruth, 1985).

Other immune-mediated diseases identified in ECS dogs are lymphocytic thyroiditis (Kennedy *et al*., 2002), systemic lupus erythematous (Day, 1996; Foster *et al*., 2000), non-erosive polyarthritis, and keratoconjunctivitis sicca (KCS) (Kaswan *et al*., 1983). Recently it has been shown that chronic pancreatitis is a common disease in ECS and the clinical and histological appearance suggests it is also immune-mediated (Watson *et al*., 2011).

1.3.2. Canine Chronic Pancreatitis

Chronic pancreatitis (CP) is defined as a continuing inflammatory disease of the pancreas, distinguished by irreversible morphological changes that commonly cause permanent loss of function, usually leading to irreversible impairment of pancreatic exocrine and endocrine function (Etemad and Whitcomb, 2001). Recent pathological and clinical studies have confirmed that CP is a common and clinically significant disease in dogs (Bostrom *et al*., 2013; Newman *et al*., 2004; Watson, 2012; Watson *et al*., 2011, 2007). However, clinically, the condition is diagnosed much less frequently than it is observed in pathological studies, which might imply that many chronic pancreatitis cases are missed or remain subclinical (Xenoulis *et al*., 2008). This supports the suggestion that the disease is under-diagnosed because of the difficulty of obtaining a non-invasive diagnosis.

There is a lack of information concerning the aetiology of pancreatitis in dogs. The majority of cases are considered idiopathic because the initiating factor cannot be determined. There is also uncertainty whether CP results from recurrent attacks of acute pancreatitis or whether it is a different disease (Xenoulis *et al*., 2008). The relationship between recurrent acute and chronic disease is likely to be complex in dogs as it is in humans and dependent on interaction of genes and environment (Watson, 2015).

Clinical signs are non-specific and usually transient. These signs can include anorexia, weakness, vomiting, diarrhoea, and abdominal pain. Particularly, the combination of vomiting and cranial abdominal pain is considered suggestive of the disease. Severe systemic complications, such as cardiovascular shock, disseminated intravascular coagulation or multi-organ failure, are not commonly found in the chronic form of the disease. Other

clinical signs may be consequences of concurrent diseases, for instance, polyuria and polydipsia in animals with diabetes mellitus or diarrhoea, weight loss and polyphagia in animals with exocrine pancreatic insufficiency. In most cases, CP is considered to be mild, and many remain subclinical (Xenoulis *et al*., 2008). It has been reported that the presence of clinical signs in dogs with CP is associated with a histopathologically more active disease determined by the findings of pancreatic and peri-pancreatic fat necrosis. Thus, CP cases that are not associated with these findings are less likely to develop clinical signs, escaping clinical diagnosis more easily (Bostrom *et al*., 2013). However, when animals reach end stage disease with a large loss of tissue mass, they may develop signs of exocrine and endocrine insufficiency (EPI and DM respectively) (Watson, 2003; Watson *et al*., 2010).

The diagnosis of chronic pancreatitis in dogs is challenging. Non-invasive diagnosis relies on ultrasound imaging and pancreatic-specific blood tests. Serum Spec canine pancreatic lipase immunoreactivity (cPLI) concentrations are currently considered the most useful serum markers for the diagnosis of canine pancreatitis but they have some limitations (Xenoulis and Steiner, 2012). Pancreatic-specific serum enzyme tests have a lower sensitivity compared to the acute disease, probably because of the loss of acinar mass in the chronic disease (Watson, 2012). It has been shown that dogs with pancreatic acinar atrophy have a low serum cPLI (Steiner et al., 2006). This suggests that dogs with advanced CP and marked loss of acinar tissue will not show elevations in cPLI. In order to establish a definitive diagnosis, it is necessary to obtain a pancreatic biopsy. However, this is not recommended in the majority of the cases until there is an effective treatment; biopsies are an invasive procedure and do not currently change treatment decisions (Watson, 2015). Although histopathological examination is the gold standard for the diagnosis, it also has some limitations. Pancreatic inflammation tends to be localised in discrete areas *i.e.* focal lesions within the pancreas instead of being spread throughout the whole organ. A single normal biopsy is insufficient to exclude pancreatitis because it can omit the affected area causing under recognition of the disease. In addition, there are no preferred sites for pancreatic biopsy collection unless gross lesions are apparent (Newman *et al*., 2004).

Chronic pancreatitis typically affects middle-aged to old dogs. In Britain, studies suggest that breeds such as Cavalier King Charles Spaniel, English Cocker Spaniel, Collie and Boxer have a particularly high prevalence of CP (Watson *et al*., 2010, 2007). Other studies from the US suggest that non-sporting and toy breeds, such as Yorkshire Terriers also have a high risk of

CP (Bostrom *et al*., 2013). Furthermore, pancreatitis in different breeds has been shown to have different histological appearances raising the possibility of breed-related differences in aetiology (Watson *et al*., 2007). An example of this is the case of the English Cocker Spaniel (Watson *et al*., 2011).

1.3.3. Chronic Pancreatitis In English Cocker Spaniels

CP is a common disease in ECS and an underlying genetic predisposition is suspected given the frequent occurrence of CP in this breed. A recent genetic study showed an increased frequency of DLA-DQB1*00701, a particular dog leukocyte antigen (DLA) haplotype, in ECS dogs with CP and a lower frequency of the DLA-DQB1*02001 haplotype, compared to controls (Bazelle *et al*., 2013).

Moreover, it has been shown that ECS dogs with CP present a clinically and histologically different pattern of the disease compared with other breeds (Watson *et al*., 2011). Clinically, CP in ECSs is often associated with an enlarged, hypoechoic pancreas on ultrasound examination, and in some cases, pancreatic mass-like lesions can be found. Histologically, pancreatic sections of ECS with CP are characterised by duct destruction, interlobular and periductular fibrosis and inflammation with dense lymphocytic infiltrates around venules and ducts, predominantly mediated by $CD3^+$ T cells. On the contrary, in the majority of other dog breeds, CP is a non-duct destructive disease, mediated by a mix of T and B cells, only presenting with ductular hyperplasia in the end-stages of disease (Watson *et al*., 2011). A recent study also revealed that sections of pancreas from ECS affected by CP present frequently with an increased number of infiltrating $IgG4^+$ plasma cells and an $IgG4^+$/total IgG^+ plasma cell ratio higher that 40 % (Figure 1.1). This was observed also in several affected organs such as kidney, liver, salivary and lachrymal glands and anal sacs from ECS with chronic inflammatory disease (Coddou, 2015 MPhil thesis; Coddou *et al*., 2020).

These distinctive features of CP in ECS dogs show similarities with human autoimmune pancreatitis (AIP) type 1, which is part of a fibro-inflammatory, multi-systemic syndrome, currently known as IgG4-Related Disease (IgG4-RD) (Stone *et al*., 2012). Patients with IgG4-RD frequently show an increased serum level of immunoglobulin G4 (IgG4) and a dense infiltration of $IgG4^+$ plasma cells in affected tissues (Culver and Bateman, 2013).

Similarly, as human IgG4-RD, the disease in ECS also shows a multi-organ nature and seems to affect kidneys, tear glands, salivary glands (Coddou *et al*., 2020) and anal sacs (Coddou, 2015). In humans, the biliary tract is also involved but convincing IgG4-associated cholangitis has not been demonstrated in ECS yet.

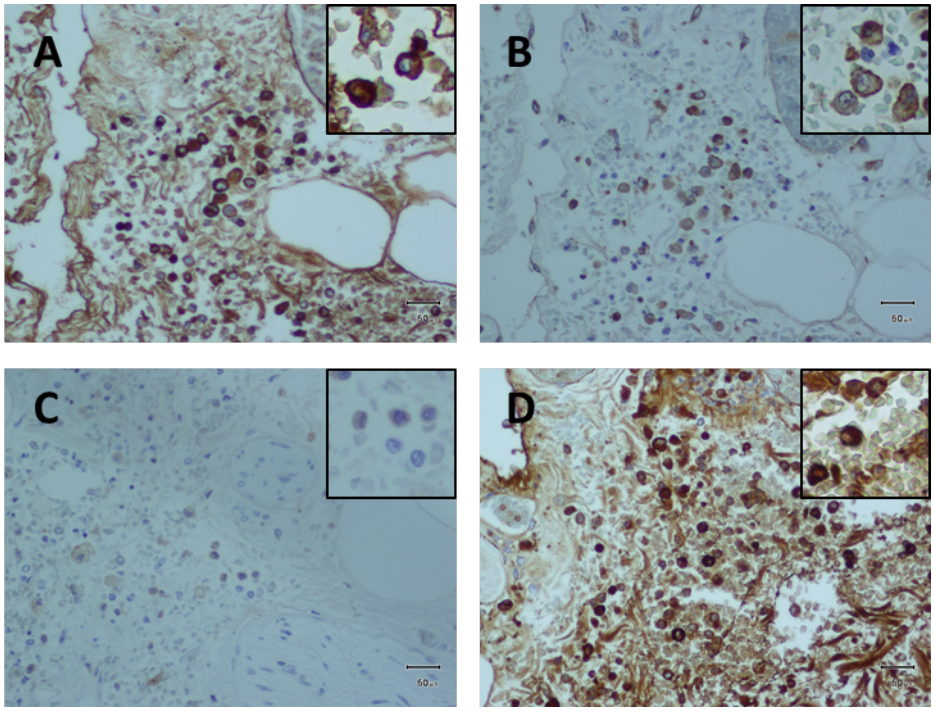

Figure 1.1: Immunohistochemistry of pancreas sections from a 6-year-old female ECS with chronic pancreatitis. Histological sections were immunostained for: (A) Total IgG^+ plasma cells, (B) $IgG2^+$ plasma cells (C) $IgG3^+$ plasma cells, and (D) $IgG4^+$ plasma cells. Note the greater infiltration of $IgG4^+$ plasma cells compared with other IgG subclasses. Bar = 50 µm. Original magnification x200. Inset: magnification x400 (Adapted from Coddou *et al*., 2020).

1.3.4. IgG Subclasses in Dogs and Humans

Immunoglobulins (Ig) or antibodies are the rearranged B cell receptor, which is secreted by B cells (plasma cells) as their major effector molecule to help eliminate foreign molecules or pathogens. There are several subclasses or isotypes of immunoglobulin, denoted by different alphabetic names (M, G, A, E, D), which have different effector properties (for example in their interaction with cellular receptors or complement components). The most abundant immunoglobulin in canine and human serum from healthy individuals is IgG. This is a protein complex composed of four peptide chains, consisting of two identical light and two identical heavy chains (Mian *et al*., 1991). It is the heavy chain that encodes the

different isotype grouping in the Fc domains. There are several different variants of IgG in mammalian species. In humans there are four subclasses IgG1, IgG2, IgG3 and IgG4 defined by their serum concentrations in normal individuals and their biochemical properties, such as electrophoretic mobility (Schur, 1987).

Four IgG subclasses have been also described in dogs (Mazza *et al*., 1993; Bergeron *et al*., 2014). These IgG subclasses have been labelled IgG1, IgG2, IgG3 and IgG4, according to a comparison with the relative concentration and electrophoretic mobility of human IgG subclasses (Mazza *et al*., 1993). The relative concentration of the IgG subclasses in normal human serum is IgG1 > IgG2 > IgG3 > IgG4, with IgG1 being the most abundant and IgG4 the least abundant (Schur, 1987). This is similar in dogs for IgG1 and IgG2. However, IgG4 appears to be more abundant than IgG3 although both IgG4 and IgG3 have a much lower concentration than IgG1 and IgG2 (Mazza *et al*., 1993, 1994a). In healthy dogs, serum concentrations of IgG subclasses do not show differences between different breeds (Mazza *et al*., 1994a). More recent studies have identified the four canine IgG subclasses γ chain RNAs, labelled them as IgG-A, IgG-B, IgG-C, IgG-D, and demonstrated a modest sequence homology and receptor binding characteristics with the four human IgG subclasses *(*Tang *et al*., 2001; Bergeron *et al*., 2014).

It is well known that in humans the different IgG subclasses are functionally different; for example, IgG3 is most efficient at complement fixation via the classical pathway while IgG4 does not activate this pathway effectively and has been traditionally considered to play only a limited role in the activation of the immune system (Day and Mazza, 1995; Stone *et al*., 2012). Due to the absence of disulphide bonds between the heavy chains, IgG4 has the unique characteristic of exchanging half-molecules with other molecules of IgG4 ("Fab-arm exchange"), which results in bispecific antibodies with two different antigen-binding sites (Fig. 1.2). Therefore, they cannot cross-link antigen to form immune complexes, contributing to its anti-inflammatory activity (van der Neut Kolfschoten *et al*., 2007).

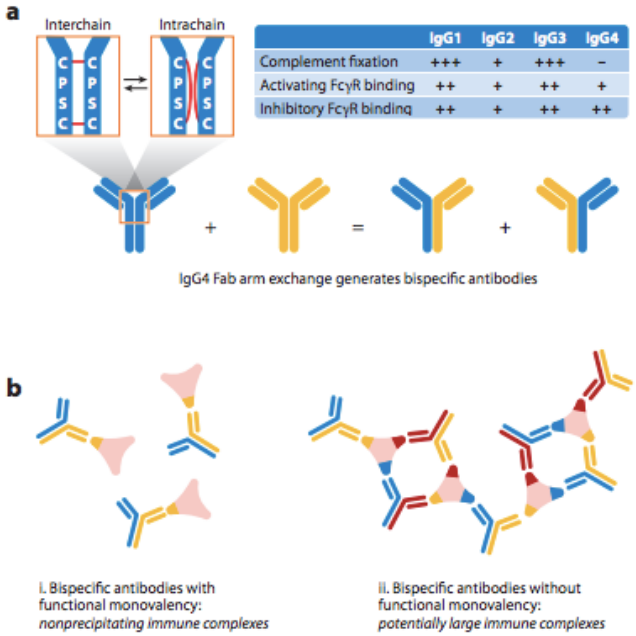

Figure 1.2: Distinct features of IgG4 antibodies. (a) Different distinct features have made IgG4 a non-inflammatory antibody: 1) it cannot fix complement; 2) it has poor binding to activating FcγRI; 3) it has the capacity to engage the inhibitory FcγR (FcγRIIB); and 4) presents Fab arm exchange. IgG4's heavy chains are able to switch from inter to intrachain disulphide bonded configurations and vice versa. Intrachain disulphide bonded IgG4 is composed of non-covalently associated hemi-IgG4 molecules, which can be exchanged *in vitro*, under reducing conditions, or *in vivo*, potentially aided by FcRn recycling. This process, named Fab arm exchange, can produce bispecific IgG4 antibodies. (b) Theoretical immune complexes that can be formed by bispecific IgG4 antibodies. The nature of the immune complexes formed *in vivo* in IgG4-related disease is unknown. There are two possibilities: (i) Fab arm exchange of antigenspecific IgG4 with antibodies of irrelevant specificity may result in functionally monovalent antibodies. Such antibodies would give rise to small, nonprecipitating immune complexes. (ii) Fab arm exchange between IgG4 antibodies that react with different epitopes on the same antigen would result in functionally bivalent antibodies, and may result in the formation of large immune complexes. Such immune complexes may be inefficiently cleared due to lack of complement binding and may account for the IgG4 deposits seen in some cases of IgG4-related disease (Mahajan *et al*., 2014) .

Recently, it was found that IgG subclasses in dogs share some functional properties with the four human IgG subclasses (Bergeron *et al*., 2014). IgG subclass expression in specific immune responses relies on the nature of the stimulating antigen and the local tissue microenvironment, in which certain immunoregulatory cytokines may be expressed selectively and promote the differential class switching of heavy chain genes; for example, switching to IgG4 is controlled primarily by type II helper T cells (Th2) that express interleukin (IL-) 4 and IL-13 (Brière *et al*., 1994).

In humans, IgG subclass screening kits are commercially available for the specific and quantitative measurement of different subclasses in serum. In dogs, monoclonal antibodies

which can selectively identify each IgG subclass in serological assays have been also developed (Mazza *et al*., 1993, 1994b). Using these antibodies, it has been possible to characterise serum concentrations of the canine IgG subclasses in healthy and affected dogs with a variety of clinical diseases (Mazza *et al*., 1994a). Using these monoclonal antibodies (mAbs) in immunohistochemical studies, different IgG subclass-positive plasma cell distributions have also been described in the skin of normal dogs and dogs affected with immune-mediated dermatopathy lesions (Day and Mazza, 1995) and in histiocytic ulcerative colitis (HUC) lesions in Boxer dogs (German *et al*., 2000). These antibodies were also used by the author in immunohistochemical studies of pancreas and other organs in ECS and other breeds (Coddou, *et al*., 2020).

These and other studies provide evidence that particular IgG subclasses show increased levels in certain inflammatory and immune-mediated diseases in humans and dogs. Of most relevance to this thesis, IgG4 levels (representing 3 % to 6 % of total IgG in healthy adult serum) increase in patients with IgG4-RD. Human patients with IgG4-RD also frequently present with an increased number of $IgG4^+$ plasma cells in affected tissues. (Stone *et al*., 2012).

1.3.5. IgG4-Related Disease

IgG4-related disease (IgG4-RD) is a recently recognised human steroid-responsive systemic fibro-inflammatory condition. It is clinically characterised by a tendency to form tumefactive lesions at multiple sites. It derives its name from the fact that most patients present with increased numbers of $IgG4^+$ plasma cells in affected tissues and elevated levels of IgG4 in serum.

IgG4-RD may affect one or several organs. The most common manifestation of IgG4-RD is within the pancreas, where the disease was initially recognised (Hamano *et al*., 2001). However, most organ systems have been reported to be involved. This has led to the recognition of IgG4-RD as a systemic condition (Kamisawa *et al*., 2003). Until now, this disease has been seen in pancreas, bile duct, liver, gastrointestinal tract, salivary and lacrimal glands, orbit, thyroid, retroperitoneum and mesentery, aorta, lung, kidneys, breast, skin, pituitary gland, meninges, prostate, lymph nodes, and pericardium (Kamisawa *et al*., 2015). IgG4-RD presents distinctive histopathological features, distinguished by dense

lymphoplasmacytic infiltrates rich in IgG4 producing plasma cells with an IgG4+/total IgG+ plasma cells ratio > 40%, often accompanied by storiform fibrosis, obliterative phlebitis, and the variable presence of eosinophils (Culver and Bateman, 2013).

The current gold standard for the diagnosis of human IgG4-RD relies on the identification of these characteristic histopathological features and correlation with the clinicopathological scenario (Deshpande *et al*., 2012). Serological and radiological findings lack adequate sensitivity and are largely non-specific for diagnostic purposes (Della-Torre *et al*., 2015).

The aetiology of this disease remains uncertain, however it is known that autoimmunity plays an important role (Stone *et al*., 2012). The physiopathology remains poorly characterised. However, a Th2-driven immunological mechanism and increased numbers of regulatory T-cells ($CD4^+CD25^+FoxP3^+$ T cells) have been proposed in its pathogenesis. Cytokine production such as interleukin IL-4, IL-5, IL-10, IL-13 and transforming growth factor (TGF)-beta causes skewing of B-cells to production of IgG4 and fibrosis. This is most probably a result of an unknown antigen triggering the immune system (Fig. 1.3). A recent study suggested there might be aberrant immunological control of the overall IgG4 response in affected humans controlled by multiple antigens, rather than a single antigen-controlled response (Culver *et al*., 2015).

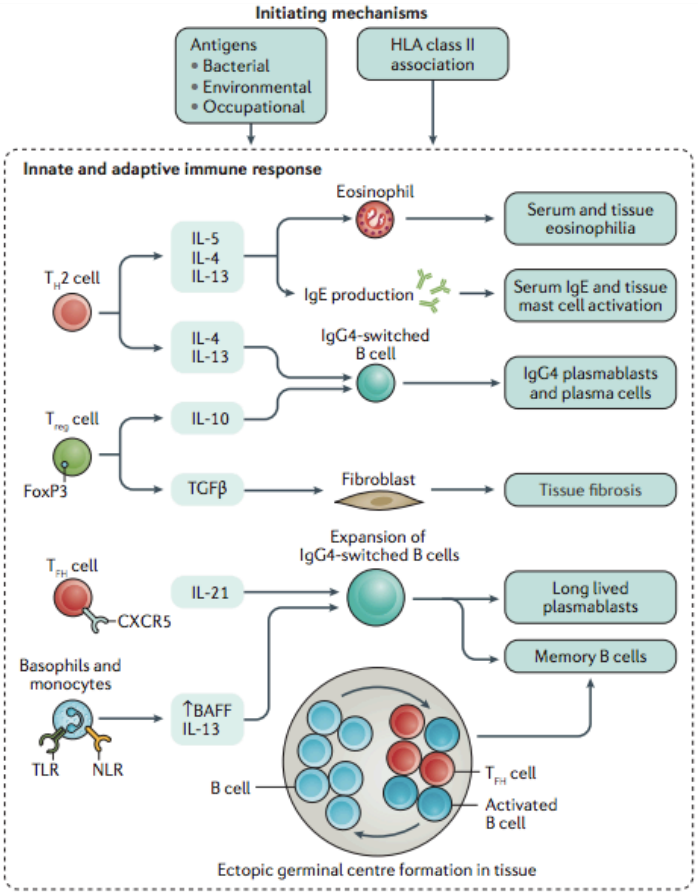

Figure 1.3: Proposed pathogenesis of IgG4-related disease. Chronic exposure to environmental and occupational antigens results in the rapid expansion of pre-existing IgG4-switched B cells in genetically susceptible individuals. These IgG4-positive memory B cells and plasmablasts traffic through the blood to lymphoid organs. The presence of T helper 2 (T_H2)-derived cytokines (IL-4 and IL-13) and T-regulatory cell (T_{reg})-derived cytokines (IL-10 and transforming growth factor (TGF) β, promote a switch to the IgG4 subclass and fibrogenesis. In the presence of mast cell-derived cytokines (IL-4 and IL-5), an elevated serum IgE, eosinophilia and history of allergy or atopy is seen. Ectopic germinal centres in tissues are formed, with T follicular helper (T_{FH}) cells providing cognate help for B cells and the production of IL-21, which drives proliferation of the IgG4-switched cells. Toll-like receptors (TLR) and nucleotide-binding oligomerization domain-like receptors (NLR) on monocytes and basophils enhance these IgG4 responses in the presence of B cell activating factor (BAFF) and IL-13, indicating crosstalk between the innate and acquired immune system. Distinct immunoregulatory functions of the IgG4-switched cells themselves lead to a dampening of immune surveillance, unchecked inflammation and progression to fibrosis (Culver and Chapman, 2016).

1.3.5.1. IgG4 serum levels in IgG4-RD and diagnosis of IgG4-RD

Since human IgG4-RD was recognised as a systemic condition in 2003, many proposals for providing guidelines for the diagnosis have been conducted. The latest guidelines for diagnostic and treatment of this condition were proposed in 2015 at the International Consensus Guidance Statement on the management and treatment of IgG4-RD, which was

composed of 42 IgG4-RD experts (Khosroshahi *et al.*, 2015). According to the consensus, the most accurate assessment of IgG4-RD is based on a full clinical history, physical examination, selected laboratory investigations in particular IgG4 serum levels, appropriate radiology studies, and it is strongly recommended a confirmation by biopsy for the exclusion of malignancies and other IgG4-RD mimics. Figure 1.4 summarises the diagnostic criteria according the International Consensus Guidance Statement on the management and treatment of IgG4-RD.

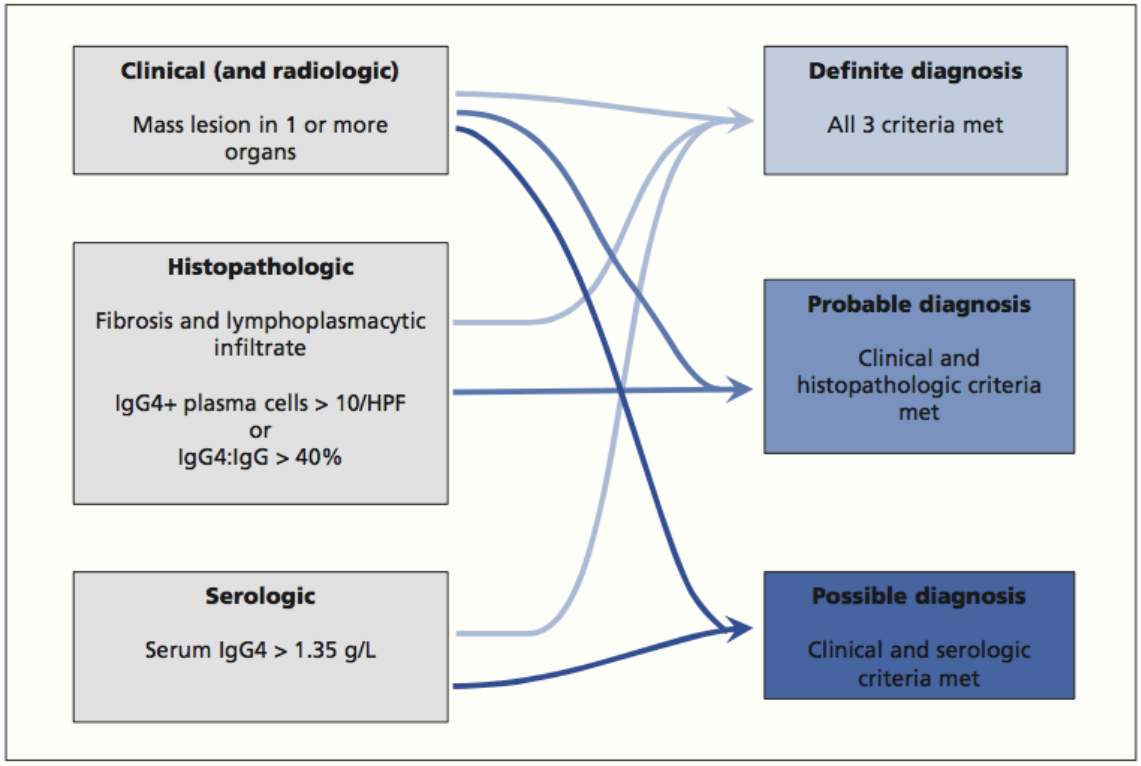

Figure 1.4: Diagnostic criteria for IgG4-related disease according the International Consensus Guidance Statement on the management and treatment of IgG4-RD by Khosroshahi *et al.*, 2015. HPF = high-powered field (Haldar *et al*., 2016)**.**

It has been described that the majority of human patients with IgG4-RD show increased serum IgG4 levels (> 135 mg/dl) (Hamano *et al*., 2001). However, a normal serum IgG4 level does not rule out the disease; almost 20 % of IgG4-RD patients are seronegative for IgG4 (Kamisawa *et al*., 2011). A more recent publication showed that nearly 50 % of a 125-patient cohort with biopsy-proven and clinically active IgG4-RD had normal serum IgG4 concentrations, suggesting potential shortcomings of using serum IgG4 concentrations in the diagnosis of IgG4-RD. The same study identified that higher concentrations of IgG4 in serum are present in a sub-type of patients with more inflammatory and active IgG4-RD. This sub-

type of patients is characterised by a greater likelihood of multiple organ involvement, high levels of inflammation markers, and low complement levels (Wallace *et al*., 2015a). This suggests that the measurement of serum IgG4 in IgG4-RD patients can be used as an indicator of disease activity. A previous study also revealed that patients with multiple organ involvement often have higher serum IgG4 than patients with the disease confined only to a single organ such as pancreas (Kamisawa *et al*., 2005).

Increased serum IgG4 levels are not specific to IgG4-RD. Multiple conditions have been associated with this, such as pancreatic carcinoma, allergic diseases, parasitic infections, and other autoimmune and inflammatory diseases (Ebbo *et al*., 2012). In addition, a recent study showed that elevated levels of IgG subclasses other than IgG4 were common among IgG4-RD patients with active disease, especially those with an elevated serum IgG4 concentrations (Wallace *et al*., 2015a). Therefore, IgG4-RD diagnosis cannot rely exclusively on the measurement of serum IgG4 levels (Su *et al*., 2015). Thus, it is important to recognise that the appropriate diagnosis of human IgG4-RD depends on the combination of clinical features, laboratory studies, and histopathologic findings. Pathologic findings represent the basis for a definitive diagnosis of IgG4-RD, and pathologists should be aware of the clinicopathologic spectrum of this condition, its diagnostic criteria, and the differential diagnosis (Bledsoe *et al*., 2018).

1.3.5.2. Circulating plasmablast and T regulatory cells levels in IgG4-RD

Recent studies using flow cytometry have described that circulating plasmablasts, precursors of plasma cells characterised as $CD19^{low}CD20^{-}CD38^{+}CD27^{+}$, are found at elevated levels in IgG4-RD patients (Mattoo *et al*., 2014; Wallace *et al*., 2015b). These circulating plasmablasts are oligoclonal, exhibit extensive somatic hypermutation (Mattoo *et al*., 2014), and are independent of serum IgG4 concentrations (Wallace *et al*., 2015b). In other words, plasmablast concentrations can be elevated even when IgG4 serum concentrations are found to be normal in patients with active IgG4-RD (Mattoo *et al*., 2014; Wallace *et al*., 2015a, 2015b). This suggests the identification by flow cytometry of elevated numbers of plasmablasts in peripheral blood is a good biomarker for the diagnosis of IgG4-RD and a reliable indicator of disease activity which is more sensitive than serum IgG4 concentrations (Kamisawa *et al*., 2015). Although plasmablast concentrations might improve diagnosis of IgG4-RD, larger studies are still required to validate this test (Della-Torre *et al*., 2015).

Normally, plasmablasts are unusual in the peripheral blood of healthy individuals (Harada *et al*., 1996). However, their level is raised briefly during infection or vaccination responses (Fink, 2012). In contrast, plasmablasts are observed for prolonged periods in the presence of autoimmune diseases or persistent antigen triggers (Harada *et al*., 1996; Wallace *et al*., 2015b). In humans, altered concentrations of plasmablasts in peripheral blood have been described in inflammatory bowel disease, rheumatoid arthritis, systemic lupus erythematosus, and multiple myeloma (Harada *et al*., 1996; Odendahl *et al*., 2000). Patients with other inflammatory conditions can present elevated plasmablast concentrations. However, the increased plasmablast concentrations seen in patients with IgG4-RD are notably greater. Concentrations over 2000 cells/ml are highly specific and have a high positive predictive value for the diagnosis (Wallace *et al*., 2015b). Furthermore, an extreme elevation in the plasmablast count along with a substantial elevation in the serum IgG4 concentration indicates a high probability of multi-organ disease. This should alert the clinician to consider more comprehensive evaluations. Instead, a low plasmablast concentration dismisses an active IgG4-RD diagnosis and may suggest another disease (Wallace *et al*., 2015b).

The activation of regulatory T cells is another immunologic characteristic of IgG4-RD in contrast to classic autoimmune conditions, in which the function of Treg cells is impaired (Stone *et al*., 2012). T regulatory $CD4^+CD25^+FoxP3^+$ cells are significantly enhanced in IgG4-RD when analysed in both affected tissues and peripheral blood. IL-10, which can be produced by Treg cells, may play a role in the promotion of fibrosis in IgG4-RD (Islam *et al*., 2015).

Immunophenotyping using flow cytometry is becoming more widely available in veterinary medicine. This allows the search for abnormal plasmablasts or T cell concentrations in peripheral blood. For instance, using flow cytometry it is possible to immunophenotype aberrant lymphocyte populations in dogs with leukaemia and lymphoma, helping to increase the accuracy of diagnosis (Villiers *et al*., 2006; Comazzi and Gelain, 2011; Kol *et al*., 2013). Identification of canine lymphocytes can be performed using antibodies against CD3 (expressed by all T cells), CD4 (expressed by helper T cells, but also by canine neutrophils), CD5 (expressed by T cells and a subset of B cells), CD8 (expressed by cytotoxic T cells), CD21 (expressed by B cells), and CD79a (expressed by B cells) (Villiers *et al*., 2006).

1.3.5.3. Treatment of human IgG4-RD

The treatment of IgG4-RD relies mainly on corticosteroids. These are effective and show dramatic clinical responses in the majority of cases, at least in the short term. Most clinical manifestations of IgG4-related disease respond well to corticosteroids. A decline in serum IgG4 levels, lessening of the lymphoplasmacytic infiltrate, and a reduction in the number of IgG4-positive plasma cells in affected tissues are usually seen in response to steroid therapy (Guma and Firestein, 2012). Some patients are refractory to glucocorticoid therapy. Treatment refractoriness appears to be more common in patients with multi-organ disease. Relapses can occur in 25 % - 50 % of patients (Khosroshahi *et al*., 2012; Bledsoe *et al*., 2018). High frequency of relapse and also long-term adverse effects with the use of corticosteroids has triggered a search for better treatments. Immunosuppressive agents, such as azathioprine, have been employed in patients with refractory or recurrent disease, but an assessment of their efficacy needs to be analysed thoroughly (Brito-Zerón *et al*., 2016).

Recently it has been demonstrated that IgG4-RD in humans responds to depletion of peripheral CD20 cells and B cells using the monoclonal antibody Rituximab (RTX). It has been shown that after B cell depletion using RTX, $IgG4^+$ plasmablast levels decline dramatically, correlating with disease remission. This supports the hypothesis that B cells play an important role in the pathogenesis of this disease (Wallace *et al*., 2015b). Serum IgG4 concentration also decreased significantly following RTX (Wallace *et al*., 2015b). The treatment with RTX is being evaluated for patients with corticosteroid resistance, glucocorticoid dependency or relapses and appears to be a promising treatment approach (Khosroshahi *et al*., 2012). Rituximab is human-specific, therefore does not bind to canine CD20, probably due to a lack of homology of the RTX epitope in the canine protein (Jubala *et al*., 2005; Impellizeri *et al*., 2006). However, a canine-specific anti-CD20 mAb is undergoing trials for treatment of canine B-cell lymphoma (Rue *et al*., 2015; Mizuno *et al*., 2020).

1.3.5.4. Genetic Background of IgG4-related disease

The genetic factors involved in the aetiology and pathophysiology of human IgG4-related disease or type 1 autoimmune pancreatitis (AIP) remain unclear. Only a few genetic studies on Asian patients have been published and most of these studies have been undertaken with insufficient sized populations and need to be validated in non-Asian countries to

produce robust results. A study on Japanese populations showed increased frequencies of DRB1*0405 and DQB1*0401 human leukocyte antigen (HLA) haplotypes in patients with type 1 AIP (Kawa *et al*., 2002). Other non-HLA genetic factors have also been reported. A study on Japanese people found evidence that the KCNA3 gene, which is involved in immunomodulation of auto-reactive effector and memory T cell–mediated autoimmune diseases, is associated with AIP. This suggests that KCNA3 may influence the risk for AIP (Ota *et al*., 2011).

Several positive associations between cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) single-nucleotide polymorphisms (SNPs) and various autoimmune diseases have been identified. CTLA-4 is an inhibitory receptor expressed on the activated memory T cells and $CD4^+$ $CD25^+$ regulatory T cells that acts as an important negative regulator of the T cell-mediated immune response (Gough *et al*., 2005). CTLA-4 contributes to the maintenance of peripheral tolerance, induction of apoptosis in activated T cells and suppression of T cell proliferation and inflammatory cytokine production (Chang *et al*., 2007). Because of its inhibitory role, CTLA-4 constitutes a potential gene candidate to determine genetic predisposition of autoimmune diseases. Studies have suggested that AIP is also associated with a genetic polymorphism in the CTLA-4 gene in Japanese (Umemura *et al*., 2008) and Chinese patients (Chang *et al*., 2007). In addition, it has been identified in Korean patients, that the substitution of aspartic acid at position 57 of DQβ1 appears to represent an important genetic factor for the relapse of AIP (Park *et al*., 2008).

In ECS dogs, the common presentation of chronic pancreatitis suggests an underlying genetic predisposition. A recent genetic study using 47 affected ECS and 82 controls, showed that dog leukocyte antigen (DLA) haplotypes are a contributory factor in the development of CP in ECSs (Bazelle *et al*., 2013). This study identified an increased frequency of DLA-DQB1*00701 haplotype, in ECSs with CP, and a lower frequency of the DLA-DQB1*02001 haplotype, compared to controls. This DLA association has also been found in ECSs with immune-mediated haemolytic anaemia (Kennedy *et al*., 2006) and anal sac carcinoma (Aguirre-Hernández *et al*., 2010). It is suggested that chronic anal sacculitis may act as a risk factor for anal sac carcinoma. These DLA associations suggest a common underlying risk factor for a variety of immune-mediated diseases in ECSs. Autoimmune disease inheritance is a complex and multifactorial process. Other non-DLA genetic factors must be also contributing in the genetic predisposition to CP in ECS.

A pilot unpublished genome-wide association study (GWAS) carried out by Dr David Sargan and Dr Jesus Aguirre-Hernandez in the Department of Veterinary Medicine, University of Cambridge, showed two loci possibly associated with CP in ECSs, one on chromosome 16 and another on chromosome 12. This study used 14 affected ECSs and 47 non-affected age-matched controls, as well as 126 different controls from the general canine population with unknown disease status. Using a mixed model approach, population stratification was corrected. It was found that neither locus showed genome-wide significance, but both retained interest (best SNP *p* values = 4.03E-06 and 7.62E-06). The first locus overlies a region containing a group of T-cell receptor beta variable loci and the anionic trypsinogen gene. The second locus overlies several genes including the branched-chain alpha-keto acid dehydrogenase BCKDHB, which is necessary for the metabolism of leucine, valine and isoleucine, which, when mutated in humans, causes maple syrup urine disease (Chuang and Shih, 2001). Mutation in BCKDHB is also associated with acute and chronic pancreatitis in children (Kahler *et al*., 1994), thus partial loss of function at this locus could contribute to CP.

1.4. Aim of study

1. Characterise the clinical features of chronic pancreatitis in English cocker spaniels.
2. Develop a serum canine-specific IgG4 test.
3. Determine the usefulness of IgG4 serum concentration in dogs as a diagnostic tool for IgG4 disease.
4. Genotypic and phenotypic evaluation of dogs to try to identify risk genes.

It is hypothesized that ECS may suffer from an IgG4-related disease; similar to the human disease, where affected dogs present elevated concentrations of IgG4 in serum.

The first objective of this study was to better characterise in a greater number of dogs, the clinical features seen in ECS affected by CP and identify similarities and differences to human IgG4-related disease (IgG4-RD).

The second and third objectives were to develop and validate a serum canine-specific IgG4 diagnostic test, an ELISA and use it to determine serum IgG4 concentrations in dogs with suspected IgG4-RD compared to normal dogs. The predictive value of serum IgG4 concentrations in diagnosing IgG4-RD in ECSs and other dog breeds was assessed. Whether it may also be used as an indicator of disease activity was also determined. Moreover, this test was used to compare levels of serum IgG4 concentrations with normal control dogs and dogs with inflammatory diseases with another known aetiology different from immune-mediated disease.

Finally, a genetic study of the disease in ECS was performed. DNA samples from affected and control ECS were used to fine map genetic loci on chromosome 16 and chromosome 12 identified in a previous pilot GWAS study as potentially linked to CP.

Therefore, the purpose of this PhD project was to contribute to the understanding of the mechanisms and genetics of IgG4-RD in ECS, which could allow trials of new treatments and potentially disease prevention by selective breeding.

CHAPTER 2: DOG RECRUITMENT AND CHARACTERIZATION OF CLINICAL MANIFESTATIONS OF CHRONIC PANCREATITIS IN ENGLISH COCKER SPANIELS

2.1. Summary

This chapter describes the process of recruiting English cocker spaniels (ECS) affected by chronic pancreatitis (CP) and control ECS non-affected by CP. It also characterises the clinical manifestations of CP in this particular breed and compares their similarities and differences to human IgG4-related disease (IgG4-RD).

One hundred and four ECS affected by CP and 44 control ECSs were recruited for this study. The affected dogs were split into 2 groups according to the methodology used in the diagnosis of CP. Case group 1 was diagnosed based either on presenting pancreatic histology (n=9) or with both elevated canine pancreatic lipase immunoreactivity (cPLI) and abnormal pancreatic ultrasound (n= 47). Case group 2 was diagnosed based on less robust diagnostic methods, including elevated cPLI or 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester (DGGR) lipase, and elevated lipase/amylase without ultrasonographic changes (n=48). Control dogs were also split into 2 groups. Control group 1 was composed of healthy dogs (n=15) and control group 2 dogs that presented with any other disease with no known association to CP in ECS and with no immune-mediated aetiology (n=29). Case records of affected and healthy control dogs were searched for details of signalment, clinical and clinicopathological findings, and evidence of keratoconjunctivitis sicca (KCS); xerostomia; proteinuria; anal sacculitis; and other immune-mediated diseases.

This study showed remarkable clinical similarities between CP in ECSs and human IgG4-RD, including a high prevalence of multi-organ manifestation. Affected ECS presented with a high prevalence of KCS (n=49), proteinuria (n=47), anal gland disease (n=36), xerostomia (n=26), atopy (n=21) and other immune-mediated diseases (n=16). ECS with parti-colour coats, particularly blue roan, showed a strong association with CP, suggesting a link between coat colour and autoimmune conditions in this breed.

2.2. Introduction

ECS suffer from a distinctive form of CP histologically characterised by duct destruction, interlobular fibrosis and dense periductular and perivenous lymphocytic aggregates (Watson *et al*., 2011). A recent study by Watson *et al.* (2018) that included a small number of ECS affected by CP (n=12) showed a high prevalence of KCS, xerostomia, proteinuria, and presence of other immune-mediated diseases in the affected dogs. In addition, a more recent study showed that several ECS affected by CP and multi-organ chronic inflammatory disease presented a predominance of IgG4 positive plasma cells on immunohistochemistry in numerous organs including pancreas, kidney, salivary glands and orbital tissue (Coddou *et al*., 2020).

These distinctive features found in ECS affected by CP show similarities with human autoimmune pancreatitis type 1, which is part of a steroid-responsive fibro-inflammatory multi-systemic syndrome, currently known as IgG4-Related Disease (IgG4-RD) (Stone *et al*., 2012). Human IgG4-RD affects one or several organs and essentially any organ can be involved in this disease, often showing a predominance of $IgG4^+$ plasma cells histologically, with an $IgG4^+$/total IgG^+ plasma cell ratio > 40 % (Culver and Bateman, 2013). Pancreas, bile ducts, lacrimal glands, major salivary glands (parotid, submandibular, sublingual), lungs, kidneys, aorta, meninges, and thyroid gland are the most commonly affected organs. Moreover, IgG4-RD patients often show an increased serum level of IgG4 and older men are more frequently affected. Commonly, this disease causes inflammatory mass lesions in the pancreas and other affected organs. When salivary and lacrimal glands are affected (IgG4-related sialoadenitis/dacryodenitis), patients often present with continuous or recurrent painless swelling and mild symptoms of dry mouth/dry eyes (Vasaitis, 2016).

The aims of this study are to better characterise with a greater number of dogs, the clinical features seen in ECS affected by CP and identify similarities and differences to human IgG4-related disease (IgG4-RD).

2.3. Material and methods

2.3.1. Selection of cases and controls

ECS affected by CP and control ECS, with no suspicion of CP and/or immune-mediated diseases, were recruited for this study. EDTA blood and serum samples were obtained from these dogs and stored at −80 ° C until further analysis. The cases were obtained from patients from the Queen's Veterinary School Hospital and from private veterinary practices across the United Kingdom and were used with fully informed owner consent (Appendix 1). Clinical records from these dogs were also provided and were used to collect data regarding signalment and clinical status. The study was approved by the Department of Veterinary Medicine, University of Cambridge Ethics Committee (codes: CR153 and CR291).

Affected and control dogs were recruited with the support from The Cocker Spaniel Club and The Kennel Club via their Breed Health Coordinators. Our study was publicised on the BARC (Bio-Acquisition Research Collaboration) page on The Kennel Club website, on The Kennel Club Dog Health Facebook page and a recruitment email was sent out to ECSs' owners. In addition, advertising brochures were created for this purpose (Appendix 2) and displayed at several dog shows in the UK and sent to the veterinary surgeons and owners who contacted us at The Queen's Veterinary School Hospital, Department of Veterinary Medicine, University of Cambridge.

Cocker Spaniels affected by chronic pancreatitis.

CP was diagnosed either with pancreatic histology or with elevated cPLI test and/or a DGGR lipase assay, abnormal pancreatic ultrasound and presence of recurrent consistent clinical signs. The cases were divided into two groups because the methods to obtain a diagnosis of CP varied between patients:

- *Case group 1*: *Definite chronic pancreatitis*, diagnosed based on pancreatic histology, or *highly probable chronic pancreatitis* with very strong clinical suspicion*,* diagnosed based on an elevated cPLI and/or DGGR lipase and pancreatic ultrasound consistent with pancreatitis.

- *Case group 2*: *Likely chronic pancreatitis,* with clinical suspicion of CP based on other less robust diagnostics including elevated cPLI in the absence of ultrasound

evidence, elevated DGGR lipase, elevated amylase/lipase, and clinical signs consistent with CP.

Signalment details were recorded and case records were particularly examined for the presence of any other disease conditions and with particular criteria to search for evidence of diseases with reported associations with CP in ECS. The conditions were recorded as it follows: keratoconjuctivitis sicca (KCS), which was diagnosed, based on the Schirmer Tear Test (STT < 15mm/min), glomerulonephritis (definite, with renal histology, or suspected, with a urine protein:creatinine (UPC) ratio of >1), xerostomia (suspected, with recurrent dental treatments used as a marker and dry oral mucous membranes at clinical examination) anal sacculitis (history of recurrent anal gland expression or infection), anal sac adenocarcinoma, atopic dermatitis, other immune-mediated diseases, and diseases caused by end-stage chronic pancreatitis such as exocrine pancreatic insufficiency (EPI) and diabetes mellitus (DM). Other concomitant diseases were also recorded. Exclusion criteria for cases consisted of steroid or other immunosuppressive treatment prior to blood tests and dogs diagnosed also with lymphoma.

Control dogs

Age-matched control dogs were included for this study and they were also divided into 2 groups:

- *Control group 1*, composed of healthy ECS. Spare blood samples were obtained from ECS having pre-anaesthetic blood screens for routine procedures such as dentals or skin or mammary mass removal.

- *Control group 2*, composed of ECS affected by other conditions not related to CP and with no immune-mediated aetiology.

Inclusion criteria for control dogs consisted of no evidence of CP, glomerulonephritis (GN), KCS, anal sac disease and/or any other immune-mediated diseases. DGGR lipase was measured on the blood samples submitted. Also, referring veterinary surgeons were asked to perform Schirmer tear tests (STT) on control cases to rule out KCS and to submit a urine sample for the calculation of UPC ratio. Only dogs with normal DGGR lipase and/or cPLI and no previous clinical signs suggestive of CP, normal tear production, and UPC < 0.4 were included as controls. Exclusion criteria for control dogs consisted of corticosteroids or any immunosuppressant treatment within the previous 6 months of obtaining the blood sample.

DGGR lipase test, UPC ratio, packed cell volume (PCV) and platelet count were offered free of charge for cases and controls sending blood samples from external practices. Results were communicated back to the referring veterinary surgeon and, via the first opinion veterinarian, to the owners. Dogs that presented with slightly increased levels of DGGR lipase were used as controls only if they did not present any signs or history compatible with CP and the diseases mentioned above.

2.3.2. Statistical Analysis

Median age of cases and controls was compared using Mann-Whitney U test. Odds ratios (OR) and 95 % confidence intervals (CI) were calculated for disease association with particular colour coat. The Fisher's exact test was performed to determine if the OR was statistically significant or not. The level of statistical significance was set at $p < 0.05$. Statistical analysis was performed using SPSS® software (IBM, version 22.0).

2.4. Results

2.4.1. Clinical features in cases

One hundred and four ECS affected by CP were recruited for this study. There were 51 females and 53 males. Table 2.1 provides the signalment and clinical details of the 104 ECS cases included in this study. Median age was 9.1 years (range 2.7-16 years). Group 1 included 56/103 (53.8 %) cases (*definite* [n=9] or *highly probable* [n=47] chronic pancreatitis) and group 2 (*likely chronic pancreatitis*) included 48/104 (46.1 %) cases. A heterogeneous mass-like lesion in the pancreas was reported in 5/47 (10.6 %) ECS that had ultrasonography performed. Lethargy, weight loss, inappetence, cranial abdominal pain, diarrhoea, and intermittent vomiting were the most common clinical signs presented in these dogs.

Forty-nine cases were diagnosed with KCS (STT results available [n=32]; history of KCS confirmed by referring vet [n=17]). Thirty-eight of these cases had bilateral KCS and the remaining 11 cases had unilateral KCS. Forty-seven dogs presented with glomerulonephritis (suspicion with UPC > 1 [n=34] or with history of proteinuria [n=10]; and confirmed on histology [n=3]). Anal sac disease was seen in 36 dogs (anal sacculitis [n= 31]; anal sac adenocarcinoma [n=5]). Twenty-six dogs had recurrent dental treatments and dry oral

mucous membranes suggesting xerostomia. Twenty-one dogs had a history of atopy. Other immune-mediated conditions such as hypothyroidism (n=6), IMTP (n=4), IMHA (n=4), IMPA (n=2), IBD (n=4) and hypoadrenocorticism (n=2) were presented in 16 cases. Table 2.2 summarises the prevalence of these conditions in affected ECSs from group 1 and group 2. The proportion of cases presented with these conditions in the case study population is shown in Fig 2.1.

Table 2.1: Signalment and clinical details of the 104 English cocker spaniel cases affected by chronic pancreatitis included in this study.

Dog Number	*Category*	*Breed*	*Age (years)*	*Sex*	*Coat colour*	*Diagnosis*
1	Case	ECS	9.3	MN	Blue roan	CP, KCS, EPI
2	Case	ECS	12.3	ME	Tricolour	CP
3	Case	ECS	7.4	MN	Black & white	CP, KCS
4	Case	ECS	5	FE	NR	CP
5	Case	ECS	11.5	FN	NR	CP, hepatic failure
6	Case	ECS	11.25	FN	Chocolate & white	CP
7	Case	ECS	12	FN	Orange roan	CP, KCS, GN, AS, Xerostomia
8	Case	ECS	10.7	MN	Blue roan	CP, KCS, GN, AS, Xerostomia, EHBO
9	Case	ECS	8.2	FN	NR	CP, GN
10	Case	ECS	7	MN	Blue roan	CP, KCS, GN, AS, Xerostomia
11	Case	ECS	12.75	FN	NR	CP, KCS, AS, Xerostomia
12	Case	ECS	13	MN	Blue roan	CP, KCS, GN, AS, Xerostomia, Horner's syndrome, Otitis externa
13	Case	ECS	9	FN	Golden	CP, KCS, PLE
14	Case	ECS	8.5	FN	Blue roan	CP, KCS, GN, ASAC, Hepatitis, EPI
15	Case	ECS	5.4	MN	Tricolour	CP, KCS, Atopy, Cryptorchidism
16	Case	ECS	11.5	MN	Blue roan	CP, GN, Xerostomia, DM
17	Case	ECS	8	MN	Chocolate & white	CP, KCS, Xerostomia, Atopy, Otitis, Thalamic lesion, Hepatitis
18	Case	ECS	9.1	FN	Golden	CP, KCS, GN, AS, Xerostomia, IBD
19	Case	ECS	13	FN	Blue roan	CP, GN
20	Case	ECS	9.6	FN	Golden	CP, KCS, GN, AS, IVDD, Glaucoma
21	Case	ECS	7	FN	Blue roan	CP, KCS, GN, AS, Xerostomia, EPI
22	Case	ECS	12.1	F	Blue roan	CP, GN, EPI, Cushings
23	Case	ECS	7.5	FN	Blue roan	CP, KCS, GN, Hypoadrenocorticism
24	Case	ECS	8.2	FE	Blue roan	CP, KCS, AS, Atopy, Hypothyroidism
25	Case	ECS	9	FN	Blue roan	CP, ASAC
26	Case	ECS	7	FN	Blue roan	CP, AS
27	Case	ECS	4	FN	Blue roan	CP
28	Case	ECS	9.5	ME	Blue roan	CP, KCS, IMHA
29	Case	ECS	8.3	FN	Blue roan	CP, GN, Hypothyroidism, DM
30	Case	ECS	10	FN	Chocolate	CP, KCS, GN, ASAC, Atopy
31	Case	ECS	5.2	MN	Black & white	CP, KCS, AS
32	Case	ECS	11	FN	Chocolate & white	CP, KCS, GN, Vacuolar hepatopathy
33	Case	ECS	8.1	MN	Black & white	CP, KCS, ASAC
34	Case	ECS	3	MN	Tricolour	CP, IMTP, IMHA
35	Case	ECS	8.75	FN	Blue roan	CP, GN, IMTP
36	Case	ECS	11.8	FN	Blue roan	CP, EPI, Mammary mass
37	Case	ECS	9.1	FN	Blue roan	CP, Xerostomia
38	Case	ECS	13.7	FN	Blue roan	CP, Xerostomia, Hypothyroidism
39	Case	ECS	12	MN	Blue roan	CP, KCS, AS, EPI
40	Case	ECS	10	MN	Blue roan	CP, KCS, GN, Xerostomia, Atopy, Cryptorchidism
41	Case	ECS	3	ME	Black & white	CP, KCS, AS, Atopy
42	Case	ECS	4	FN	Blue roan	CP
43	Case	ECS	10	MN	Golden	CP, KCS, AS, Atopy, DM
44	Case	ECS	8.1	M	Blue roan	CP, GN
45	Case	ECS	8.6	FN	Blue roan	CP, GN
46	Case	ECS	14.9	FN	NR	CP, AS, IBD
47	Case	ECS	16	M	Blue roan	CP, KCS, Atopy, Lymphoma
48	Case	ECS	9	FN	Blue roan	CP, KCS, GN, AS
49	Case	ECS	12.6	M	Blue roan	CP, GN, AS, DM
50	Case	ECS	12.2	FN	NR	CP, Mammary mass
51	Case	ECS	11.4	FN	Blue roan	CP, Xerostomia, Atopy, Epilepsy
52	Case	ECS	9.25	FN	Blue roan	CP, KCS, GN, AS, Atopy, IMTP, Vestibular disease

Dog Number	*Category*	*Breed*	*Age (years)*	*Sex*	*Coat colour*	*Diagnosis*
53	Case	ECS	4	FN	NR	CP
54	Case	ECS	10.4	MN	Golden	CP, GN, Xerostomia
55	Case	ECS	3.7	MN	Blue roan	CP, KCS, GN, Atopy, IBD, Epilepsy
56	Case	ECS	11	FN	NR	CP, KCS, GN, AS, Osteoarthritis, Vacuolar hepatopathy
57	Case	ECS	8.75	MN	NR	CP, KCS
58	Case	ECS	10.5	MN	Black & white	CP, EPI
59	Case	ECS	3.7	MN	Blue roan	CP, Atopy
60	Case	ECS	5	MN	Blue roan	CP
61	Case	ECS	6	MN	Tricolour	CP, KCS, GN, EPI, DM
62	Case	ECS	6.4	FN	Black & white	CP
63	Case	ECS		FN	NR	CP
64	Case	ECS	10.9	ME	Black & white	CP
65	Case	ECS	12.5	FN	Blue roan	CP, KCS, GN, AS, Xerostomia, Mitral valve disease
66	Case	ECS	7	FN	Blue roan	CP, GN,
67	Case	ECS	9	FN	Blue roan	CP, KCS, GN,
68	Case	ECS	9	M	Red roan	CP, KCS, Hypothyroidism, DM
69	Case	ECS	9.6	MN	Golden	CP, AS, Xerostomia, Arthritis
70	Case	ECS	10.8	M	Red	CP, Xerostomia
71	Case	ECS	5.5	MN	Blue roan	CP, EPI
72	Case	ECS	13.75	FN	Blue roan	CP, GN, Xerostomia, EPI
73	Case	ECS	5.2	F	Black & white	CP, Epilepsy
74	Case	ECS	10.25	ME	Black & white	CP, KCS, GN, AS
75	Case	ECS	8.25	M	Blue roan	CP
76	Case	ECS	7	FN	NR	CP, KCS, GN, Atopy
77	Case	ECS	2.75	M	Tricolour	CP, IMPA
78	Case	ECS	9.2	MN	NR	CP, AS, IVDD
79	Case	ECS	11	MN	Blue/tan	CP, KCS, GN, Atopy, Hypothyroidism
80	Case	ECS	9.25	MN	Orange roan	CP, KCS, GN, Xerostomia, Osteoarthritis
81	Case	ECS	9.75	FN	Black & white	CP, KCS, GN, Atopy, Hypothyroidism, Urothelial carcinoma
82	Case	ECS	10.75	ME	NR	CP, GN, IVDD
83	Case	ECS	7	MN	Chocolate	CP
84	Case	ECS	7.4	MN	Red roan	CP, Xerostomia, IMTP, IMPA, Chronic hepatitis
85	Case	ECS	11	ME	Golden	CP, KCS, GN, Xerostomia
86	Case	ECS	7.75	MN	Blue roan	CP, GN, ASAC, Xerostomia
87	Case	ECS	9.9	FN	Orange roan	CP, GN
88	Case	ECS	9.7	MN	NR	CP, GN, Peripheral neuropathy, Chronic hepatitis
89	Case	ECS	5	MN	Blue roan	CP, AS
90	Case	ECS	8.25	F	NR	CP
91	Case	ECS	6	MN	Red	CP, AS, Atopy, Otitis
92	Case	ECS	11.6	MN	Blue roan	CP, KCS, AS
93	Case	ECS	9.9	FN	Blue roan	CP, KCS, AS, Xerostomia, Atopy
94	Case	ECS*	8.5	FN	Black	CP, GN
95	Case	ECS	8.25	MN	Chocolate	CP, KCS, AS, Xerostomia, Atopy, IMHA, Maxillary fibrosarcoma
96	Case	ECS	6.25	MN	Black	CP, GN, AS, Peripheral vestibular syndrome
97	Case	ECS	12.3	MN	Blue roan	CP; KCS, GN, AS, IMHA, IBD
98	Case	ECS	7.6	FN	Chocolate & white	CP, Addison's disease
99	Case	ECS	11.7	FN	Orange	CP, KCS, Atopy, Chronic bronchitis
100	Case	ECS	14.25	FN	Black	CP, KCS, GN, Xerostomia, Atopy, EPI, Otitis
101	Case	ECS	13	M	Blue roan	CP
102	Case	ECS	9.8	MN	Blue roan	CP, KCS, GN, Atopy, DM
103	Case	ECS	4.3	MN	Black	CP
104	Case	ECS	10.8	FN	Chocolate roan	CP, KCS, GN, AS, Xerostomia

ECS, English cocker spaniel; F, female; FN, neutered female; M, male; MN, neutered male; CP, chronic pancreatitis; GN, glomerulonephritis; KCS, keratoconjunctivitis sicca; AS, anal sacculitis; ASAC, anal sac adenocarcinoma; EPI, exocrine pancreatic insufficiency; EHBO, extra hepatic biliary tract obstruction; DM, diabetes mellitus; IBD, inflammatory bowel disease; IMHA, immune mediated haemolytic anaemia; IMPA, immune mediated polyarthritis; IMTP, immune mediated thrombocytopenia; PLE, protein-losing enteropathy; NR, not recorded. * Working cocker spaniel.

Table 2.2: Number of dogs that presented with concurrent conditions associated with CP in ECS.

Condition		Number of cases group 1 (n=56)	Number of cases group 2 (n=48)	*Total cases* (n=104)
Keratoconjunctivitis Sicca	STT <15mm/min	20	12	32
	History of dry eye	8	9	17
Glomerulonephritis	Biopsy/ UPC > 1	24	13	37
	History of Proteinuria	3	7	10
Anal sac disease	Anal sacculitis	16	15	31
	Anal sac adenocarcinoma	3	2	5
Other Immune-mediated disease	Hypothyroidism	2	4	6
	Hypoadrenocorticism	2	0	2
	IMTP	1	3	4
	IMHA	0	4	4
	IMPA	0	2	2
	IBD	3	1	4
Xerostomia		14	12	26
Atopy		8	13	21

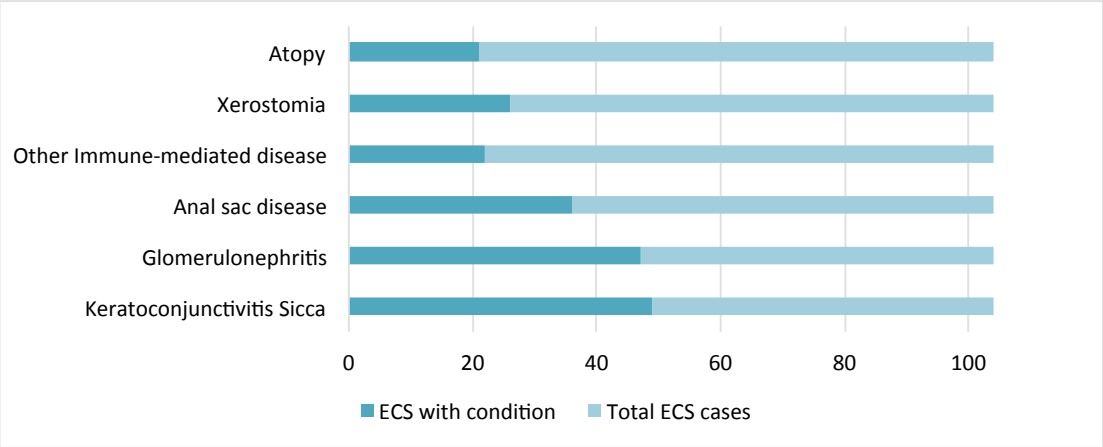

Figure 2.1: Proportion of cases presenting with concurrent conditions associated with CP in ECS.

Seventy-eight ECS affected by CP (75 %) had involvement of other organ/s in addition to pancreas, suggesting multi-organ chronic inflammatory disease (KCS n=49; GN n=47; anal sacculitis n=31 and presence of xerostomia, suggesting sialadenitis n=26). Thirty-two ECSs had 1 extra organ involved, 25 ECSs had 2 organs involved, 12 ECSs had 3 organs involved and 9 ECSs had 4 organs involved in addition to pancreas (Fig. 2.2).

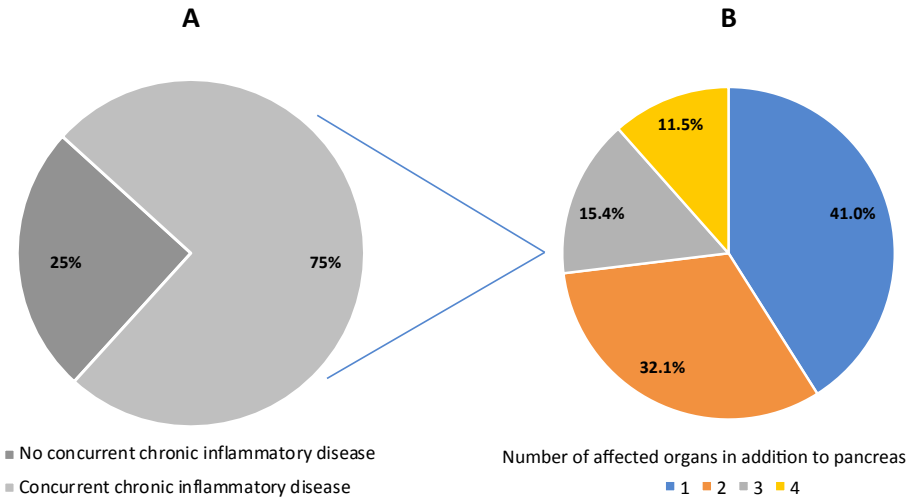

Figure 2.2: Concurrent chronic inflammatory disease in English cocker spaniels affected by chronic pancreatitis. A: pie chart represents the percentage of ECSs affected by CP presenting with concurrent chronic inflammatory disease in addition to CP. B: pie chart indicates the number of organs affected by chronic inflammatory disease in addition to pancreas in ECSs presented with multi-organ involvement.

Venn diagram (Fig. 2.3) represents the number and percentage of cases having more than one organ affected by chronic inflammatory disease, showing an overlap in some affected organs.

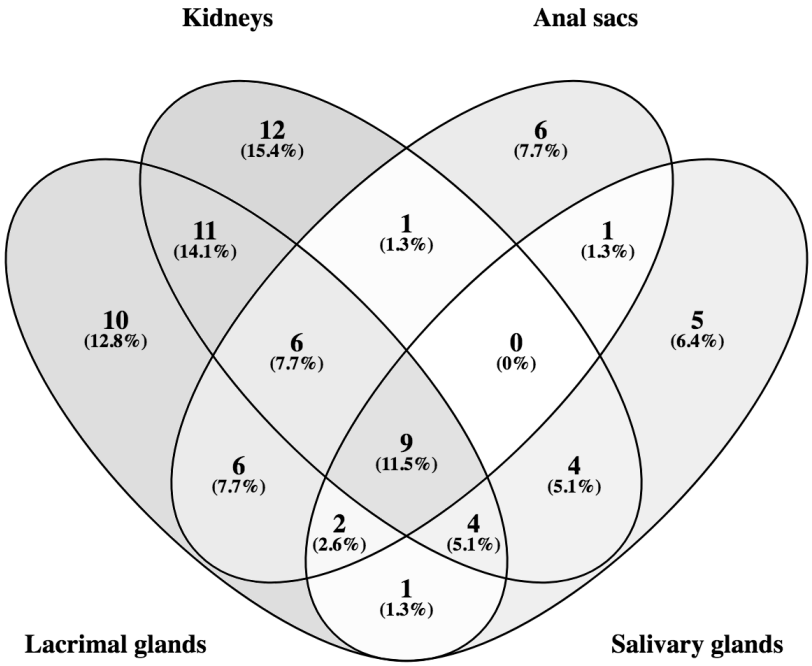

Figure 2.3: Venn diagram representing the number and percentage of English cocker spaniels affected by chronic pancreatitis that presented with multi-organ pathology in this study.

Regarding diseases caused by end-stage CP, concurrent diabetes mellitus was present in 7/104 (6.7 %) dogs. EPI was present in 11/104 (10.6 %). Only 1 dog from the study had both DM and EPI diagnosed (case 61).

Regarding other diseases, 4/104 (3.8 %) dogs had chronic hepatitis, 3/104 (2.9 %) dogs had intervertebral disc disease, 3/104 (2.9 %) dogs had idiopathic epilepsy, 3/104 (2.9 %) had chronic bronchitis, 2/104 (1.9 %) suffered from osteoarthritis, 2/104 (1.9 %) dogs had mammary masses, 2/104 (1.9 %) dogs suffered from cryptorchidism, and 2/104 (1.9 %) had enlarged prostate.

Information regarding coat colour was provided for 89/104 (85.6 %) affected dogs. From those dogs with colour coat recorded, 72 (80.9 %) had parti-colour coats (blue roan n=47 [52.8 %]; black and white n= 10 [11.2 %]; tricolour n=5 [5.6 %]; chocolate and white n=4 [4.5 %]; orange roan n=3 [3.4 %]; red roan n=2 [2.2 %]; chocolate roan n=1 [1.1 %]) and 17 (19.1 %) had solid colours (golden n=8 [9.0 %]; black n=4 [4.5 %]; chocolate n=3 [3.4 %]; red n=2 [2.2 %]) (Fig. 2.4).

2.4.2. Clinical features in control dogs

Forty-four ECS that fulfilled the strict inclusion criteria were recruited as controls. Fifteen dogs belonged to *Control group 1* (healthy ECSs), and 29 belonged to *Control group 2* (diseased control group). Group 1 was composed of 7 females and 8 males and group 2 of 10 females and 19 males. Median age was 10.0 years for group 1 (range 7.0-11.25 years) and 9.2 years for group 2 (range 7.0-13.3 years). The signalment, in addition to clinical details of the control dogs from group 1 and 2 included in this study, are summarised in Table 2.3 and 2.4, respectively.

Table 2.3: Signalment and clinical details of the 15 healthy control English cocker spaniels (control group 1) included in this study.

Dog Number	*Category*	*Breed*	*Age (years)*	*Sex*	*Coat colour*	*Diagnosis*
1	Control	ECS	10	MN	Chocolate	Healthy
2	Control	ECS	9	MN	Lemon & white	Healthy
3	Control	ECS	9	MN	Orange roan	Healthy
4	Control	ECS	9	MN	NR	Healthy
5	Control	ECS	9.5	MN	Golden	Healthy
6	Control	ECS	10.5	FE	Golden	Healthy
7	Control	ECS	10	FN	Blue roan	Healthy
8	Control	ECS	10.3	MN	Lemon roan	Healthy
9	Control	ECS	7	MN	Chocolate & white	Healthy
10	Control	ECS	10	FN	Black	Healthy
11	Control	ECS	8.9	FN	Golden	Healthy
12	Control	ECS	10.4	FN	Chocolate	Healthy
13	Control	ECS	11.25	M	Orange roan	Healthy
14	Control	ECS	9.6	F	Black	Healthy
15	Control	ECS	10.75	FN	Blue roan	Healthy

ECS, English cocker spaniel; F, female; FN, neutered female; M, male; MN, neutered male; NR, not recorded.

Table 2.4: Signalment and clinical details of the 29 control English cocker spaniels affected by other conditions unrelated to chronic pancreatitis and with no immune-mediated aetiology (control group 2) included in this study.

Dog Number	*Category*	*Breed*	*Age (years)*	*Sex*	*Coat colour*	*Diagnosis*
1	Control	ECS	8.1	ME	Red	DCM, MVD
2	Control	ECS	9	M	NR	IVDD
3	Control	ECS	7	M	Orange roan	Epilepsy
4	Control	ECS	10.6	MN	Golden	Nasal squamous cell carcinoma
5	Control	ECS	7.25	FN	Liver & white	USMI
6	Control	ECS	11.8	MN	Tricolour	Cranial cruciate ligament disease
7	Control	ECS	9	FN	Orange roan	Obesity
8	Control	ECS	8	FN	Golden	IVDD
9	Control	ECS	9.7	MN	NR	Bronchogenic pulmonary carcinoma
10	Control	ECS	10.75	FN	NR	IVDD
11	Control	ECS	7.3	MN	Black	High-grade mandibular sarcoma
12	Control	ECS	9.2	MN	NR	Bronchogenic pulmonary carcinoma
13	Control	ECS	8.1	MN	NR	Chronic bilateral otitis externa
14	Control	ECS	13	ME	NR	IVDD
15	Control	ECS	10	FN	NR	Pulmonary Oedema
16	Control	ECS	12.9	MN	Black	Bronchial carcinoma
17	Control	ECS	8.9	FN	Tricolour	IVDD
18	Control	ECS	8.3	MN	Black & white	Small intestinal disease
19	Control	ECS	11.6	FN	Blue roan	IVDD
20	Control	ECS	13.3	FN	Black	Oral melanoma
21	Control	ECS	9.7	MN	Black	Prostatic carcinoma
22	Control	ECS	8.4	MN	Golden	Low-grade soft tissue sarcoma
23	Control	ECS	8.75	ME	Lemon & white	IVDD
24	Control	ECS	12.5	MN	Liver & white	IVDD
25	Control	ECS	8.1	MN	Blue roan	Cranial cruciate ligament disease
26	Control	ECS	10.9	FN	Brown sable	Cranial cruciate ligament disease
27	Control	ECS	11.1	ME	Black & white	Heart-base tumour
28	Control	ECS	10.1	MN	NR	IVDD
29	Control	ECS	9	FN	NR	Bacterial hepatitis

ECS, English cocker spaniel; F, female; FN, neutered female; M, male; MN, neutered male; DCM, dilated cardiomyopathy; MVD, mitral valve disease; USMI, urethral sphincter mechanism incompetence; IVDD, intervertebral disc disease; TCC, transitional cell carcinoma; NR, not recorded.

There was no a significant difference in age between CP cases and controls (median age 9.1 *vs.* 9.7 years, Mann-Whitney U test, *P*= 0.276).

Regarding diagnosis for control group 2, neurological (10/29) and oncological (9/29) cases were overrepresented in this study population. Seven dogs from group 1 had slightly increased DGGR lipase but they did not present other signs of pancreatitis. Eight dogs from group 2 had slightly increased DGGR lipase with no other signs of pancreatitis. Five of them were diagnosed with IVDD.

Information regarding coat colour was obtained for 14/15 ECS from control group 1. Seven dogs had solid colour coat (golden [n=3], black [n=2], chocolate [n=2]) and 7 had parti-colour coats (orange roan [n=2], blue roan [n=2], lemon roan [n= 1], lemon and white [n= 1], chocolate and white [n= 1]).

Information about coat colour in control group 2 was available for 20/29 dogs. The vast majority of them had parti-colour coats (blue roan [n=2], black and white [n=2], orange roan [n=2], liver and white [n=2], tricolour [n=2], lemon & white [n=1], brown sable [n= 1]), and 8 dogs had solid colour coats (black [n=4], golden [n=3], and red [n= 1]).

Figure 2.4 illustrates the incidence of the different coat colours in the cases and controls from group 1 and 2 included in this study.

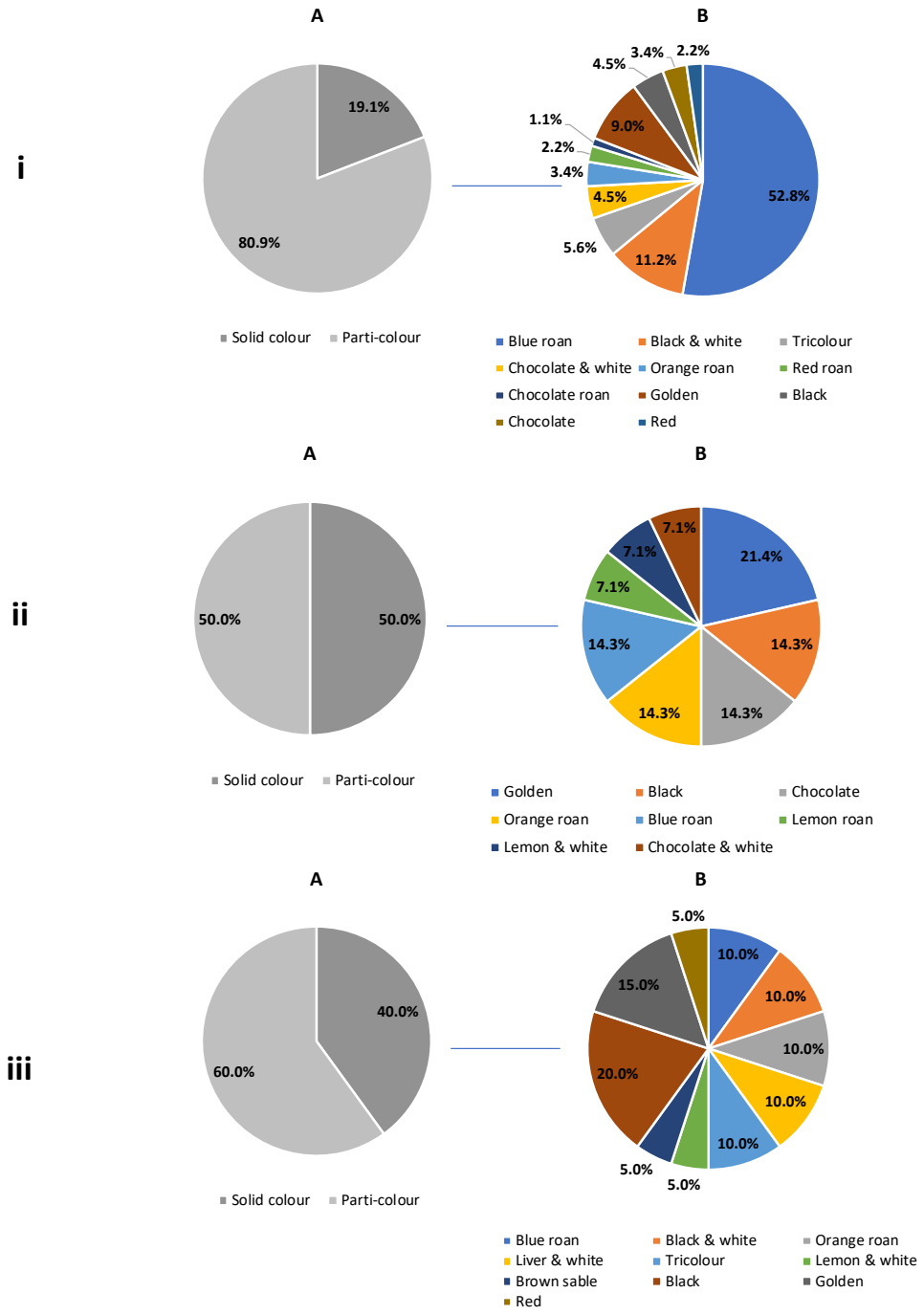

Figure 2.4: Coat colour distribution in (i) English cocker spaniels affected by chronic pancreatitis, (ii) healthy English cocker spaniels (control group 1) and (iii) English cocker spaniels affected by diverse non-immune mediated conditions unrelated with CP (control group 2). A: Pie chart shows incidence of parti-colour (roan coats and mixes of two or three colours) and solid colours. B: Individual coat colours presented in this study are represented.

Parti-colour coats showed a higher frequency in cases than in control dogs (80.9 % *vs.* 55.9 %, *P* = 0.006). Particularly, the blue roan parti-colour coat was highly overrepresented in cases than in controls (52.8 % *vs.* 11.8 %), indicating a significant association with CP in ECS (OR 8.39, 95% CI 2.73–25.80, *P* <0.001) (Fig. 2.5).

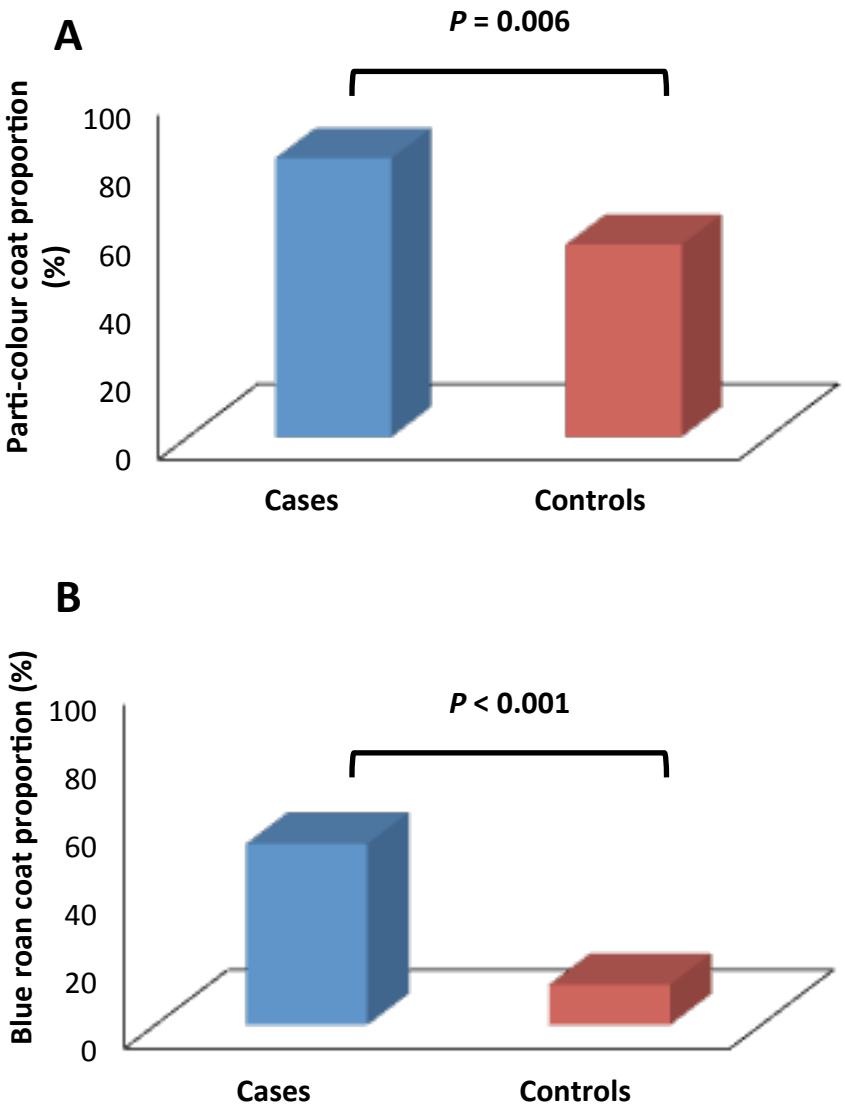

Figure 2.5: Comparison of coat colour proportion between cases and controls. A) Parti-colour coat proportion and B) Blue roan coat proportion in cases (blue) and controls (red). *P* values indicate statistical significance of differences.

2.5. Discussion and conclusions

This chapter describes for the first time a clinical study of a large number of ECSs affected with chronic pancreatitis compared with an age-matched control group of the same breed without any evidence of chronic pancreatitis. Affected dogs were significantly more likely to have parti-coloured coats compared with unaffected dogs and showed some similarities with human IgG4-related disease in the high prevalence of concurrent diseases.

This chapter has given a number of insights into the clinical presentations of CP in ECS dogs, showing some similarities with human IgG4-related disease. Keratoconjunctivitis sicca (KCS), more commonly known as dry eye, showed a high prevalence in the ECS affected by CP included in this study (47.1 %), while other studies have shown a prevalence of KCS of 4 % in the general dog population (Williams *et al*., 2008). In humans the orbital tissue is frequently involved during the course of IgG4-RD. These patients often present with dry eye, which may be associated with lacrimal gland and/or orbital nerve involvement (Kocabeyoglu *et al.,* 2016). The high prevalence of KCS observed in this study suggests a potential link between CP and KCS in ECS. Therefore, it is relevant to evaluate tear production by the use of Schirmer's tear test to determine the presence of dry eye in these animals. However, it is important to acknowledge that the prevalence of KCS in this breed is unknown, this may be high and a co-occurrence of two independent disease entities rather than multi-organ disease could be present.

In addition, concurrent glomerulonephritis (GN) was seen overrepresented in ECS affected by CP (45.2 %). Diagnosis of GN in the vast majority of the dogs was made based on suspicion with a UPC > 1 (32.7 %) or with history of proteinuria (9.6 %) and was confirmed on histology in just a few dogs (2.9 %). GN, in a similar manner to chronic pancreatitis, can be diagnosed on histopathology although renal biopsies are rarely performed ante mortem in either humans or dogs since it is invasive procedure. It is known that persistent proteinuria is a clinicopathological sign of GN, which was the reason why a protein:creatinine ratio (UPC) of > 1 was used as a marker of GN. However, this is not a specific marker for the diagnosis of GN and it could be increased for other possible causes, predominantly hypertension and amyloidosis, therefore the diagnosis is only presumptive without a biopsy. It is important to mention that several clinical records did not have a UPC performed and simply mentioned the existence of proteinuria. Proteinuria alone is difficult to interpret as it varies with urine concentration.

Suspected xerostomia, defined as dry mouth resulting from reduced or absent saliva flow, showed a high prevalence in ECS affected by CP (25 %). In human IgG4-RD, the involvement of salivary glands (IgG4-related sialadenitis) is observed in 27 % to 53 % of patients and submandibular glands are more frequently affected, but parotid, sublingual, and labial salivary glands are also involved. Usually, salivary secretion is normal or slightly reduced and xerostomia is present in 30 % of the IgG4- RD patients (Puxeddu *et al*., 2018). Xerostomia is rarely evaluated in dogs; however, it is known that it is a cause of persistent dental disease in humans (Cassolato *et al*., 2003). This is one of the reasons why repeated dental treatments were used as a marker for xerostomia, although it was difficult to determine the real cause of a dog's persistent dental disease, which is a study limitation. The commonest test used in humans to measure salivary flow rate cannot be applicable in dogs since stimulated saliva production is required to be spat out while chewing a piece of sterile wax for five minutes. Further investigation should aim to obtain a reliable method to measure salivary production in order to reach a conclusion about the involvement of dry mouth in this disease.

In this study it was also seen that 29.8 % of the ECS affected by CP presented with a history of anal sacculitis, while other studies have shown a prevalence of 12.5 % in the general dog population (Halnan *et al*., 1976). A previous histological study of anal sac tissue from ECS presenting with anal sacculitis showed histopathological findings similar morphologically to human IgG4-RD, with the presence of lymphocytic and plasmacytic infiltration, fibrosis and increased number of IgG4+ plasma cells (Coddou, 2015. MPhil Thesis). This suggests that anal sacculitis in ECS could be one of the IgG4-RD sequelae, which it is obviously not reported in humans.

Involvement of other organ(s) (kidney, salivary and lacrimal glands, and anal sacs) in addition to pancreas was seen in 75 % of ECS affected by CP, suggesting a multi-systemic chronic inflammatory disease, similar to IgG4-RD. However, It is important to consider the possibility of co-occurrence of independent disease entities rather than multi-organ disease or also that the extent of involvement of other organs may have been underestimated due to lack of relevant imaging studies or biopsy specimens.

Atopy and atopic dermatitis were present in 20.2 % of the study population versus approximately 10 % of the general dog population (Scott *et al*., 2001). A potential link has been described between atopy and human IgG4-RD, although this remains controversial. A

study in the UK has reported a frequent clinical history of allergy (63 %) and atopy (40 %) in human patients affected by IgG4-RD (Culver *et al*., 2017) and this has been supported by retrospective data in Japanese AIP patients (Kamisawa *et al*., 2009) but not by other groups (Della Torre *et al*., 2014).

Concurrent immune-mediated conditions such as hypothyroidism, IMTP, IMHA, IMPA, IBD, and hypoadrenocorticism were seen in 16 (15.4 %) cases affected by CP, implying a predisposition in ECS to immune-mediated disease. A previous genetic study showed an increased frequency of one dog leukocyte antigen haplotype in ECSs with CP (Bazelle et al., 2013). This haplotype has also been demonstrated to be more frequent in ECSs with IMHA (Kennedy et al., 2006), suggesting that CP may be part of a multi-organ immune-mediated disease in ECSs in which this DLA haplotype may play an important role.

Other studies have demonstrated that neutering in dogs is associated with increased risk for certain autoimmune disorders such as atopic dermatitis, autoimmune haemolytic anaemia, hypoadrenocorticism, hypothyroidism, immune-mediated thrombocytopenia, inflammatory bowel disease, and systemic lupus erythematosus (Sundburg *et al*., 2016; Oberbauer *et al*., 2019). It would be interesting as a future work, to determine whether neutered dogs included in this study are more predisposed to have CP and suspicion of IgG4-RD.

Chronic pancreatitis causes a permanent destruction of pancreatic tissue and progressive loss of exocrine and endocrine function, which can eventually progress to exocrine pancreatic insufficiency (EPI) and/or diabetes mellitus (DM) at end-stage. These diseases can reportedly develop only if 80-90 % of functional mass is lost, since the pancreas has an extensive functional reserve capacity. Thus, many dogs will not reach end stage by the end of their lives (Watson, 2012). A recent study in primary-care clinics in the UK indicated that pancreatitis is one of the factors associated with the risk of dogs developing DM. That study reported an apparent annual prevalence of DM of 0.26% and an annual incidence risk of 0.09% in dogs aged ≥3 years. The same study showed an increased hazard of death following diagnosis of DM in cocker spaniels compared to crossbreeds (Heeley *et al*., 2020). There is currently no data available on the prevalence of EPI. In this thesis, 6.7 % and 10.6 % of ECS affected by CP presented concurrent DM and EPI, respectively. This high prevalence observed, suggests the importance of actively evaluating their presence in ECS affected by CP.

The inflammatory lesions present in IgG4-related pancreatic disease in humans frequently causes diffuse enlargement of the organ or in some cases a benign pancreatic mass, making differentiation from pancreatic cancer difficult (Stone *et al*., 2012). In this study only five affected dogs presented with mass-like pancreatic lesions identified on ultrasound. This pancreatic feature appears to be caused because of fibrosis and inflammation. This study suggests it is not as common in ECS as in humans.

The English Cocker Spaniel breed health coordinator, when asked about the prevalence of parti-colour coats in the general population, provided an estimate that the ECS population in the UK is currently made up of around 60% parti-colours and 40% solid colours (personal communication). Surprisingly, a strong association between CP and dogs with parti-colour coats was observed in this study (80.9 % of the affected ECSs were parti-colour). In particular, the blue roan parti-colour coat was highly overrepresented in cases than in controls (52.8 % vs. 11.8 %), indicating a significant association with CP in ECS (OR 8.39, 95% CI 2.73–25.80, $P < 0.001$). This suggests a candidate gene in linkage disequilibrium with the gene that determines parti-colour coats. It has been demonstrated that pigment type switching in dogs is controlled by a gene that has been called the *K locus*, which is located on dog chromosome 16 (Kerns *et al*., 2007). This could be an interesting candidate gene to be further investigated.

This study had limitations inherent in its initial design and control selection. Control dogs were included if they did not present evidence of CP but also if they did not have evidence of GN, KCS, anal sac disease and any other immune-mediated diseases. Therefore, it was not possible to compare the prevalence of these diseases between groups. In addition, due to the difficulties of obtaining healthy age-matched controls, some cases of benign mammary masses and dental removal cases were included in this group. Future work including a control group without chronic pancreatitis but also without exclusion of the conditions mentioned above would be needed in order to determine if these conditions are part of a multi-organ disease or if they are coexisting independent disease entities.

This chapter has characterised the clinical manifestations of CP in ECSs and has suggests potential clinical similarities to human IgG4-RD, including a high ocurrence of KCS, xerostomia, proteinuria, atopy and other concurrent immune-mediated diseases that could be acting as part of a multi-inflammatory syndrome. Anal sacculitis is also recurrent in affected dogs and particularly interesting is the clear predisposition of parti-colour ECS, in

particular blue roans, to CP. Future work, involving a following up of these dogs in order to obtain up-to-date clinical records could help to further strengthen any associations found and improve understanding of this disease and its manifestations.

CHAPTER 3: DEVELOPMENT OF A CANINE SERUM IgG4 TEST

3.1. Summary

Human IgG4-RD is diagnosed on clinical presentation, diagnostic imaging findings, histological criteria and serum IgG4 elevation. There is currently no routine serum IgG4 test for dogs and diagnosis is based on organ biopsy, which is invasive and rarely justified in clinical cases.

This chapter describes the development of a non-invasive test capable of measuring IgG4 levels in canine serum. The purpose of this test was as a diagnostic tool for the recognition of suspected IgG4-related disease in affected dogs and to monitor efficacy of treatment. Firstly, monoclonal antibodies against dog IgG4 were produced, an essential step in order to develop an ELISA for the detection of dog IgG4 in serum. The original hybridomas producing these antibodies (Mazza *et al*., 1993, 1994b) were grown in tissue culture and are capable of producing mAbs reactive with dog IgG4 in serological and immunohistochemical assays. Different strategies for the isolation of IgG4 from dog serum using affinity and anion exchange chromatography, and the validation of the ELISAs are also reported in this section. After several attempts at isolating IgG4 from frozen dog plasma and fresh serum, mass spectrometry results showed that IgG4 was only partially purified. A competitive ELISA for the detection of dog IgG4 in serum was developed. Standard curves for IgG4 measurement showed highly reliable results. However, levels of IgG4 detected in a plasma pool from healthy dogs were demonstrated to be higher than previously reported for normal serum IgG4 ranges and higher than total IgG.

3.2. Introduction

IgG4 is one of four IgG subclasses that are recognized in humans. In normal human serum, the concentrations of these subclasses decrease in numerical order, with IgG1 being the most abundant and IgG4 the least abundant. Four subclasses of canine IgG have also been defined and named on the basis of their relative serum concentration and electrophoretic mobility in accordance with the equivalent human molecules (Mazza *et al*., 1994a). A more recent study has identified canine IgG subclass γ chain RNAs and labelled them as IgG-A, IgG-B, IgG-C, IgG-D, and demonstrated a modest sequence homology with the four human IgG subclasses (Tang *et al*., 2001). Another study using canine B cell lymphomas as the source of RNA, also identified 4 IgG heavy chains in dogs. This study made expression constructs with cDNAs of these RNAs and performed functional Fc binding assays on the canine IgG subclasses to confirm they share many functional features with human subclasses (Bergeron *et al*., 2014).

Human IgG4-RD is diagnosed based on clinical presentation, histological criteria, diagnostic imaging findings and serum IgG4 elevation. Currently, there is not a routine serum IgG4 test for dogs and there are no studies describing levels of serum IgG4 in dogs affected by CP. Diagnosis is based on organ biopsy, using immunohistochemistry with previously published canine monoclonal antibodies against IgG4 (Mazza *et al*., 1993, 1994b; Coddou *et al*., 2020). However, biopsies are invasive and rarely justified in clinical cases, for these reasons it is important to develop a reliable test able to measure IgG4 in canine serum and determine its usefulness in diagnosing IgG4-RD in dogs.

Purified dog IgG subclasses were used to prepare a set of monoclonal antibodies (mAbs) by Mazza *et al*. (1993), which have been used worldwide for studies of canine IgG subclass specificity in autoimmune, allergic and infectious diseases. These purified mAbs and the purified IgG fractions were no longer available; therefore, in order to develop an ELISA test capable of measuring IgG4 in dog serum it was essential to produce them. Thus, the aims of this chapter were to develop a quantitative canine serum IgG4 test, and to produce all the materials needed for this, including purified dog IgG standards.

3.3. Materials and methods

3.3.1. Production of monoclonal antibodies against canine IgG subclasses

Hybridoma cells B6, E5, A3G4, and A5 produce mouse mAbs against canine IgG subclasses IgG1, IgG2, IgG3 and IgG4 respectively. They were previously developed and kindly provided by Professor Michael Day (School of Veterinary Science, University of Bristol) (Mazza *et al*., 1993, 1994b).

These hybridomas were produced from the fusion of splenocytes, obtained from mice previously immunized with the respective purified dog IgG subclasses, and P3X63Ag8.653 mouse BALB/c myeloma cells (Mazza *et al*., 1994b). The A5 hybridoma, specific for IgG4 and producing a mouse IgG1 antibody (Mazza *et al*., 1993, 1994b) was grown as below and mAbs purified from saturated supernatant.

3.3.1.1. Hybridoma cell culture

i. Media Preparation

Hybridoma cells were grown using growth media consisting of 50 % (v/v) RPMI 1640 (Sigma-Aldrich) supplemented with 15 % (v/v) heat inactivated foetal bovine serum (FBS) (Gibco®) and 50 % (v/v) Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) medium supplemented with 10 % (v/v) heat inactivated FBS (Gibco®). The medium was supplemented also with 2 mM L-glutamine (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), 100 IU/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma-Aldrich), 0.05 mM β-mercaptoethanol (Sigma-Aldrich), and 0.5 µg/ml Amphotericin B (Gibco®). Conditioned media, obtained from the centrifugation and filtration (0.22 µm filter) of supernatant obtained from the culture of BV-2 murine microglial cell line, was added to 10 % (v/v) to the cell media to provide cell growth factors.

Media formulation and cell manipulation were carried out under a sterile laminar flow microbiological safety cabinet (Bio 2+, Envair).

ii. Thawing of hybridoma cells

Twenty-four hours before hybridoma cells were thawed, mouse peritoneal macrophages were seeded in 24-well plates at a concentration of 1 x 10^5 cells/well (volume of 0.5 ml) using RPMI 1640 (Sigma-Aldrich) medium supplemented with 10 % FBS in 24 well plates.

Hybridoma cells were removed from a liquid nitrogen storage tank. Cells were immediately thawed in a 37 ° C in a water bath with continuous and mild agitation for approximately 1 min. Cells were transferred to a conical tube and 10 ml cold DMEM was added and centrifuged at 400 x ***g*** for 5 min at 8 ° C. After centrifugation, the supernatant was discarded and 2 ml of media was added to the cell pellet to resuspend the cells. Subsequently, cell density was measured by counting the cells using a haemocytometer with Trypan blue.

Cells were added in a ½ dilution series to the 24-well plate, with 1 ml of warm medium, with an initial seeding density of 5 x 10^5 cells/ml. Cells were then incubated at 37 ° C and 5 % CO_2 in a humidified atmosphere using a cell culture incubator (Incusafe MCO-17AIC, Sanyo).

iii. Cell culture and passage

Hybridoma cells were cultured in fresh medium as described above. When cells became confluent (approximately 1 x 10^6 cells/ml), they were seeded in 25 cm^2, 75 cm^2 or 175 cm^2 tissue culture flasks supplied by Corning (UK), at a concentration of 3 x 10^5 cells/ml. Cells were grown in the same conditions as described previously.

iv. Saturated supernatant

Cells were allowed to overgrow in 175 cm^2 tissue culture flasks containing 200 ml of medium until 50-80 % of the cells were dead. This was observed by phase-contrast microscopy. Then, cells and medium were centrifuged at 1,139 x ***g*** for 10 min at 4 ° C to remove cell debris. Finally, the supernatant was divided into aliquots and stored at -20 ° C.

3.3.1.2. Testing hybridoma antibody production

Hybridoma antibody production was tested using intracellular flow cytometry staining. Briefly, 3 x 10^6 hybridoma cells were incubated with 1 ml of 4 % formaldehyde solution for 15 min at room temperature. Cells were pelleted (400 x ***g***, 5 min) and washed in PBS with 0.1 % bovine serum albumin and 0.01 % sodium azide (PBA), then treated with 0.1 %

saponin in PBA (saponin/PBA) for 30 min at room temperature. After detergent treatment, cells were incubated for 30 min at room temperature in the dark with polyclonal rabbit $F(ab')_2$ anti-mouse immunoglobulins:FITC (Code: F0313, Dako, 1:100 dilution), polyclonal rabbit $F(ab')_2$ anti-mouse immunoglobulins:RPE (Code: R0439, Dako, 1:50 dilution), or goat anti-mouse IgG1:FITC (Code: M32001, Caltag Laboratories, 1:100 dilution). Goat IgG:FITC (Code: F7381, Sigma, 1:500 dilution) was used as a negative control. All antibodies were prepared in saponin/PBA. Then, cells were washed twice in saponin/PBA and centrifuged at 1,139 x ***g*** for 3 min. Finally, cells were resuspended in 150 µl of PBA and analysed by flow cytometry using a BD Accuri™ C6 flow cytometer (BD Biosciences).

3.3.1.3. Antibody Purification using a Protein G column

Saturated cell culture supernatant, obtained from the A5 hybridoma, was pre-filtered by using a 0.45 µm Minisart® filter (Sartorius) and then purified by affinity chromatography using a Protein G column (HiTrap® Protein G High Performance column, GE Healthcare). Briefly, the Protein G column was washed with 5 volumes of 20 mM NaH_2PO_4 (pH 7.0). The saturated supernatant was then loaded onto the column. The column was washed with 5 volumes of 20 mM NaH_2PO_4 (pH 7.0). Subsequently, the antibody was eluted and collected from the column using 3 volumes of 0.1 M glycine (pH 2.7). Eluted fractions were collected in 1 ml tubes containing 40 µl of 1 M Tris-HCl (pH 9.0). The column was immediately washed using 5 volumes of 20 mM NaH_2PO_4 (pH 7.0) and 5 volumes of 20 % Ethanol and stored at 4 ° C. Fractions containing mAb were determined by absorption at OD_{280} using a NanoDrop® Spectrophotometer (ND-1000, Thermo Scientific). Those with mAb were pooled and finally, the concentration of purified antibody was measured by OD_{280}. Pooled mAb solution was dialysed overnight at 4 ° C against 2 litres of PBS and the concentration re-determined. The antibody was aliquoted and stored at -80 ° C.

3.3.1.4. Reactivity of mAb against canine IgG4 using immunohistochemistry

The functionality of the monoclonal antibody against canine IgG4 (A5) was also evaluated using immunohistochemistry by determining staining of IgG4-positive plasma cells in canine lymph node sections. Enzymatic antigen retrieval using Proteinase K (Dako) was used according to the manufacturer's instructions to optimise the antibody staining intensity. Immunohistochemistry for the detection of IgG4 positive plasma cells was carried out using an automated slide processing system (Autostainer plus Link, Dako). Endogenous peroxidase

activity was blocked using peroxidase-blocking solution (EnVision™ FLEX Peroxidase-Blocking Reagent, Dako) that was incubated for 10 min. Subsequently, canine lymph node sections were incubated for 1 h with mAb A5 against canine IgG4 (1.9 mg/ml) at 1:100, 1:200, 1:400, 1:800 and 1:1600 dilutions in antibody diluent (EnVision™ FLEX Antibody Diluent, Dako). Negative control consisted of histological sections of canine lymph nodes incubated without primary antibodies, but with antibody diluent (EnVision™ FLEX Antibody Diluent, Dako) and isotype-matched non-immune antibody ZG4 mouse IgG1 (Lowe *et al.*, 1982), using the same concentration as the primary antibodies. Goat secondary antibody against mouse immunoglobulin conjugated with horseradish peroxidase (EnVision™ FLEX /HRP, Dako) was applied for 30 min. Chromogen diaminobenzidine (EnVision™ FLEX DAB+ Chromogen, Dako) was used as substrate to visualise the immunostaining. Tissue sections were counterstained using haematoxylin (EnVision™ FLEX Haematoxylin, Dako). Between each step a rinse with wash buffer (EnVision™ FLEX Wash Buffer 20x) was carried out. Subsequently, the sections were dehydrated, cleared, and mounted. Finally, canine lymph node samples were analysed by light microscopy (Eclipse E400 Biological Microscope, Nikon), determining the staining of IgG4 positive plasma cells. Bright field images were recorded on a DP20-E5 Microscope digital camera (Olympus).

3.3.2. ELISA test for the detection of IgG4 in canine plasma

Frozen plasma (canine cryo-supernatant in CPD anticoagulant) from normal dogs, kindly donated by Pet Blood Bank UK, was used to validate the anti-canine IgG4 mAb using two capture enzyme-linked immunosorbent assays (ELISAs).

i. Sandwich or capture direct ELISA – capture anti-dog IgG4 (A5)

ELISA plates (Maxisorp, Nunc) were coated with 100 µl/well of anti-canine IgG4 (A5) mAb (incubated overnight at 4 °C), as a capture antibody. Several concentrations (0.19 to 10 µg/ml in PBS) in a ½ dilution series were tested. After incubation, plates were washed 4 times with 0.05 % Tween 20/PBS, blocked with 0.25 % gelatin (Bio-Rad) in PBS, and incubated for 30 min at room temperature. After washing again with 0.05 % Tween 20/PBS, 100 µl/well of dog plasma in a ½ dilution series (diluted in PBS/gelatin as above) were incubated for 1 h at room temperature. Control wells with no dog plasma but PBS/gelatin were used. After plasma incubation, plates were washed and goat anti-dog IgG:HRP (horseradish peroxidase) conjugated detection antibody (PA1-29738, Thermo-Fisher) was incubated in dilution series (1:5,000-1:20,000) for 1 h at room temperature. After washing,

TMB (3,3', 5,5;-tetramethylbenzidine) chromogen solution (Life Technologies) was used as a substrate for HRP. Then, 100 µl of TMB were added to the wells, and plates were incubated in the dark for 15-30 min at room temperature. Optical densities were measured at OD_{650} in an iMark microplate absorbance reader (Bio-Rad).

ii. Sandwich or capture indirect ELISA – capture anti-dog IgG

ELISA plates (Maxisorp, Nunc) were incubated overnight at 4 ° C with 100 µl/well of goat anti-dog IgG (Fc) (SAB3700101-2mg, Sigma) in a dilution series ranging from a concentration of 0.10 to 5.0 µg/ml PBS. After incubation, plates were washed and blocked using the same procedure described for the capture direct ELISA. Later, plates were incubated for 1 h at room temperature with dog plasma (100 µl/well) using ½ dilution series in PBS/gelatin. Then plates were washed again and incubated for 1 h at room temperature with 100 µl/well mouse anti-dog IgG4 (A5) mAb at a concentration of 2 µg/ml PBS/gelatin. After washing, plates were incubated with 100 µl/well of goat anti-mouse IgG (Fc) HRP conjugated (A2554, Sigma) as a detection antibody, using ½ serial dilutions ranging from 1:10,000 to 1:320,000. Then plates were washed and TMB was incubated, as described before. Finally, optical densities were measured at OD650 nm.

3.3.3. Isolation of canine IgG4 from dog plasma

Several attempts for purifying canine IgG4 from dog plasma (Pet Blood Bank UK) using affinity chromatography were conducted.

i. Isolation of IgG4 from dog plasma using affinity chromatography

Purification of dog IgG4 was performed as described in Mazza *et al*. (1993) with minor modifications. Briefly, in order to obtain separated crude dog immunoglobulin, 20 ml of normal dog plasma was initially precipitated at a 45 % saturation of ammonium sulphate at 20 ° C. The obtained Ig fraction was then dialysed into 0.1 M sodium phosphate pH 8.0, overnight at 4 ° C, and protein concentration was measured using a NanoDrop® Spectrophotometer (ND-1000, Thermo Scientific) at 280 nm.

Then, 2 ml of this precipitated dog Ig (30 mg) were subjected to protein A and then protein G affinity chromatography performed at 4 ° C using the fast protein liquid chromatography (FPLC) system (ÄKTAFPLC, GE Healthcare Life Sciences).

Protein A affinity chromatography was carried out on a ProSep®-vA Ultra (4 ml column volume) (Millipore), and Protein G affinity chromatography on a ProSep®-G (3.5 ml column volume) (Millipore) porous glass chromatography resin. Starting material was always 0.22 µm filtered (Millex GP filter unit, Millipore) prior to being loaded onto the chromatography columns.

The columns were connected to a monitor for the combined measurement of UV absorption, pH and conductivity (UPC-900, Amersham Biosciences) and the UNICORN 5.31 software (GE Healthcare Life Sciences) was used to control and supervise the chromatography system. The starting elution buffer (0 % gradient) was 0.1 M sodium phosphate (pH 8.0), and the final elution buffer (100 % gradient) 0.1 M sodium citrate (pH 2.5). All chromatographic buffers were formulated using high purity grade reagents (Sigma-Aldrich) in Milli-Q water, and were 0.2 µm filtered before use using a Nalgene Rapid-Flow™ sterile disposable filter unit (Thermo Scientific). The start material was loaded onto the column (1 vol. of precipitated dog Ig in 10 vol. of starting buffer) at 0.5 ml/min then the column was washed with 5 column volumes of 0.1 M sodium phosphate (pH 8.0).

Two ml fractions were collected using a Gilson ® FC 203 fraction collector, at a flow rate of 0.5 ml/min. Neutralising buffer 2 M Tris-HCL, pH 8.5 was added to the eluted fractions and the protein peaks were detected by reading each fraction at OD_{280} using a ND-1000 NanoDrop® Spectrophotometer.

After Protein A and G chromatography, the presence of IgG4 was tested in the collected fractions using a sandwich or capture indirect ELISA as described above. Fractions that showed higher OD were pooled, dialysed overnight at 4 ° C in 0.1 M sodium phosphate (pH 8.0). In order to confirm purity, 3 µg of pooled fractions containing the purified dog IgG4 were run on a 4-12 % Bis-Tris SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis), and stained with Coomassie Blue. Then, the gels were submitted to the Cambridge Centre for Proteomics at the Biochemistry Department, University of Cambridge for mass spectrometry analysis of the heavy chain band.

ii. Affinity purification with A5 mAb linked onto column beads

Preparation of the affinity column:

Mouse anti-dog IgG4 monoclonal antibody (A5) was coupled onto Ultralink™ Biosupport beads (bisacrylamide/azlactone copolymer) (Thermo Scientific) as described by the manufacturer. Briefly, 1.9 mg of A5 mAb was dissolved in 2 ml of 1.2 M sodium citrate, 0.2 M MOPS (3-(*N*-morpholino)propanesulfonic acid) (pH 7.5). Then, the antibody solution was added directly to 0.25 g of dry Ultralink™ Biosupport beads, and mixed for two hours at room temperature. This was followed by centrifugation for 10 min at 1,200 x ***g*** and supernatant was removed. Then, any remaining reactive sites on the beads were blocked by incubation with 3 M ethanolamine (pH 9.0) for 2.5 h at room temperature. Finally, the beads were packed in a column and washed thoroughly with phosphate buffered saline (1 M NaCl, 25 mM phosphate, pH 7.0) and stored at 4 ° C.

IgG4 purification using the coupled column:

The mouse anti-dog IgG4 mAb (A5) Ultralink™ Biosupport column was connected to a peristaltic pump (Watson-Marlow 520S) and set at 2.0 rpm for washing with 5 volumes of PBS/EDTA before being loaded with the dog IgG at 0.2 rpm. Starting material was 6.5 ml of dog IgG (4 mg) previously subjected to ProSep G affinity column (as above) and containing 10 mM EDTA, which had been filtered using a 0.22 µm Millex GP filter unit (Millipore). Then it was equilibrated in PBS, 10 mM EDTA (pH 7.0) and loaded onto the column that was washed again with 5 volumes of PBS/EDTA at 0.2 rpm. Then 6 volumes of 0.1 M sodium phosphate (pH 8.0) were added at 4.1 rpm. Then, after the addition of 5 volumes of PBS/EDTA, elution buffer consisting of 0.1 M Sodium Citrate (pH 2.5) was added at 1.0 rpm. Subsequently, eluted fractions were obtained and collected in 1 ml tubes containing 200 µl Tris (pH 8.5) as neutralising buffer. Fractions containing protein were determined by absorption at OD_{280} using a NanoDrop® Spectrophotometer.

iii. Affinity purification with anti-dog IgG1 linked on to column beads

This protocol used an affinity column coupled to a goat anti-dog IgG1 antibody (Bethyl labs) in an attempt to bind both IgG1 and IgG4 selectively as showed in Bergeron *et al*. (2014). It then used a Protein A column to remove IgG1 with high salt (3 M NaCl) buffer. This second step would allow IgG1 to remain bound to the column, so in theory the flow through would be mostly IgG4.

The preparation of the affinity column linked 1.5 mg of goat anti-dog IgG1 (Bethyl Laboratories) previously dialysed into 0.6 M sodium citrate, 0.1M MOPS (pH 7.5) to 0.19 g of Ultralink™ Biosupport beads (Thermo Scientific), following the steps described previously. The size of the prepared column was 1.5 ml. After preparation of the column, 8 ml of dog serum, previously dialysed overnight into PBS, 10 mM EDTA (pH 7.0) and filtered, was loaded onto the affinity column. This was a low-pressure purification carried out at 4 ° C using a peristaltic pump unit (Watson Marlow 520s) to deliver the serum sample at a flow rate of 0.1 ml/min. Then, the column was washed with 5 column volumes of 0.1 M sodium phosphate (pH 8.0) at a flow rate of 1 ml/min. The elution buffer consisted of 0.2 M glycine (pH 2.5) (1 ml/min). Eluted fractions were collected in 1 ml tubes containing 100 µl 2 M Tris/HCL (pH 8.6). Protein detection was determined by absorption at OD_{280} using a NanoDrop® Spectrophotometer.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Purified dog IgG4 (3 µg) and a pre-stained protein standard used as molecular weight markers (Novex™ Sharp, Invitrogen) were loaded on NuPAGE™ 4–12 % Bis-Tris, 1.0 mm SDS-PAGE gels (Invitrogen) wells. The sample and protein standard were previously mixed with 4X sample buffer containing 106 mM Tris-HCl, 141 mM Tris base, 2.2 % SDS, 10 % glycerol, 0.51 mM EDTA, 50 mM DTT, 0.22 mM Commassie Blue, 0.175 mM Phenol Red (pH 8.5), diluted to obtain a final concentration of 1X, and then heat-denatured at 70 ° C for 10 min (PTC-100 Programmable Thermal Controller, MJ Research). Afterwards, electrophoresis was carried out at 200 V for 45 min in MES-SDS running buffer (50 mM MES (2-(*N*-morpholino)ethanesulfonic acid) , 50 mM Tris base, 0.1 % SDS, 1 mM EDTA (ethylenediaminetetraacetic acid), pH 7.3) using an XCell SureLock™ Mini-Cell Apparatus (Invitrogen), which was connected to an EPS 600 Electrophoresis Power Supply (Pharmacia Biotech). After electrophoresis, the gels were stained using Coomassie blue stain or transferred to nitrocellulose for western blot analysis. The Coomassie staining method consisted of adding the gels into fixing solution (50 % methanol and 10 % glacial acetic acid) for 1 h with gentle agitation and then staining them using staining solution (0.5 % Coomassie Brilliant Blue R-250, 45 % methanol, 10 % acetic acid) for 20 min with gentle agitation at room temperature. Finally, the gels were distained in destaining solution (20 % methanol, 7 % acetic acid) for 2-3 h at room temperature. Afterwards, each gel was placed in a 50 ml Falcon tube containing 2 ml of distilled water and stored at 4 ° C for further liquid

chromatography-tandem mass spectrometry (LC-MS/MS) analysis of protein bands. LC-MS/MS was carried out at the Cambridge Centre for Proteomics, Biochemistry Department, University of Cambridge. Peptide and protein identification were performed using MASCOT software (Matrix Science Ltd).

Western blot analysis

After protein separation on gel electrophoresis, the proteins were transferred onto an Immuno-Blot PVDF membrane (Bio-Rad) for 1 h at 350 mA, 60 W. The transfer buffer consisted of 25 mM Tris-Base, 200 mM glycine and 20 % methanol. The membranes were then blocked overnight at 4 ° C in blocking buffer containing 5 % skimmed milk powder in PBS, 0.05 % Tween 20, and 10 mM EDTA. The membranes were then washed with TBST (tris-buffered saline, 0.1% Tween 20) 3 times with agitation for 5 min. Goat anti-dog IgG (H+L)-HRP (Thermofisher) (1/5,000 dilution) for membrane A and donkey anti-sheep IgG (H+L)-HRP (Invitrogen) (1/2,000 dilution) for membrane B, prepared in 0.5 % milk/TBST were incubated for 1 h at room temperature in a roller mixer (Fig. 3.9). This was followed by 3 washes with TBST for 5 min each. Next, the proteins were detected after the addition of chemiluminescence substrates ECL™ (Western Blotting Detection Reagents Kit, GE Healthcare). Finally, reactive protein bands were visualised using a Chemidoc MP Imaging System (Bio-Rad).

3.3.4 Competitive ELISA for the quantitative measurement of canine serum IgG4 levels

A competitive inhibition ELISA able to quantitatively measure serum canine IgG4 was validated. A pooled plasma sample from normal dogs, kindly donated by Pet Blood Bank UK, was used to standardise protocols. The protocol was based on Mazza *et al.* (1994b) with minor modifications. In order to develop this assay, standard curves for IgG4 using isolated dog IgG4 fractions (above section 3.3.3) were produced**.** Briefly, ELISA plates (Maxisorp, Nunc) were coated with 100 µl/well of dog IgG4 (1 µg/ml), overnight at 4 ° C in PBS. The plates were then washed five times in PBS containing 0.05 % Tween 20 (PBS/T), and blocked by incubation with 200 µl/well of PBS/T containing 5 % marvel milk powder for 1 h at room temperature. Plates were washed again and afterwards, 75 µl of dog IgG4 at 271 µg/ml was added to duplicate wells and they were subsequently serially diluted (1/3 in PBS/T) across the plate, obtaining 50 µl/well as the final volume. In addition, 75 µl of normal dog plasma

donated by Pet Blood Bank UK (diluted 1/100 in PBST/1% gelatine) were added in duplicates into the respective wells, and then serially diluted at 1/3 across the plate leaving also a final volume of 50 µl/well. This step was quickly followed by the addition of 50 µl/well of mAb anti-dog IgG4 (A5) at 1 µg/ml. Control wells with no IgG4 but PBS/T were included. After the mAb addition, the plates were incubated in continuous gentle agitation on a microplate shaker (Titramax 100, Heidolph) for 4 h at room temperature. The plates were then washed five times as previously mentioned, and 100 µl of goat anti-mouse IgG (Fc)-HRP (Sigma) were added to each well at a 1/20,000 dilution in PBS/T. The plates were incubated for one hour at room temperature, washed and 100 µl of TMB substrate (Life Technologies) were added. The absorbance at 650 nm was read in a microplate absorbance reader after incubation in the dark at room temperature for 15 to 30 minutes. The IgG4 concentrations were determined from the standard curve obtained in each ELISA and corrected for the appropriate sample dilution factors.

3.3.5 Competitive ELISA for the quantitative measurement of canine total IgG serum levels

Total dog IgG serum levels were measured using a non-commercial competitive ELISA as follows. Microtitre ELISA plates (Maxisorp, Nunc) were coated with 100 µl/well of dog IgG at a concentration of 1 µg/ml, previously purified by gel filtration chromatography, and incubated overnight at 4 ° C. After incubation, plates were washed 5 times with washing solution containing 0.05 % Tween 20/PBS. Then, unbound sites were blocked with 200 µl/well of PBST containing 5 % marvel milk powder, and incubated for 1 h at room temperature. After the washing step, 75 µl of purified dog IgG standard (271 µg/ml) were added to duplicate wells and then serially diluted (1/3 in PBST with 1 % gelatine) across the plate, leaving a final volume of 50 µl/well. In addition, 75 µl of plasma or serum samples (diluted 1/100 in PBST/1% gelatine) were added in duplicates into the respective wells, and then serially diluted at 1/3 across the plate leaving also a final volume of 50 µl/well. This step was immediately followed by the addition of 50 µl/well of rabbit anti-dog IgG (Sigma-Aldrich) in a dilution of 1/10,000 in PBST/gelatine 1%. After 4 h of incubation at room temperature on a plate shaker, the plates were washed and then 100 µl/well of goat anti-rabbit (H+L) IgG-HRP (Thermo Fisher) in a dilution of 1/10,000 were incubated for 1 h at room temperature. After washing, 100 µl/well of TMB were added and incubated for 15 min at room temperature and optical densities were measured at 655 nm.

3.4. Results

3.4.1. Production of mouse anti-canine IgG4 mAb (A5)

As described previously, mouse anti-canine IgG4 mAb was produced from A5 hybridoma cells that were kindly donated by Professor Michael Day, University of Bristol (Mazza e*t al*., 1993). A5 cells were tested for antibody production by intracellular staining using different anti-mouse immunoglobulin reagents (Fig. 3.1). The data indicated that the A5 cells contained mouse immunoglobulin that was mouse IgG1 as published. The polyclonal rabbit F(ab')2 anti-mouse Immunoglobulins:RPE displayed the greatest staining (98.4 % positive cells).

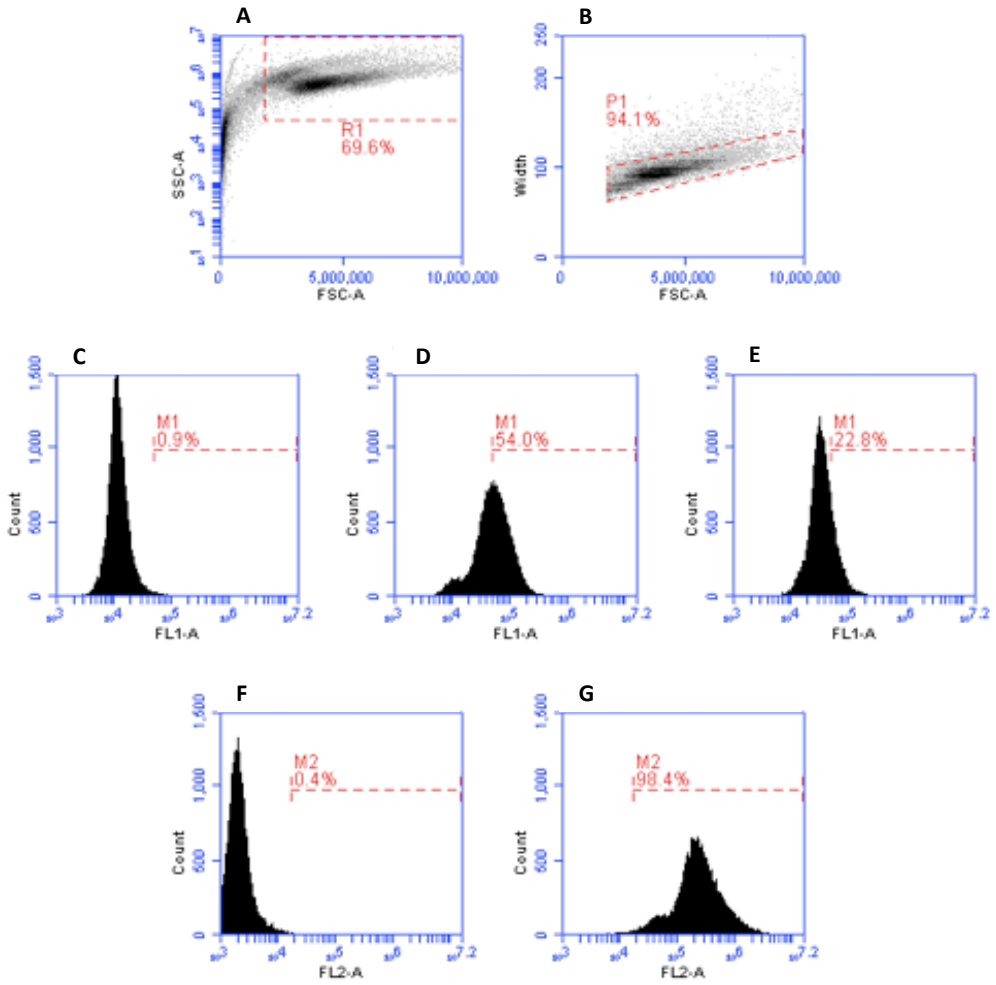

Figure 3.1: Screening of mouse A5 hybridoma cells for antibody production by intracellular staining for immunoglobulin. (A) Gating of cells by forward and side scatter which was then used in (B) Gating to exclude doublets using forward scatter area *vs* FSC-width. Histograms of fluorescence in the green (FL1A-FITC) and orange (FL2A-PE) channels: (D) anti-mouse Ig:FITC; (E) anti-mouse IgG1:FITC; (G) anti-mouse Ig:PE. In panels C and F, the fluorescence of the negative control (goat Ig:FITC) is reported for comparison.

Saturated supernatant was produced after the hybridoma cells were allowed to over-grow. The final amount of saturated supernatant obtained was 600 ml. This amount was collected during 12 cell passages for about 3 months. This mAb was purified from the supernatant using a Protein G column. After purification, the amount of antibody obtained was 15 mg of mouse anti-canine IgG4 in 6 ml. The mAb solution was dialysed into PBS and after dialysis, the protein concentration had reduced from 2.5 mg/ml to 1.9 mg/ml.

The mAb hybridoma E5 (mouse anti-canine IgG2) was also able to grow and produced purified antibody as above of 1.5 mg/ml. The production of mouse anti-canine IgG1 and IgG3 mAbs was not achieved. Hybridomas specific for these subtypes (B6 and A3G4, respectively) did not grow under the same conditions as for A5 and E5. Attempts to resuscitate and grow these hybridoma cells were done using feeder peritoneal macrophages and the addition of conditioned medium from a macrophage cell line but they were not able to grow and expand *in vitro*. The cell stocks were old (had been frozen for >20 years) and appeared not to grow in the laboratory of Professor Day either (personal communication).

3.4.2. Reactivity of mAb against canine IgG4 using immunohistochemistry

A5 mouse anti-canine IgG4 mAb was tested for reactivity in an immunohistochemistry assay in canine lymph nodes. All A5 dilutions tested were capable of detecting $IgG4^+$ plasma cells. However, the 1:100 dilution showed the best staining and thus was selected for future analysis using formalin-fixed tissues (Fig. 3.2).

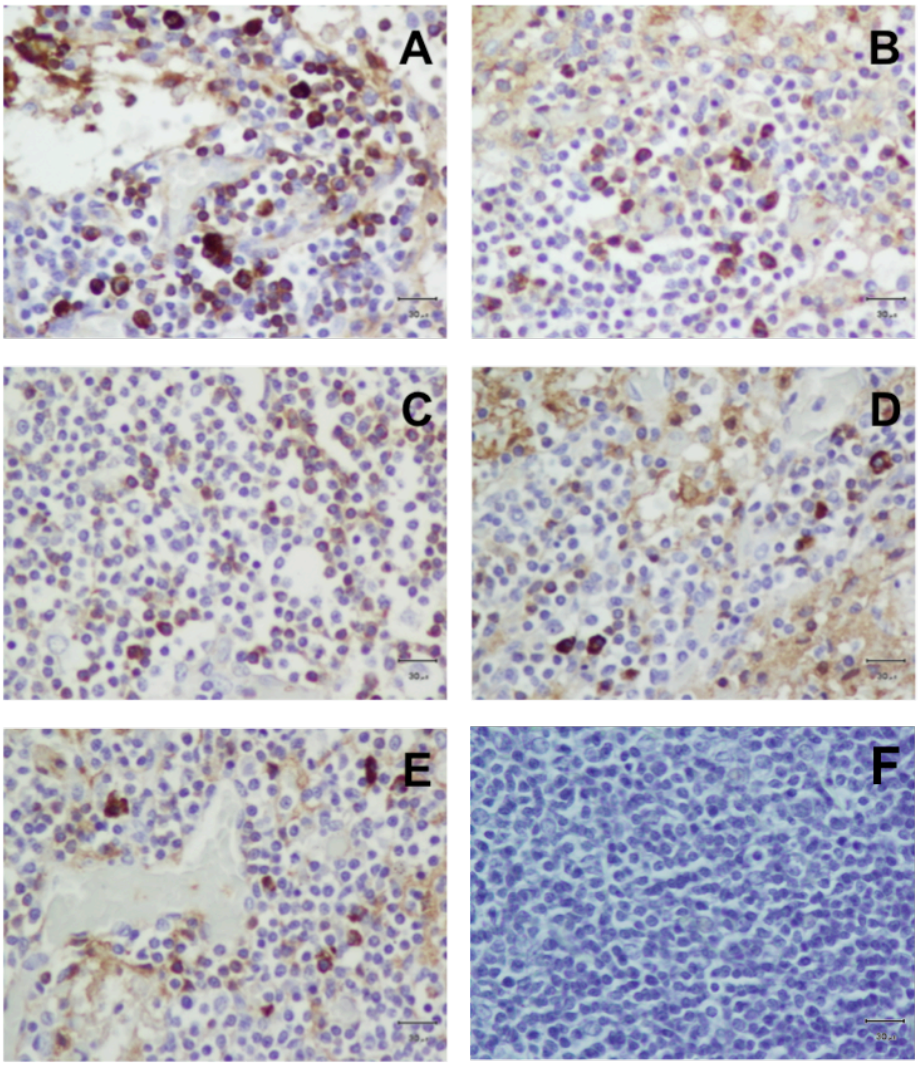

Figure 3.2: A5 mouse anti-canine IgG4 mAb detecting IgG4$^+$ plasma cells in sections of canine lymph nodes. A5 mAb was tested by immunohistochemistry on canine lymph nodes using a dilution series (A) 1:100, (B) 1:200, (C) 1:400, (D) 1:800, (E) 1:1600 and (F) negative control with isotype-matched non-immune antibody ZG4 mouse IgG1 (1:100). Note a more intense staining in (A) using a dilution of 1:100. Bar= 30 µm, magnification x400.

3.4.3. Validation of ELISAs for the detection of IgG4 in canine serum

i. Sandwich or capture direct ELISA – capture anti-canine IgG4

A5 mouse anti-canine IgG4 was able to detect IgG4 in canine serum when used as the capture antibody in a direct sandwich ELISA. A chequerboard test was initially trialled and the combination of antibody concentrations that showed the greatest reactivity with dog serum at 1:20 after subtracting blank control values was: 1.25 µg A5/ml as a capture antibody, and 1:10,000 dilution of detection antibody (anti-dog HRP) (Fig. 3.3).

ii. Sandwich or capture indirect ELISA – capture anti-canine IgG

An indirect capture ELISA in which A5 mouse-anti canine IgG4 was used to detect captured IgG4 was also able to detect IgG4 in canine serum. The combination of antibody and serum concentrations that showed greatest reactivity (subtracting blank control values) using serum at 1:20 and 2 µg A5/ml was: 0.63 µg goat anti-dog IgG (Fc specific) as a capture antibody, and 1:20,000 goat anti-mouse IgG-HRP (Fig. 3.4).

The latter ELISA method used less amount of A5 antibody compared with the sandwich or capture direct ELISA, for this reason it was selected as a routine ELISA to determine the presence of IgG4 in further serum IgG4 isolation attempts.

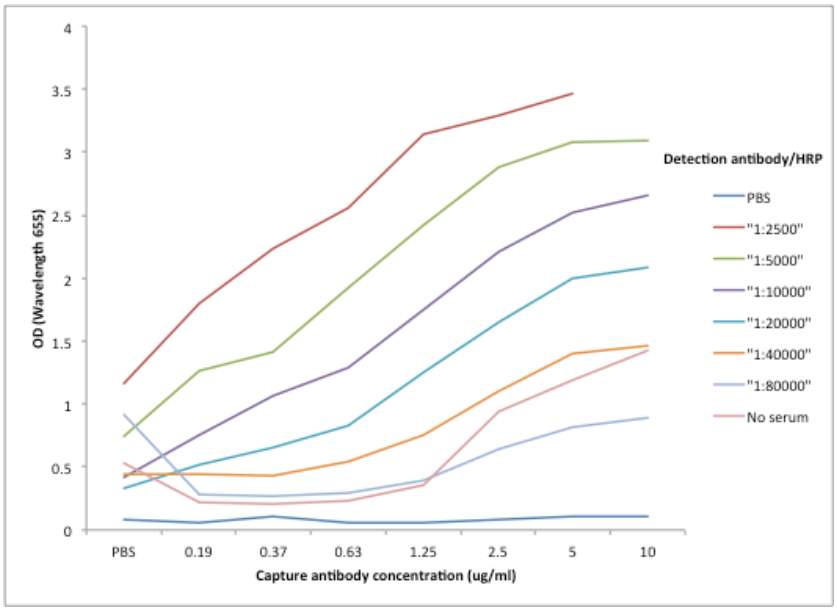

Figure 3.3: Sandwich ELISA trial for the detection of IgG4 in dog plasma - canine IgG4 capture. Purified A5 mouse-anti canine IgG4 was used as the capture antibody at different coating concentrations (0.19- 10 µg/ml), dog plasma at 1:20, and goat anti-canine IgG:HRP in dilutions from 1:2,500 to 1:80,000. Controls where capture and detection antibodies and serum were omitted are shown.

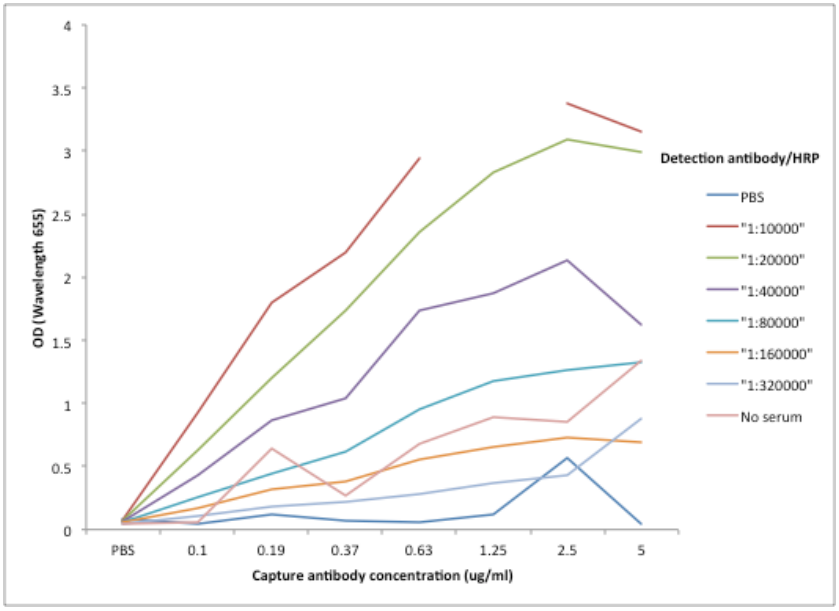

Figure 3.4: Sandwich ELISA trial for the detection of IgG4 in dog plasma - canine IgG capture. Optical densities are shown when using goat anti-canine IgG as a capture antibody in different concentrations (0.1 - 5 µg/ml), dog plasma at 1:20, 2 µg/ml of A5 mouse-anti canine IgG4, and goat anti-mouse IgG:HRP in dilutions from 1:10,000 to 1:320,000. Controls where capture and detection antibodies and serum were omitted are shown.

3.4.4. Attempts to purify canine IgG4

i. Attempt 1 - Isolation of 'IgG4' from dog plasma using affinity chromatography

Dog immunoglobulins were initially separated from dog plasma by precipitation in 45 % ammonium sulphate, obtaining a concentration of 15.45 mg/ml after overnight dialysis in phosphate buffer. Afterwards, 2 ml of crude immunoglobulins (30.9 mg) were purified on protein A affinity chromatography, obtaining 3 peaks (Fig. 3.5A), similar to those described by Mazza *et al*. (1993). Peak 1 consisted of flow-through material, which did not bind to protein A. Peaks 2 and 3 contained bound material, which was eluted by using 0.1 M sodium citrate (pH 2.5). Sandwich ELISAs were performed in order to determine the presence of dog 'IgG4' in the eluted fractions, using mAb A5 to capture the antibody (Fig. 3.5B). The ELISA results showed a major peak of reactivity from the eluted fractions that belonged to peak 3 from the protein A purification. These eluted fractions were pooled, then dialysed into phosphate buffer and subsequently subjected to protein G affinity chromatography (Fig. 3.5C). Most protein bound onto Protein G and was eluted as a major peak with shoulders after fraction number 15. Fractions after 15 were also tested for the presence of dog 'IgG4' by sandwich ELISA using anti-canine IgG to capture the Ig (Fig. 3.5D). Fractions 16-23 were

pooled and a concentration of 0.61 mg/ml of dog 'IgG4' was obtained after dialysis in phosphate buffer.

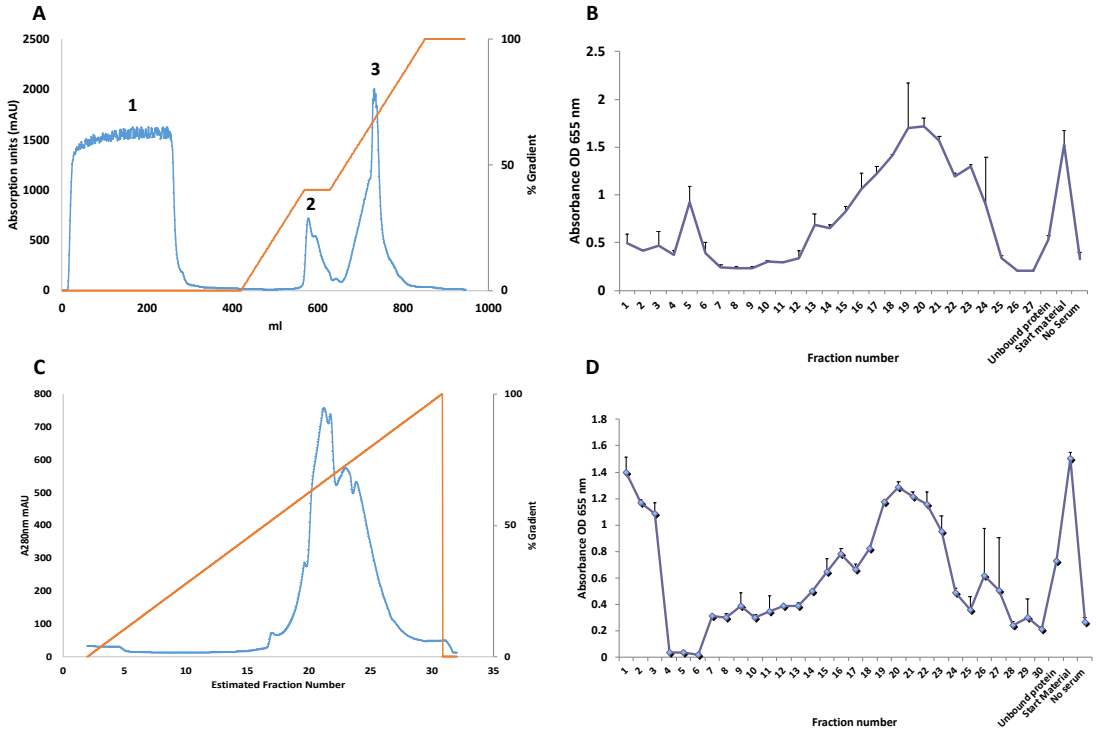

Figure 3.5: Purification of IgG4 from dog plasma. A) Protein A affinity chromatography of 2 ml dog plasma previously precipitated in 45 % ammonium sulphate. Peak 1 was material, which did not bind to protein A. Peaks 2, and 3 were bound protein, which was eluted after using 0.1M sodium citrate (pH 2.5); B) Detection of dog IgG4 by a sandwich ELISA (A5 mAb capture) in the eluted fractions after protein A affinity chromatography; C) Gradient fractions with higher ODs on ELISA in B (fractions number 15-24) were pooled, dialysed, and then purified again, using protein G affinity chromatography, obtaining a single protein peak after elution; D) fractions obtained in C were tested again for IgG4 by a sandwich ELISA (anti-canine IgG capture). Fractions 16-23 were pooled, and dialysed. Blue line: Absorbance. Orange line: % gradient.

SDS-PAGE of purified dog 'IgG4' from above was performed, obtaining 3 bands of different molecular weight (MW) (Fig. 3.6). Bands of approximately 53 kDa and 25 kDa represent the IgG heavy (H) and light (L) chains, respectively. Bands of approximately 78 kDa suggest the formation of half molecules (H-L dimer). Only the heavy chain band was sent for mass spectrometry analysis.

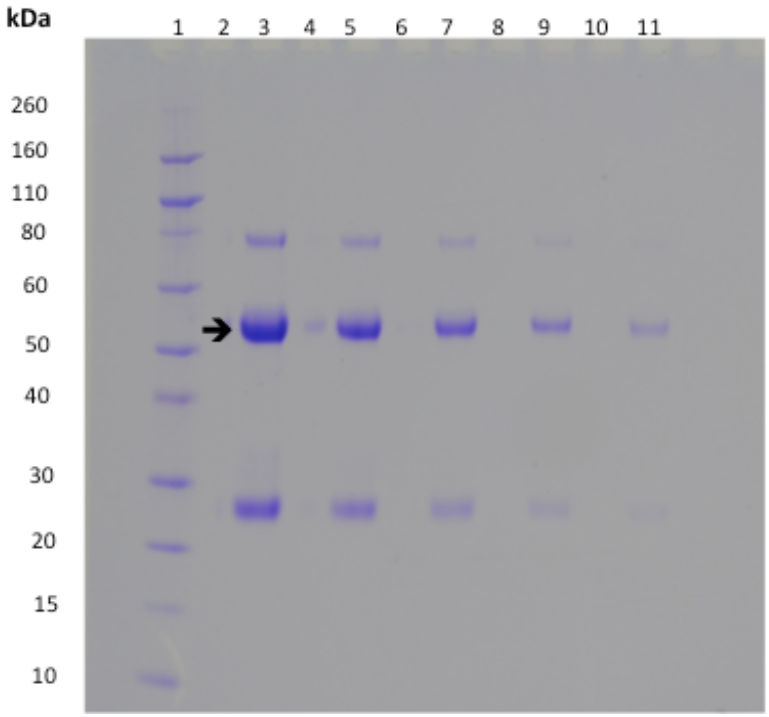

Figure 3.6: 4-12% Bis-Tris SDS-PAGE of purified dog 'IgG4' using Protein A and Protein G affinity chromatography loaded in 5 gel tracks. Track 1: pre-stained protein standard. Track 3: 4.5 µg purified dog IgG4. Subsequent tracks contain 1/2 serial dilutions of the same protein. Arrow indicates the IgG heavy chain (MW 53 kDa approx.) sent for LC-MS/MS analysis. Bands of approximately MW 25 kDa represent the IgG light chains. Bands of approximately 78 kDa suggest the formation of half molecules (H-L dimer).

LC-MS/MS analysis identified the gel band as a mixture of dog IgG2 and IgG3 heavy chain proteins, with 50 % and 31 % of protein coverage respectively (Figs. 3.7A and 3.7B). Dog IgG1 heavy chain was not identified as a component of the gel band. Only a few peptides were identified as a match to IgG4 heavy chain protein (Fig. 3.7C) and for this reason other methods for purifying dog IgG4 were evaluated. The results were also analysed against a common contaminant database on the MASCOT search. Contaminants such as *Homo sapiens* Cytoskeletal Keratin type I and II, *Bos taurus* serum albumin and Beta-casein were also identified as components of the analysed gel band.

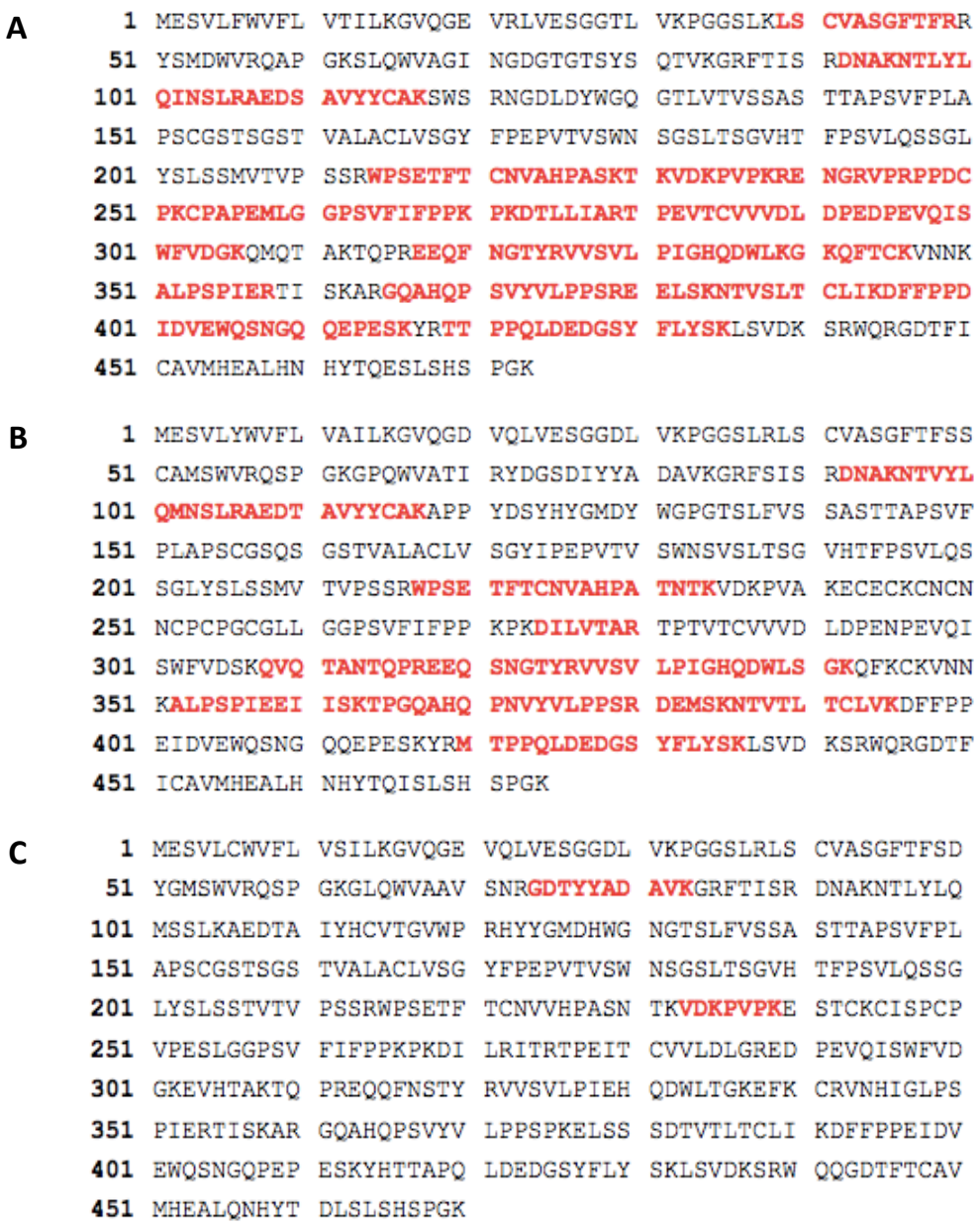

Figure 3.7: MASCOT search results for dog immunoglobulin gamma heavy chain 2 (panel A), 3 (panel B) and 4 (panel C) in the purified heavy chain of approximately 53 kDa. Results obtained after LC-MS/MS analysis (Cambridge Centre for Proteomics, Biochemistry Department, University of Cambridge), showed protein sequence coverage of 50 %, 31 % and 3 % for dog IgG2, IgG3 and IgG4, respectively. Matched peptides with *Canis lupus familiaris* immunoglobulin gamma heavy chain 2 (NCBI GenBank: AAL35302.1), 3 (NCBI GenBank: AAL35303.1) and 4 (NCBI GenBank: AAL35304.1) are shown in bold red.

ii. Attempt 2 - Isolation of dog 'IgG4' using an affinity column coupled with mouse anti-dog IgG4 mAb (A5)

After the lack of success in purifying dog IgG4 using protein A and G affinity columns, a purification strategy using an affinity column coupled with mouse anti-dog IgG4 mAb (A5)

was attempted. Thus, 2 mg of A5 mAb was coupled to UltralinkTM Biosupport column. In order to determine whether the mAb was properly coupled, a sample of the column beads was loaded onto an SDS-PAGE gel where it was possible to detect the heavy (approx. 53 KDa) and light (approx. 26 kDa) chains of the mAb (Fig. 3.8), demonstrating that the A5 mAb was coupled to the beads.

Afterwards, dog IgG (4 mg) previously purified using Protein A and then G affinity columns was mixed with 10 mM EDTA (pH 7.3) and then loaded onto the UltralinkTM Biosupport column previously linked with the A5 mAb. After elution with 0.1 M Citrate buffer (pH 2.5), 0.5 ml fractions were collected and neutralised with 100 µl Tris-HCl (pH 8.5). Then the A_{280} was measured but no protein was detected in the elution fractions. A second attempt was made, this time re-loading onto this column 22.5 ml of dog IgG previously precipitated from 45 % ammonium sulphate at a concentration of 13.23 mg/ml. Fractions were again eluted using 0.1 M citrate buffer (pH 2.5), and measurement of protein concentration on the Nanodrop again showed that dog immunoglobulins did not elute from the column. Other attempts were made re-loading the column with fresh dog serum and using different high salt elution buffers, such as 1 M sodium chloride, 2 M magnesium chloride, 4 M magnesium chloride, 0.1 M glycine-sodium hydroxide (pH 10), and 3 M potassium thiocyonate, at a flow rate of 2.1 ml/min with no success after measuring A_{280} on the Nanodrop.

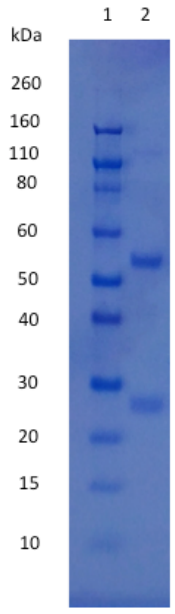

Figure 3.8: 4-12% Bis-Tris SDS-PAGE of Ultralink^{TM} Biosupport resin linked with mouse anti-dog IgG4 mAb A5. 1. Pre-stained protein standard 2. Ultralink Biosupport resin linked with mouse anti-dog IgG4 mAb (A5) before IgG4 purification. Mouse IgG light chain (25 kDa approx.) and mouse IgG heavy chain (53 kDa approx.) are shown in track 2.

iii. Attempt 3 - Isolation of dog 'IgG4' using an affinity column coupled with goat anti-dog IgG1 antibody

A new attempt to purify dog IgG4 from serum was made, this time following the approach that dog IgG4 does not bind protein A and goat anti-dog IgG1 (Bethyl labs) binds to both IgG1 and IgG4 selectively (Bergeron *et al*., 2014). Thus, commercial goat anti-dog IgG1 polyclonal antibody was coupled onto the affinity purification column. Then, dog serum was loaded onto this column, attempting to bind dog IgG1 and IgG4 to then use a protein A column to remove dog IgG1 in a high salt buffer containing 3 M NaCl, assuming that the IgG1 will bind to protein A column and the IgG4 will not, obtaining dog IgG4 in the flow-through.

After elution with 0.2 M glycine (pH 2.5) from the prepared column, no protein was obtained when measuring absorbance at OD_{280} in Nanodrop. Several elution buffers were then used to try to elute immunoglobulins from the column such as 0.2 M glycine (pH 3), 0.2 M glycine (pH 10), 0.2 M glycine (pH 11), and a high salt elution buffer, 4 M magnesium chloride (pH 6.5), with no success. Loading, washing and elution flow rates were decreased as well (0.1 ml/min, 0.3 ml/min and 0.3 ml/min, respectively). Then, the column was also reloaded with 1.5 ml of fresh dog serum that was heat inactivated for 30 minutes at 56 ^{o}C before loading it onto the column and diluted in the same amount of PBS, keeping EDTA away this time. Previously named elution buffers were used but again with no success in obtaining any immunoglobulins from the column. Therefore, it was not possible to perform the second step using the Protein A column.

A western blot was performed to determine if the goat anti-dog IgG1 antibody was properly coupled to the column resin, and if it was able to bind dog immunoglobulins. A sample of the resin beads before and after being used to purify dog IgG was subjected to a western blot, using for the detection, antibodies against sheep (cross-react with goat) and dog IgG, respectively. The anti-dog IgG reagent recognised heavy and light chains (Fig. 3.9A) and the anti-sheep reagent (Fig. 3.9B) recognised just heavy chains. Apparently, there was dog IgG binding in the column linked with anti-dog-IgG1 (Fig. 3.9A, track 6), which was not able to elute from the column. However, the anti-dog antibody showed cross-reactivity with goat IgG and anti-sheep antibody showed cross-reactivity with dog IgG. Thus, it was not possible to confirm the binding of dog IgG.

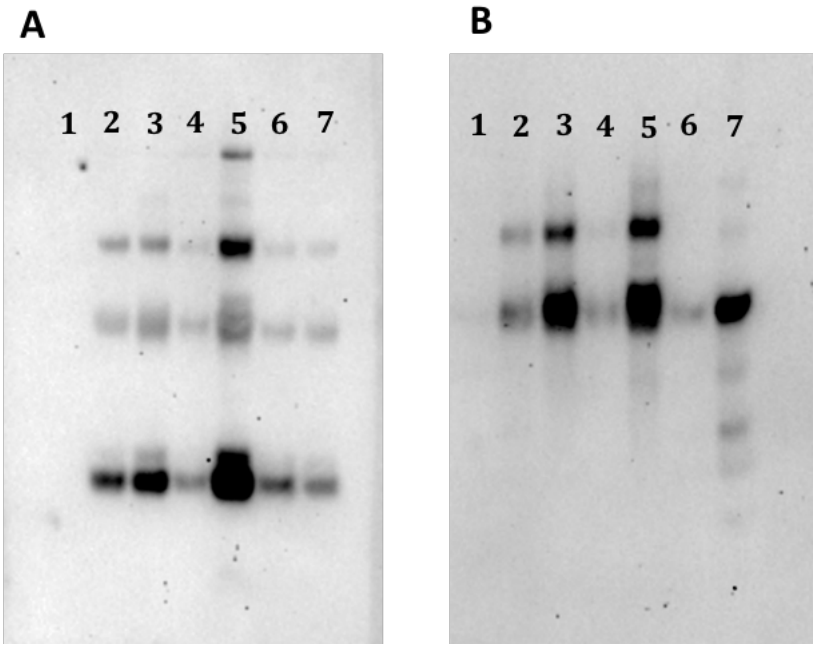

Figure 3.9: Western Blot of Ultralink™ Biosupport resin linked with goat anti-dog IgG1 polyclonal antibody. 1: Pre-stained protein standard, 2: Dog IgG standard after protein A and G affinity purification, 3: Dog IgG standard (after precipitation at 45% saturation of ammonium sulphate), 4: Fresh dog serum, 5: Ammonium sulphate start material, 6: Beads eluted with SDS (after being used for dog IgG purification), 7: Beads in reducing buffer (before being used to purify dog IgG). Approximately 100 ng of heavy chains were loaded in each track. Antibodies: A) Goat anti-dog IgG (H+L)-HRP (PA1-29738, Thermofisher) dilution 1:5,000; B) Donkey anti-sheep IgG (H+L)-HRP (A16047, Invitrogen) dilution 1:2,000.

iv. Attempt 4 – Isolation of dog 'IgG4' using the approach that IgG4 does not bind to Protein A

As it was not possible to make a pure dog IgG4 preparation as a standard using the above approaches, another purification plan following the approach that dog IgG4 does not bind to Protein A was conducted. Thus, 5 ml of dog IgG at 15.35 mg/ml previously precipitated with ammonium sulphate were loaded onto a Prosep A column. Loading buffer was PBS and elution buffer was 3 M sodium chloride. Loading and elution flow rates were 0.2 ml/min and 0.5 ml/min, respectively. Five peaks were obtained using this column (Fig. 3.10). Peak 1 (Fractions 5, 6, 7, 8) and peak 2 (fractions 12 and 13) consisted of run through material. Peak 3 (fractions 31, 32) consisted of elution fractions after using 3 M sodium chloride. Peak 4 (fractions 53,54, 55) and peak 5 (fractions 57,58,59) consisted of elution fractions after using 0.1 M sodium citrate (pH 2.5). The peaks fractions were pooled and protein concentration was measured (peak 1: 3.21 mg/ml; peak 2: 1.16 mg/ml; peak 3: 0.12 mg/ml; peak 4: 2.9 mg/ml and peak 5: 1.64 mg/ml). Then, 1 µg of each pool were loaded onto a NuPAGE4-12% gel (Fig. 3.11). The heavy chain of pool 3 was sent for mass spectrophotometry analysis.

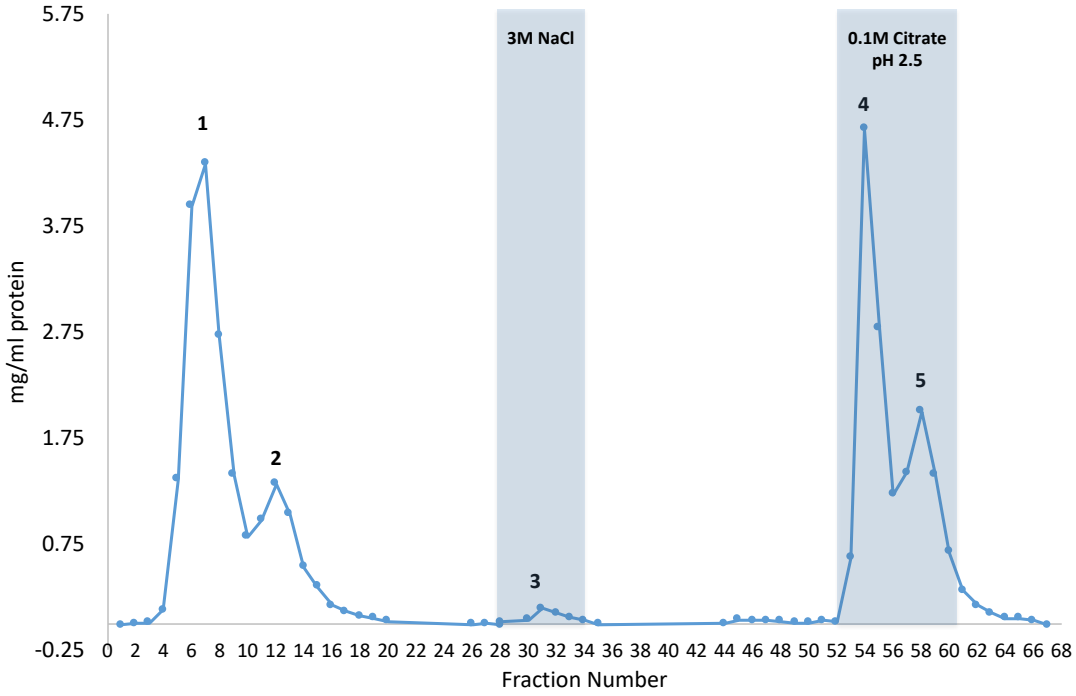

Figure 3.10: Protein A affinity chromatography of 5 ml dog plasma previously precipitated at 45% saturation of ammonium sulphate. Numbers 1-5 indicate the different peaks obtained after Protein A affinity chromatography.

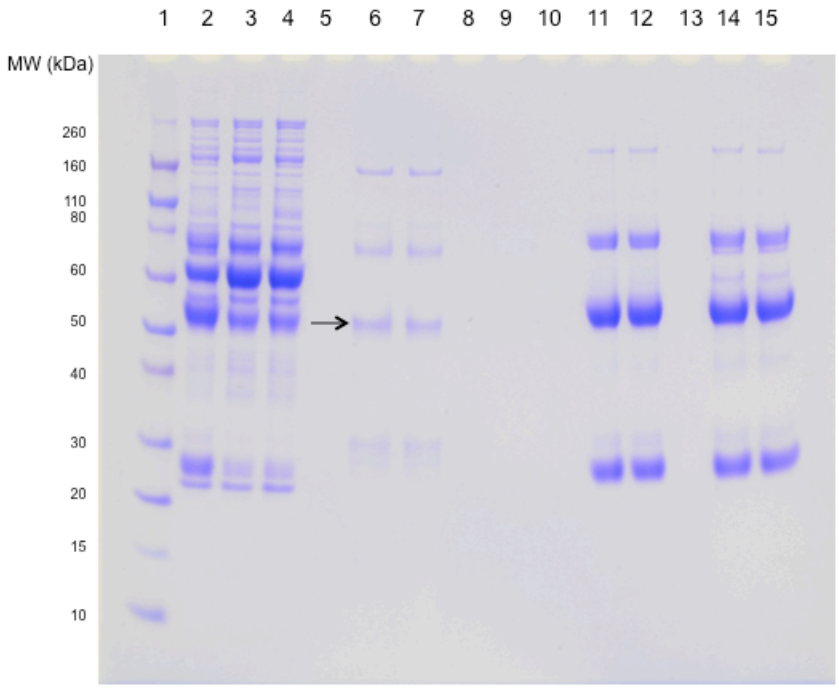

Figure 3.11: 4-12% Bis-Tris SDS-PAGE for mass spectrometry analysis to determine identity of dog IgG heavy chain band. An attempt to purify dog immunoglobulin gamma heavy chain D (IgG4) from dog serum used exclusion from a Protein A column. Pooled elution fractions were loaded into gel tracks (1µg per track approx.). **Tracks 1**: pre-stained protein standard, **2**: start material, **3**: pool 1, **4**: pool 2, **6 & 7**: Pool 3, **9 & 10**: Pool 4, **11 &12**: pool 5, **14 & 15**: pool 6. The heavy chain (MW 53kDa approx.) in track 6 was sent for mass spec analysis to determine its identity and purity. An arrow indicates this band.

LC-MS/MS analysis identified the components of the gel band as a mixture of dog IgG1, IgG2, IgG3 and IgG4 heavy chains, with protein coverage of 29 %, 12 %, 32 % and 17 %, respectively (Fig. 3.12).

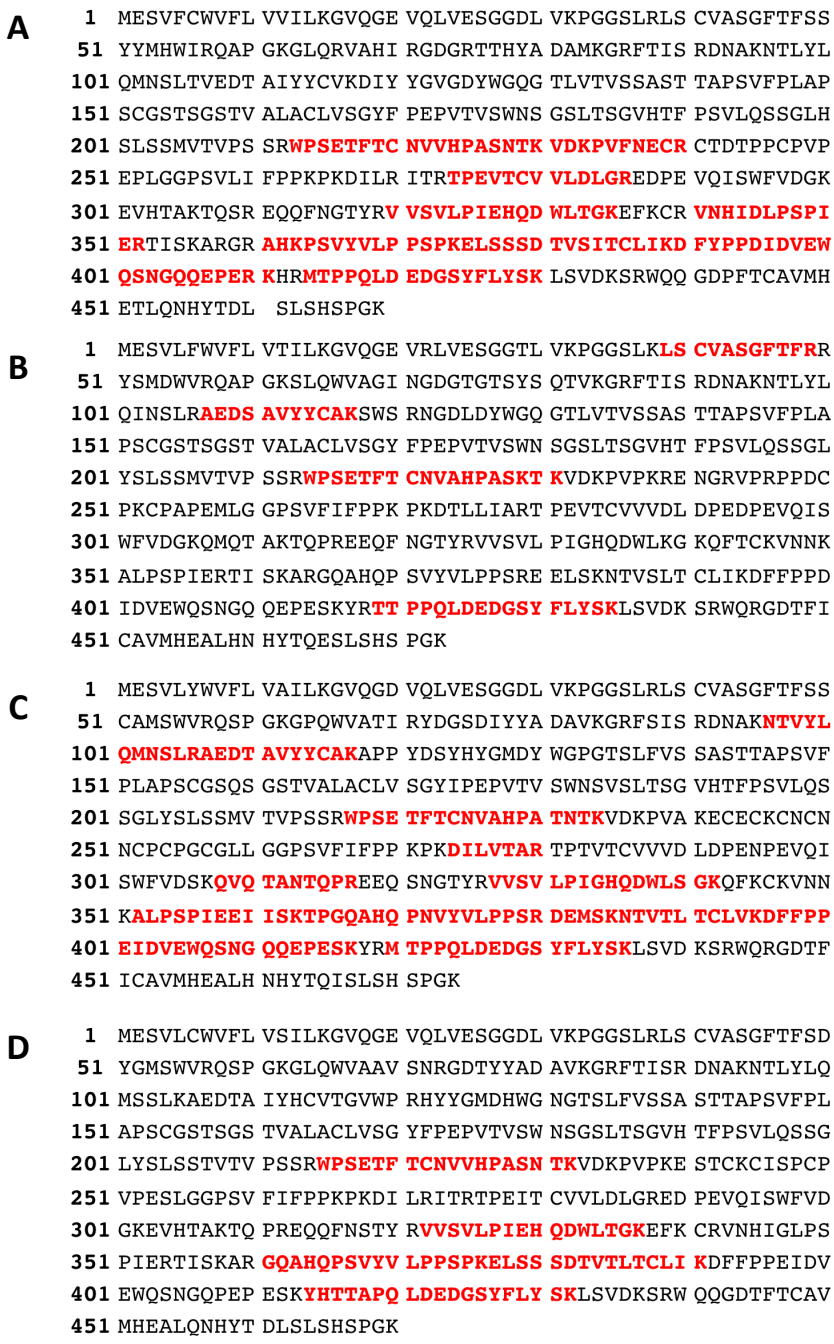

Figure 3.12: MASCOT search results for dog immunoglobulin gamma heavy chain 1 (panel A), 2 (panel B), 3 (panel C) and 4 (panel D) in the purified heavy chain of approximately 53 kDa. Results obtained after LC-MS/MS analysis (Cambridge Centre for Proteomics, Biochemistry Department, University of Cambridge), showed a protein sequence coverage of 29 %, 12 %, 32 % and 17 % for dog IgG1, IgG2, IgG3 and IgG4, respectively. Matched peptides with *Canis lupus familiaris* immunoglobulin gamma heavy chain 1 (NCBI GenBank: AAL35301.1), 2 (NCBI GenBank: AAL35302.1), 3 (NCBI GenBank: AAL35303.1) and 4 (NCBI GenBank: AAL35304.1) are shown in bold red.

v. Attempt 5 – Isolation of dog 'IgG4' using Protein A followed by Protein G affinity chromatography

The last method used to try to purify dog IgG4 consisted of subjecting 2 ml of fresh dog serum previously filtered and diluted with 0.1 M sodium phosphate (pH 9.0), 10 mM EDTA buffer to a Protein A affinity chromatography. Protein was eluted using 0.1 M sodium phosphate/3 M sodium chloride (pH 8.0), then 0.1 M sodium phosphate/3 M Sodium Chloride (pH 7.0), and finally 0.1 M sodium citrate (pH 2.5). Elution fractions were subjected to a sandwich ELISA (A5 mAb capture) in order to detect dog IgG4 (Fig. 3.13). The flow through material was subjected to Protein G affinity chromatography.

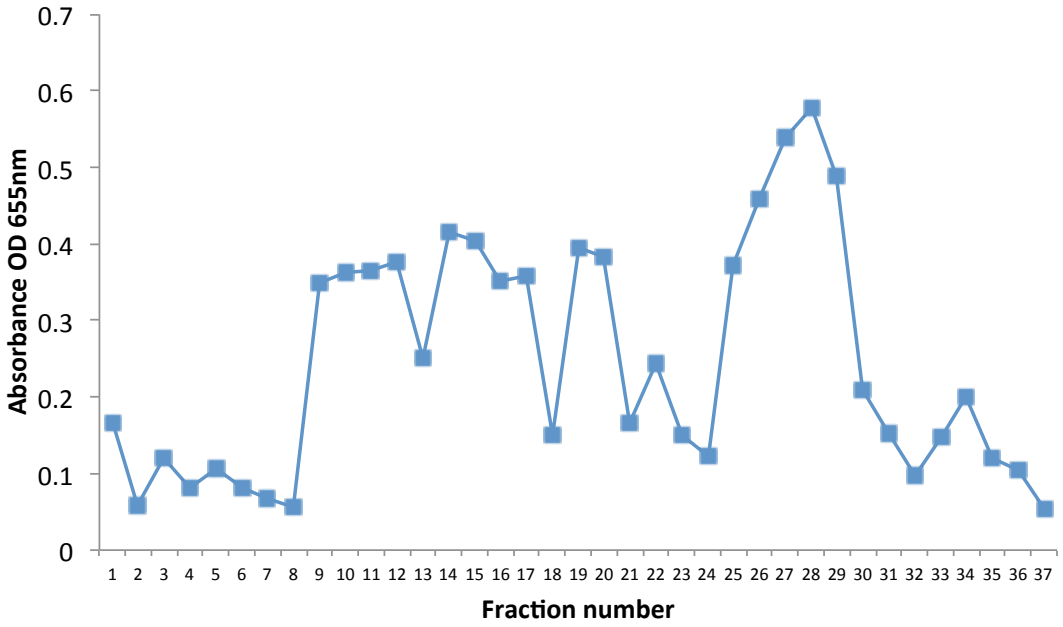

Figure 3.13: Sandwich ELISA (A5 mAb capture) for the detection of dog IgG4 of a flow through serum sample from protein A and then eluated from G chromatography.

Fraction numbers 7 to 50 were pooled (pool 1) and protein concentration was measured on Nanodrop, obtaining 0.04 mg/ml. Fraction numbers 53 to 63 were also pooled (pool 2), obtaining a concentration of 0.08 mg/ml. Protein from both pools was concentrated using Amicon® Bioseparation Stirred Cells (Millipore), with ultrafiltration cellulose membrane discs of 44.5 mm diameter and nominal molecular weight limit (NMWL) of 30,000 Da (Millipore) and applying gas pressure directly to the ultrafiltration cell. After this procedure, the final concentration was 0.16 mg/ml for pool 1 and 0.38 mg/ml for pool 2. Then, 3 µg of each pool

were loaded in a NuPAGE4-12% gel (Fig. 3.14). The heavy chain band of pool 1 and 2 were sent for LC-MS/MS analysis.

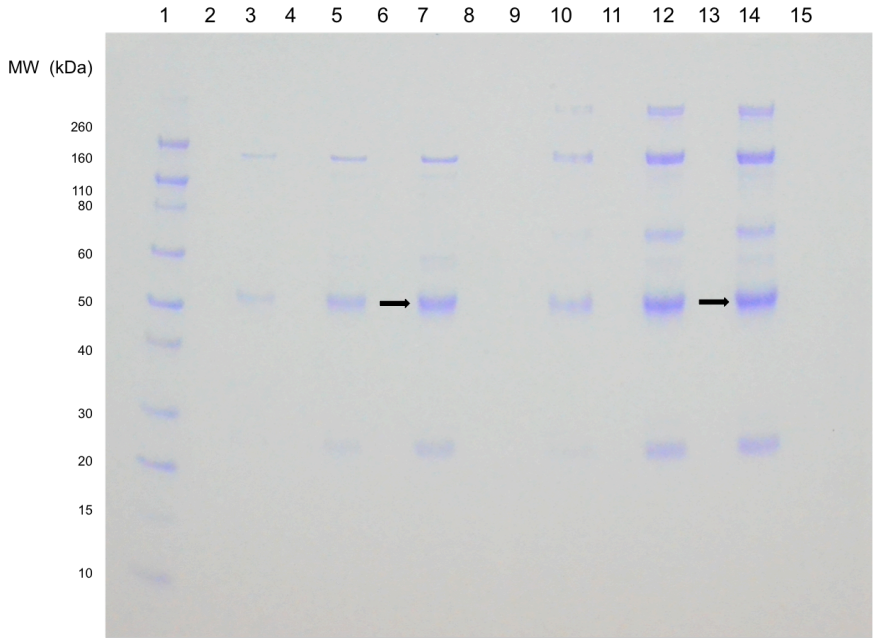

Figure 3.14: 4-12% Bis-Tris SDS-PAGE to determine the identity of Dog IgG heavy chain bands from pool 1 and pool 2. Dog IgG4 heavy chain was attempted to be purified from dog serum and loaded in gel track number 14 (3 µg per track approx.). Track 1 contains pre-stained protein standard. The heavy chain (MW 53kDa approx.) in track 14 was sent for LC-MS/MS to determine its identity and purity. This band was run in triplicate in track number 10 and 12. The heavy chain of the dog immunoglobulin loaded in track number 7 (also run in triplicate in tracks number 3 and 5) was also sent mass spec analysis. The arrows indicate these bands.

LC-MS/MS analysis identified the components of the gel band as a mixture of dog IgG1, IgG2, IgG3 and IgG4 heavy chains in pools 1 and 2. Pool 1 had protein coverage for dog IgG1, IgG2, IgG3 and IgG4 heavy chains of 38 %, 12 %, 56 % and 27 %, respectively (data not shown and Fig. 3.15). Pool 2 had protein coverage for dog IgG1, IgG2, IgG3 and IgG4 heavy chains of 28%, 43%, 52% and 31%, respectively (data not shown and Fig. 3.15).

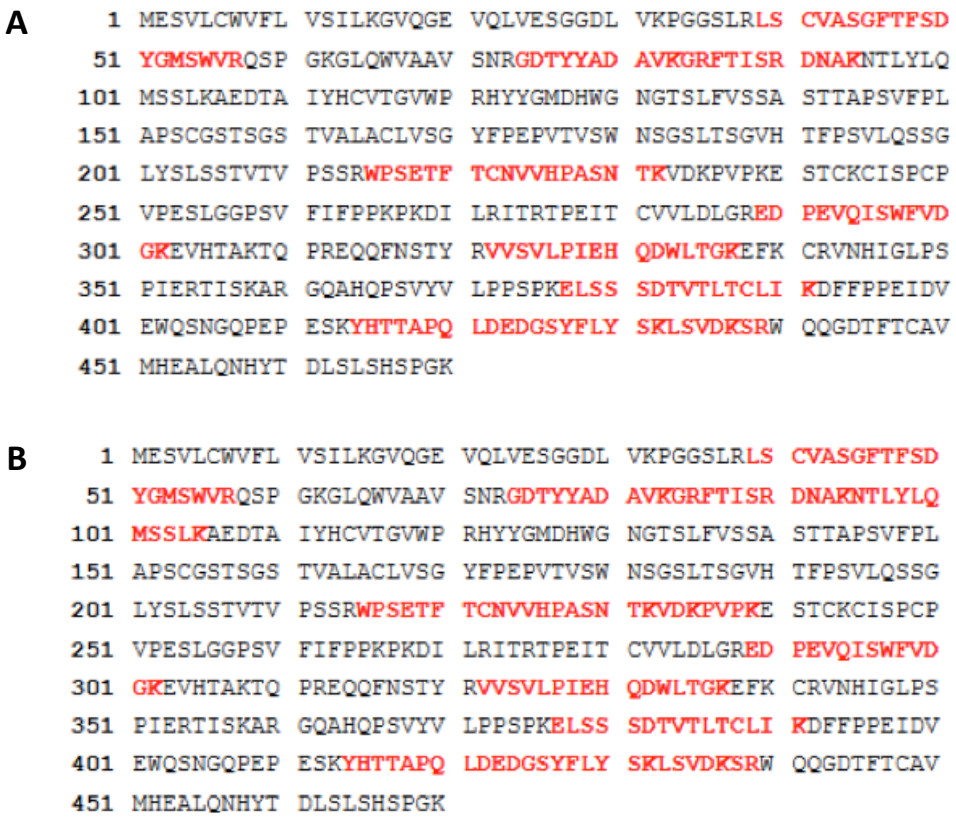

Figure 3.15: MASCOT search results for dog immunoglobulin gamma heavy chain 4 in the purified heavy chain of approximately 53 kDa obtained from pool 1 and 2 after been subjected to Protein G column. Results obtained after LC-MS/MS analysis (Cambridge Centre for Proteomics, Biochemistry Department, University of Cambridge), showed protein sequence coverage of 27% and 31% for dog IgG4 in pool 1 (panel A) and pool 2 (panel B), respectively. Matched peptides with *Canis lupus familiaris* immunoglobulin gamma heavy chain 4 (NCBI GenBank: AAL35304.1) are shown in bold red.

3.4.3.4 Competitive ELISA for the quantitative measurement of canine serum IgG4 levels

A competitive ELISA was developed for the purpose of measuring canine serum IgG4 levels. Protein that was subjected to chromatography and showed the highest protein coverage for dog IgG-D (31%) after LC-MS/MS analysis was used as standard. Stable standard curves were obtained using this competitive ELISA. The coefficient of determination (R^2) calculated for the standard curves was in the range of 0.973 to 0.999 for all standard curves generated with this competitive ELISA. Fig. 3.16 shows a typical standard curve obtained for the measurement of dog 'IgG4'.

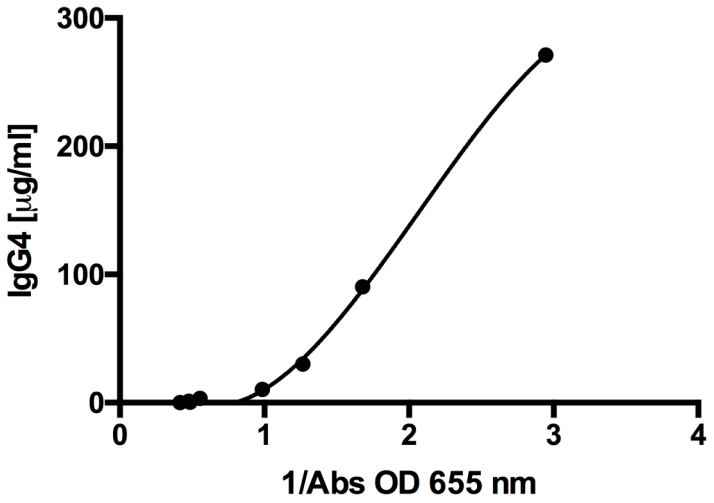

Figure 3.16: Standard curve for the detection of dog IgG4 using a competitive ELISA. Standard curve for the measurement of serum IgG4 generated the following equation: y = -26.145x3 + 162.48x2 - 177.08x + 51.171 (R^2= 0.99904). Each point of the curve represents the mean absorbance of two wells.

The concentration of 'IgG4' was determined in a pooled plasma sample from normal dogs (Pet Blood Bank UK) by choosing absorbance values, which lay within the straight region of the standard curves. The average concentration of 'IgG4' in the pooled plasma sample from normal dogs was 66.8 mg/ml (*SD*=34.3). This value was the average obtained in repetitions that were done in 13 different ELISAs. The values ranged between 12.7 mg/ml to 100.3 mg/ml, using the same plasma sample each time (Fig. 3.17).

Total IgG concentration was also measured, using a competitive ELISA. The normal plasma pool had a total IgG concentration of 12.48 mg/ml (*SD*=2.4) (Fig. 3.17).

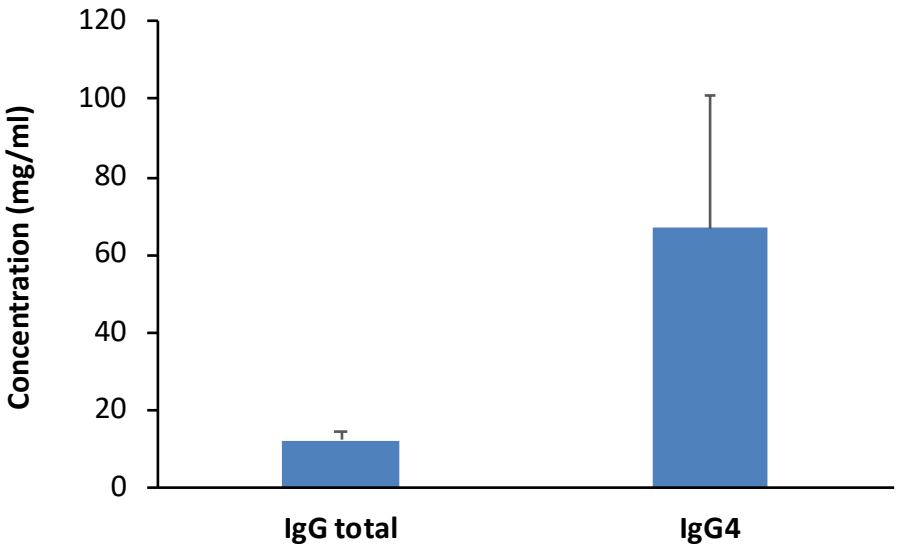

Figure 3.17: Concentration of IgG and 'IgG4' in pool of plasma from normal dogs. The bars indicate the standard deviation.

3.5. Discussion and conclusions

This chapter described the development of a competitive ELISA to try to detect dog IgG4. For this purpose, a mouse anti-canine IgG4 mAb (A5) (Mazza *et al*., 1993) was successfully produced. This antibody was seen as crucial for generating an ELISA test capable of recognising IgG4 in serum from ECS dogs with chronic pancreatitis in order to determine if affected ECS dogs have elevated concentrations of IgG4 in serum, as seen in humans with IgG4-RD. Since there is not a specific commercial mAb for canine IgG4 and the antibodies purified by Mazza *et al*. (1993) are no longer available, there was the need to produce more mAbs using the original hybridomas. It was seen that the A5 anti-canine IgG4 mAb apparently detected IgG4 in the plasma of normal dogs by ELISA, and $IgG4^+$ plasma cells in canine lymph nodes by IHC. Thus, mouse anti-canine IgG4 was thought to be useful for the detection of normal and abnormal concentrations of IgG4 in canine serum. It has been reported that the A5 mAb has restricted specificity for IgG4 and IgG2, but it is IgG4 specific in serological and IHC assays (Mazza *et al*., 1994a; Day and Mazza, 1995). However, it is necessary to make sure if the A5 mAb was specifically recognising dog IgG4 using the competitive ELISA shown in this study. This was not possible to demonstrate in this study, since dog IgG4 was not available.

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The production of mouse anti-canine IgG1, IgG2, and IgG3 was not achieved. Hybridomas for these subtypes did not grow in the same conditions as for A5. The frozen vials supplied by University of Bristol were 23 years old. It was attempted to resuscitate the hybridoma cells using feeder peritoneal macrophages and the addition of conditioned medium from a macrophage cell line. Using these conditions, the E5 hybridoma specific for IgG2 was able to grow but the ones against IgG1 and IgG3 (B6 and A3G4) were not. There can be numerous reasons for this: 1) a low number of cells was initially frozen per vial, 2) age of the cells and 3) freezing/thawing process of the vials resulting in a loss of viability.

Antibodies against IgG1, IgG2 and IgG3 would have allowed to determine the concentration of these subclasses in canine serum and compare them with concentrations of IgG4 in normal and dogs with clinical disease. However, this was not completely necessary for this project as total IgG and the IgG4/total IgG ratio could be measured using commercial antibodies against total canine IgG, as done in humans with IgG4-RD.

Purification of dog IgG4 from plasma to be used as a standard in the ELISAs using size and ion-exchange chromatography was not achieved. Several purification strategies were attempted in order to isolate dog IgG4 from dog plasma. The purification strategies used here, were firstly based on (Mazza *et al*., 1993) studies, where IgG4 was isolated using protein A and then protein G. However, in the present study, after mass spectrometry analysing of the apparently pure IgG4, it was found that only a few peptides were identified as a match to immunoglobulin gamma heavy chain D protein (IgG-D). IgG-D has been published as being the equivalent to IgG4 as this subclass lacks effector functions and functions similarly to human IgG4 (Bergeron *et al*., 2014). The subsequent purification strategies were then based on this more recent study, which describes that IgG4 canine subclass does not bind to protein A (Bergeron *et al*., 2014). However, after using that strategy, mass spectrophotometry analysis identified that dog IgG4 was found in combination with the other subclasses. After not succeeding in purifying dog IgG4, Dr Lisa Bergeron (Zoetis, Global Therapeutic Research, Veterinary Medicine Research and Development) was contacted to ask to obtain the plasmid constructs that were used in her comparative functional characterization of canine IgG subclasses study (Bergeron *et al*., 2014) to try to use them to produce a recombinant IgG4. However, because of patent issues the plasmids were not released from Zoetis. Due to the limited time and because generating a recombinant IgG4 to be used as a standard for the ELISAs was not the main purpose of this project, making a recombinant IgG4 will be considered as future work. For this purpose a commercial cloning plasmid that expresses the constant region of the canine IgG4 heavy chain (pFUSE-CHIg-dG4, InvivoGen) could be used.

The purified sample that was found to have the highest protein sequence coverage (31 %), matching peptides with dog IgG4 on mass spectrophotometry was used as a standard comparator across plates allowing the quantitative competitive ELISA to be performed. Thus, a competitive ELISA for the measurement of IgG4 was developed. Stable standard 'IgG4' curves were obtained, with the correlation coefficient of the regression equation found to rise to 0.999. However, when using this competitive 'IgG4' ELISA, the average IgG4 concentration detected in normal plasma was 75.08 mg/ml, which was much higher than the reported normal reference values for dog IgG4 in serum (<0.4-2.0 mg/ml) (Mazza *et al*., 1994a). Moreover, the IgG4 concentration using this ELISA method was also higher than the total IgG levels measured in the same plasma sample, which cannot be correct, as IgG4 is a

minor fraction of the total IgG. For these reasons, it was decided to measure the serum IgG4 levels in affected and control ECS by using a commercial IgG4 ELISA kit (Chapter 4).

In humans, serum IgG4 levels are measured by using nephelometric assays as a routine test. One study has shown an established ELISA system using commercial antibodies for the measurement of serum IgG4 in humans with clinical applications in autoimmune diseases, IgG4-RD included. That study demonstrated reliability of the ELISA system, showing serum IgG4 levels consistent with results that were obtained by using nephelometric assays (Sun *et al*., 2013).

Measurement of canine IgG4 is challenging in the absence of commercially available reagents specific for this subclass. Sequence of the four canine IgG sub-classes documented from cDNA data has demonstrated modest sequence homology and receptor binding characteristics with the four human IgG subclasses (Tang *et al*., 2001; Bergeron *et al*., 2014). It would be interesting to explore the potential for cross-reactivity of human reagents with the canine subclasses. A recent work showed increased serum IgG4 concentrations in a Husky mixed breed dog with suspected IgG4-RD using immunofixation (IF). IF is a diagnostic and research tool used for characterizing the electrophoretic location of immunoglobulin fractions in serum and urine but does not allow direct quantification. It can only inform a semiquantitative assessment of protein abundance (Colopy et al., 2019). This technique uses of a commercial polyclonal canine anti-IgG1 antibody, which is suggested to cross-react with canine IgG4 (Colopy et al., 2019). However, this commercially available polyclonal IgG1 antisera can be used to positively but possibly not selectively identify canine IgG4 by immunofixation (Donaghy and Moore, 2020).

This chapter has described several attempts for the development of a non-invasive test capable to measure IgG4 levels in canine serum. The A5 mAb against dog IgG4 was produced for this purpose. Different strategies for the isolation of IgG4 from dog serum using affinity and anion exchange chromatography were shown in this chapter. Pure dog IgG4 was not possible to obtain. Future work making a recombinant IgG4 and demonstrating evidence that the anti-dog IgG4 mAb (A5) specifically recognises dog IgG4 in serological assays is needed.

CHAPTER 4: CONCENTRATION OF TOTAL SERUM IgG, IgG4 AND IgE IN ENGLISH COCKER SPANIELS AFFECTED BY CHRONIC PANCREATITIS

4.1. Summary

The aim of this chapter was to determine whether ECS affected by chronic pancreatitis and other associated chronic inflammatory disease in different organs had increased levels of serum IgG4, as has been described in human patients affected by IgG4-RD. Total serum IgG concentrations were measured in order to determine the serum IgG4/IgG ratio. Total serum IgE concentration was also measured to determine if affected ECSs had increased levels, as seen in some human patients affected by IgG4-RD. In order to complete this objective, serum samples from 96 English cocker spaniels affected by chronic pancreatitis and 41 control ECS (14 healthy and 27 affected by other diseases unrelated to CP) were measured for total IgG, IgG4 and IgE, using enzyme-linked immunosorbent assays (ELISA). Median concentration of total serum IgG in CP cases, healthy controls and control dogs affected by other diseases unrelated to CP was 12.03 mg/ml, 13.87 mg/ml and 11.01 mg/ml, respectively. Median concentration of serum IgG4 was 1.16 mg/ml in CP cases, 0.62 mg/ml in healthy controls and 0.76 mg/ml in control ECSs affected by other diseases. Median serum IgG4/IgG ratio was 11.13 % for affected ECSs, 4.36 % for healthy controls and 6.16 % for diseased controls. Median concentration of total serum IgE was 4.82 ng/µl in CP cases, 3.44 ng/µl in healthy controls and 2.81 ng/µl in control dogs affected by other diseases. IgG4 levels were significantly higher in ECSs affected by CP compared with healthy dogs and dogs affected by other diseases ($P < 0.01$). In addition, cases that presented with a clinical inflammatory manifestation in more than one organ had significantly higher levels of serum IgG4 than the ones with just pancreatic manifestation ($P < 0.05$). A ROC curve indicated a fair diagnostic accuracy of the serum IgG4 test (AUC= 0.701, 95% CI, 0.599 - 0.802, P < 0.001). A serum IgG4 cut-off of 0.85 mg/ml had a sensitivity of 70.2 %, specificity of 63.4 %, positive predictive value (PPV) was 81.48 % and negative predictive value (NPV) was 48.15 % for diagnosing IgG4-RD in dogs with CP. The serum IgG4/IgG ratio was significantly higher in cases than in healthy controls ($P < 0.01$) and diseased controls ($P < 0.05$). There was no significant difference in total serum IgG and IgE concentrations between cases and control dogs.

4.2. Introduction

Serum IgG4 concentrations in human patients affected by IgG4-RD are often elevated. In a systematic review, in which nearly two thousand IgG4-RD human patients were analysed, 87 % of these patients had elevated serum IgG4 (Brito-Zerón *et al*., 2016). The serum IgG4 concentration is correlated with the number of organs involved, thus the more organs affected, the higher the serum concentration (Carruthers *et al*., 2014). It has also been seen that patients with pancreatic involvement are more likely to have an elevated serum IgG4 concentration compared to patients without pancreatic involvement (Wallace *et al*., 2015a).

Serum concentrations of IgG4 >1.35 mg/ml have been widely accepted as a cut-off value, and have demonstrated a sensitivity of 97.0 % and a specificity of 79.6 % in diagnosing human IgG4-RD (Masaki *et al*., 2012). However, high levels of serum IgG4 are not observed in some patients, especially those with early and/or limited stage IgG4-RD (Vasaitis, 2016).

Increased levels of IgG4 are not specific for IgG4-RD. It has been reported that other diseases such as pancreatic carcinoma, cholangiocarcinoma, sclerosing cholangitis, recurrent infections, autoimmune diseases, allergic conditions, and skin diseases including atopic dermatitis and pemphigus vulgaris, among others, can present with increased levels of serum IgG4 (Ghazale *et al*., 2007; Carruthers *et al*., 2014; Su *et al*., 2015). Also, it has been seen that serum IgG4 levels can be elevated in 5 % of the normal population (Chari, 2011). However, the measurement of serum IgG4 in human patients affected by IgG4-RD is still considered useful for diagnosis of this disease, and especially to determine multi-organ manifestation and responsiveness to therapy.

Human IgG4-RD has also been associated with allergy, atopy, eosinophilia, increased serum IgE levels, and IgE^+ mast cells in lymphoid, biliary, and pancreatic tissue. This suggests that most patients with IgG4-RD may develop an IgE-mediated allergic response and that serum IgE levels might be used in diagnosis and to predict relapse (Culver *et al*., 2017). Peripheral eosinophilia and elevated serum IgE are often present among human patients affected by IgG4-RD, especially those patients with elevated serum IgG4 and a greater number of organs involved (Wallace *et al*., 2015a).

In dogs, normal levels of serum IgG4 range between <0.4 to 2.0 mg/ml (Mazza *et al*., 1994a). Certain inflammatory or immune-mediated disorders such as anal furunculosis, otitis externa, IMHA and hypothyroidism in dogs, can present with increased serum levels of IgG4 (Mazza *et al*., 1994a). In a recent study, it was seen that ECS with suspicion of IgG4-RD had increased numbers of $IgG4^+$ infiltrating plasma cells in affected organs (Coddou *et al*., 2020). Anecdotally, it has been seen that some ECS with suspected IgG4-RD present with a history of atopy. In humans, total serum IgE is increased in most patients with atopic disease such as allergic rhinitis, asthma, and atopic dermatitis (Zetterström and Johansson, 1981). In dogs, total serum IgE concentrations could not discriminate between healthy and atopic dog populations, suggesting that quantification of total serum IgE would not be valuable as a screening test for canine atopy (Hill *et al*., 1995). Whether IgG, IgG4 and IgE serum levels are altered in ECS diagnosed with CP and suspicion of IgG4-RD is not known until now.

The aims of the work reported in this chapter were to assess serum IgG, IgG4 and IgE levels and serum IgG4/IgG ratios in ECS affected by chronic pancreatitis and multi-organ disease, suspected of presenting with IgG4-RD, and determine its correlation with disease. Moreover, assessing the predictive value of serum IgG4 concentrations in diagnosing IgG4-RD and determining whether these serological tests may also be used as indicators of disease activity. In addition, these tests were used to compare levels of serum IgG, IgG4 and IgE concentrations with normal control dogs and dogs with inflammatory diseases with another known aetiology different from immune-mediated disease.

4.3. Materials and methods

4.3.1. Dogs and serum sample collection

As described in Chapter 2, one hundred and four ECS affected by chronic pancreatitis and 44 ECS control dogs with no suspicion of CP and/or immune-mediated diseases were recruited from The Queen's Veterinary School Hospital, University of Cambridge, and private veterinary practices across the UK. Serum samples were available from 100/104 cases and 41/44 controls. Cases number 52, 74, 75 and 85, control 9 from group 1 and controls 7 and 14 from group 2 did not have serum samples available, only EDTA/blood was provided.

Cases were split into 2 groups according to the methodology used in the diagnosis of CP. *Case group 1* was diagnosed based either on presenting with pancreatic histology (n=9) or with both elevated cPLI and abnormal pancreatic ultrasound (n= 47). *Case group 2* was diagnosed based on less robust diagnostic methods, including elevated cPLI or DGGR lipase, and elevated lipase/amylase without ultrasonographic changes (n=48).

Control dogs were also divided in two groups. *Control group 1* included 15 healthy ECS and *control group 2* included 29 ECS presenting with several diseases with non-immune mediated aetiology.

Inclusion criteria for control dogs are described on Chapter 2 (materials and methods). Briefly, they consisted of no evidence of CP, glomerulonephritis, KCS, anal sac issues and/or any other immune-mediated diseases. Exclusion criteria for cases and control dogs consisted of corticosteroids or any immunosuppressant treatment within the previous 6 months of obtaining blood samples.

The signalment, in addition to the clinical details of the cases and control dogs included in this study, are summarised on Chapter 2 (Tables 1, 2, and 3).

This study was approved by the Ethics Committee at the Department of Veterinary Medicine, University of Cambridge (ethical reviews CR153 and CR291) and serum samples were used with fully informed owner consent.

4.3.2. Total IgG serum levels

Total dog IgG serum levels were measured using a non-commercial competitive ELISA. The protocol can be seen on Chapter 3 (Materials and Methods, section 3.3.5).

4.3.3. IgG4 serum levels

The IgG4 ELISA developed in Chapter 3 was not accurate for reasons explained in that chapter. Instead, dog serum IgG4 concentration was measured using a commercially available canine Immunoglobulin G4 (IgG4) competitive ELISA kit (BlueGene, Shanghai, China) according to the manufacturer's instructions. Briefly, 100 µl of the standards provided in the kit and serum samples from cases and control dogs were added in duplicates to the appropriate wells. Serum samples were previously diluted in PBS (pH 7.0), using a 1/5 dilution for cases and 1/2 dilution for controls. Immediately, 50 µl of conjugate were added to each well except in blank control well. Wells were then mixed and plates were covered and incubated for 1 h at 37 ° C. Afterwards, plates were washed 5 times using the wash solution provided in the kit. Then, 50 µl of substrate A and same amount of substrate B were added to each well. Plates were incubated in the dark and after 15 min of incubation at 37 ° C, 50 µl of stop solution were added to each well. Finally, optical density (O.D.) was determined at 450 nm using an iMark microplate absorbance reader (Bio-Rad).

4.3.4. IgE serum levels

The quantitative measurement of dog IgE in serum was determined by using a commercially available sandwich dog IgE ELISA kit (Abcam, Cambridge, United Kingdom) according to the instructions given by the manufacturer. Briefly, ELISA plates containing anti-IgE antibodies previously adsorbed to the surface of polystyrene microtiter wells, received 100 µl of each standard provided and serum samples from cases and control dogs in a 1/2,000 dilution, these were added in duplicates into the designated wells and incubated at room temperature for 30 min. Following incubation, the plates were then washed four times using 1X Wash Buffer to remove unbound proteins. This was followed by the addition of 100 µl/well of 1X anti-IgE antibodies conjugated with horseradish peroxidase (HRP) and incubated at room temperature for 30 min in the dark. Plates were then washed again four times and 100 µl of TMB chromogenic substrate solution were added into each well. After

incubation in the dark for 10 min at room temperature, 100 µl/well of stop solution were added and absorbance at 450 nm was immediately determined.

4.3.5. Calculation of results

The obtained absorbance readings of the standards and samples duplicates were averaged. The mean value of the blank control was then subtracted. Standard curves were produced by plotting the average absorbance for each standard against their concentrations and lines were fitted using linear regression selecting the best fit line of each data point. As in competitive ELISAs there is an inverse relationship between the signal obtained and the concentration of the target protein in the sample, the standard curves for them were generated by plotting the reciprocal of the absorbance measurements. The serum immunoglobulin concentrations of all samples were interpolated from the standard curves and corrected for the appropriate sample dilution factors.

4.3.6. Statistical analysis

Mann-Whitney *U* and Kruskal–Wallis tests were used to compare the serum immunoglobulin concentrations (IgG, IgG4 and IgE) and IgG4/IgG ratio between cases and control dogs in individual and multiple groups, respectively. In addition, a receiver operator characteristic (ROC) curve was calculated to determine cut-off points and sensitivity and specificity of the serum IgG4 test and IgG4/IgG ratio. Statistical analysis was performed using SPSS® software (IBM, version 22.0). *P* values of < 0.05 were considered statistically significant.

4.4. Results

4.4.1. Selection of cases and controls

4.4.1.1. CP Cases

A total of 138 serum samples were included in this study. Ninety-six serum samples belonged to ECS affected by CP and with suspicion of IgG4-RD. Four cases out of the 100 ECS cases that had available serum samples were excluded from this study since they were on immunosuppressive treatment with corticosteroids for other concurrent immune-mediated conditions (cases number 34, 77, 84, and 95). The age of the cases included ranged from 3.0 to 14.9 years at diagnosis (median age 9.2 years), and they comprised 50 females and 46 males.

Seventy-five out of the 96 cases affected by CP included in this study had evidence of other disease associated with CP in ECS, such as KCS (n=45); anal sac disease (anal sacculitis n=28, anal sac adenocarcinoma n=5); xerostomia (n=23); glomerulonephritis (n=44); atopy (n=20) or other immune-mediated diseases (n=11). Seventy-three cases had involvement of other organ(s) in addition to pancreas, suggesting multi-organ chronic inflammatory disease.

4.4.1.2. Controls

Forty-one serum samples included in this study belonged to control ECS that had no suspicion of CP or other immune-mediated condition. Fourteen samples belonged to healthy ECS (*control group 1*). The age of these healthy controls ranged from 8.9 to 11.25 years (median age 10.0 years) and they included 7 females and 7 males. Twenty-seven serum samples belonged to ECS diagnosed with other non-immune-mediated conditions (*control group 2*). The age of the control dogs from group 2 ranged from 7.0 to 13.3 years (median 9.2 years), with 18 males and 9 females. The most common diagnoses for the diseased control group were IVDD (n=8), carcinoma (n=5), and cranial cruciate ligament disease (n=3). None of the control dogs were on corticosteroids or other immunosuppressive treatments.

There was no significant difference in age between CP cases, healthy controls and diseased controls (Kruskal-Wallis test, P= 0.548).

Clinical details of cases and controls are described in detail in chapter 2 (Tables 1, 2, and 3).

4.4.2. Serum IgG, IgG4, and IgE measurements

4.4.2.1. Standard curves

The concentration of the total IgG, IgG4, and IgE in each serum sample was determined by choosing absorbance values, which lay within the sloping region of the standard curves. In the case of the IgG and IgE measurements, 2 serum samples for each respective test produced signals that were lower than the lowest standard, lying outside the standard curve. For this reason, it was not possible to obtain IgG and IgE concentrations from those samples.

The coefficient of determination (R^2) calculated for the IgG, IgG4, and IgE standard curves was in the range of 0.993 to 1.0 for all standard curves.

Figure 4.1 shows typical standard curves obtained for dog IgG, IgG4 and IgE ELISAs, respectively.

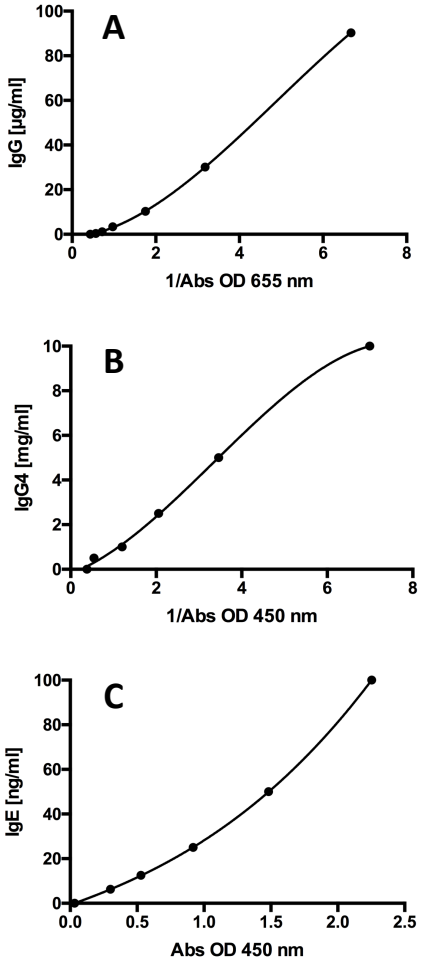

Figure 4.1: Standard curves for the detection of dog IgG (A), IgG4 (B) and IgE (C) in serum. Standard curves for the measurement of total serum IgG, IgG4 and IgE, generated the following equations: A) $y = -0.228x^3 + 3.3487x^2 + 1.5688x - 1.4272$ (R^2= 0.99997). B) $y = -0.0339x^3 + 0.3416x^2 + 0.7275x - 0.1927$ (R^2= 0.99897) C) $y = 2.2393x^3 + 5.3386x^2 + 21.318x - 0.6886$ (R^2 = 1.0). Each point of the curve represents the mean absorbance.

4.4.2.2. Total IgG serum levels

The median concentration of total IgG in serum of affected ECS was 12.03 mg/ml. The values ranged between 3.94 mg/ml to 52.66 mg/ml (Fig. 4.2). Median serum IgG concentration did not show a significant difference between cases with definite diagnosis of CP (case group 1) and cases with possible diagnosis (case group 2) (12.26 *vs.* 11.23 mg/ml, Mann-Whitney U test: U= 1032, *P*= 0.952). Moreover, there was no significant difference in serum IgG concentration between cases that presented with clinical inflammatory manifestation in more than one organ and the ones with pancreatic manifestation only (12.11 mg/ml *vs*.

10.52 mg/ml, Mann-Whitney U test: U= 672, *P*= 0.248) (Fig. 4.6). The case with the highest serum concentration of IgG was a 9-year-old, blue roan, female ECS that presented with CP and concurrent ASAC (case 25). It is unknown if this case was hypercalcaemic and whether this could have interfered with the measurement of immunoglobulins.

The median concentration of IgG in serum of healthy control ECSs (control group 1) was 13.87 mg/ml and values ranged from 7.04 mg/ml to 29.88 mg/ml (Fig. 4.2). The control dog from group 1 with the highest serum concentration of IgG was a healthy 9-year-old male ECS that had a lip fold surgical excision (control 2).

The median concentration of total IgG in serum of diseased control ECS (control group 2) was 11.01 mg/ml, ranging from 2.71 mg/ml to 23.26 mg/ml (Fig. 4.2). The dog from control group 2 with the highest IgG serum concentration was a 10.6-year-old male ECS diagnosed with nasal squamous cell carcinoma (control 4).

There was no significant difference between cases, healthy controls and diseased controls regarding the serum concentration of total IgG (Kruskal–Wallis test, *P*= 0.317) (Fig. 4.2).

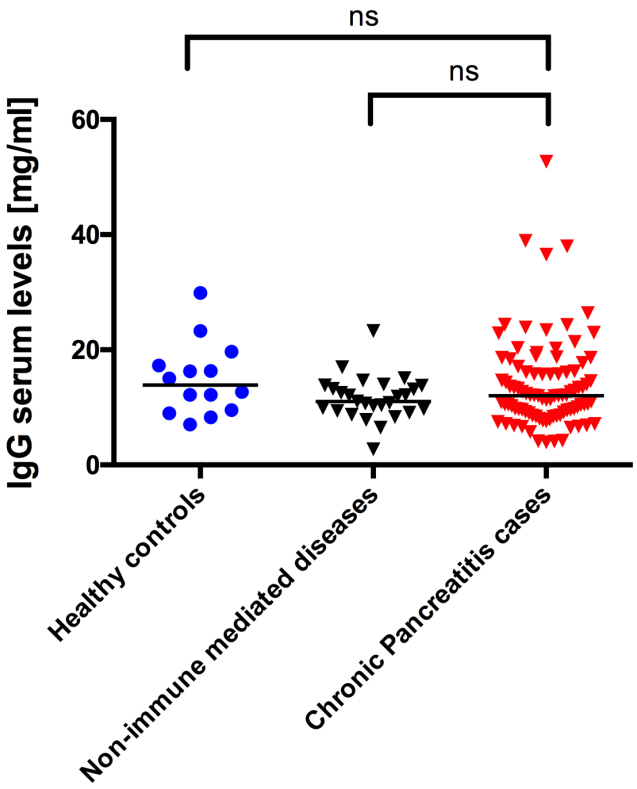

Figure 4.2: Total IgG serum levels in ECS affected by chronic pancreatitis compared with control ECS with no evidence of chronic pancreatitis. Serum IgG concentration is shown for healthy ECS (control group 1) in blue, for ECS affected by other disease with no known association to CP and with no immune-mediated aetiology (control group 2) in black and for ECS affected by CP in red. Results show

no significant difference in the level of serum IgG between cases and controls from groups 1 and 2 (Kruskal–Wallis test, *P*= 0.317). The horizontal lines represent the median from each group of dogs.

4.4.2.3. IgG4 serum levels

The median concentration of IgG4 in serum of affected ECSs was 1.16 mg/ml. The values in the cases ranged between 0.29 mg/ml to 11.61 mg/ml (Fig. 4.3). Median serum IgG4 concentration did not show significant differences between cases with definite diagnosis of CP (case group 1) and cases with possible diagnosis (case group 2) (1.23 *vs.* 1.11 mg/ml, Mann-Whitney U test: U= 1028.5, *P*= 0.631). Thirty cases had serum IgG4 concentrations higher than the reported normal reference values (<0.4-2.0 mg/ml). Twenty-seven out of those 30 cases presented with multi-organ inflammatory disease, the remaining 3 cases had only pancreatic inflammatory manifestation. In addition, cases that presented with clinical inflammatory manifestations in more than one organ had significantly higher levels of serum IgG4 than the ones with pancreatic manifestation only (1.31 mg/ml *vs.* 1.12 mg/ml, Mann-Whitney U test: U= 551, *P*= 0.025). The affected dog with the highest serum IgG4 concentration (11.61 mg/ml) was a 12.2-year-old female ECS that presented with CP and concurrent mammary mass (case 50).

The median concentration of IgG4 in serum of healthy controls was 0.62 mg/ml, ranging from 0.29 mg/ml to 4.43 mg/ml (Fig. 4.3). Two healthy controls (controls 12 and 13) had serum IgG4 concentrations higher than reported reference values. The control dog from this group that had the highest concentration of serum IgG4 (4.43 mg/ml) was a healthy 10.4-year-old female ECS that had a dental procedure and presented with slightly increased DGGR lipase but no other signs of CP (control 12).

Control dogs presenting with diseases with no immune-mediated aetiology had a median IgG4 serum concentration of 0.76 mg/ml and values ranged from 0.30 mg/ml to 7.41 mg/ml (Fig. 4.3). Five controls from group 2 had serum IgG4 concentration higher than 2 mg/ml and they were diagnosed with bronchogenic carcinoma (control 12 and 16), small intestinal disease (control 18), IVDD (control 19) and heart-base tumour (control 27), respectively. The dog from control group 2 with the highest serum IgG4 concentration (7.41 mg/ml) was an 11.6-year-old female blue roan ECS that presented with IVDD and had increased levels of DGGR lipase but no other clinical signs of CP (control 19).

There was a significant difference between cases, healthy controls and diseased controls regarding the serum IgG4 concentration (Kruskal–Wallis test, *P*= 0.001). The serum IgG4 level was significantly higher in cases than in control group 1 (Mann-Whitney U test: U= 327, *P*= 0.001) and control group 2 (Mann-Whitney U test: U= 830.5, *P*= 0.003) (Fig. 4.3). There was no significant difference in serum IgG4 concentration between control group 1 and control group 2 (Mann-Whitney U test: U= 141, *P*= 0.095).

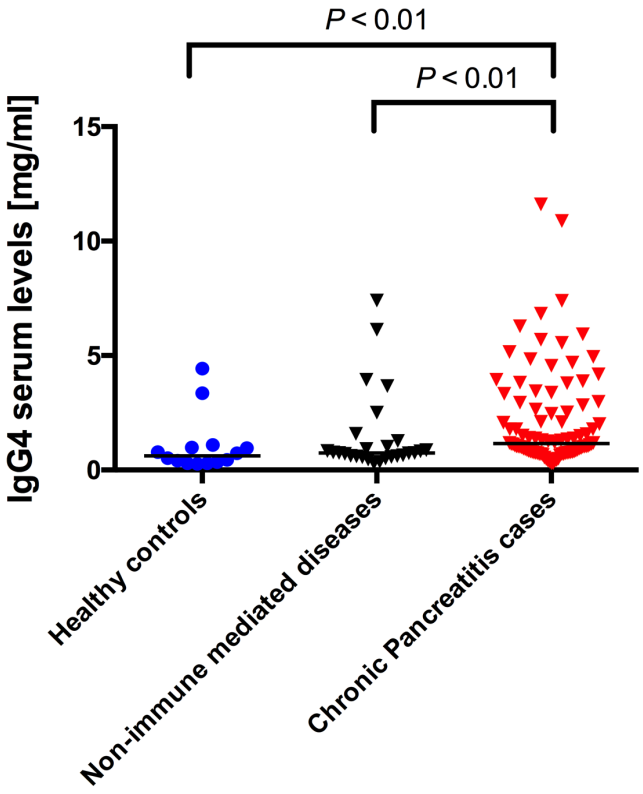

Figure 4.3: Serum IgG4 levels in ECS affected by chronic pancreatitis compared with control ECS with no evidence of chronic pancreatitis. Serum IgG4 concentration is shown for healthy ECS (control group 1) in blue, for ECSs affected by other disease with no known association to CP and with no immune-mediated aetiology (control group 2) in black and for ECS affected by CP in red. Results show significantly higher serum IgG4 levels in ECSs affected by CP versus ECS with no evidence of chronic pancreatitis (control group 1 [*P*= 0.001] and control group 2 [*P*= 0.003]). The horizontal lines represent the median from each group of dogs.

4.4.2.4. Serum IgG4/IgG ratio

The median serum IgG4/IgG ratio was 11.13 % for affected ECS, ranging from 0.77 % to 96.86 % (Fig. 4.4). Median serum IgG4/IgG ratio did not show a significant difference between cases from group 1 and group 2 (11.09 % *vs.* 10.70 %, Mann-Whitney U test: U= 980, *P*= 0.638). Cases with single pancreatic involvement and cases with multi-organ

manifestation did not show significantly difference in serum IgG4/IgG ratio (9.40 % *vs.* 11.68 %, Mann-Whitney U test: U= 596, *P*= 0.082). Healthy controls had a median serum IgG4/IgG ratio of 4.36 % and the range was from 0.97 % to 22.56 % (Fig. 4.4). Diseased controls had a median serum IgG4/IgG ratio of 6.16 %, ranging from 2.71 % to 78.61 % (Fig. 4.4).

There was a significant difference between cases, healthy controls and diseased controls regarding the serum IgG4/IgG ratio (Kruskal–Wallis test, *P*= 0.004). The median serum IgG4/IgG ratio was significantly higher in cases than in healthy controls (Mann-Whitney U test: U= 319, *P*= 0.001) and diseased controls (Mann-Whitney U test: U= 959, *P*= 0.037) (Fig. 4.4). In addition, serum IgG4/IgG ratio was significantly higher in control group 2 than in control group 1 (Mann-Whitney U test: U= 124.5, *P*= 0.039).

Cases 37 and 78 were removed from IgG, IgG4 and IgG4/IgG ratio statistical analysis because of having an IgG4 concentration, which was higher than the total IgG concentration (25.32 mg/ml IgG4 *vs.* 11.38 mg/ml IgG in case 37 and 22.84 mg/ml IgG4 *vs.* 11.98 mg/ml IgG in case 78), suggesting interference with the assay.

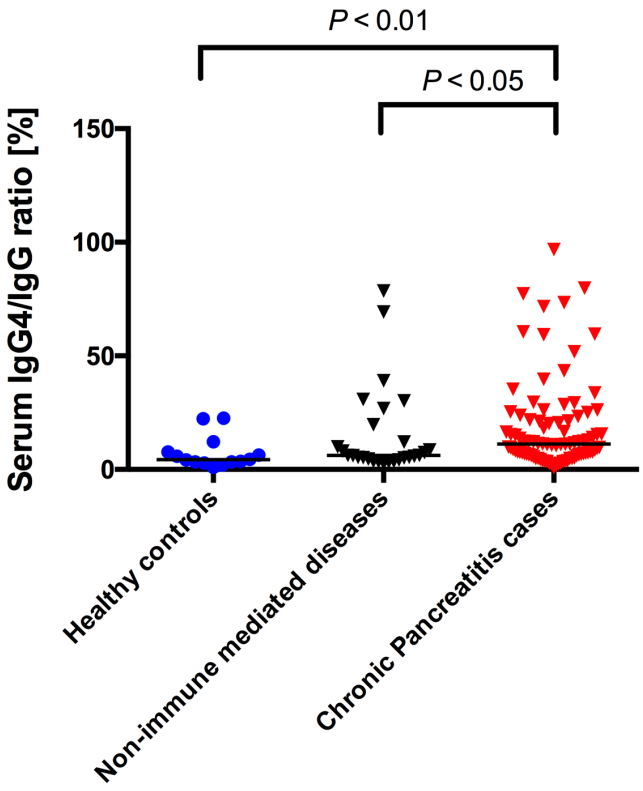

Figure 4.4: Serum IgG4/IgG ratio in ECS affected by chronic pancreatitis compared with control ECS with no evidence of chronic pancreatitis. Serum IgG4/IgG ratio is shown for healthy ECS (control group 1) in blue, for ECS affected by other diseases with no known association to CP and with no immune-mediated aetiology (control group 2) in black and for ECS affected by CP in red. Results show significantly higher serum IgG4/IgG ratio in ECS affected by CP versus control group 1 ($P < 0.01$) and between CP cases and control group 2 ($P < 0.05$). The horizontal lines represent the median from each group of dogs.

4.4.2.5. IgE serum levels

The median concentration of IgE in serum of affected ECSs was 4.82 ng/µL. The concentration values ranged between 0.01 ng/µL to 87.97 ng/µL in cases (Fig. 4.5). Median serum IgE concentration did not show significant differences between cases from group 1 and group 2 (4.24 ng/µL *vs.* 5.16 ng/µL, Mann-Whitney U test: U= 990.5, *P*= 0.441). In addition, serum IgE concentration did not show significant differences between cases that presented with clinical inflammatory manifestations in more than one organ and those with pancreatic manifestations only (4.52 ng/µL *vs.* 6.26 ng/µL, Mann-Whitney U test: U= 711, *P*= 0.617). The case with the highest serum IgE concentration was a 5.2-year old female black and white ECS affected by CP and concurrent epilepsy (case 73).

The median concentration of IgE in the serum of healthy control ECSs was 3.44 ng/µL, ranging from 0.04 ng/µL to 15.79 ng/µL (Fig. 4.5). The control dog from this group that presented with the highest concentration of serum IgE was a healthy 10.5-year-old golden female ECS that had a benign mass removed (control 6).

Control dogs presenting with diseases with no immune-mediated aetiology had a median IgE serum concentration of 2.81 ng/µL, ranging from 0.24 ng/µL to 32.95 ng/µL (Fig. 4.5). The control dog from the diseased group that presented with the highest concentration of serum IgE was a 7.3-year-old black male ECS that was diagnosed with high-grade mandibular sarcoma (control 11).

There was no significant difference between cases, healthy controls and diseased controls regarding the serum IgE concentration (Kruskal–Wallis test, *P*= 0.842) (Fig. 4.5).

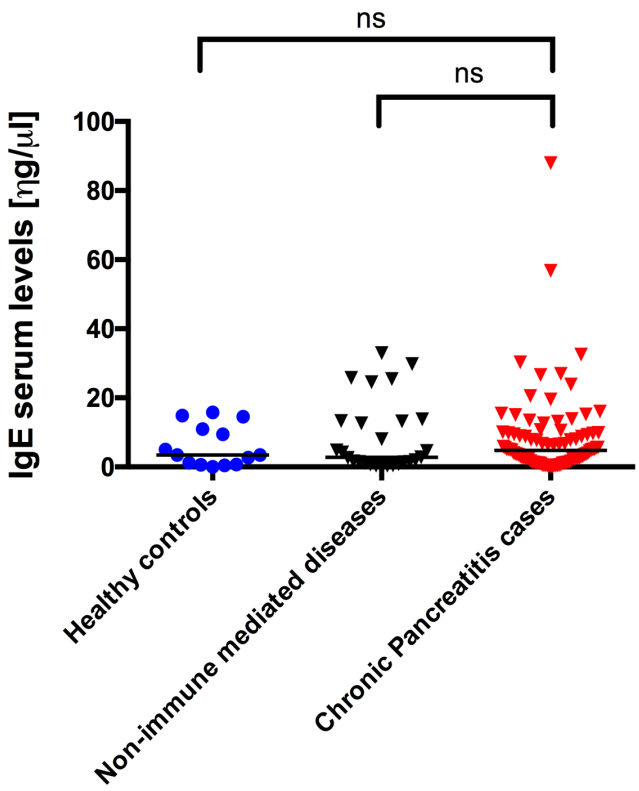

Figure 4.5: Serum IgE levels in ECS affected by chronic pancreatitis compared with control ECS with no evidence of chronic pancreatitis. Serum IgE concentration is shown for healthy ECS (control group 1) in blue, for ECS affected by other disease with no known association to CP and with no immune-mediated aetiology (control group 2) in black and for ECS affected by CP in red. Results show no significant difference in levels of serum IgE between cases, healthy controls and diseased controls (*P*= 0.842). The horizontal lines represent the median serum IgE concentration from each group of dogs.

Figures 4.6 and 4.7 show the serum IgG, IgG4, IgE levels and IgG4/IgG ratios found in ECS affected by CP divided by case groups and in ECS affected by CP with single or multi-organ clinical manifestations, respectively.

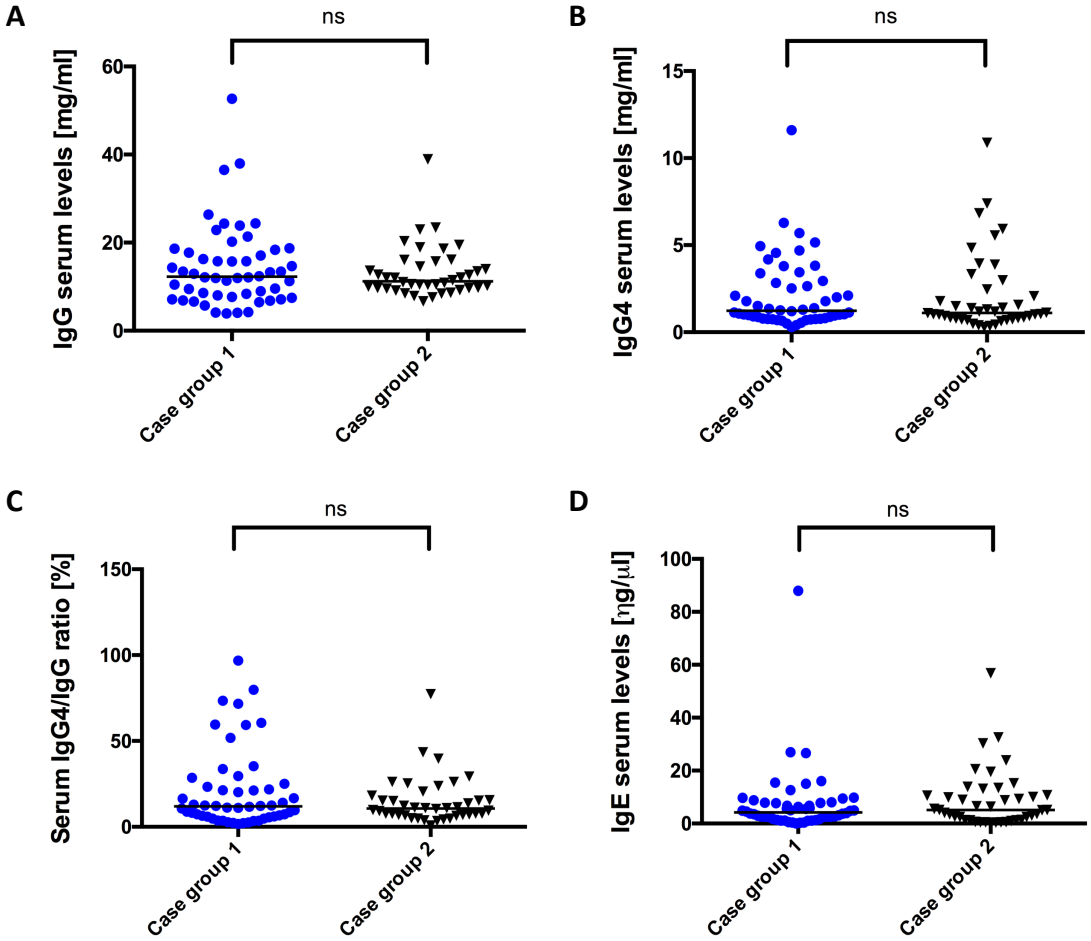

Figure 4.6: Serum IgG, IgG4, IgE levels and IgG4/IgG ratio in English cocker spaniels affected by chronic pancreatitis in case groups 1 and 2. Figures show in A: total serum IgG levels; B: serum IgG4 levels; C: serum IgG4/IgG ratio (%); and D: serum IgE levels in ECS cases with a definite diagnose of CP (case group 1) in blue and cases with possible diagnose of CP (case group 2) in black. The horizontal lines represent the median from each group of dogs. Ns: no statistically significant difference.

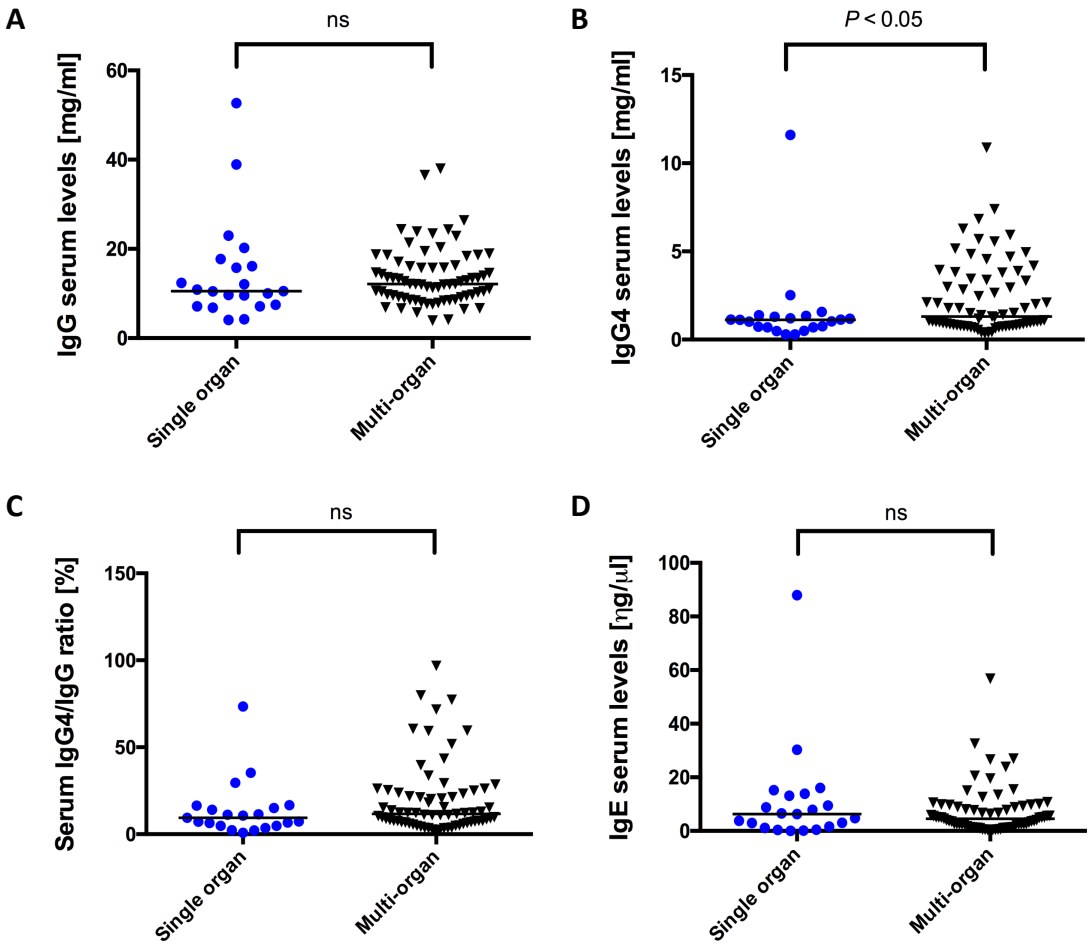

Figure 4.7: Serum IgG, IgG4, IgE levels and IgG4/IgG ratio in English cocker spaniels affected by chronic pancreatitis with single or multi-organ clinical manifestations. Figures show in A: total serum IgG levels; B: serum IgG4 levels; C: serum IgG4/IgG ratio (%); and D: serum IgE levels in ECS cases with single pancreatic manifestation in blue and cases with multi-organ manifestation in black. The horizontal lines represent the median from each group of dogs. Ns: no statistically significant difference.

Table 4.1 outlines demographic features, serum IgG, IgG4, IgE levels and serum IgG4/IgG ratio obtained in cases and control ECSs.

Table 4.1: Summary of serology data of ECS affected by chronic pancreatitis and controls.

	Cases	Control Group 1	Control Group 2	Kruskal-Wallis test *P* Value
n	96	14	27	
Age, median and range (years)	9.2 (3.0-14.9)	10.0 (8.9-11.25)	9.2 (7.0-13.3)	0.548
Females	50	7	9	
Males	46	7	18	
Serum total IgG, median and range (mg/ml)	12.03 (3.94-52.66)	13.87 (7.04-29.88)	11.01 (2.71-23.26)	0.317
Serum IgG4, median and range (mg/ml)	1.16 (0.29-11.61)	0.62 (0.29-4.43)	0.76 (0.30-7.41)	0.001
Serum IgE, median and range (ng/µl)	4.82 (0.01-87.97)	3.44 (0.04-15.79)	2.81 (0.24-32.95)	0.842
Serum IgG4/IgG ratio, median and range (%)	11.13 (0.77-96.86)	4.36 (0.97-22.56)	6.16 (2.71-78.61)	0.004

4.4.2.6. Receiver operating characteristic (ROC) curve for a diagnosis of IgG4-RD in English cocker spaniels affected by chronic pancreatitis

A ROC curve was obtained in order to analyse the utility of the serum IgG4 concentration as a diagnostic test for IgG4-RD in ECS affected by CP. All the ECS affected by chronic pancreatitis that had IgG4 levels measured, with the exception of cases 37 and 78, were included in the positive group, assuming they have IgG4-RD. Cases 37 and 78 were excluded since they had an IgG4 concentration which was higher than the total IgG concentration, suggesting interference with the assays. All the control dogs from group 1 and 2 that had serum IgG4 concentration measured were considered in the negative group. The included samples gave an area under the curve (AUC) of 0.701 (95% CI, 0.599 - 0.802, P<0.001), which indicates a fair diagnostic accuracy of the serum IgG4 test to distinguish among subjects that have the condition and those that do not present with it (Fig. 4.8). In addition, it was found that at a serum IgG4 cut-off of 0.85 mg/ml, the sensitivity was 70.2 %, specificity was 63.4 %, positive predictive value (PPV) was 81.48 % and negative predictive value (NPV) was 48.15 % in diagnosing IgG4-RD in dogs with CP. Sixty-six out of 94 cases, 5/14 healthy controls and 10/27 diseased controls had serum IgG4 levels >0.85 mg/ml. A higher IgG4 cut-off to diagnose the disease was also evaluated. By increasing the IgG4 cut-off to 2.08 mg/ml, the sensitivity obtained decreased to 30.8 % and the specificity increased to 82.9 %, the PPV was 80.5 % and NPV was 34.34 %. Twenty-nine out of 94 cases, 2/14 healthy controls and 5/27 diseased controls had serum IgG4 levels >2.08 mg/ml.

Moreover, another ROC curve was generated in order to analyse the utility of the serum IgG4/IgG ratio in diagnosing IgG4-RD in ECSs. The AUC for the serum IgG4/IgG ratio was 0.661 (95 % CI, 0.556 - 0.767, P=0.003). A serum IgG4/IgG ratio of 7.09 % had a sensitivity of 70.7 %, a specificity of 61.0 %, a PPV of 80.25 %, and an NPV of 48.08 %. Sixty-five out of 94 cases, 4/14 healthy controls and 12/27 diseased controls had serum IgG4/IgG ratios >7.09 %. A higher serum IgG4/IgG ratio cut-off of 11.13 % had a sensitivity of 50.0 %, a specificity of 73.2 %, a PPV of 80.7 %, and an NPV of 39.47 %. Forty-six out of 92 cases, 3/14 healthy controls and 8/27 diseased controls had serum IgG4/IgG ratios >11.13 %.

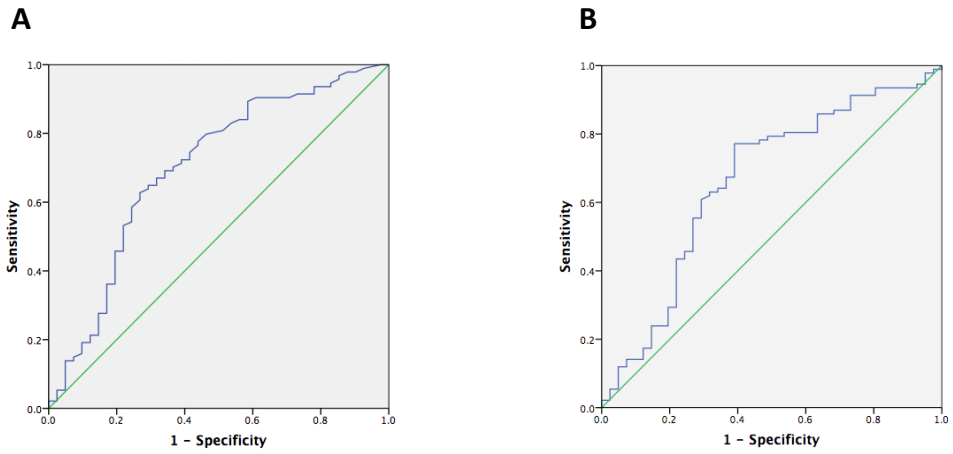

Figure 4.8: ROC curve analysis of the utility of using IgG4 concentration and IgG4/IgG ratio in diagnosis of IgG4-RD in English cocker spaniels. ROC curve analysis performed for the serum IgG4 levels (A) and IgG4/IgG ratio (B) that were measured in ECS affected by CP and control dogs with no evidence of CP. Each point on the curve (blue line) represents the true-positive rate and false-positive rate associated with the test value. The green line represents the null hypothesis. A: AUC= 0.701 (*P* <0.001), B: AUC= 0.661 (*P*=0.003).

4.5. Discussion and conclusions

In this study, total serum IgG, IgG4, IgE levels, and IgG4/IgG ratios were measured in English cocker spaniels affected by chronic pancreatitis and control dogs of the same breed, both healthy and diseased. The main aim of this study was to investigate the role of serum IgG4 levels in the diagnosis of CP in ECS cases with suspicion of IgG4-RD.

It was demonstrated that affected ECSs had significantly higher serum levels of IgG4 compared with age-matched healthy controls (median 1.16 mg/ml *vs.* 0.62 mg/ml, $P < 0.01$) and compared with ECS presenting with different diseases with no immune-mediated aetiology and with no evidence of chronic pancreatitis (median 1.16 mg/ml *vs.* 0.76 mg/ml, $P < 0.01$).

A commercial ELISA kit was used to determine serum IgG4 concentrations. Differing dilutions were used for cases and controls. Serum samples from cases were more diluted than control samples but both used the manufacturer's recommended dilutions and the results were corrected by the dilution factor. The reason for this discrepancy was the assumption that cases would have increased concentration of IgG4 in serum; therefore if they were less diluted, they may be not detected by the ELISA reader due to saturation. In a diagnostic context when it would not be possible to know whether a patient is a possible case or control, a unique dilution would be used. Since both dilutions worked well, the dilution used for cases would be recommended because less serum sample is needed for the detection of IgG4.

Increased levels of serum IgG4 have been associated with human autoimmune pancreatitis and IgG4-RD. IgG4 is the least prevalent of the four IgG subclasses in humans and dogs. In humans, IgG4 represents 3–6 % of total IgG in the serum of healthy adults. It has been seen that serum IgG4 can be elevated up to 50 times the upper limit of the normal range in patients diagnosed with IgG4-RD (Stone *et al*., 2012). Although elevated serum IgG4 is not specific for a diagnosis of human IgG4-RD, it is a good tool to determine disease activity, multi-organ manifestation, and risk of relapse in patients. Most patients with IgG4-RD show multiple organ involvement at diagnosis, with both high absolute serum IgG4 concentrations and serum IgG4/IgG ratios.

As seen on human IgG4-RD, the cases in this study that presented with clinical inflammatory manifestations in more than one organ had significantly higher levels of serum IgG4 than the ones with just pancreatic manifestation (1.31 mg/ml *vs.* 1.12 mg/ml $P < 0.05$). These results suggest that the measurement of serum IgG4 could be useful to predict disease activity and multi-organ manifestation.

Using previously reported normal serum IgG4 ranges is however not wise when using a different test. For this reason, in the current study it was generated a new range using our own available cases and controls. A ROC curve indicated a fair accuracy of this serum IgG4 test in diagnosing IgG4-RD in ECS affected by CP. It was demonstrated that a cut-off value of 0.85 mg/ml had a sensitivity of 70.2 %, specificity of 63.4 %, PPV of 81.48 % and NPV of 48.15% in diagnosing IgG4-RD in dogs with CP. These results indicate that reliance upon serum IgG4 concentrations for establishing the diagnosis of IgG4-RD is not recommended, since elevated serum IgG4 concentrations can also be present in patients with non-IgG4-RD diagnoses. As these CP cases did not have histological analysis and IgG4+ plasma cell staining performed to check IgG4-RD features in affected organs, a higher IgG4 cut-off to diagnose the disease was also evaluated. Thus, increasing the cut-off value for serum IgG4 to 2.08 mg/ml improved the specificity from 63.4 % to 82.9 % but produced an unacceptable reduction in sensitivity, from 70.2 % to 30.8%. In humans, serum concentrations of IgG4 > 1.35 mg/ml have been widely accepted as a cut-off value, and have demonstrated a sensitivity of 97.0 % and a specificity of 79.6 % in diagnosing IgG4-RD and differentiating it from other disease conditions (Masaki *et al*., 2012). Some authors have also suggested that a serum IgG4 concentration that is more than twice the upper limit of normal (> 2.8 mg/ml) is more highly specific for human IgG-RD (Ghazale *et al*., 2007). Regarding serum IgG4 in dogs, we would need to look at IgG4 concentrations in larger numbers of affected and unaffected dogs before being sure of the ideal cut-off value to use for diagnostic purposes.

Some studies have shown that between 10 % and 30 % of human IgG4-RD patients have normal serum IgG4 concentrations, even in the presence of classic histopathological and immunohistochemical staining features of the disease (Wallace *et al*., 2015b). In the current study, 28/94 (29.8 %) and 65/94 (69.1 %) ECS affected by CP had serum IgG4 levels < 0.85 mg/ml and < 2.08 mg/ml, respectively. These dogs did not have increase serum IgG4 concentrations, and because they did not have histology performed, it was not possible to categorically prove that they could be affected with IgG4-RD.

As seen on human IgG4-RD, this study showed that serum IgG4 levels could be also elevated in other non-IgG4-RD conditions. Five control dogs presenting with diseases with no immune-mediated aetiology had increased serum IgG4 concentration. The control dog with the highest serum IgG4 concentration was an 11.6-year-old female blue roan ECS that presented with IVDD and had increased levels of DGGR lipase but no other clinical signs of CP. It has been observed that dogs with IVDD can present with increased canine pancreatic lipase immunoreactivity (cPLI) levels without presenting a diagnosis of CP (Schueler *et al*., 2018). However, it would be interesting to do further examination or observation in this patient in order to determine if the increased DGGR lipase was associated with an underlying diagnosis of CP and the increased IgG4 levels were as a consequence of the latter.

In addition, it has been reported in humans that serum IgG4 level can be elevated in 5 % of the normal population (Ryu *et al*., 2011). In the current study, 2/14 (14.3 %) healthy controls had elevated serum IgG4 levels (> 2.08 mg/ml). The healthy control dog that had the highest concentration of serum IgG4 (4.43 mg/ml) was a healthy 10.4-year-old female ECS that had a dental procedure and presented with slightly increased DGGR lipase but no other signs of CP. Following up this dog could be useful to obtain important information to determine in more detail whether the increased DGGR lipase was actually not associated with an underlying diagnosis of CP in this dog.

Serum IgG levels and IgG4/IgG ratios in ECS presenting with CP and controls were also evaluated. No significant difference was observed regarding concentrations of total IgG in serum between cases and healthy controls and between cases and controls presenting with other conditions. Studies have shown that serum IgG4/IgG ratios > 8 % had a sensitivity and specificity of 95.5 % and 87.5 %, respectively, for the diagnosis of human IgG4-RD *(Masaki et al*., 2012). In our study, the median serum IgG4/IgG ratio was significantly higher in cases than in healthy controls ($P < 0.01$) and in diseased controls ($P < 0.05$). After generating a ROC curve to analyse the utility of the serum IgG4/IgG ratio in diagnosing IgG4-RD in ECS, it was concluded that the test had a poor accuracy to distinguish among subjects that have the condition and those that do not. A serum IgG4/IgG ratio of 7.09 % had a sensitivity of 70.7 % and a specificity of 61.0 %. A higher serum IgG4/IgG ratio cut-off of 11.13 % was also evaluated, decreasing the sensitivity to 50 % and increasing the specificity to 73.2 %. These results indicate that the increase in serum IgG4 levels and IgG4/IgG ratios are not fully

specific to detect this condition in dogs. However, if the measurement of serum IgG4 levels and IgG4/IgG ratios are used in combination with evidence of pathological manifestations in affected organs and clinical symptoms, the sensitivity and specificity in diagnosing IgG4-RD in affected dogs could increase as seen in human IgG4-RD.

Corticosteroids are the first-line treatment for human IgG4-RD patients. It has been observed that elevated serum IgG4 levels in IgG4-RD fall with corticosteroid treatment, with significant declines at 8 and 12 weeks of therapy (Culver *et al*., 2016). Here, four cases recruited for this study were on immunosuppressive treatment with corticosteroids for other concurrent immune-mediated conditions. These cases have been excluded to avoid bias. As a future work, it will be evaluated if affected dogs receiving immunosuppressive therapy have a reduction in their serum IgG4 levels after treatment determining if the test is able to identify treatment response.

Since increased serum IgG4 levels can be observed in other disorders, it is necessary to diagnose IgG4-related disease carefully and correctly. It is important to bear in mind that the diagnosis of IgG4-RD does not depend on serum IgG4 levels in isolation. The clinical scenario must always be considered when diagnosing this disease, and should be supported by other criteria, including histological evidence, clinical or radiological evidence of organ involvement, and serological or radiological response to steroids.

Different publications have suggested a causative role for T-helper type 2 (Th2) cells, however the pathogenesis of human IgG4-RD is not yet fully understood. In addition, some studies have reported that a high proportion of patients with IgG4-RD present with long standing allergies, peripheral blood eosinophilia, and increased serum IgE levels (Kamisawa e*t al*., 2009; Culver *et al*., 2017). Allergic immune responses can be induced by specific Th2 cytokines, such as IL-4, IL-5, and IL-13, which promote peripheral blood eosinophilia and the secretion of IgG4 and IgE (Torre *et al*., 2014). In this study we evaluated the serum concentration of IgE in CP cases and controls. It was observed that the median serum IgE concentration was not significantly different between cases and healthy controls (4.82 ng/µL *vs.* 3.44 ng/µL, *P*= 0.631) and dogs presenting with other conditions (4.82 ng/µL *vs.* 2.81 ng/µL, *P*= 0.697). These results are similar as previous studies in dogs that showed that total serum IgE concentrations could not discriminate between healthy and atopic dog

populations, suggesting that quantification of total serum IgE would not be valuable as a screening test for canine atopy (Hill *et al*., 1995).

This study had limitations inherent in its initial design and selection of cases. In order to confirm the diagnosis of IgG4-RD, characteristic histological features such as dense lymphoplasmacytic infiltrate, storiform fibrosis, obliterative phlebitis and an increase number of IgG4+ plasma cells have to be present. The CP cases included in this study did not have histology performed; therefore it was not possible to confirm categorically that they suffer from IgG4-RD. Also, some lipaemic serum samples were included and they could be interfering with the measurement of immunoglobulin on the ELISA.

In conclusion, this study has shown that ECS affected by CP can present with increased serum IgG4 levels and IgG4/IgG ratios. Moreover, serum IgG4 levels are higher in patients that presented with clinical inflammatory manifestation in more than one organ, demonstrating similarities to human IgG4-RD. A serum IgG4 test seems a promising non-invasive diagnostic test that could allow early recognition and timely immunosuppressive treatment, preventing or delaying the development of further signs in other organs, thus improving quality of life in dogs with IgG4-RD. Future studies are necessary in order to determine serum IgG4 concentration in dogs with histologically proven diagnosis of IgG4-RD to establish an ideal cut-off value for diagnostic purposes with high specificity and sensitivity and determine whether serum IgG4 concentration in ECSs with CP is decreased after immunosuppressive treatment.

CHAPTER 5: GENOTYPIC EVALUATION OF ENGLISH COCKER SPANIELS AFFECTED BY CHRONIC PANCREATITIS

5.1. Summary

Chronic pancreatitis (CP) is common in English cocker spaniels (ECS) and has similar clinical and histological features to human IgG4-related disease (IgG4-RD). The genetics of human IgG4-RD is poorly understood but it is likely a multifactorial disease comprising both the effects of genetic susceptibility and environmental factors. Human leukocyte antigen (HLA) and also non-HLA genes have been suggested as risk factors for IgG4-RD, particularly in the pancreatic manifestation. The common occurrence of CP in ECS suggests an underlying genetic predisposition. A previous genetic study of dog leukocyte antigen (DLA) in ECS affected by CP showed an increased frequency of the haplotype DLA-DQB1*00701 among cases and a lower frequency of the DLA-DQB1*02001 haplotype, compared to controls. An unpublished pilot genome-wide association study (GWAS) showed two loci containing candidates that might also contribute to disease causation, one on chromosome 16 and the other on chromosome 12. The purpose of this chapter was to confirm or repudiate the genetic associations from the pilot GWAS. In order to do this, PCR amplified the loci that contained the SNPs using a larger cohort of well-phenotyped CP cases and control ECS. This allowed analysis of SNP frequency between the case and control groups. Results indicated that there was no overall significant difference in the SNPs analysed on chromosome 12 and 16 using the total comparison groups (total controls versus total cases) although homozygosity in certain positions could be shown to be associated with disease when only healthy controls and cases were compared.

5.2. Introduction

Different studies in human Asian populations using small sample sizes, have suggested that different variants in genes could be associated to IgG4-RD susceptibility. HLA serotypes DRB1*0405 and DQB1*0401 increase the susceptibility to IgG4-RD disease in Japanese populations, whereas DQβ1-57 without aspartic acid is associated with disease relapse in Korean populations (Kawa *et al*., 2002; Park *et al*., 2008). In addition, it has been seen that single-nucleotide polymorphisms in non-HLA genes such as CTLA-4 (cytotoxic T-lymphocyte antigen-4) (Umemura *et al*., 2008), KCNA3 (potassium voltage-gated channel subfamily A member 3) (Ota *et al*., 2011) and FCRL3 (Fc receptor-like 3) (Umemura *et al*., 2006) play a role in disease susceptibility .

One of the most valuable genetic studies to date is the one obtained by the first Genome-Wide Association Study (GWAS) from Japan including 857 patients affected by IgG4-RD and 2,082 healthy controls (Terao *et al*., 2019). Herein, a significant association was found for two loci, HLA-DRB1 and FCGR2B. The strongest disease association found in HLA-DRB1 was probably caused by the amino acid variation at position 7 of the β domain of the peptide-binding groove of HLA-DRB1 that plays a key role in antigen presentation. Amino acid variants at this position have been reported in other autoimmune diseases such as rheumatoid arthritis (Raychaudhuri *et al*., 2012). The single nucleotide variant in FCGR2B (rs1340976) showed an association with increased FCGR2B expression. Moreover, the same single nucleotide variant was associated with clinical features of IgG4-RD including the number of organs in the body which are involved, and serum IgG4 levels at diagnosis. FCGR2B encodes Fcγ receptor IIB (FcγRIIB), which is expressed by B lymphocytes, monocytes, and dendritic cells. It is the only known inhibitory Fcγ receptor and has a vital role in the elimination of auto reactive B cells (Xiang *et al*., 2007). This suggested that stronger inhibitory signals from FcγIIB might predispose to IgG4-RD. However, the mechanism involved in susceptibility to IgG4-RD remains unknown and efforts to incorporate information related to variability of this Fcγ receptor have not been incorporated in the pathogenesis model of the disease.

In ECS dogs, the common presentation of chronic pancreatitis (CP) suggests an underlying genetic predisposition. A previous genetic study using 47 affected ECS and 82 controls, showed that dog leukocyte antigen (DLA) haplotypes are a contributory factor in the

development of CP in ECS (Bazelle *et al*., 2013). This study identified an increased frequency of the DLA-DQB1*00701 haplotype in ECS with CP, and a lower frequency of the DLA-DQB1*02001 haplotype, compared to controls. This DLA association has also been found in ECS with immune-mediated haemolytic anaemia (Kennedy *et al*., 2006), suggesting an underlying inherited susceptibility to a variety of immune-mediated diseases in the ECS breed.

A recent unpublished pilot genome-wide association study (GWAS) on the 167k Illumina SNP array carried out by Dr David Sargan and Dr Jesus Aguirre-Hernandez in the Department of Veterinary Medicine, University of Cambridge (personal communication), showed two loci as potentially linked to the disease with CP in ECS, one on chromosome 16 and another on chromosome 12. This study included 14 affected ECS and 47 non-affected age-matched controls, as well as 126 other controls from the general canine population with unknown disease status. After correction for population stratification using a mixed model approach, neither locus retained genome-wide significance, but both retained interest (best SNP p values = 4.03E-06 and 7.62E-06). The first locus overlies a region containing a group of T-cell receptor beta (TCR-β) variable loci and the anionic trypsinogen (PRSS2) gene. The second locus overlies several genes including the branched-chain alpha-keto acid dehydrogenase (BCKDHB), which is necessary for the metabolism of leucine, valine and isoleucine, and when mutated in humans, causes maple syrup urine disease (Chuang and Shih, 2001). Mutation in BCKDHB is also associated with acute and chronic pancreatitis in children (Kahler *et al*., 1994), thus partial loss of function at this locus could contribute to CP.

The aim of this chapter was to sequence the regions that contain two different SNP variants within the two loci that showed genome-wide interest in the pilot GWAS, using a larger number of cases and controls, to confirm or repudiate the associations previously found.

5.3. Materials and methods

5.3.1. Dog recruitment and EDTA blood sample collection

Residual peripheral blood samples from diagnostic tests of 104 ECS affected by CP and 44 age-matched control ECS with no suspicion of CP and/or immune-mediated disease were collected in ethylene diaminetetraacetic acid (EDTA) tubes and stored at − 20 ° C until further analysis. The samples were obtained from patients at the Queen's Veterinary School Hospital and from first opinion veterinary practices and referral centres throughout the UK. The dog owners were informed about the study and they signed a consent form for using surplus blood for DNA extraction and for collection of the medical records. None of the dogs recruited here were used in previous genetic studies. This study was approved by the Ethics Committee at the Department of Veterinary Medicine, University of Cambridge (ethical review CR291).

As described in chapter 2, cases were split into 2 groups according to the methodology used in the diagnosis of CP. *Case group 1* was diagnosed based either on presenting pancreatic histology confirming CP (n=9) or with both elevated cPLI and abnormal pancreatic ultrasound (n= 47). *Case group 2* was diagnosed based on less robust diagnostic methods, including suggestive clinical signs with elevated cPLI or DGGR lipase, and elevated lipase/amylase without ultrasonographic changes (n=48). Cases on corticosteroids or any immunosuppressant treatment were not excluded from this genetic study.

Control dogs were also divided in two groups. *Control group 1* included 15 healthy ECS and *control group 2* included 29 ECS presenting with several diseases unrelated with CP and with non-immune mediated aetiology. Inclusion criteria for control dogs consisted on no evidence of CP, glomerulonephritis, KCS, anal sac issues and/or any other immune-mediated diseases.

The signalment, in addition to the clinical details of the cases and control dogs included in this study, are summarised in Chapter 2 (Tables 2.1, 2.2, and 2.3).

5.3.2. Genomic DNA extraction

Genomic DNA of cases and controls was isolated from EDTA blood using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the instructions provided by the manufacturer. Briefly, 100 µl of each anticoagulant-treated blood sample were lysed using 20 µl of proteinase K. Then, 200 µl of buffer AL were added, and this mixture was incubated at 56 ° C for 10 min. This was followed by the addition of 200 µl ethanol (98 %). Then, the lysate was loaded onto the DNeasy mini spin column and centrifuged at 6,000 x ***g*** for 1 min, allowing the selective binding of the DNA to the DNeasy membrane. Remaining contaminants were removed in two wash steps and finally the DNA was eluted using 100 µl of buffer AE. The DNA concentration was quantified using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific) and all samples were normalised to 5 ng/µL. Extracted DNA samples were stored at – 20 ° C until used.

5.3.3. Primer design

Reference DNA sequences for the loci of interest on canine chromosome 12 (CFA12) and 16 (CFA16) containing the GWAS identified SNPs were retrieved from the Ensembl and UCSD genome browsers. Primers targeting sequences for rs22196038 (also known as BICF2P1267895) and rs852916308 (also known as BICF2P552398) that spanned the SNPs located on CFA12: 41298146 and CFA16: 6783605 of the genome build CanFam3.1, respectively, were designed using Primer3 online software (http://primer3.ut.ee/). Primers were checked for uniqueness using primer3_masker. Designed primers are shown in Table 5.1.

Table 5.1: Primers used for PCR assays

Target	*Primer name*	*Primer sequence (5'–3')*	*Length (bp)*	*Manufacturer Annealing temperature*	*Optimal Annealing Temperature with Touchdown*	*Amplicon size (bp)*
rs22196038 SNP	*Chr12_ SNP*	*Forward:* GCAAGAGTCGCACAGATCTG	20	64 °C	66 ° C	217
		Reverse: CGCCTCGGGTAAACAAACTC	20			
rs852916308	*Chr16_SNP*	*Forward*: ATATTCCACCTCGCACCTGG	20	64 °C	66 ° C	247
		Reverse: TGTTCTTCTCCTTCTCTGCTACA	23			

5.3.4. Polymerase chain reactions (PCR)

PCR amplifications targeting the region containing the rs22196038 (on CAF12) and rs852916308 SNPs (on CFA16) were carried out separately using the designed primers shown in Table 1, in a Mastercycler® EP thermal cycler (Eppendorf). Phusion High-fidelity DNA polymerase (Thermofisher Scientific) was used for all the PCRs. Master mix was prepared according to the manufacturer's instructions. PCR amplification was performed using 15 ng genomic DNA in a 15 µl reaction. A negative control containing no DNA template (NTC) was included in each PCR run to monitor possible contamination and primer-dimer formation. The same cycling conditions were used for all DNA samples and the NTC.

Initially, the PCR cycling protocol used the annealing temperature suggested by the primers' manufacturer (Sigma-Aldrich, 64 °C), but this needed to be optimised. Thus, a gradient PCR was performed to identify the optimum annealing temperature for each primer set. PCRs were also found to be cleaner if an initial set of touchdown cycles were added. Based on the results of the gradient PCR, the final PCR protocols for both sets of primers were as it follows (Table 1): a denaturation cycle of 98 °C for 2 min, followed by 6 touchdown cycles starting with an annealing temperature of 72 °C, that decreased by 1 degree per cycle, then 30 cycles of 98 °C for 10 seconds, 66 °C for 15 seconds, 72 °C for 15 seconds, followed by a final extension cycle of 72 °C for 10 min.

5.3.5. Agarose gels

In order to determine specificity of DNA fragment amplification, 5 µL of each PCR product were loaded onto 1.5 % agarose gels, stained with ethidium bromide solution (Sigma). TrackIt™ Cyan/Orange Loading Buffer (Invitrogen) was added to the samples before loading. TrackIt™ 100 base pair DNA ladder (Invitrogen) was also loaded and used as a DNA size marker. Agarose gel electrophoresis was run at 100 V, 250 mA for 45 min, using 1 X Tris-acetate-EDTA (TAE) as a running buffer. DNA gel bands were visualised using an ultraviolet transilluminator (UVP).

5.3.6. PCR Product DNA purification

DNA was purified from PCR amplification products and/or from agarose gel bands, using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) following the

manufacturer's instructions. Briefly, 10 µL of Membrane Binding Solution were added to each 10 µL of PCR amplification product. This mixture was then transferred to individual Wizard® SV mini-columns. This was followed by incubation at room temperature for 1 min, and centrifugation at 16,000 × ***g*** for 1 min. In order to increase yield, flow-through was loaded back onto the columns and then centrifuged again. The columns were washed twice by adding Membrane Wash Solution, containing ethanol. Finally, DNA was eluted with 50 µl of nuclease-free water and stored at 4 ° C for subsequent sequencing analysis.

When DNA sequencing was of poor quality, the PCR was repeated and all the product run through an agarose gel and purified from the excised DNA gel band. In that case, the individual gel slices were placed in separate 1.5 ml microcentrifuge tubes, and 10 µL of Membrane Binding Solution were added per 10 mg of gel slice. Then, tubes were vortexed and incubated at 60 ° C until the gel slice was completely dissolved. The gel mixture was then transferred to a mini-column as above and the manufacturer's instruction followed as above. However, in this case, DNA was eluted using less nuclease-free water (30 µl) to give the required DNA concentration for sequencing.

5.3.7. DNA sequencing

Purified DNA products were submitted for Sanger sequencing to Source Bioscience (Source Genomics, Cambridge, UK) using the primers above (Table 1). Each DNA fragment was bi-directionally sequenced. Sequencing results were visualised by using the 4 Peaks software. EMBOSS Explorer Matcher (http://www.bioinformatics.nl/emboss-explorer/) was used to perform a local alignment against the specific canine sequences, identifying local similarities and allowing easier recognition of the SNP. Multiple DNA sequence alignment was carried out using Clustal Omega (https://www.ebi.ac.uk). Multiple sequence alignments were then visualised using Seaview software v4.6.2 (Gouy *et al.*, 2010; Galtier *et al.*, 1996).

5.3.8 Restriction Enzyme Digestion

PCR amplification products for the region containing the SNP on CAF12 that after sequencing were categorised as containing a heterozygous genotype were subjected to restriction enzyme digestion to confirm the genotype. In order to find the correct enzyme, EMBOSS Explorer Remap was used to display restriction enzyme cleavage sites in the predicted PCR

product sequence. After using this software, BseMII (BspCNI) enzyme (Thermo Fisher), which recognizes CTCAG(10/8)^ sites, was selected for this purpose. Predicted cut sites for this particular restriction enzyme are shown in Appendix 3. All restriction enzyme reactions were performed for 2 h at 55 ° C and used the buffer and conditions recommended by the manufacturer. Digested DNA was analysed by gel electrophoresis on 2 % agarose gels and visualised on a UV transilluminator (UVP).

Statistical analysis

The genotype and allele frequencies were compared between groups using Fisher's exact test. Odds ratios (OR) and 95 % confidence intervals (CI) were calculated for disease association of individual alleles and genotypes. A two-proportion Z-test was performed to test the significance of allele frequencies when the control group was divided into two groups (healthy controls and controls presenting with other diseases different than CP). Statistical analyses were performed using STATA software package (StataCorp LLC, Texas, USA). A p value of < 0.05 was considered statistically significant.

5.4. Results

5.4.1. Dog recruitment and EDTA blood sample collection

EDTA blood samples from a total of 104 cases and 44 control ECS dogs were collected for this study. The age of the affected dogs ranged from 2.7 to 16 years at diagnosis (median 9.1 years), and they comprised 51 females and 53 males. Affected ECS all had confirmed or strongly suspected CP and also a high prevalence of KCS (n=49), proteinuria (n=49), anal gland disease (n=36), xerostomia (n=27), atopy (n=21) and other immune-mediated diseases (n=16).

The age of the control dogs ranged from 7.0 to 12.9 years (median 9.7 years), with 27 males and 17 females. Fifteen control dogs were considered healthy (median age 10 years) and 29 had other non-immune conditions unrelated to CP (median age 9.2 years).

The cases and controls included in this chapter are the same ones included in chapter 2. Phenotypic and clinical data were analysed in that chapter.

5.4.2. Genotyping

In order to determine whether ECS affected by CP had specific genotypes leading to disease expression, the genetic loci already identified in a preliminary GWAS in ECS with CP were evaluated. Thus, PCRs were set up to look at the SNPs within the two loci in the new cases and controls. For this purpose, primers were designed to amplify the regions that contained SNPs of interest (Table 5.1). PCRs were optimized using a temperature gradient and touch down reactions and PCR product size was confirmed on 1.5 % agarose gels (Figs. 5.1 and 5.2). PCR product sizes using these primers were approximately 217 bp for region containing the *rs22196038 SNP* located on CFA12 (Fig. 5.1) and 247 bp for region containing the *rs852916308 SNP* located on CFA16 (Fig. 5.2).

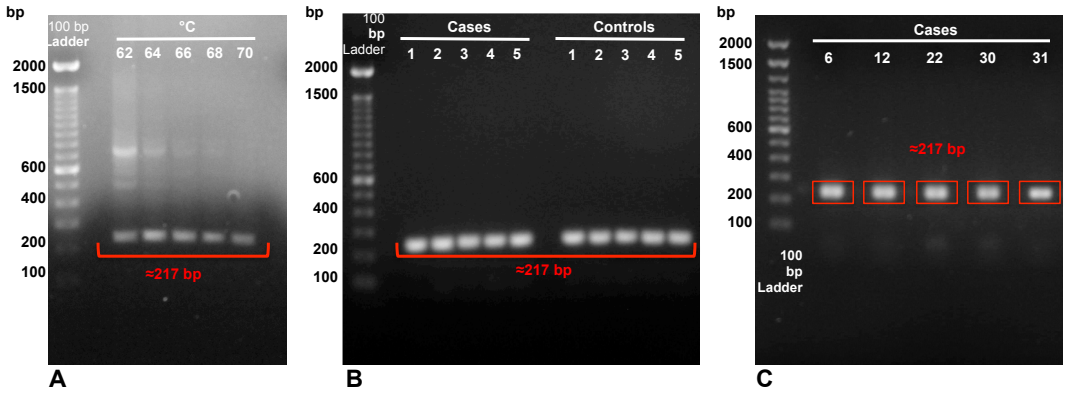

Figure 5.1: 1.5 % Agarose gel electrophoresis of PCR amplification products from canine DNA using *rs22196038 SNP* primers. (A) Gradient PCR for optimization, (B) Optimised touch down PCR (C) Touch down PCR showing DNA samples that were purified from the gel for further sequencing. The PCR products that were purified from the gel band are indicated in red squares.

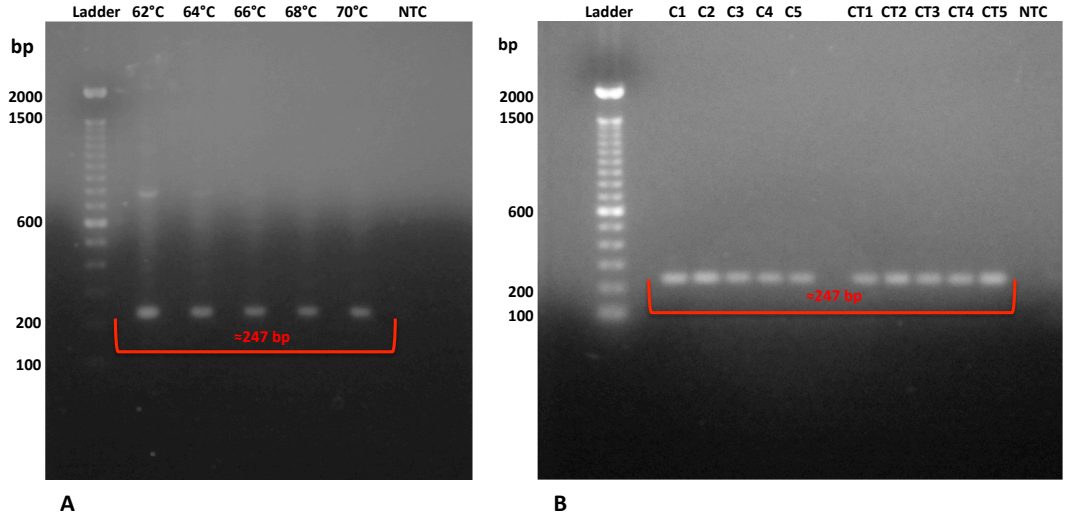

Figure 5.2: 1.5 % Agarose gel electrophoresis of PCR amplification products of canine DNA using *rs852916308 SNP* primers. (A) Gradient PCR for optimization, (B) Optimised touch down PCR of DNA samples that were purified from PCR products for further sequencing. The sizes of the PCR products are indicated in red colour. NTC= non-template control.

Subsequently, PCR products from cases and controls were sequenced and genotyped. Firstly, the candidate SNP on chromosome 16 was sequenced in 20 CP cases and 20 controls. The results showed no differences in the distribution of alleles and genotypes at this candidate SNP when the cases and controls were compared. In addition, no significant association was observed between CP and this SNP since all the cases and controls analysed presented exactly the same SNP variant (variant G) (Fig. 5.3). Figure 5.4 shows the alignment for the sequenced DNA in cases 1-20 and controls (CT) included against the reference genome CanFam3.1. Although the reference genome (boxer breed) had a C in the SNP position, all the ECS samples had a G. After obtaining these results, it was decided not to continue

sequencing the SNP on chromosome 16 in the remaining cases and controls and start sequencing the SNP candidate on chromosome 12 that showed interest in the pilot GWAS.

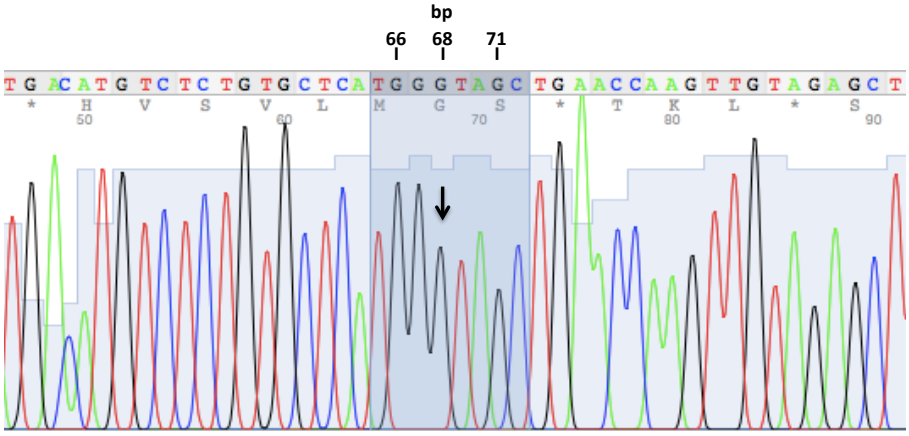

Figure 5.3: DNA sequencing plots showing a homozygous G genotype. The arrow indicates the rs852916308 SNP variant G.

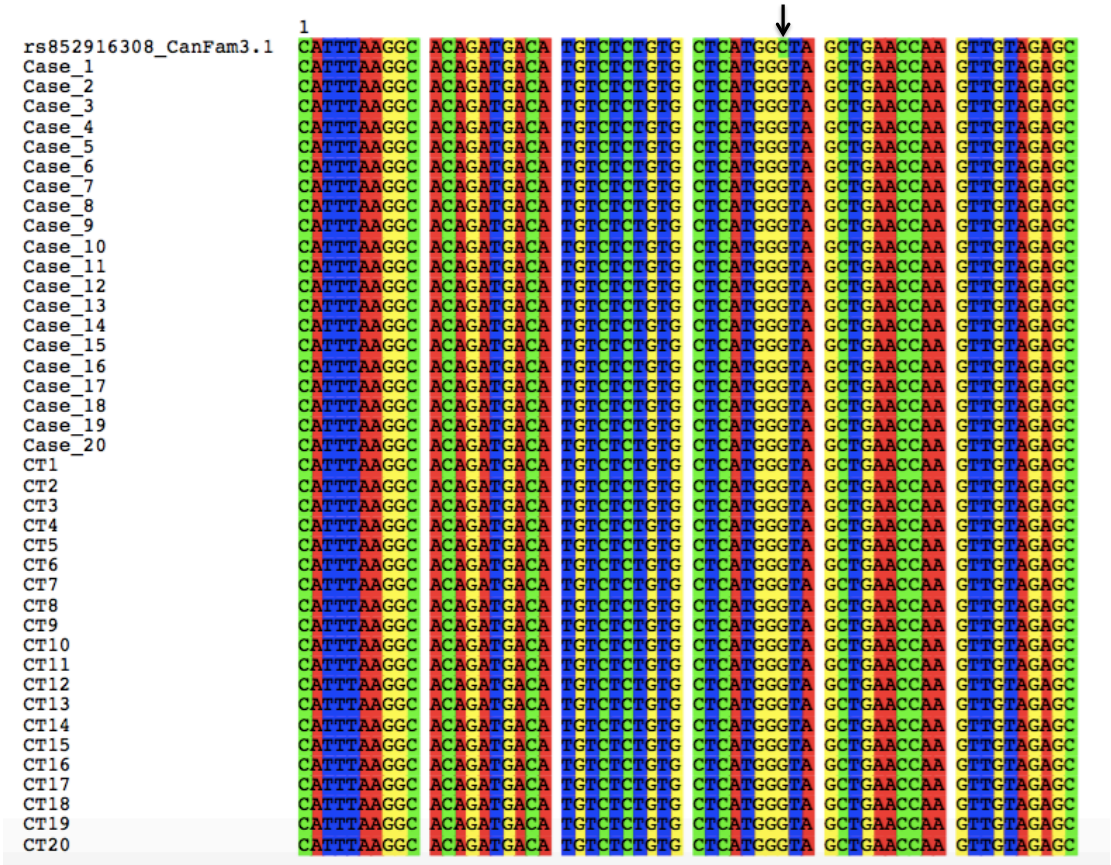

Figure 5.4: Multiple DNA sequence alignment for the region containing the rs852916308 SNP located on canine chromosome 16. DNA was sequenced in 20 cases and 20 controls (CT). An arrow indicates the position of the variant, which is C for the reference sequence (first line) and G for all the cases and controls evaluated.

Thus, the region around the candidate SNP rs22196038 located on CFA12 was sequenced in 104 CP cases and 44 controls. The genotypes obtained were homozygous T or C and heterozygous. Fig. 5.5 shows the DNA sequencing plots with the 3 different genotypes obtained and Fig. 5.6 a multiple DNA alignment for the region containing the rs22196038 SNP in 12 cases and 12 controls (CT), respectively.

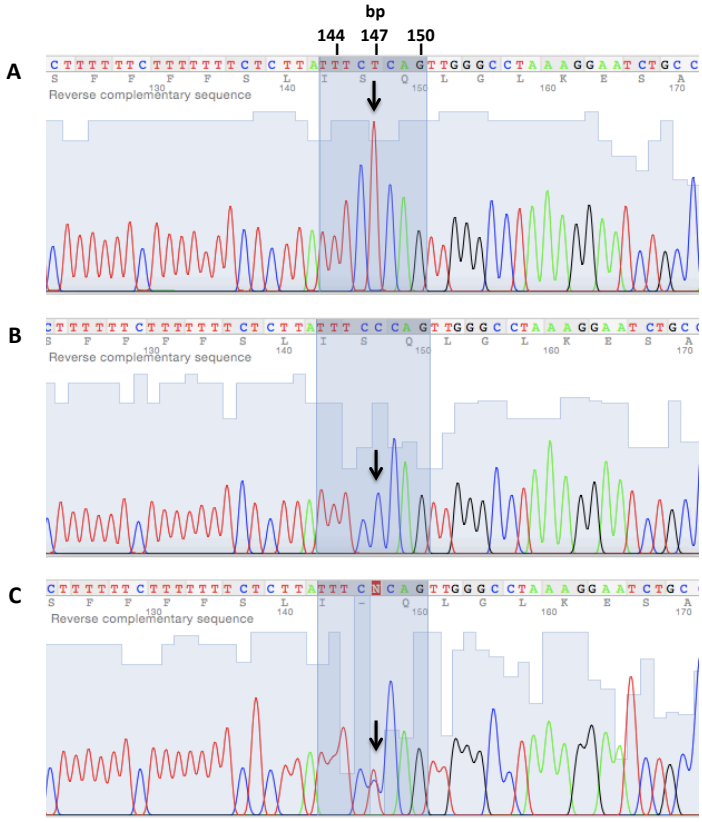

Figure 5.5: DNA sequencing plots of SNP rs22196038 showing the three different genotypes. (A) Homozygous T (B) Homozygous C (C) Heterozygous. The arrows indicate the rs22196038 SNP variant position.

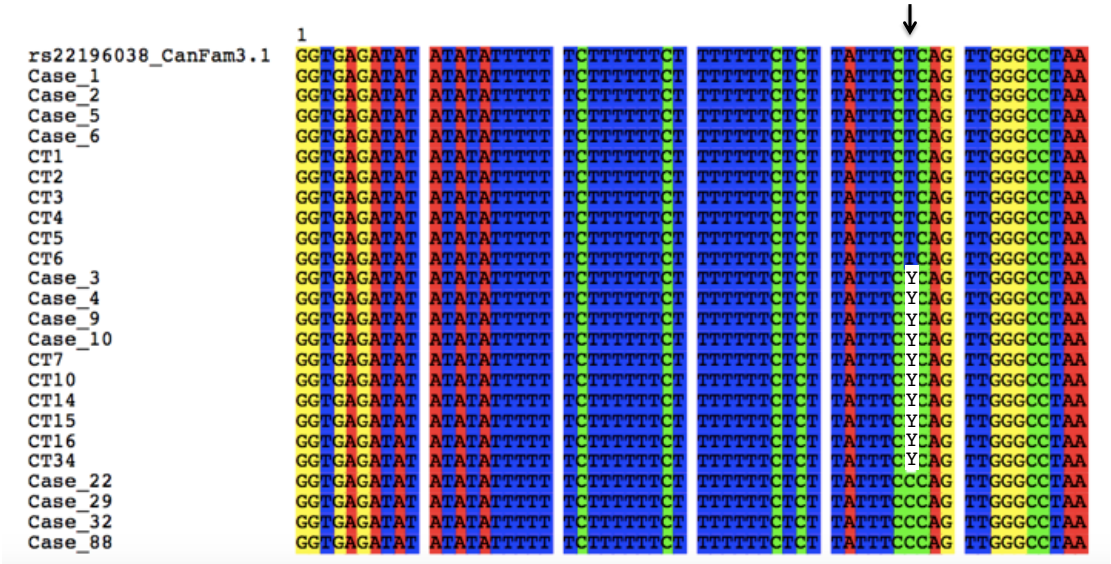

Figure 5.6: Multiple DNA sequence alignment for the region containing the rs22196038 SNP located on canine chromosome 12. DNA was sequenced in 104 cases and 44 controls. The figure shows a multiple alignment of 12 cases and 12 controls (CT). An arrow indicates the position of the variant, which can be T (blue) or C (green). Heterozygous genotypes are represented with the letter Y.

In order to confirm heterozygosity, restriction enzyme digestion was carried out on the samples that were difficult to determine if they had a heterozygous genotype after sequencing them in both directions. Examples of heterozygous genotypes that were subjected to restriction enzyme digestion to confirm heterozygosity are shown in Fig. 5.7. Following enzyme digestion, the digested DNA was analysed by gel electrophoresis on 2 % agarose gels (Fig. 5.8). DNA samples that had a heterozygous genotype showed 3 gel bands on the agarose gels with sizes of 82 bp (half intensity), 135 bp (half intensity) and 217 bp (stronger intensity). DNA samples with a homozygous T genotype showed 2 gel bands (135 bp and 82 bp each) and homozygous C genotype showed 1 gel band of 217 bp.

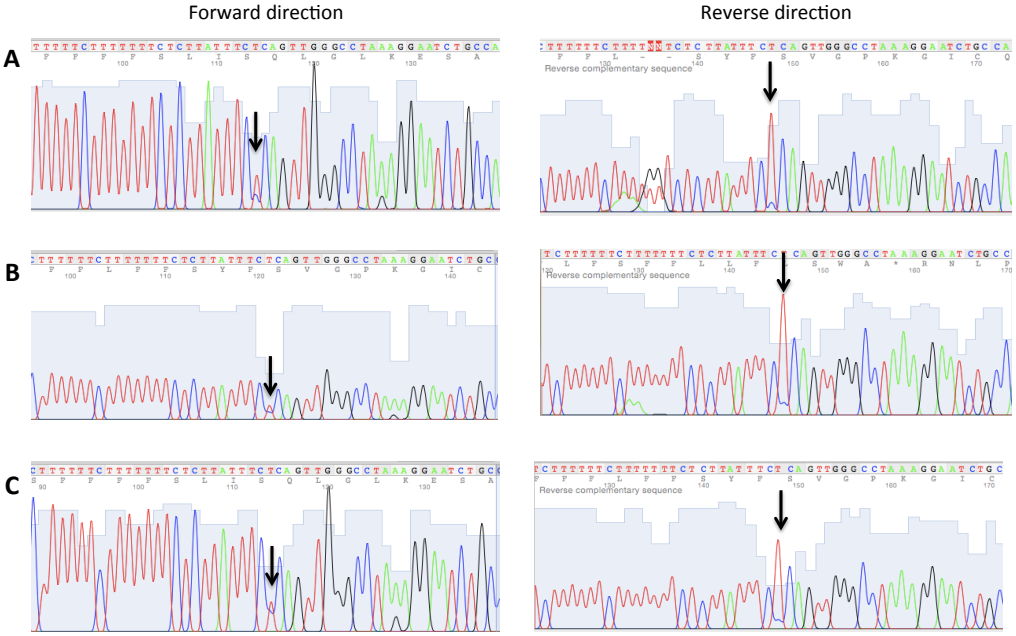

Figure 5.7: DNA sequencing plots showing heterozygosity in the region containing the rs22196038 SNP located on canine chromosome 12 from (A) DNA of a control ECS, and (B) and (C) DNA from two different cases. These are examples of heterozygous genotypes that were subjected to restriction enzyme digestion in order to confirm genotype. The arrows indicate the rs22196038 SNP variant position.

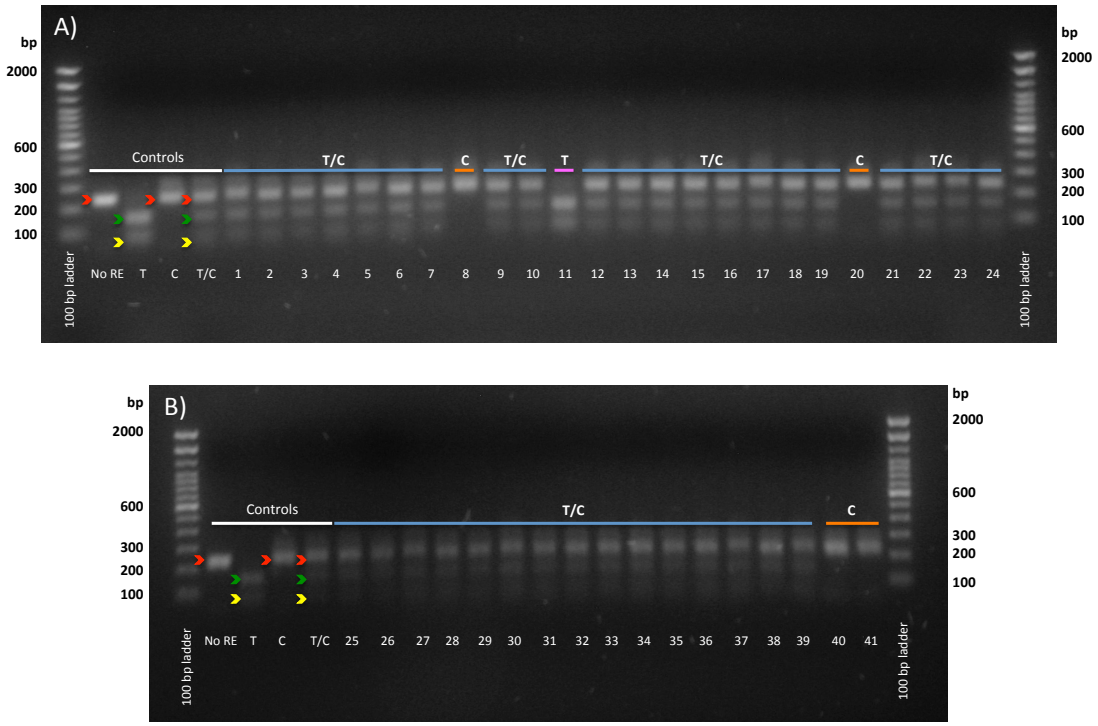

Figure 5.8: 2 % Agarose gel electrophoresis of restriction enzyme-digested PCR amplification products of the region containing the rs22196038 SNP located on canine chromosome 12. BseMII (BspCNI) digestion was used to confirm heterozygosity. No RE: no restriction enzyme digestion applied, C: homozygous C, T: homozygous T, T/C: heterozygous.

The allele frequencies observed in the cases were 77.9 % and 22.1 % for the variant T and C, respectively (Fig. 5.9). In controls, the allele frequencies observed were 80.7 % for the variant T and 19.3 % for the variant C (Fig. 5.9). No differences were found regarding T and C allele frequencies between cases and controls. However, when dividing controls into two groups (healthy or non-CP related disease) and comparing them with the cases, the T allele frequency was significantly higher in healthy controls than in cases (P = 0.024) and diseased controls (P=0.030) but no significant difference was found between cases and diseased controls (P = 0.274) (Table 5.2). Regarding C allele frequency, this was significantly lower in healthy controls than cases (P = 0.024) and diseased controls (P = 0.030). However, no differences were found in C allele frequencies between cases and diseased controls (P= 0.274) (Table 5.2).

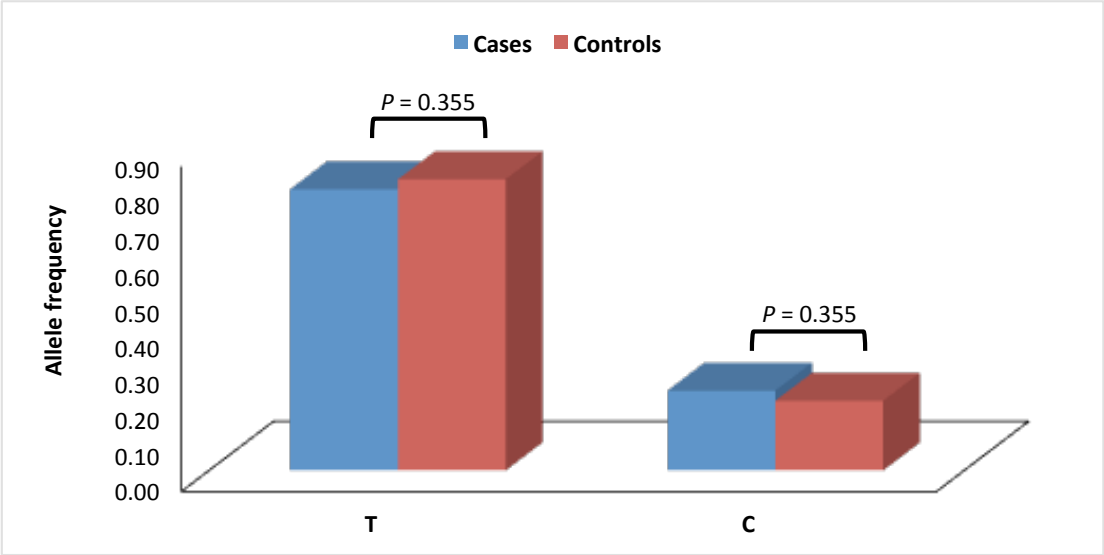

Figure 5.9: Allele frequencies for the candidate SNP rs22196038 located on CFA12 locus in cases and controls. Graphs show a comparison of the frequencies of T and C alleles between cases (in blue) and controls (in red). Results demonstrated that there was not a significant difference in the allele frequencies between both dog groups (Fisher's exact test).

Table 5.2: Summary of allele frequencies in cases and control ECS.

Allele	*CP cases*		*Controls*				*Two Proportion Z-Test*		
			Healthy		*Other diseases*		*P-value*		
	Number of Alleles	*Frequency (%)*	*Number of Alleles*	*Frequency (%)*	*Number of Alleles*	*Frequency (%)*	*Cases vs. healthy*	*Cases vs. other disease*	*Healthy vs. other disease*
T	162	77.9%	28	93.3%	43	74.1%	0.024	0.274	0.03
C	46	22.1%	2	6.7%	15	25.9%	0.024	0.274	0.03

The homozygous T genotype showed the highest frequency in cases and controls (62.5% and 61.4%, respectively). The heterozygous genotype was the second most common in both groups (30.8% and 38.6%, respectively), while the homozygous C genotype showed the lowest frequency in both groups (6.7% and 0%, respectively) (Table 5.3). There was not a significant difference between cases and controls regarding the frequency of heterozygous and homozygous T genotypes (*P*= 0.229 and 0.519, respectively). When the frequency of the homozygous C genotype was compared between cases and controls there was a *P* value of 0.079 (Table 5.3 and Fig. 5.10).

Table 5.3: Genotype frequencies and association with CP in cases and control ECS.

Genotype	*CP cases (n=104)*		*Controls (n=44)*		*Odds ratio (95% CI)*	*Fisher's exact test P-value*
	Number of dogs	*Frequency (%)*	*Number of dogs*	*Frequency (%)*		
Homozygous T	65	62.5%	27	61.4%	1.05 (0.51-2.17)	0.519
Homozygous C	7	6.7%	0	0%	3.10 (0.37-26.00)	0.079
Heterozygous T/C	32	30.8%	17	38.6%	0.71 (0.34-1.47)	0.229

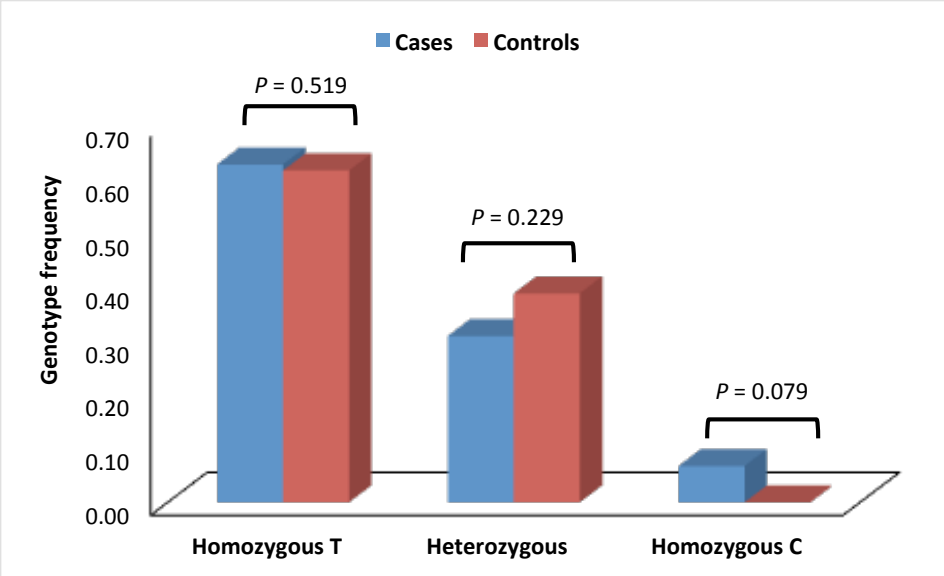

Figure 5.10: Genotype frequencies for the candidate SNP rs22196038 located on CFA12 locus in cases and controls. Graphs show a comparison in the genotype frequencies between cases (in blue) and controls (in red). Results demonstrated that there is not a significant difference in the genotype frequencies between both dog groups (Fisher's exact test).

A significant association between genotype profile and CP was seen when dividing the controls into two different groups (healthy and diseased controls) and comparing them with cases (*P*= 0.049). Under this scenario, differences were observed in the frequency of heterozygous and homozygous T genotypes (*P*=0.026 and 0.042, respectively) but no differences were observed in the frequencies of homozygous C genotype (*P*= 0.356) (Fig. 5.11).

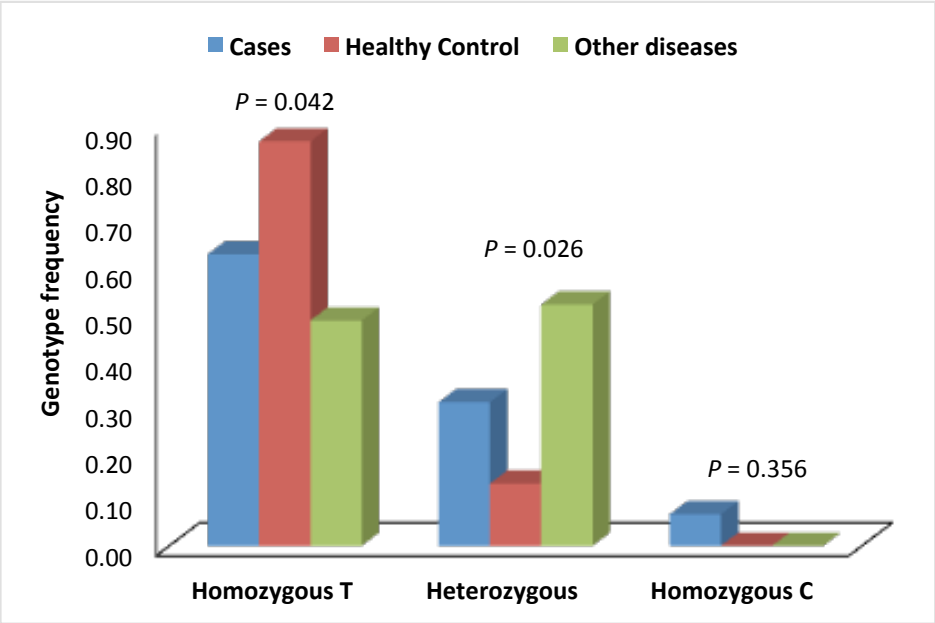

Figure 5.11: Genotype frequencies for the candidate SNP rs22196038 located on CFA12 locus in cases, and healthy and diseased controls. Graphs show a comparison of the genotype frequencies between cases (in blue), healthy (in red) and diseased (in green) controls. Results demonstrated that there was a significant difference in the heterozygous and homozygous T genotypes between groups, when the control group was divided into healthy and diseased dogs (Fisher's exact test).

5.5. Discussion and conclusions

This chapter describes a genotypic evaluation of ECS affected by CP aiming to confirm or reject the genetic associations that showed loci of interest in a previous unpublished pilot GWAS. The candidate SNPs in the loci of interest were amplified by PCR and the allele and genotype frequencies obtained were compared between cases and controls. No association with disease and no difference between cases and controls were observed in the distribution of alleles and genotypes at the rs852916308 SNP located on chromosome 16, which overlies a region containing a group of T-cell receptor beta variable loci and the anionic trypsinogen gene. Sequencing of this locus was performed in only 20 cases and 20 controls and since the results in that subset of dogs showed that all the cases and controls analysed presented exactly the same SNP variant, it was decided not to continue sequencing this locus in the remaining cases and controls. The reported allele alternatives for the rs852916308 SNP are G and C. The Boxer variant C is relatively rare in other breeds, the allele frequencies observed in the general canine population for these variants are 99 % for G and 1 % for C (European Variation Archive, EVA study PRJEB24066). Our results showed a G allele frequency of 100 % in cases and controls.

The rs22196038 SNP located on chromosome 12, which is downstream of the BCKDHB gene, was sequenced and genotyped in the 104 cases and 44 controls. The reported allele alternatives for the rs22196038 SNP are T or C and the allele frequencies observed in the general canine population for these variants are 77 % and 23 %, respectively (European Variation Archive, EVA study PRJEB24066). The allele frequencies observed in our study for the cases were 77.9 % and 22.1 % for the variant T and C, respectively. In controls, the allele frequencies observed were 80.7 % for the variant T and 19.3 % for the variant C. The allele and genotype frequencies at this SNP did not show significant differences between cases and controls. Regarding the homozygous C genotype, its frequency did not show a significant difference between cases and controls (P= 0.079). However, none of the control dogs from both groups presented the homozygous C genotype, whereas 7/104 (6.7%) affected dogs did present it. This is interesting and might suggest that this genotype is important for disease in some dogs but larger numbers of controls would be needed to confirm this finding. When control dogs were separated into two different groups, it was possible to see allele frequencies of 93.3 % and 6.67 % for the allele T and C, respectively, in completely healthy dogs. In diseased controls, allele frequencies were 74.1 % for the allele T and 25.9 % for the allele C. A significant association between genotype profile and dog groups was seen for this SNP when dividing the controls into healthy and diseased controls and comparing them with the cases (P= 0.049). Under

this scenario, allele T frequencies were significantly higher in healthy controls than in cases (*P* = 0.024) and diseased controls (*P*=0.030) and allele C frequencies were significantly lower in healthy controls than cases (*P* = 0.024) and diseased controls (*P* = 0.030). In addition, differences were observed in the frequency of heterozygous and homozygous T genotypes (*P*= 0.026 and 0.042, respectively) but no differences were observed in the frequencies of homozygous C genotype (*P*= 0.356) when controls were divided. This could suggest that the chromosome 12 locus may partially contribute to the development of CP in ECS. However, co-dominance or multiple loci may be also involved in disease causation. Thus, further studies including a much larger number of dogs should be carried out in order to demonstrate whether the chromosome 12 locus could be contributing to the development of CP in ECS.

The evaluated SNP located on chromosome 12 is associated with a genomic region containing the BCKDHB gene. The BCKDHB gene is needed for metabolism of leucine, valine and isoleucine, and when mutated in humans, causes maple syrup urine disease (Chuang and Shih, 2001). In addition, BCKDHB is associated with acute and chronic pancreatitis in human infants, so partial loss of function at this locus could contribute to chronic pancreatitis (Kahler *et al*., 1994). Further research to establish whether the BCKDHB gene is indeed involved in determining disease susceptibility is needed. For this purpose other SNP variants for this gene should be evaluated.

An important limitation of this study was the number of controls included. It has been recognized that in order for genetic studies to achieve sufficient power, very large numbers of patients and controls need to be analysed. In this study, a sample set of 104 ECS affected by CP was included, which was much larger than the control group. The purpose was to include at least 100 controls but they were difficult to recruit due to the strict selection criterion. As future work, another GWAS and exome sequencing will be performed to try to find possible candidate loci associated with CP in ECS. The cases and controls included in this study will be used for this purpose but in order to increase statistical power, additional controls will be recruited. In pedigree dogs, it is possible to undertake a GWAS with fewer cases and controls than would be necessary in people (Lindblad-Toh *et al*., 2005; Karlsson *et al*, 2007). Genomic mapping within a single dog breed can often be accomplished using relatively small numbers of samples because of long Linkage Disequilibrium (LD) within the genome, as well as reduced diversity in the genetic origin of many inherited diseases, giving genetic homogeneity and strong signals even in diseases showing high genetic diversity in humans. However, it is also possible that ECS are relatively homozygous for a major susceptibility gene variant, but this

has incomplete penetrance, requiring additional genetic modifiers and environmental risk factors. In this instance a case/control study will not reveal any differences.

One of the most valuable genetic studies on IgG4-RD to date is the one obtained by the first GWAS from Japanese researchers, where it was found that locus FCGR2B is significantly associated with susceptibility to IgG4-related disease (Terao *et al*., 2019). Fc fragment of IgG receptor IIB is coded by the FCGR2B gene and it is a low affinity inhibitory receptor for the Fc region of IgG with a critical role in immune regulation. FCGR2B participates in the phagocytosis of immune complexes and in the regulation of antibody production by B lymphocytes that plays a role in the development of autoimmunity (Kyogoku *et al*., 2002). In dogs, this gene is located on chromosome 38. It would be interesting to evaluate if variants of this gene could be associated with susceptibility to CP in ECS.

Moreover, since a strong association between CP and dogs with parti-colour coats was observed in Chapter 2, it would be interesting as future work to determine a candidate gene in linkage disequilibrium with the gene that determines parti-colour coats. Studies have demonstrated that pigment type switching in dogs is controlled by a gene named the *K* locus, which is located on dog chromosome 16 (Kerns et al., 2007). This could be an interesting candidate gene to be further investigated.

To summarise, this chapter rejected the genetic association previously found between CP in the ECS and the SNP rs852916308 located on dog chromosome 16. This SNP was not polymorphic in ECS. Results found on the SNP rs22196038 located on chromosome 12, did not show significant differences of allele and genotype frequencies between cases and total number of controls. However, when allele frequencies were compared between cases and healthy controls, significant differences were shown. This suggests that this locus may act as a potential contributory factor in the development of CP in ECS. However, it is very likely that multiple loci are involved in disease causation. Further studies, including a greater number of healthy ECS could confirm this association.

CHAPTER 6: CONCLUSION AND FUTURE WORK

6.1. Conclusions

This dissertation presented an investigation on chronic pancreatitis in the English cocker spaniel with a focus on its immunology and genetics, increasing our understanding of its diagnosis and pathogenesis and demonstrating similarities with human IgG4-related disease.

Firstly, clinical features in ECS affected by CP were characterized. The study showed remarkable clinical similarities between CP in ECS and human IgG4-RD, including a high prevalence of multi-organ manifestation. Affected ECS presented with a high prevalence of KCS, proteinuria, anal gland disease, suspected xerostomia, atopy and other immune-mediated diseases. Moreover, a strong association between CP and ECSs presenting blue roan coat colour was demonstrated, suggesting a link between coat colour and immune-mediated conditions in this breed. While there is no obvious way these can be linked from a pathophysiological perspective, it suggests some of the genetic determinants for coat colour and immune-mediated disease are segregating together in the breed.

Secondly, several attempts to develop a non-invasive test capable of measuring IgG4 concentrations in canine serum were described. A monoclonal antibody that was published to be against dog IgG4 was produced for this purpose and it was demonstrated that the antibody worked in ELISA and IHC. In addition, different strategies for the isolation of IgG4 from dog serum using affinity and anion exchange chromatography were described. However, isolation of pure dog IgG4 was not achieved. A competitive ELISA for the detection of dog IgG4 in serum was developed using the monoclonal antibody. Its standard curves for IgG4 measurement showed highly reliable results. However, concentrations of IgG4 detected in a plasma pool from healthy dogs were demonstrated to be higher than previously reported ranges for normal serum IgG4 and higher than total IgG, suggesting that the test was not specifically recognising dog IgG4 in serum. Much more work will be needed to optimise this IgG4 test, which was beyond the scope and timescale of this PhD. For these reasons, a commercial IgG4 ELISA test was used to measure IgG4 concentrations in affected and control dogs.

Thus, serum IgG4 concentrations were assessed and it was demonstrated that affected ECS had significantly higher IgG4 serum concentrations compared with age-matched healthy ECS and ECS presenting with different diseases unrelated to CP and with no known immune-mediated aetiology. Moreover, it was demonstrated that cases presenting with clinical inflammatory manifestations in

more than one organ had significantly higher concentrations of serum IgG4 than those with just pancreatic manifestation, which is the same as seen in human IgG4-RD. A cut-off value for serum IgG4 concentration for the diagnosis of IgG4-RD in ECS with CP was suggested. Using this cut-off value of 0.85 mg/ml, a sensitivity of 70.2 %, specificity of 63.4 %, PPV of 81.48 % and NPV of 48.15% in diagnosing the disease was achieved. Moreover, total serum IgG, IgE concentrations and IgG4/IgG ratios were also measured in cases and controls. No significant difference was observed regarding concentrations of total IgG and IgE in serum between cases and controls. Serum IgG4/IgG ratio was significantly higher in cases than controls. The measurement of serum IgG4 seems to be a promising non-invasive diagnostic test for early recognition of disease and timely immunosuppressive treatment, preventing or delaying the development of further signs in other organs and hopefully resulting in the improvement of the quality of life in dogs with IgG4-RD. In the absence of biopsies to confirm histological features of IgG4-RD in affected dogs, the measurement of serum IgG4 concentrations may be a useful tool to approach diagnosis of IgG4-RD in dogs. However, since increased serum IgG4 concentrations can be observed in dogs affected by other conditions, it is important to always consider the general clinical scenario before diagnosing this disease, putting special emphasis in the determination of involvement in other organs as well to the pancreas.

Finally, a genetic study trying to identify risk factors in affected dogs was performed. In this dissertation, the potential genetic association previously found between CP in the ECS and the SNP rs852916308 located on dog chromosome 16, which overlies a region containing a group of T-cell receptor beta variable loci and the anionic trypsinogen gene, was rejected. Results found on the SNP rs22196038 located on chromosome 12, demonstrated some interesting potential genotype associations. Differences in allele and genotype frequencies were observed when only healthy controls and cases were compared but the study was hampered by the difficulty of obtaining non-diseased controls. This locus overlies the BCKDHB gene, and when mutated is associated with human maple syrup urine disease (Chuang and Shih, 2001) and chronic pancreatitis in children (Kahler et al., 1994), thus partial loss of function at this locus could contribute to CP in ECS. However, a higher number of non-diseased controls would be needed in order to validate these potential associations.

6.2. Future work

6.2.1. Flow cytometry studies

Flow cytometry is becoming a commonly used technique to characterise a variety of cells. It provides a powerful application to rapidly determine the relative percentages of T-lymphocyte subsets and B-lymphocytes. Recent studies have shown that human patients affected by IgG4-RD often present an oligoclonal expansion of IgG4+ B cells in peripheral blood identified as CD19+CD20-CD27+CD38+ plasmablasts on flow cytometry (Mattoo *et al*., 2014). Plasmablast concentrations can be elevated even when IgG4 serum concentrations are found to be normal in patients with active IgG4-RD, demonstrating to be a disease marker of great usefulness for the diagnosis of this disease in humans. Future work will investigate the immune status of ECS with CP and suspected IgG4-RD using flow cytometry to look at a range of B and T cell subsets with a validated panel of antibodies including CD3, CD4, CD5, CD8, CD21, CD25, CD79a, FoxP3 and specially evaluating if plasmablasts are elevated in affected dogs. Although flow cytometry contributes with numerous insights, this technology still presents some disadvantages. Potential limitations of flow cytometry studies in veterinary medicine are the lower availability of commercially available canine specific antibodies and restricted options for fluorochrome labels commercially available. This is one of the main reasons why designing flow cytometry staining panels is harder for dog cell samples than for human cell samples. Differentiation into plasmablasts is most commonly monitored with surface markers CD19, CD20, CD27, CD38, and CD138 and intracellular marker Ki-67 (Fink, 2012). Antibodies against CD19 and CD20 are available for use in dogs, and will be validated in this study but canine specific antibodies against CD27, CD38, CD138 and Ki-67 are not available for flow cytometry, which is a disadvantage for this study; it will be evaluated whether human or mouse antibodies can present cross-reactivity. Other potential limitation of flow cytometry studies is the difficulty of obtaining fresh blood samples. Samples need to be processed within hours of collection, reducing the clinical practicability of this methodology.

6.2.2. Cytokine studies

Cytokine and chemokine research plays a significant role in achieving a deeper understanding of immune mediated diseases. A Type 2 helper T-cell (Th2)-driven immunological mechanism has been proposed in the pathogenesis of human IgG4-RD. Th2 cytokines, such as interleukin (IL)-4, IL-5, and IL-13, next to T-regulatory-associated cytokines, such as IL-10, and transforming growth factor beta (TGF-β) are involved in IgG4-RD (Della-Torre *et al*., 2015). It would be interesting to see whether the

same cytokines are participating in affected ECS. Recent developments involving highly sensitive Multiplex detection technologies have presented clinical investigators with unprecedented opportunities to measure large numbers of cytokines within a limited sample volume (Zhou *et al*., 2010). Cytokine studies have the advantage of presenting an easier access to appropriate clinical material (frozen serum or plasma) but they also have potential limitations because fibrosis and a Th2 microenvironment may be associated with a number of other conditions. Therefore, this study could inform about the possible immune mechanism of the ECS disease but could not be useful as a potential diagnostic tool. A commercially available canine cytokine panel kit (MILLIPLEX® MAP, EMD Millipore) that uses the Luminex xMAP technology and enables to make the simultaneous quantification of cytokines could be used for this purpose.

6.2.3. Responsiveness to immunosuppressive treatment

Human IgG4-RD responds to corticosteroids and it has been seen that serum IgG4 concentrations declines substantially after treatment in most patients. Future studies will evaluate if affected dogs receiving immunosuppressive therapy have a reduction in their serum IgG4 concentrations after treatment determining if the test is able to identify treatment response. An anti-CD20 antibody, rituximab, is used successfully in humans with steroid resistant disease (Wallace *et al.*, 2016). It would be good to look at the efficacy of this more specific immunosuppressive treatment in dogs. However, the human antibody does not cross react with dogs but a canine specific anti-CD20 mAb is undergoing trials for treatment of canine B-cell lymphoma (Rue *et al*., 2015; Mizuno *et al.,* 2020).

6.2.4. Genetic Studies

As future work, another GWAS will be performed to try to find other possible candidate loci associated with CP in ECS. The cases and controls included in this study will be used for this purpose but in order to increase statistical power, additional controls will be recruited, aiming to obtain at least 100 control dogs. Particularly, since the blue roan parti-colour coat was highly overrepresented in ECS affected by CP, It would be interesting to determine a candidate gene in linkage disequilibrium with one of the genes that determines coat pigmentation. The gene named *K* locus, located on dog chromosome 16, has demonstrated a role on controlling pigment type switching (Kerns et al., 2007). This could be an interesting candidate gene to be further investigated.

6.2.5. Development of IgG4 ELISA test

Development of a recombinant dog IgG4 to use it as standard and validation of the specificity of the anti-dog IgG4 antibody to allow the development of our own serological IgG4 test.

Collectively, the studies in this thesis have shed light on multi-organ immune mediated disease in ECS and demonstrated convincingly that immune-mediated diseases in different breeds of dog are likely to have different mechanisms and genetic predispositions. Understanding these better in the future should also result in better targeted treatment with more specific immunosuppressive reagents and potentially selective breeding to reduce breed incidence.

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Appendix

1) Consent forms

The Queen's Veterinary School Hospital
University of Cambridge
Madingley Road Cambridge CB3 0ES

Tel: 01223 337621 / 01223 337669 Fax: 01223 330848

Penny J Watson MA, VetMD, CertVR, DSAM, Dip ECVIM, FRCVS
RCVS and European Specialist in Small Animal Internal Medicine
Senior Lecturer in Small Animal Medicine and Clinical Nutrition

IMMUNE-MEDIATED DISEASES IN ENGLISH COCKER SPANIELS:
Owner information and consent form

Thank you for helping us with our study into immune-mediated diseases in Cocker Spaniels. We hope that the results of this study will help us to diagnose and treat this condition in Cockers better in the future. Cocker Spaniels are predisposed to a variety of immune-mediated diseases. An immune-mediated disease is a disease where a dog's own immune system starts to attack part of the dog's own body 'by mistake'. This is because the immune system believes that part of the dog is 'foreign' such as an infection and tries to remove it. Immune-mediated diseases tend to be focussed on one type of cell and the signs the dog shows reflect that: so for example, immune-mediated attack on red blood cells causes anaemia due to destruction of the dog's own red blood cells whereas immune-mediated arthritis causes lameness due to immune attack on the joints.

We believe that cocker spaniels have an increased tendency to immune-mediated diseases in a number of organs: sometimes one at a time and sometimes several together. These include, but are not restricted to:

- Immune mediated haemolytic anaemia: which is immune cell attack on red blood cells
- Immune mediated thrombocytopenia: which is immune cell attack on the blood clotting cells ('platelets') which means the dog is prone to bleeding
- Immune mediated glomerulonephritis: which is immune cell attack on the kidney which leads to protein loss in the urine and, in severe cases, to kidney failure
- Keratoconjunctivitis sicca ('dry eye') where the immune system attacks the lacrimal gland and causes the eyes to become dry.

We also believe that some cases of chronic pancreatitis; chronic liver disease and anal sac disease in cocker spaniels are due to immune mediated disease.

We are working on studies to develop a blood test, which we hope will help us diagnose immune-mediated disease in cocker spaniels more reliably. We are also working to understand the cause and genetics of the conditions better, which we hope, will lead to better treatment. At the moment, the diagnosis is difficult and relies on a combination of blood tests and biopsy findings. Treatment often involves high doses of systemic steroids, which are usually effective but also cause marked side effects, so new treatments would be welcome.

For our study we are recruiting cocker spaniels with immune-mediated disease, and/or chronic pancreatitis, and also healthy dogs.

With your consent, we would like:

- To use any blood left over from any blood tests that your vet might possibly do, and eventually freeze the remainder for future analysis. Your dog will remain anonymous and just the case number and breed will be recorded.
- To analyse his/her urine that you will kindly collect by free catch or having your dog urinate on a clean floor.

We offer free of charge: a DGGR lipase test to identify chronic pancreatitis, and also a urine protein: creatinine test to identify disease in the kidneys. Results will be communicated to the referring veterinary surgeon.

Owner consent:

- **I consent to use of any blood leftover from any blood that the vet may need to take from my dog**
- **I consent to long term storage of any spare blood from the above**
- **I consent to provide a free catch sample of his/her urine**
- **I also consent to provide my veterinarian's contact details to contact him/her about the results of our tests**

Signed…….. Date……

The Queen's Veterinary School Hospital
University of Cambridge
Madingley Road Cambridge CB3 0ES

Tel: 01223 337621 / 01223 337669 Fax: 01223 330848

Penny J Watson MA, VetMD, CertVR, DSAM, Dip ECVIM, FRCVS
RCVS and European Specialist in Small Animal Internal Medicine
Senior Lecturer in Small Animal Medicine and Clinical Nutrition

IMMUNE-MEDIATED DISEASES IN ENGLISH COCKER SPANIELS:
Veterinary Surgeon Study Description

Immune-mediated diseases, including immune-mediated haemolytic anaemia (IMHA) and immune mediated thrombocytopenia (IMTP), are particularly problematic in susceptible dog breeds such as the English Cocker Spaniel (ECS).

Chronic pancreatitis (CP) is a common disease in ECSs that is thought to be an immune-mediated condition. CP shows a distinctive clinical and histopathological appearance in ECSs. It typically affects middle-aged to older dogs, with a higher prevalence in males, and at least 50% of affected dogs subsequently develop Diabetes Mellitus, exocrine pancreatic insufficiency, or both. Dogs also often have other concurrent autoimmune diseases, particularly keratoconjunctivitis sicca and glomerulonephritis. Histologically, it is a duct-destructive disease with predominantly interlobular and periductular distribution of fibrosis and inflammation, with locally dense lymphoplasmacytic infiltrates, the presence of multi-organ manifestation, and increased numbers of IgG4+ plasma cells in the pancreas and other affected organs. These characteristics are also seen in human autoimmune pancreatitis (AIP). Human AIP is part of a steroid-responsive multi-organ syndrome, newly recognized as IgG4-Related Disease (IgG4-RD). Human IgG4-RD may affect one or several organs, pancreatitis being the most common manifestation. It is often seen that serum IgG4 levels and IgG4+ plasma cell numbers are increased in the affected tissues. The evidence above therefore suggests that CP in ECS is one of the manifestations of IgG4-RD.

Much remains unknown about the mechanisms and genetics underlying IgG4-RD in both species. The behaviour of IgG4 *in vivo*, the participation of this molecule in the disease, and whether its role in IgG4-RD is primary or secondary are still poorly understood.

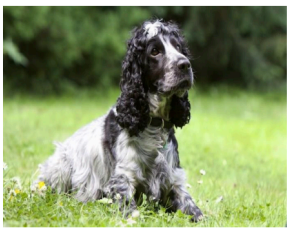
Our study aims firstly to develop a reliable and non-invasive diagnostic test for canine IgG4-RD, useful for diagnosis and to monitor the efficacy of steroid treatment in affected dogs. The second aim of the project is to investigate the immune status of affected dogs using flow cytometry to look for a range of B and T cell subsets. Finally, studies into the genetics of the disease will be undertaken. These studies will subsequently increase the understanding of the immune response and genetics of affected ECSs; information obtained will help to prevent the disease and inform about future treatments in ECSs and other breeds predisposed to immune-mediated disease.

In order to undertake this study, we are recruiting blood and serum samples from English Cocker Spaniels affected with immune-mediated disease (IMHA, and/or IMTP), and/or chronic pancreatitis, and/or glomerulonephritis. In addition, we are recruiting samples from middle-aged to older healthy dogs (over 9 years old) from dogs having pre-anaesthetic blood screens for routine procedures such as dentals or skin mass removal. Healthy control dogs must not have dry eye and anal sac disorders. Dogs on immunosuppressive medication (such as steroids or cyclosporine (Atopica)), or diagnosed with lymphoma or cancer will not be suitable for this study. We would like to use any blood and serum left over from any blood tests that veterinarians might possibly do, and eventually freeze the remainder for future analysis. In addition, we would like to perform a urine protein:creatinine test from urine collected by free catch. The dogs will remain anonymous and just the case number and breed will be recorded. Results will be communicated to the primary care veterinary surgeon and owner. Our study has passed University of Cambridge ethical review.

Veterinarians sending a blood sample +/- urine sample from practice will be offered free of charge: a DGGR lipase test; a urine protein:creatinine test; a packed cell volume (PCV) or platelet count, and CRP test (inflammatory marker).

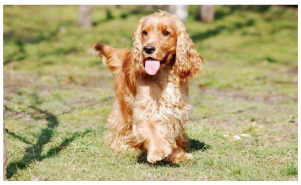
Thank you for helping us with our study about immune mediated diseases in Cocker spaniels. We hope that the results of this study will help us to better diagnose and treat this condition in Cockers in the future.

2) Brochure



For our study we are recruiting healthy cockers and cockers with immune-mediated disease, and/or chronic pancreatitis.

Sample type: urine and blood (left over from routine blood tests) samples taken by your vet. Please note, your vet should contact the researchers first for further info and consent forms.



Thank you for helping us with our study into immune-mediated diseases. We hope that the results of this study will help us to better diagnose and treat this condition in Cockers in the future.

Contacts

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Immune-mediated diseases in the English Cocker Spaniel Study

UNIVERSITY OF CAMBRIDGE

Immune-mediated diseases are very problematic in susceptible dog breeds such as the English Cocker Spaniel.

An immune-mediated disease refers to a condition where a dog's own immune system begins to attack and reject the body's own tissues by mistake.
This can be specific to one type of tissue or generalized.

We believe that cockers have an increased tendency to immune-mediated diseases in a number of organs, sometimes one at a time and sometimes several together. These include, but are not restricted to:

- Immune-mediated haemolytic anaemia: where the immune system attacks red blood cells.

- Immune-mediated thrombocytopenia: where the immune system attacks the blood clotting cells ('platelets') which means the dog is prone to bleeding.

- Immune-mediated glomerulonephritis: where the immune system attacks the kidneys.

- Keratoconjunctivitis sicca ('dry eye') where the immune system attacks the lacrimal gland and causes the eyes to become dry.

Chronic pancreatitis is a common disease in Cockers and it seems to be immune-mediated. We also believe that some cases of chronic liver disease and anal sac disease in Cockers are caused by immune-mediated disease.

Our research study

We are working on studies to develop a blood test, which we hope will help us diagnose immune-mediated disease in cocker spaniels more reliably. We are also working to understand the cause and genetics of the conditions better, which we hope will lead to better prevention and treatment of this disease.

UNIVERSITY OF CAMBRIDGE

Petplan CHARITABLE TRUST

We would like to use any blood and serum left over from any blood tests that your vet might possibly do.

In addition, we would like to analyse your dog's urine that you will kindly collect by free catch or having your dog urinate on a clean floor. Your dog will remain anonymous and just the case number and breed will be recorded.

We offer free of charge: a lipase test to identify chronic pancreatitis, and also a urine protein: creatinine test to identify disease in the kidneys. Results will be communicated to the primary care veterinary surgeon and owner.

Criteria for healthy dogs:

• >9 years old

•Male or female

•No history of pancreatitis, kidney disease, dry eye or recurrent anal sacculitis

Criteria for affected dogs:

• Any age

• Male or female

• History of chronic pancreatitis and/or immune-mediated disease

Exclusion criteria: dogs on any immunosuppressive medication or with lymphoma.

3) Restriction enzyme cut sites

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predicted_PCR_C


          GTTTTTTCCCTAAGACCTCAAATTTCATACAATAGGTTCAGAAAGTGAATGCTCCCCCCA
                   10        20        30        40        50        60
          ----:----|----:----|----:----|----:----|----:----|----:----|

          GGGGTCAGGCAAAAGAGGTGAGATATATATATTTTTTCTTTTTTTCTTTTTTTCTCTTATT
                   70        80        90        100       110       120
          ----:----|----:----|----:----|----:----|----:----|----:----|

          TCCCAGTTGGGCCTAAAGGAATCTGCCAAGGGAAAATCCCTGCTTAGTCATCTCCGAGGT
                   130       140       150       160       170       180
          ----:----|----:----|----:----|----:----|----:----|----:----|

          CTCTTGAGGATCCAGATGAGTTTGTTTACCCGAGGCG
                   190       200       210
          ----:----|----:----|----:----|----:--


# Enzymes that cut  Frequency   Isoschizomers


# Enzymes which cut less frequently than the MINCUTS criterion
# Enzymes < MINCUTS Frequency   Isoschizomers


# Enzymes which cut more frequently than the MAXCUTS criterion
# Enzymes > MAXCUTS Frequency   Isoschizomers


# Enzymes that do not cut

BspCNI


# No. of cutting enzymes which do not match the
# SITELEN, BLUNT, STICKY, COMMERCIAL, AMBIGUOUS criteria

0
```

```
predicted_PCR_T


          GTTTTTTCCCTAAGACCTCAAATTTCATACAATAGGTTCAGAAAGTGAATGCTCCCCCCA
                   10        20        30        40        50        60
          ----:----|----:----|----:----|----:----|----:----|----:----|

          GGGGTCAGGCAAAAGAGGTGAGATATATATATTTTTTCTTTTTTCTTTTTTTTCTCTTATT
                   70        80        90        100       110       120
          ----:----|----:----|----:----|----:----|----:----|----:----|

                        BspCNI
                        \
          TCTCAGTTGGGCCTAAAGGAATCTGCCAAGGGAAAATCCCTGCTTAGTCATCTCCGAGGT
                   130       140       150       160       170       180
          ----:----|----:----|----:----|----:----|----:----|----:----|

          CTCTTGAGGATCCAGATGAGTTTGTTTACCCGAGGCG
                   190       200       210
          ----:----|----:----|----:----|----:--


# Enzymes that cut  Frequency   Isoschizomers
    BspCNI          1


# Enzymes which cut less frequently than the MINCUTS criterion
# Enzymes < MINCUTS Frequency   Isoschizomers


# Enzymes which cut more frequently than the MAXCUTS criterion
# Enzymes > MAXCUTS Frequency   Isoschizomers


# Enzymes that do not cut



# No. of cutting enzymes which do not match the
# SITELEN, BLUNT, STICKY, COMMERCIAL, AMBIGUOUS criteria

0
```