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**Natural history and Pathogenesis
of IgG4-related disease**

DPhil Clinical Medicine

Prologue

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Abstract

Natural history and pathogenesis of IgG4 related disease.

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DPhil Clinical Medicine. Trinity Term, Year 4.

IgG4-related disease (IgG4-RD) is a systemic fibro-inflammatory condition characterised by elevated serum IgG4 and an abundance of IgG4 plasma cells in involved organs. The natural history of disease and pathogenic mechanisms are poorly understood, and are explored in this thesis.

The diagnosis of IgG4-RD is a challenge. Evidence to support a serum IgG4 level of 2.8g/l in differentiating IgG4-RD from non-IgG4-RD conditions, a serum IgG1:IgG4 ratio of 0.24 in differentiating IgG4-sclerosing cholangitis from primary sclerosing cholangitis with elevated serum IgG4, and the role of serum IgE in those with a history of allergy and atopy is provided. Furthermore, observational data highlighting new environmental risk factors and disease associations are revealed. Despite being considered a benign corticosteroid-responsive condition, evidence for disease relapse, organ dysfunction and failure, malignancy and mortality in a prospective cohort is shown. Patterns of disease presentation and levels of serum IgG4 and IgE at diagnosis are used to identify those who relapse and develop multi-organ disease.

A single antigen initiating disease has yet to be found. Polyclonal IgG4 responses to multiple environmental antigens in IgG4-RD are reported, and evidence against *Helicobacter pylori* plasminogen binding peptide as a microbial antigen is shown. Novel HLA class II associations, linked to disease susceptibility in a UK cohort provide support for immune-mediated pathogenesis. Gene expression analysis implicates cytokines in driving IgG4 switch and proliferation, chemokines in trafficking and homing of lymphocytes to end organs, complement proteins in the classical and lectin pathways, and members of the TGF-beta pathway as putative immune drivers of disease. Differences in the phenotype of IgG1 and IgG4 B cells in health and IgG4-RD are reported, including responses to complement activation and immune complexes. Finally, elevated IgE levels, the presence of IgE-positive mast cells in involved tissues, and up-regulation of the Fc-Epsilon receptor on the surface of IgG4 cells, support the role of an IgE-mediated response.

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For this thesis and DPhil I was responsible for:

- securing grants and consumables funding as an individual applicant and co-supervisor for 3 academic students,
 - applying for Oxfordshire research ethical approval and NIHR portfolio support.
 - clinical cohort identification and recruitment, database design, clinical data collection.
 - blood and tissue sample acquisition, and processing of samples.
 - design and preparation of *Helicobacter pylori* plasminogen binding peptide overlapping epitopes, performing and teaching B and T cell ELISpots, Luminex cytokine array, IgG subclass ELISA's, RNA and DNA extraction, PCR-based assays, B cell isolation, purification and culture, B and T cell surface staining and functional assays, FACS-based sorting of IgG1 and IgG4 cells, and B cell receptor sequencing.
 - interpretation of tissue morphology and immunostaining for IgG, IgG4 and IgE including mean counts and ratios.
 - design of oligo-microarray, selection of whole blood samples, performing literature review, analysis of data using 3 types of software to interpret gene expression profiles and pathway analysis, and use of gene set enrichment analysis.
 - statistical analysis using the use of SPSS and Graphpad prism.
- presentations at loco-regional meetings, national courses and international conferences, and publications in peer-reviewed journals and book chapters.
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 - set up and presentation of patients at the monthly IgG4 multi-disciplinary meetings, where patients with suspected or definite IgG4-RD and disease mimics are discussed.
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Publications

The following manuscripts have been written during my DPhil and are relevant to my thesis. * denotes equal contributing first authorship.

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PRESENTATIONS

I have been invited as a speaker to present work directly related to my thesis at the following societies, conferences and courses.

- Systemic manifestations of IgG4-RD. Pancreatic Society of Great Britain and Ireland (PSGBI). John Innes Conference Centre, Norwich, Nov 2015.
- IgG4, IBD and Liver. Oxford Masterclass: New concepts and care in IBD and the liver. Examination School, Oxford, Sept 2014
- General overview of IgG4-RD. British Society of Gastrointestinal and Abdominal Radiology (BSGAR). The Randolph Hotel, Oxford, Feb 2014.
- Concepts in IgG4-RD. Vlaamse Vereniging voor Gastroenterologie (VVGE) Flemish Gastroenterology Association Fall Symposium. Crowne Plaza Hotel, Brugge, Nov 2013.
- Immunological mechanisms underlying IgG4-RD. Centre for Chronic Immunodeficiency (CCID) Translational Conference. Freiburg, Germany, Nov 2012.
- Pathogenesis of IgG4-RD. Pancreatobiliary Section. British Society of Gastroenterology (BSG). Glasgow, June 2013.
- General overview of IgG4-RD. Immune complex disease and Vasculitis. British Society of Rheumatology (BSR). Convention Centre, Birmingham, April 2013.

- IgG4-RD: an enigmatic disease. Academic Research In Progress Talk. Translational Centre, Birmingham, Jan 2013.
- Unravelling the mysteries of IgG4-RD. Wetherall Institute of Molecular Medicine (WIMM) Lecture. WIMM Lecture Theatre, Oxford, Sept 2013 and June 2011.
- Pathogenic mechanisms of IgG4-related disease. Grand Round, Oxford, Feb 2011 and 2013 and Royal Berkshire Hospital, Sept 2015.
- New insights into IgG4-RD. Hepatology and Gastroenterology Oxford Masterclass. The Examination School, Oxford, Sept 2012.
- UK IgG4-RD Science Day Symposium. Post Graduate Education Centre, John Radcliffe Hospital, Oxford. July 2012.
- Elevated serum and tissue IgG4 in PSC is associated with a worse clinical outcome. SE Regional GUT Club. John Radcliffe Hospital, Oxford, Aug 2012.
- IgG4-RD: an immune-mediated disease. Academic Medical Forum, Oxford, March 2012 and TGU Seminar Series, John Radcliffe Hospital, Oxford, April 2012.
- B and T cell immunology in IgG4-RD. B Cell Meeting, Sanquin Institute for Immunology, Amsterdam. Dec 2011.
- IgG4 is not useful or necessary in the diagnosis of IgG4-RD. Controversies and Changes in IgG assays. ACB Spotlight Meeting, Royal College of Pathologists, London, Sept 2011.
- IgG4-sialadenitis and its manifestations. ENT Meeting. West Wing, John Radcliffe Hospital, Oxford, Nov 2011.
- IgG4-cholangitis. Cholestatic Liver Disease Course. European Association for the study of Liver Disease (EASL). Wadham College, Oxford, July 2010.

Chapter 1 Introduction

Extracts from Chapter 1 have been published:

- **EL Culver**, AC Bateman. General principles of IgG4-related disease. *Diagn Histopathol* 2013;19:111-118
- **EL Culver** & AC Bateman. IgG4-related disease. C Probert, Editor. *Recent Advances in Gastroenterology*. JP Medical Ltd. Feb 2014:93-112.

1.1 BACKGROUND

IgG4-related disease (IgG4-RD) is a recently described systemic fibro-inflammatory condition characterised by the development of mass lesions, with similar histopathological findings and an abundant IgG4-positive plasma cell infiltrate in involved organs (Stone et al, N Engl J Med 2012). The disease can mimic malignancy and other inflammatory diseases, resulting in misdiagnosis, delays in treatment and unnecessary surgery. To date IgG4-RD has been reported in every organ system except the brain parenchyma. Autoimmune pancreatitis (type I AIP) was the first described pancreatic manifestation of the disease, upon which the early diagnostic criteria were based (Chari et al, Clin Gastroenterol Hepatol 2006; Otsuki et al, J Gastroenterol 2008). Biliary disease (IgG4-related sclerosing cholangitis; IgG4- SC) is one of the most frequent extra-pancreatic manifestations (Ghazale et al, Gastroenterol 2008). The disease has been described in Asia, the US and throughout Europe, with geographical variations in its clinical presentation, the utility of serum IgG4 in diagnosis, presence of extra-pancreatic manifestations, treatment regimens and outcome (Sah et al, Curr Opin Rheumatol 2012; Hart et al, GUT 2012). A separate histological variant, named idiopathic duct centric pancreatitis (IDCP) or type II AIP has been described

(Deshpande et al, Am J Surg Pathol 2011), but is not considered part of the IgG4-RD spectrum with distinct histopathology, and is not discussed in this thesis.

IgG4-RD is thought to be immune-mediated. This is suggested by a human leukocyte antigen (HLA) type II association described in Japanese patients with AIP, the presence of serological abnormalities (e.g. presence of autoantibodies and elevated levels of IgG and IgG4) and a dramatic response to corticosteroid therapy (Hamano et al, N Engl J Med 2001; Kawa et al, Gastroenterol 2002; Kamisawa et al, GUT 2009). However, the pathogenic mechanisms and the precise nature of the underlying immune response have not been clarified.

1.2 HISTORICAL PERSPECTIVE

IgG4-RD was first described as a chronic inflammatory sclerosis of the pancreas in 1961. However, it was not until 1995 that the term ‘autoimmune pancreatitis’ (AIP) was suggested (Yoshida et al, Dig Dis Sci 1995) and in 2001 the potential utility of serum IgG4 in distinguishing AIP from other pancreatic conditions became clear (Hamano et al, N Engl J Med 2001). The systemic nature of the disease was recognised in 2003 when multiple extra-pancreatic lesions were described in patients with AIP, all with similar histopathological characteristics (Kamisawa et al, J Gastroenterol 2003). These extra-pancreatic manifestations could precede, be synchronous with, follow or occur in the absence of pancreatic disease. Additional evidence came from biopsies of uninvolved organs in patients with AIP, such as the stomach and duodenum, in which there was an abundance of IgG4-positive plasma cells (Kamisawa et al, World J Gastroenterol 2006).

The nomenclature for the disease continues to evolve – the name IgG4-RD was agreed at the first international consensus meeting in Boston in 2011 (Stone et al, Semin Diagn Pathol 2012) - however the disease has been referred to by a variety of terms over the years in the literature (**Table 1**).

Table 1: Nomenclature in the literature for IgG4-RD

IgG4-related systemic disease
IgG4-related sclerosing disease
IgG4 disease
IgG4 syndrome
IgG4-related systemic sclerosing disease
IgG4-related autoimmune disease
Hyper-IgG4 disease
Systemic IgG4-related plasmacytic syndrome (SIPS)
IgG4-positive multi-organ lymphoproliferative syndrome (MOLPS)

A broader understanding of this disease has led to the realisation that many medical conditions long viewed as separate entities are actually part of the IgG4-RD spectrum (**Table 2**).

1.3 EPIDEMIOLOGY

Epidemiological data on the global incidence and prevalence of IgG4-RD is sparse. In 2002, the incidence and prevalence of AIP in Japan was estimated to be 0.28-1.08 cases/100,000 people and 0.8 cases/100,000 people respectively (Nishimori et al, J Gastroenterol 2007). In 2008, a further study in Japan described AIP in 2709 patients and estimated IgG4-RD in 8000 patients (Uchida et al, Int J Rheum 2012). A US study

revealed AIP in 11% of patients undergoing pancreatic resection for benign disease (Yadav et al, Clin Gastroenterol Hepatol 2003). There has been no such data from the UK. As recognition increases, the reported prevalence will inevitably grow.

Table 2: Diseases attributed to organ manifestations of IgG4-RD.

Original terminology	Organ target
Autoimmune pancreatitis	Pancreas
Sclerosing cholangitis	Biliary tree
Mikulicz's syndrome	Salivary and lacrimal glands
Kuttner's tumour	Submandibular glands
Riedel's thyroiditis	Thyroid
Retroperitoneal fibrosis (Ormond's disease)	Retroperitoneum
Mediastinal fibrosis	Mediastinum
Chronic sclerosing aortitis	Aorta
Inflammatory abdominal aortitis	Abdominal aorta
Orbital pseudotumour	Orbital adnexa
Eosinophilic angiocentric fibrosis	Orbits, sinuses and nasal cavities
Multifocal fibrosclerosis	Orbits, thyroid gland, retroperitoneum, mediastinum and other tissues
Idiopathic hypocomplementaemic tubulointerstitial nephritis with extensive tubulointerstitial deposits	Kidney

1.4 DEMOGRAPHICS

IgG4-RD has striking demographic features – the majority of patients are men (80%), and are in the 6th decade of life (Kamisawa et al, J Gastroenterol 2003). This gender predominance is particularly evident in kidney and retroperitoneal disease and less marked in sialoadenitis - where Japanese series suggest a more equal distribution. This

contrasts with autoimmune conditions that mimic IgG4-RD, such as Sjogren's syndrome mimicking IgG4-related sialoadenitis, which have a female predominance.

1.5 CLINICAL FEATURES

As IgG4-RD is a multisystem disorder, its clinical features are variable. Common sites of involvement are: pancreas, bile ducts, liver, GI tract, salivary and lacrimal glands, orbit, retroperitoneum and mesentery, aorta, thyroid, lung, kidneys, breast, skin, pituitary gland, meninges, prostate, lymph nodes and pericardium. The pattern of organ involvement and activity of disease usually determines the initial presentation. It can involve organs and tissues in a tumefactive or diffuse manner. The former can produce clinically obvious effects such as obstructive jaundice (a pancreatic mass and biliary involvement), proptosis (with orbital involvement) and a glandular mass (with parotid or salivary gland involvement), which may closely simulate malignancy. The latter is more likely to affect organs such as the skin or meninges, or can be clinically silent and detected on radiological imaging or on examination of a resection specimen (such as a diffusely enlarged pancreas or kidney) (Raissian et al, J Am Soc Nephrol 2011; Zen et al, Am J Surg Pathol 2009). Constitutional symptoms such as fever are rare. A history of allergy or atopy - such as eczema, asthma and hay fever - has been described in up to 40% of patients in one series, but the relevance of this is unknown (Kamisawa et al, Eur J Gastroenterol Hepatol 2009). There have been case reports of organ dysfunction in patients with fibrotic disease, although the frequency of this and factors that lead to disease progression have not been investigated.

1.6 SEROLOGICAL EVALUATION

Patients with IgG4-RD have polyclonal elevation of serum IgG4, even up to 50 times the upper limit of normal (ULN). The normal range for serum IgG4 in children and adults was established in Europe in the 1980's using radioimmunoassay (Furic et al, Clin Rev Allerg 1983). Subsequently, radioimmunoassay was replaced by serum nephelometry, although normal ranges for serum IgG4 were not re-validated using this modality. At our institute, the normal range for serum IgG4 is 0.3g/l to 1.3g/l, as determined by regional testing using the Siemens BNII Nephelometer and IgG4 monoclonal antibody (Cambridge Biosciences) at the Department of Clinical Immunology, Churchill Hospital, Oxford. However, in accordance with other laboratories and the Mayo HISORT diagnostic criteria for AIP (Chari et al, Clin Gastroenterol Hepatol 2006), we have considered the serum IgG4 level to be increased above an upper limit of normal (ULN) of 1.4g/l.

The serum IgG4 level is reportedly increased in around 80% of AIP patients. The reported sensitivities of an elevated serum IgG4 for AIP vary in the literature from 68 to 95% (Sah et al, Curr Opin Rheumatol 2011). A serum IgG4 twice the ULN (2.8g/l) has been reported to be specific in the differentiation of AIP from pancreatic cancer (Ghazale et al, Am J Gastroenterol 2007), but its value in differentiating systemic IgG4-RD, AIP or IgG4-SC from other inflammatory conditions or other malignancies is unknown. A clinical phenotype of AIP with a normal serum IgG4 has been described in Japan, with fewer extra-pancreatic lesions and a better clinical course (Matsubayashi et al, Dig Liver Dis 2007), although subsequent studies suggest this group may have included patients with IDCP or type II AIP (Kamisawa et al, J Gastroenterol 2011).

Once a diagnosis of IgG4-RD has been established, the serum IgG4 has been proposed to be a marker of disease activity and/or organ involvement (Kamisawa et al, Pancreas 2005), although the data here are contentious. Serum IgG4 levels are reported to fall with corticosteroid treatment, but do not always return to normal. The relevance of a persistently elevated IgG4 level in patients with IgG4-RD and other inflammatory conditions is not certain.

Other serum autoantibodies are non-specific in this disease e.g. anti-nuclear antibody (present in almost 50%) and rheumatoid factor (present in 20%). Serum inflammatory markers such as the erythrocyte sedimentation rate and C-reactive protein concentration are often elevated. Several autoantibodies specific and non-specific to the pancreas have been identified in AIP but none is useful as a diagnostic marker.

1.7 RADIOLOGICAL FEATURES

The radiological features of IgG4-RD are variable and depend on the organs involved. Some organs such as the pancreas have specific imaging findings, whilst most other organs such as the kidney and lung display non-specific features (Sahani et al, Radiology 2004). A combination of imaging modalities such as computerised tomography (CT) and magnetic resonance imaging (MRI) as well as functional studies can be used at diagnosis to establish involved organs and after corticosteroid treatment – to assess response, monitor disease activity and to look for evidence of relapse. Positron Emission Tomography (PET) scanning has been suggested as a useful means of looking at subclinical organ involvement and response to steroids in one prospective study (Zhang et al, Eur J Nucl Med Mol Imaging 2014), but is not widely available or disease specific.

1.8 ORGAN INVOLVEMENT IN IGG4-RD

1.8.1 Pancreas

The pancreas was the first organ in which the unique histological features of a diffuse lymphoplasmacytic infiltrate and fibrosis were described. Clinical presentation is commonly with obstructive jaundice, weight loss and mild abdominal discomfort. Cross-sectional imaging of the pancreas may show the classical features of diffuse organ involvement ('sausage-shaped pancreas') together with pancreatic and bile duct dilation (often segmental in nature), distal bile duct stricture and/or a mass lesion that may not be easily distinguishable from pancreatic adenocarcinoma on imaging features alone (Sahani et al, Radiology 2014) (**Figures 1 and 2**). Lymphadenopathy does not sway the diagnosis towards malignancy as 80% of patients that have AIP have local lymphadenopathy (Hamano et al, J Gastroenterol 2006). The diagnosis is ideally confirmed with pancreatic core biopsy usually during endoscopic ultrasound (EUS), and/or bile duct biopsy taken at endoscopic retrograde cholangiopancreatography (ERCP). Fine needle aspiration of cells from a pancreatic mass, and/or biliary brushings in those with strictures, are useful to exclude dysplasia but have limited utility in actual diagnosis. Invasive surgery still occurs in equivocal cases if there is a high suspicion of malignancy.

Two forms of AIP are recognised; type 1 AIP is a manifestation of systemic IgG4-RD and type II AIP (IDCP), which is not considered part of this spectrum. They have similar clinical presentations, radiological findings and respond to corticosteroid therapy, however the latter presents in younger patients, often with a co-existent history of inflammatory bowel disease, and has important histological differences with

granulocytic epithelial lesions and negative IgG4 immunostaining (Deshpande et al, Am J Surg Pathol 2011.)

Figure 1. CT Pancreas showing diffuse pancreatic enlargement in AIP



Figure 1: CT pancreas: The pancreas is diffusely bulky and sausage-shaped with enlarged peripancreatic and portal lymph nodes. The pancreatic duct is not dilated. Histologically confirmed AIP with the triad of morphological manifestations and abundant IgG4-positive plasma cells.

1.8.2 Biliary system

IgG4-related sclerosing cholangitis (IgG4-SC) is the name given to the biliary manifestation of IgG4-RD, although some have argued that ‘sclerosing’ be removed from this title given the usually rapid resolution of strictures with corticosteroid. It is the second most common site to be involved, following AIP. Clinical presentation is with obstructive jaundice and weight loss (Ghazale et al, Gastroenterol 2008). The imaging features in IgG4-SC are not highly specific on cholangiography - although the presence of a proximal bile duct or intrahepatic strictures in a patient with a pancreatic mass or multifocal biliary strictures is often seen (Webster et al, J Hepatol 2009).

Diagnosis should be supported histologically. Biliary aspirates, brushings and biopsies can be taken endoscopically, with/without SpyGlass (Boston Scientific) cholangioscopy where available, to aid in diagnosis and exclude dysplasia, and stents can also be placed for early therapy. Stents can often be removed after corticosteroid treatment. The key differential of a single stricture is a cholangiocarcinoma (CCA) and of multiple strictures is primary sclerosing cholangitis (PSC) (**Figure 3**). Clinical clues to differentiate IgG4-SC from CCA and PSC include the presence of spontaneously resolving or corticosteroid-responsive biliary strictures and evidence of other systemic organ involvement (Culver et al, Aliment Pharm Ther, 2011; Webster et al, J Hepatol 2009). The latter should be actively sought. IgG4-SC requires a high index of suspicion and should be excluded in all unexplained biliary strictures.

Figure 2. ERCP showing a distal common bile duct stricture in AIP



Figure 2: ERCP: A tight distal common bile duct stricture, with a guidewire placed for dilatation and 10 French stent insertion. The stent was removed after corticosteroids were given successfully to treat IgG4-related AIP and IgG4-SC.

Figure 3. ERCP showing a hilar stricture in IgG4-SC

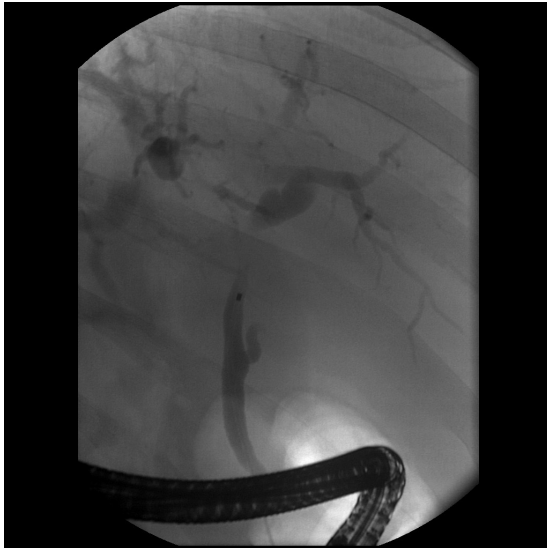


Figure 3: ERCP: A proximal cystic duct and intrahepatic duct stricture with distal right and left ductal dilatation. This patient with a normal serum IgG4 had surgery for presumed hilar cholangiocarcinoma with the resection specimen demonstrating IgG4-SC.

1.8.3 Liver

IgG4 hepatopathy is the term adopted to describe liver manifestations of IgG4-RD. Pseudotumours of the liver may be detected radiologically, after investigation of abnormal liver function tests or discovered incidentally (Nakanuma et al, Hum Pathol 1994). There are five distinct histopathological patterns described (1) portal inflammation with or without interface hepatitis, (2) large bile-duct obstructive features, (3) portal sclerosis, (4) lobular hepatitis, and (5) canalicular cholestasis. Clues to differentiate IgG4-hepatopathy from autoimmune hepatitis (AIH) include the lack of autoantibodies such as antimitochondrial antibody and smooth muscle antibody, the presence of abundant IgG4 positive plasma cells and storiform-pattern of fibrosis on liver biopsy (Umemura et al, GUT 2007). Essentially the first-line treatment of the two conditions with high-dose corticosteroids are similar, however management of IgG4-

hepatopathy should be guided by the presence of other organ manifestations and indications for second line immunosuppressive therapy differ.

1.8.4 Gallbladder

IgG4-related cholecystitis is usually asymptomatic and detected on histological examination of the gallbladder in patients with AIP (Wang et al, Histopath 2009). It is often described as an acalculous lymphoplasmacytic cholecystitis (Abraham et al, Am J Surg Pathol 2003). Histological examination of gallbladder biopsies after cholecystectomy with IgG4 immunostaining can help to support a diagnosis of AIP (Culver et al, J Clin Pathol 2012).

1.8.5 Luminal Gastrointestinal Tract (GIT)

The stomach, duodenum and colon have all been proposed to be involved in this systemic disease with high numbers of IgG4-positive plasma cells in involved tissue. However, the relevance of IgG4 positive plasma cells in either a normal-appearing or macroscopically inflamed GIT is unknown, and the concept that IgG4-RD can be diagnosed by blind biopsies of the gastrointestinal tract has by no means been validated (Strehl et al, J Clin Pathol 2011). More recently, an IgG4 infiltrate has been reported in the oesophagus in patients with eosinophilic oesophagitis, although this is not currently considered as a subset of IgG4-RD (Clayton et al, Gastroenterol 2014).

1.8.6 Lung

Pulmonary involvement may manifest with inflammatory pseudotumours, interstitial lung disease, lung nodules, consolidations, pleural effusions and thickening. Clinical

symptoms include non-specific cough, breathlessness, haemoptysis and chest discomfort but patients can be asymptomatic (Taniguchi et al, GUT 2004). Four patterns of lung involvement have been described on the basis of radiological and histological findings; solid nodular, bronchovascular, alveolar interstitial (with honeycombing, bronchiectasis, and diffuse ground-glass opacities), and round-shaped ground-glass opacities (Zen et al, Am J Surg Pathol 2009; Shrestha et al, Am J Surg Pathol 2009) (**Figure 4**). The pulmonary manifestations of IgG4-RD have a wide differential diagnosis including lung carcinoma (squamous cell, adenocarcinomas), bronchioloalveolar carcinoma, lymphoma, lymphomatoid granulomatosis, infections, granulomatosis with polyangiitis, sarcoidosis, and multicentric Castleman's disease.

Figure 4. CT Chest showing IgG4-interstitial lung disease.



Figure 4. High Resolution Computerised Tomography of the Chest: Bilateral ground glass opacification, interstitial fibrosis and extensive right-sided pleural thickening of IgG4-related interstitial disease. This patient had multiple organ involvement including the pancreas, bile ducts and salivary glands - confirmed on histology and responsive to corticosteroids.

1.8.7 Kidney

Renal IgG4 disease is most commonly recognised as IgG4-related tubulointerstitial nephritis (TIN), and less frequently as glomerular disease, especially membranous glomerulonephritis (Alexander et al, Kidney Int 2009; Saeki et al, Intern Med 2007). Proteinuria, haematuria, reduced renal function and hypocomplementaemia are features. On imaging diffuse renal enlargement, focal mass lesions and thickening of the renal pelvis have been described (Saeki et al, Kidney Int 2010) (**Figure 5**). Renal lesions are not often seen in isolation. Biopsies may be required to confirm diagnosis and importantly to exclude carcinoma and lymphoma (Cornell et al, Am J Surg Pathol 2007).

Figure 5. *CT Abdomen and Pelvis showing IgG4-related renal disease.*

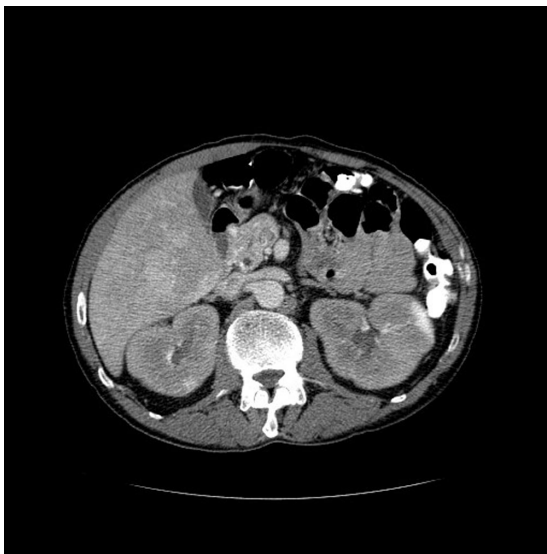


Figure 5. *Computerised Tomography of the Abdomen and Pelvis: Both kidneys are diffusely enlarged and hypoattenuated with a few peripheral foci of normal enhancing parenchyma. The pancreatic head and uncinata process was bulky (not shown in this image). Histology confirmed IgG4-tubulointerstitial nephritis (TIN) and both the kidneys and pancreas enlargement resolved with corticosteroids.*

1.8.8 Salivary and Lacrimal Glands

This includes historical cases of chronic sclerosing sialoadenitis, Kuttner's tumour and Mikulicz's disease. Salivary and lacrimal gland involvement is the fourth most common extra-pancreatic manifestation after IgG4-SC and lymphadenopathy. IgG4-siloadenitis is most likely to occur in the submandibular gland and not include the parotid (**Figure 6**) (Chow et al, Hong Kong Med J 2008). Salivary gland involvement consists of firm nodular swelling that is symmetrical and associated with pain, tenderness, and decreased saliva production.

Kuttner's tumour is a symmetrical swelling of the submandibular gland and is often IgG4-related in the absence of stones or infection. Mikulicz's disease is a bilateral symmetrical painless swelling of the lacrimal and salivary (parotid and submandibular) glands, most of whom actually have IgG4-RD (Yamamoto et al, Mod Rheumatol 2006). Minor salivary gland disease can also be present (such as in the lip) even if the major glands are macroscopically normal (Blanco et al, Ann Diagn Pathol 2003).

Figure 6. MRI Neck with gadolinium contrast showing IgG4-related sialoadenitis.

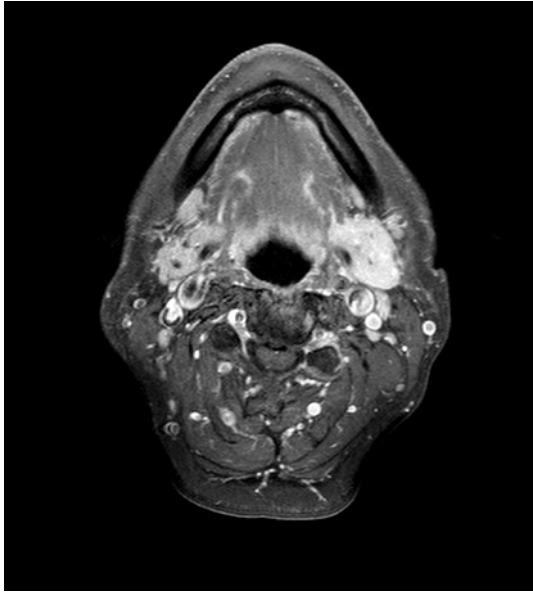


Figure 6. MRI Neck with gadolinium contrast: An enlarged left submandibular gland with uniform contrast enhancement and bilateral enlarged lymph nodes. The diagnosis was confirmed retrospectively after surgical resection.

1.8.9 Orbit

Orbital disease commonly presents as a chronic sclerosing dacryoadenitis (CSD) or an inflammatory orbital pseudotumour. Ocular structures affected by IgG4-RD include the lacrimal glands (dacryoadenitis), nasolacrimal duct, the retrobulbar region (including orbital pseudotumour), extraocular muscles (orbital myositis), uveal tract (uveitis), optic nerve sheath, superior orbital fissure, cavernous sinus and orbital fatty tissues (Sato et al, Pathol Int 2008). The process may even extend outside the orbit to the trigeminal nerve (Katsura *et al*, Am J Neuroradiol 2011). CSD consists of bilateral lacrimal gland involvement with lymphoid hyperplasia and fibrosis (Cheuk et al, Am J Surg Pathol 2007). In the majority, salivary gland disease coexists although isolated cases can occur. Orbital inflammatory pseudotumours are mass-forming lesions (**Figure 7**). Orbital IgG4-RD must be differentiated from lymphoma (particularly marginal zone

lymphoma), other malignant lesions, thyroid eye disease, sarcoidosis, granulomatosis with polyangiitis and be excluded before a diagnosis of 'idiopathic' orbital disease is made. There have been controversial reports of clonal orbital adnexal lymphoma arising from orbital IgG4-RD (Cheuk et al, Am J Surg Pathol 2008), but this concept requires further clarification.

Figure 7: Clinical examination revealing an orbital pseudotumour.

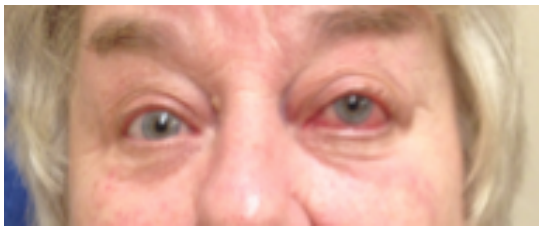


Figure 7: Clinical examination reveals proptosis of the left orbit with external ophthalmoplegia. MRI orbit confirmed enlargement and contrast-enhancement of the left peri-orbital muscles, and MRI head and neck showed minor salivary gland enlargement. Biopsy of the salivary gland was consistent with IgG4-related sialoadenitis. Prednisolone and subsequent mycophenolate mofetil resulted in improvement of the pseudotumour, but 12 weeks of cyclophosphamide was necessary for significant resolution.

1.8.10 Retroperitoneum and Mesentery

Retroperitoneal fibrosis (RF) is characterised by chronic inflammation and marked fibrosis of the retroperitoneal tissue. A proportion of cases of RF belong to the IgG4-RD spectrum (Zen et al, Am J Surg Pathol 2009). It can present with abdominal pain and obstructive symptoms, especially in advanced cases where fibrosis can encase and obstruct the ureters and other abdominal organs. Corticosteroids can reduce the early inflammatory component although advanced fibrotic disease can be resistant to treatment requiring surgical intervention and a worse outcome (Kardar et al, J Urol 2002). Mesenteric fibrosclerosis and sclerosing mediastinitis have also been described (Chen et al, J Clin Pathol 2008; Culver et al, GUT 2012) (**Figure 8A and B**).

Figure 8: A: CT Abdomen and Pelvis showing a mesenteric mass



Figure 8: B: Colonic resection specimen confirming a fibrosclerotic mass lesion.



Figure 8: (A) Contrast-enhanced CT of the abdomen: A 65mm by 54mm hyperdense mass lesion at the ileocaecal junction (thick black arrow), with local and mesenteric lymphadenopathy (thin black arrows). (B) Colonic resection specimen was fibrosclerotic, and histology was consistent with IgG4-mesenteric fibrosclerosis with storiform fibrosis and abundant IgG4 positive plasma cells.

1.8.11 Aorta

Abdominal or thoracic aortitis are IgG4-RD manifestations, which may lead to an abdominal aortic aneurysm, and can extend to peri-aortitis (Palmisano et al, Best Pract Res Clin Rheumatol 2009; Stone et al, Arthritis Care Res (Hoboken) 2010). They often

occur in the context of RF. Biopsies of the aorta are not commonplace, and therefore additional organ manifestations are sought to obtain specimens for histological confirmation. A radiological response to corticosteroids has been described.

1.8.12 Thyroid

The thyroid has been described to be affected by IgG4-RD in two different ways; a subset of Hashimoto's thyroiditis (the fibrosing variant) and Riedel's thyroiditis. IgG4 Hashimoto's thyroiditis is more closely associated with rapid progress, subclinical hypothyroidism, higher levels of circulating antibodies, and more diffuse low echogenicity than non-IgG4 Hashimoto's disease (Deshpande et al, J Clin Pathol 2012). Several studies have observed a high prevalence of hypothyroidism in patients with AIP (Komatsu et al, Dig Dis Sci 2005), but the cause for this is unclear.

1.8.13 Pituitary gland

Hypophysitis is associated with IgG4-RD. Most patients are middle-aged men who present with anterior or posterior pituitary insufficiency. Imaging studies often reveal a pituitary mass or thickened pituitary stalk. In some patients, both clinical signs and radiological changes resolve with corticosteroids (Haraguchi et al, Endocr J 2010). The presence of other organs involvement and elevated serum IgG4 concentrations are a major clue to the diagnosis. The differential diagnosis includes other infiltrative conditions such as sarcoidosis, granulomatosis with polyangiitis, histiocytosis, and lymphoma.

1.8.14 Meninges

IgG4-related meningeal disease has been termed IgG4 pachymeningitis. It has been reported to affect elderly men and present with radiculomyelopathy, headache, or cranial nerve palsies (Lindstrom et al, *Acta Neuropathol* 2010). Some cases of ‘idiopathic’ hypertrophic pachymeningitis not confirmed by biopsy have been associated with multifocal fibrosclerosis, which belongs to the IgG4-RD spectrum.

1.8.15 Lymph nodes

Involvement of lymph nodes as generalized lymphadenopathy or localised disease adjacent to the specific organ affected by IgG4-RD is a common finding. Generalized lymphadenopathy may be the sole presentation in IgG4-RD (**Figure 9**). In this instance, serial lymph nodes biopsies are often undertaken to exclude lymphoma, sarcoidosis, multicentric Castleman’s disease, and disseminated malignancies. In most cases these are reported as a reactive follicular hyperplasia and IgG4 staining is rarely done (Sato et al, *Int J Rheumatol* 2012). In AIP, almost 80% of patients have lymphadenopathy on imaging studies. Enlarged regional lymph nodes are also detected during resection of the affected organs. Five histological subtypes of IgG4-related lymphadenopathy have been described (Sato et al, *Int J Rheumatol* 2012). It is challenging to base a diagnosis of IgG4-RD solely on lymphadenopathy and polyclonal serum IgG4 elevation without other organ manifestations and thus an extensive search should be done for additional organs involved.

Figure 9. *CT Abdomen and Pelvis showing generalized lymphadenopathy.*



Figure 9. *CT Abdomen and Pelvis: Bilateral external iliac, common iliac and retroperitoneal lymphadenopathy. The right inguinal node is 13x24mm. Six months later, CT pancreas demonstrated a dilated pancreatic duct with a mass at the head of the pancreas (image not shown). Biopsies of the lymph nodes excluded lymphoma and a complete response to corticosteroids was seen. Relapse with IgG4-related sialoadenitis occurred at two years, requiring steroids and mycophenolate therapy.*

1.8.16 Breast, Prostate, Pericardium and Skin

Inflammatory pseudotumours and IgG4-related sclerosing mastitis have been described, the latter presenting as solitary or multiple painless masses in one or both breasts (Zen et al, *Am J Surg Pathol* 2005; Cheuk et al, *Am J Surg Pathol* 2009). IgG4-related prostatitis has been reported in some patients with other organ manifestations and response to corticosteroids has been described (Yoshimura et al, *Int Med* 2006; Uehara et al, *Pathol Int* 2008). Cutaneous manifestations of IgG4-RSD appear to be erythematous or flesh-coloured papules or plaques that have a tendency for localization on the face or head (Khosroshahi et al, *Am J Med* 2011). IgG4-RD has been also linked to cases of constrictive pericarditis (Sugimoto et al, *Int J Cardiol* 2008).

1.9 HISTOPATHOLOGY

The classical histopathological features of IgG4-RD have been well described (Deshpande et al, Mod Pathol 2012). The characteristic ‘triad’ are; (1) lymphoplasmacytic inflammation, (2) storiform fibrosis and (3) obliterative phlebitis. [31] All of these features can be seen on ‘routine’ haematoxylin and eosin (H&E) stained sections (**Figure 10**).

Figure 10. *The triad of histological features of IgG4-RD.*

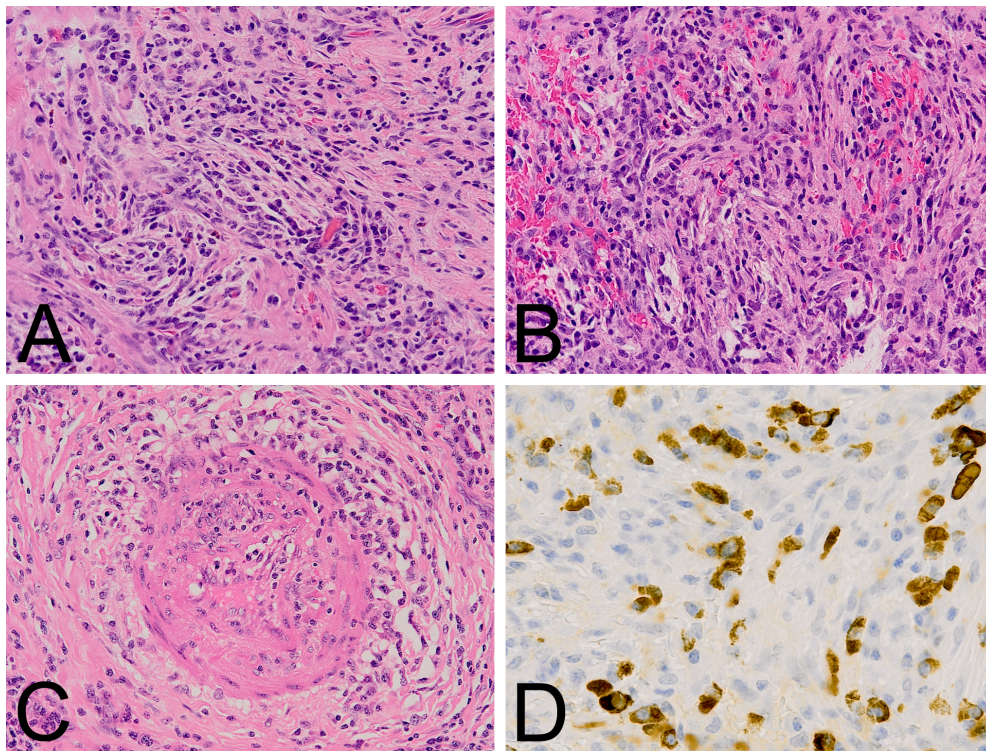


Figure 10: Histopathology in IgG4-RD: (A). Lymphoplasmacytic inflammation. Large numbers of lymphocytes and plasma cells are present – both are chronic inflammatory cells (haematoxylin and eosin stain; magnification x200). (B). Storiform fibrosis. Interwoven and radiating spindle-shaped myofibroblasts are present in association with the inflammatory cell infiltrate (haematoxylin and eosin stain; magnification x200). (C). Obliterative phlebitis. A vein showing complete fibrous obliteration of its lumen together with chronic inflammatory cells (lymphocytes) infiltrating the vein wall (haematoxylin and eosin stain; magnification x200). (D). Prominent IgG4+ plasma cells. The cells with brown stained cytoplasm are plasma cells containing IgG4 (IgG4 immunohistochemistry; magnification x400). Slides photographed by Dr. Adrian Bateman.

The lymphoplasmacytic inflammation, a chronic inflammatory cell infiltrate comprising predominantly CD4⁺ lymphocytes and CD138⁺ plasma cells, is commonly diffuse and/or dense in nature. ‘Storiform’ fibrosis refers to collagen deposition, usually with associated myofibroblasts, arranged in an interwoven or radiating pattern. Obliterative phlebitis refers to occlusion of veins by collagen, and while visible on an H&E stain can be highlighted with a connective tissue stain such as elastic Van Gieson. A variable presence of eosinophils has been described within the inflammatory infiltrate.

These appearances may constitute non-specific features of chronic inflammatory conditions other than IgG4-RD. The index of suspicion for a diagnosis of IgG4-RD is significantly higher when all three are present in an appropriate clinical setting. In addition, the presence of prominent IgG4-positive plasma cells, as identified using immunohistochemistry (a technique in which a specific antigen is identified within a tissue section via the binding of a specific antibody, with this binding process subsequently visualised usually with a coloured dye), strongly supports a diagnosis of IgG4-RD. Lastly, the number of IgG4-positive plasma cells should exceed a ratio of 40% of the total number of IgG-positive plasma cells, to differentiate it from other inflammatory conditions with a plasma cell infiltration. When all of these features are present, they can effectively lead to recognition of the condition based on histological examination alone, in accordance with the Boston Consensus Histopathological Criteria (Deshpande et al, Mod Pathol 2012) (**Table 3**). However, it is optimal to interpret histological features in the context of a clinical case.

Table 3. The Boston consensus criteria for the histological diagnosis of IgG4-RD

A. Key morphological features	
Dense lymphoplasmacytic inflammation	
Fibrosis that is at least focally storiform in nature	
Obliterative phlebitis	
B. Other morphological features that may be present	
Phlebitis without obliteration of the lumen	
Increased numbers of eosinophils	
C. Raised numbers of IgG4+ plasma cells	
Measured as the mean IgG4+ plasma cell count per high-power microscope field (HPF) within the three HPFs containing the greatest number of these cells	
The minimum mean number of IgG4+ plasma cells per HPF required varies between anatomical sites and whether biopsy or surgical material is available for examination	
The IgG4+/IgG+ plasma cell ratio must be >40%	
Overall assessment of histological features	
Histologically <i>highly suggestive</i> of IgG4-RD	<i>Two or more</i> of the key morphological features and the required number of IgG4+ plasma cells per HPF for that site
<i>Probable</i> histological features of IgG4-RD	<i>One</i> of the key morphological features and the required number of IgG4+ plasma cells per HPF for that site
<i>Insufficient</i> histopathological features of IgG4-RD	Cases with appearances falling outside of the above two categories

The histological features of IgG4-RD may be variable in different areas of a tissue section. Although inflammatory changes are often diffuse and/or dense, the disease can be patchy in distribution. Furthermore, it is believed that there is an initial inflammation-predominant phase in which IgG4-positive plasma cells are characteristically prominent, followed by a fibrosis-predominant phase in which inflammation is less dense and fibrosis dominates – including increasingly dense collagen bundles as the disease becomes more chronic. These are the main reasons why

a definitive histological diagnosis of IgG4-RD is usually easier to reach when examining tissue from resection specimens, compared to the more limited conclusions that may be drawn from biopsy material.

Prominence of IgG4-positive plasma cells may be seen in conditions other than IgG4-RD (Ravi et al, *Inflam Bowel Dis* 2009; Roberts et al, *Histopathology* 2010; Stone et al, *Curr Opin Rheumatol* 2011; Rollins-Raval et al, *Int J Surg Pathol* 2012) (**Table 4**). The presence of IgG4 cells alone, without the characteristic morphological features of IgG4-RD that are identifiable on a routine haematoxylin and eosin-stained tissue section, is not sufficient for a diagnosis of IgG4-RD (Strehl et al, *J Clin Pathol* 2011). When IgG4-positive plasma cells are prominent but one or more of the key triad of morphological features are absent, it should alert the pathologist to the possibility that they may be seeing a non-specific increase in IgG4-positive plasma cell numbers. This can inevitably lead to over-diagnosis. Further biopsies may be required to establish a diagnosis. It is important in this instance, to place the histology in the context of an appropriate clinical history and radiological investigations.

In some circumstances however, prominent IgG4-positive plasma cell numbers as an individual finding can lend support to a diagnosis of IgG4-RD, which is being considered on clinical and/or radiological grounds (Moon et al, *Gastrointest Endosc* 2010; Kawakami et al, *Gastrointest Endosc* 2010). Manifestations of IgG4-RD at certain sites may not be associated with the presence of all three of the morphological 'triad' of features. Examples include many of the lymphoreticular patterns of involvement, where prominent IgG4+ plasma cells can be present within a lymphoid

infiltrate and in the absence of storiform fibrosis or obliterative venulitis (Ferry et al, Diag Histopathol 2013).

Table 4. IgG4+ plasma cells may be prominent without IgG4-RD.

Site	Condition
Oral cavity	Epulis plasmacellularis
	Radicular cyst
Colon	Diverticulitis
Synovium	Rheumatoid arthritis
Skin	Inflammatory skin conditions
Various	Carcinoma-associated inflammatory response (esp. squamous cell carcinoma)
Lymph nodes	Castleman's disease
	Follicular hyperplasia
	Interfollicular plasmacytosis
Colon	Inflammatory bowel disease
Lung	Rosai-Dorfman disease
Aorta	Peri-aortitis
Various	Carcinoma e.g. pancreatic
	Lymphoma esp. low-grade marginal zone & follicular

1.10 DIAGNOSTIC GUIDELINES

The development of guidelines for the diagnosis of IgG4-RD has followed its increasingly worldwide recognition, the realisation of its systemic nature, and a need to differentiate it from other disease conditions. The first guidelines were created for AIP in Japan and Korea, with consensus Asian diagnostic criteria being developed (Otsuki et al, *J Gastroenterol* 2008). Subsequent guidelines for AIP and IgG4-SC came from the Mayo Clinic in the US, named the HISORt criteria (Chari et al, *Clin Gastroenterol*

Hepatol 2006) (**Table 5**). More recently, the Japanese Comprehensive Diagnostic Criteria (CDC) were created for application to all organ manifestations of systemic IgG4-RD (Umehara et al, Mod Rheumatol 2012) (**Table 6**).

Table 5. The HISORt criteria for the diagnosis of AIP.

1.	Histopathology – <i>one or both</i> criteria required	Characteristic appearances within biopsy or resection material*
		At least 10 IgG4-positive plasma cells per high power field within areas of lymphoplasmacytic infiltrate
2.	Imaging & serology – <i>all three</i> criteria required	Diffusely enlarged pancreas with delayed and ‘rim’ enhancement
		Irregular pancreatic duct
		Increased serum IgG4 concentration
3.	Response to steroid therapy – <i>all three</i> criteria required	Unexplained pancreatic disease after a full clinical workup – including exclusion of cancer
		Raised serum IgG4 concentration and/or extrapancreatic organ involvement with increased numbers of tissue IgG4-positive plasma cells
		Resolution or marked improvement in disease with steroid therapy

Table 5: The Mayo HISORt diagnostic criteria for AIP. Histopathology includes a lymphoplasmacytic infiltrate, ‘storiform’ fibrosis and obliterative phlebitis; the inflammatory cell infiltrate alone is not sufficient to meet this criterion.

Table 6. The Japanese comprehensive diagnostic criteria (CDC) for IgG4-RD.

1.	Clinical examination showing characteristic diffuse/localized swelling or masses in single or multiple organs
2.	Haematological examination shows elevated serum IgG4 concentrations (>135mg/dl)
3.	Histopathological examination shows: (1) Marked lymphocyte and plasmacytic infiltration and fibrosis (2) Infiltration of IgG4+ plasma cells: ratio of IgG4+/IgG+ cells >40% and >10 IgG4+ plasma cells/HPF
Definite: 1 + 2 + 3 Probable: 1 + 3 Possible: 1 + 2 However, it is important to differentiate IgG4-RD from malignant tumours of each organ (e.g. cancer, lymphoma) and similar diseases (e.g. Sjogren's syndrome, primary sclerosing cholangitis, Castleman's disease, secondary retroperitoneal fibrosis, Wegener's granulomatosis, sarcoidosis, Churg-Strauss syndrome) by additional histopathological examination Even when patients cannot be diagnosed using the CCD criteria, they may be diagnosed using organ-specific diagnostic criteria for IgG4-RD	

All of these criteria are based on a combination of clinical, serological, radiological and histological features. Generally, the confidence within which a diagnosis of IgG4-RD is made increases if supporting evidence is available from at least 3 of these 4 disciplines. Guidelines for the histological diagnosis of IgG4-RD depending on the organ involved and tissue sample obtained (resection or biopsy) were agreed among experts in Boston in 2011, named the Boston Consensus Histopathological Criteria (Deshpande et al, Mod Pathol 2012) (**Table 3**).

1.11 TREATMENT

The necessity and urgency of treatment for patients with IgG4-RD depends on the involvement of vital organs and risk of organ dysfunction or failure. Potentially irreversible consequences include portal hypertension and cirrhosis in IgG4-related SC, aortic dissection in IgG4-related aortitis, irreversible fibrosis and encasement of vessels within the mesentery and retroperitoneum, chronic salivary and lachrymal gland dysfunction in IgG4-sialoadenitis, and visual impairment from orbital pseudotumours. These manifestations should be treated urgently. In other cases spontaneous resolution occurs and conservative approaches can be adopted, such as in the case of lymphadenopathy and pulmonary nodules.

The majority of patients (>95%) have a rapid response to glucocorticoids (Kamisawa et al, GUT 2009). The response is most dramatic in those with early inflammatory disease. Indeed, disease response to steroids is one of the diagnostic criteria for AIP within the HISORT criteria. Despite this, it should be remembered that many inflammatory conditions may be ameliorated by steroids and therefore that a clinical response to treatment does not alone confirm a diagnosis of IgG4-RD. While the response to steroids is usually good, relapse is common on steroid withdrawal and discontinuation. Relapse rates have been documented to occur in 32% of patients within six months, 56% within one year and 92% within three years, in one retrospective Japanese series (Kamisawa et al, GUT 2009).

In those with recurrent or refractory disease, second line immunosuppressant agents have included azathioprine, mycophenolate mofetil and methotrexate (Hart et al, GUT

2012). More recently, case series confirm that B-cell depletion with the anti-CD20 monoclonal antibody rituximab has proven effective in patients whose disease has not been controlled by other drugs (Khosroshahi et al, *Medicine (Baltimore)* 2012). This is an encouraging discovery and cannot be due to a direct effect of the agent on plasma cells since CD20 is lost by B-cells on maturation to plasma cells. Treatment with immunosuppressant therapies is most effective when IgG4-RD is in its inflammation-predominant phase. Patients whose disease has become more chronic and has moved into a fibrosis-predominant phase may show a more limited response to medical therapy. In this situation, surgery may still be required in order to remove symptomatic masses e.g. to relieve obstruction.

Specific treatment-related questions include (1) whether to treat everyone with corticosteroid therapy, including those with isolated lymphadenopathy or single organ sialoadenitis? (2) how long to continue corticosteroid therapy, and if the length of treatment influences relapse, as it does in patients with AIH? (3) whom to treat and when to treat with immunosuppressant drugs - should we be considering a top-down approach with dual disease-modifying agents such as in rheumatoid arthritis, or as an additional therapy for those who relapse only? (4) which immunosuppressive agents to use, and if this depends on the organs involved? - the gastroenterologist favours azathioprine for AIP and IgG4-SC, and the rheumatologist favours mycophenolate for extra-pancreatobiliary manifestations, based on experience with these drugs with no good evidence base (5) should we consider the early use of biological therapies such as rituximab in those with multi-organ disease? if so, how do we predict those that will respond and how does it affect the underlying immune response? These questions need to be addressed by controlled studies before therapeutic options progress.

1.12 PATHOGENETIC MECHANISMS

Insights into the genetic and immunological aspects of IgG4-RD have increased over the last decade. An overview of pathogenic mechanisms is shown in **Figure 11**.

Figure 11. Overview of the pathogenic mechanisms in IgG4-RD.

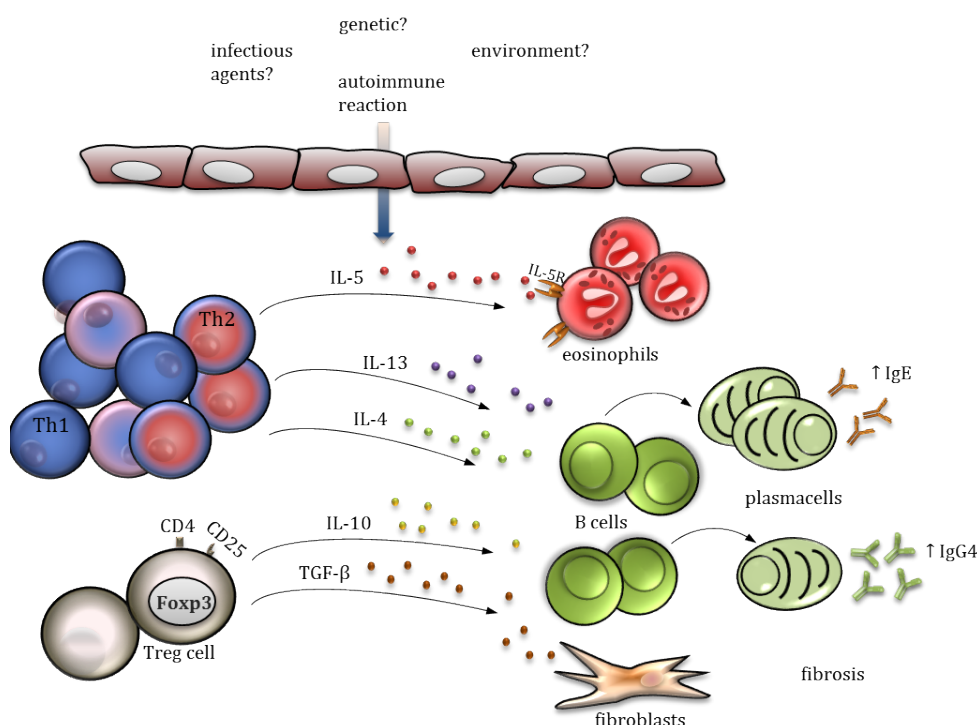


Figure 11: Mechanisms of IgG4-RD. Schematic designed with illustrative assistance from Wouter Smit (AMC, Amsterdam). Key: T reg: T regulatory cell; Th1 and Th2: T helper cells; IL5-R: IL5 receptor.

Genetic studies in Asia have suggested that several HLA and non-HLA haplotypes or genotypes are associated with susceptibility to AIP, disease relapse after steroid therapy or extra-pancreatic disease (Kawa et al, Gastroenterol 2002; Park et al, Gastroenterol 2008; Hirano et al, J Gastroenterol 2009). Of particular interest is the class II type HLA DRB1_0405 and DQB1_0401 (Kawa et al, Gastroenterol 2002). Single nucleotide polymorphisms involved in disease susceptibility or recurrence have been reported to be

present within genes encoding proteins including cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), tumour necrosis factor- α (TNF α) and Fc receptor-like 3 (FcR-3) (Umemura et al, Am J Gastroenterol 2008; Umemura et al, GUT 2006). These studies focus on the pancreatic manifestation of disease in Asian patients, and their applicability to systemic disease in the West is unknown.

Several auto antigens have been investigated, such as carbonic anhydrase (CA) II, lactoferrin (LF) and pancreatic secretory trypsin inhibitor (PSTI), based on the hypothesis that IgG4-RD is an autoimmune disorder (Okazaki et al, Gastroenterol 2000; Aparisi et al, GUT 2005; Nishimori et al, GUT 2005; Asada et al, Pancreas 2006). Autoantibodies against CAII and LF are frequently detected in the serum of patients with AIP but are non-specific (Lohr et al, Am J Gastroenterol 2010). A proteomics study identified a potential candidate auto antigen but the sequence was not clarified (Yamamoto et al, Rheumatol Int 2010). Importantly, IgG4-type autoantibodies have not been detected in IgG4-RD, although IgG4 from IgG4-RD patient's serum can bind to normal human tissue (Aoki et al, Histopath 2005).

Gastric *Helicobacter pylori* infection was proposed to trigger AIP in genetically predisposed individuals through a process of antibody cross-reactivity (molecular mimicry). This was based on sequence homology between human CAII and α -CA of *H. pylori*, the homologous segment containing the binding motif of the susceptibility HLA molecule DRB1_0405 (Guarneri et al, J Cell Mol Med 2005), and homology between the plasminogen-binding protein of *H. pylori* and ubiquitin-protein ligase E3 component n-recogin 2 expressed in pancreatic acinar cells (Frulloni et al, N Engl J Med 2009). However these findings have not been validated in systemic disease or in an external

cohort.

Convincing evidence that IgG4 is driving the pathology of IgG4-RD is lacking. IgG4 autoantibodies have been shown to play an important role in immune-mediated disorders unrelated to IgG4-RD. These include IgG4 antibodies against desmoglein 1 in pemphigus vulgaris and foliaceus (Rock et al, N Engl J Med 1989; Warren et al, J Invest Dermatol 2003), MuSK protein in myasthenia gravis, the M-type phospholipase A2 receptor in patients with idiopathic membranous glomerulonephritis (Beck et al, Kidney Int 2010) and IgG4-containing immune complexes that damage renal glomeruli in subsets of childhood membranous glomerulonephritis (Debiec et al, N Engl J Med 2011).

Immune mediated pathways may drive the IgG4-RD process. Interestingly, regulatory immune reactions are activated rather than suppressed as in many other autoimmune disorders (Miyoshi et al, Pancreas 2008; Sakaguchi et al, Immunol Rev 2006). Regulatory cytokines interleukin-10 (IL10) and transforming growth factor- β (TGF- β) have been suggested, respectively, to play important roles in IgG4 class switching and fibroplasia in the disease (Zen et al, Hepatology 2007). A Th2-dominant immune response is seen in peripheral blood and tissues (Miyake et al, Mod Rheumatol 2008; Kudo-Tanaka et al, Mod Rheumatol 2009; Kanari et al, Int Arch Allergy Immunol 2010). Chemokine expression profiles have not been well characterized, although a role of CXC chemokine receptor type 5 (CXCR5), expressed on T follicular helper cells, has been suggested (Esposito et al, PLoSOne 2008). Toll-like receptor and Nod-like receptor stimulation have been implicated in IgG4-RD, since IL10 and high levels of IgG4 are produced in response to stimulation in a B cell activating factor (BAFF)-dependent

manner (Akitake et al, GUT 2010; Lighaam et al, Int J Rheumatol 2012). The presence of peripheral and tissue eosinophilia, elevated serum IgE and a history of allergy in some patients with AIP may suggest a chronic allergic reaction in pathogenesis; however a specific allergen has not been identified (Kamisawa et al, Eur J Gastroenterol Hepatol 2009). Although IgG4-RD has been thought of as a single disease entity, different causative factors may be involved in different organs.

1.13 IGG4 BIOLOGY

IgG4 is the least prevalent of the four IgG subclasses, representing 3-6% of total IgG in the serum of adults (normal range 0.05-1.4g/l). It can however account for up to 80% of total IgG after chronic exposure to certain antigens. IgG4 is a unique antibody in both structure and function (Aalberse et al, Immunol 2002). Amino acid differences in the CH2 domain of IgG4 lead to negligible binding to the C1q protein complex and reduced binding to the Fc-gamma receptors of effector cells. This means that IgG4 is unable to activate the classical complement pathway and has a limited role in immune activation. In the core hinge region, an amino acid switch, from proline in IgG1 to serine in IgG4, allows for IgG4 to undergo a process of exchange of half-antibodies *in vivo* (Rispen et al, J Am Chem Soc 2011). This generates functionally monovalent antibodies which cannot cross-link antigens or form large immune complexes. IgG4 can bind the Fc portion of other IgG molecules at its constant domain, which may contribute to its anti-inflammatory function (Rispen et al, J Immunol 2009). It can also interfere with complement-activating and immune-precipitating activities of IgG1.

Physiological IgG4 responses can be induced by repeat or high antigen exposure and is

associated with tolerance induction such as in immunotherapy. IgG4 production is driven predominantly by type 2 helper (Th2) cytokines in particular IL4 and IL13, which induce IgG4 and IgE, and IL10, IL21 and IL12 that shifts this balance towards IgG4 – the ‘modified Th2 response’ (Wood et al, Cell Immunol 2004; de Boer et al, Infect Immun 1997).

1.14 SUMMARY

IgG4-RD remains an under-recognised systemic condition. The clinic-pathological definition continues to evolve. Overlaps with similar diseases that do not form part of the spectrum of IgG4-RD are becoming increasingly recognised and differentiation of these from ‘true’ IgG4-RD is important and challenging. Diagnostic algorithms have been developed to aid identification of this disease, and it is unlikely that any single criterion will be diagnostic in isolation. The classic morphological ‘triad’ of pathological appearances, supported by an abundant IgG4+ plasma cell count and IgG4+/IgG+ ratio, is the most reliable feature, but should be interpreted in the correct clinical context. The majority of patients are corticosteroid responsive but disease relapse occurs on withdrawal and discontinuation. Treatment options continue to expand as experience with B cell depletion therapies in refractory disease grows, however decisions on whom and when to treat, what agent to use, and the true effect of these regimens on the underlying immune activation remains uncertain.

It is evident that prospective studies of IgG4-RD patients are required to clarify the natural history and long-term outcome of patients, the utility of serum IgG4 in diagnosis and follow-up, the role of allergy and IgE in disease, and other potential diagnostic or prognostic markers. Validated outcome measures are crucial in assessing disease

activity and response to treatment. The underlying pathogenesis of the disease remains elusive. Specific questions include finding a disease-specific antigenic driver, if one exists, confirming or refuting the proposed association of AIP with *H.pylori*, understanding the immunological milieu particularly the presence of B cells and plasma cells in disease, establishing genetic HLA associations present in patients with systemic disease outside of Asia, and finding other serological and/or genetic markers of disease activity and severity. Defining the actual role of the IgG4 antibody as a bystander molecule or having a direct pathogenic influence on the disease process remains the most challenging question.

1.15 AIMS AND OBJECTIVES

In this thesis I aim to address the following questions using a prospective cohort of IgG4-RD patients established in Oxford and then extended across the UK. Specifically;

1. What is the natural history of disease in terms of organ involvement, response to therapy and disease relapse? (**Chapter 3**)
2. What is the longer-term outcome of disease in terms of complications, morbidity and mortality? (**Chapter 3**)
3. What is the diagnostic utility of serum IgG4 levels in differentiating IgG4-RD from other malignant, inflammatory and immune diseases? (**Chapter 4**)
4. What is the prognostic utility of serum IgG4 levels in determining relapse, multi-organ involvement and poor outcome (organ dysfunction and death)? (**Chapter 4**)
7. What is the prevalence of allergy and/or atopy, elevated IgE levels and eosinophilia in IgG4-RD? (**Chapter 5**)
7. What is the diagnostic utility of serum IgE levels in differentiating IgG4-RD from other malignant, inflammatory and immune diseases? (**Chapter 5**)
8. What is the prognostic utility of serum IgE levels in determining relapse, multi-organ involvement and poor outcome (organ dysfunction and death)? (**Chapter 5**)
9. What is the role of IgE and mast cells in pathogenesis of disease? (**Chapter 5**)
8. What is the prevalence of and link between occupational exposure and IgG4-RD? (**Chapter 6**)
9. Is there a single antigen involved in pathogenesis of disease? (**Chapter 7 and 8**)
10. What is the evidence that *Helicobacter pylori* antigens are involved in pathogenesis of disease? (**Chapter 8**)

11. What is the phenotype and function of IgG1 and IgG4 B cells in health and disease?

(**Chapter 9**)

12. What gene expression profiles and pathways may be involved in disease pathogenesis? (**Chapter 10**)

13. Can gene expression profiles provide clues for disease severity, in terms of relapse and organ involvement? (**Chapter 10**)

13. What HLA associations are present in a UK cohort of IgG4-RD? (**Chapter 11**)

1.16 THE HYPOTHESES

Each Chapter has a list of individual hypotheses outlined. **Figure 12** is a diagrammatic representation of how each Chapter fits under my unifying hypothesis for the natural history and pathogenesis of IgG4-RD.

Figure 12: My hypothesis for the natural history and pathogenesis of IgG4-RD.

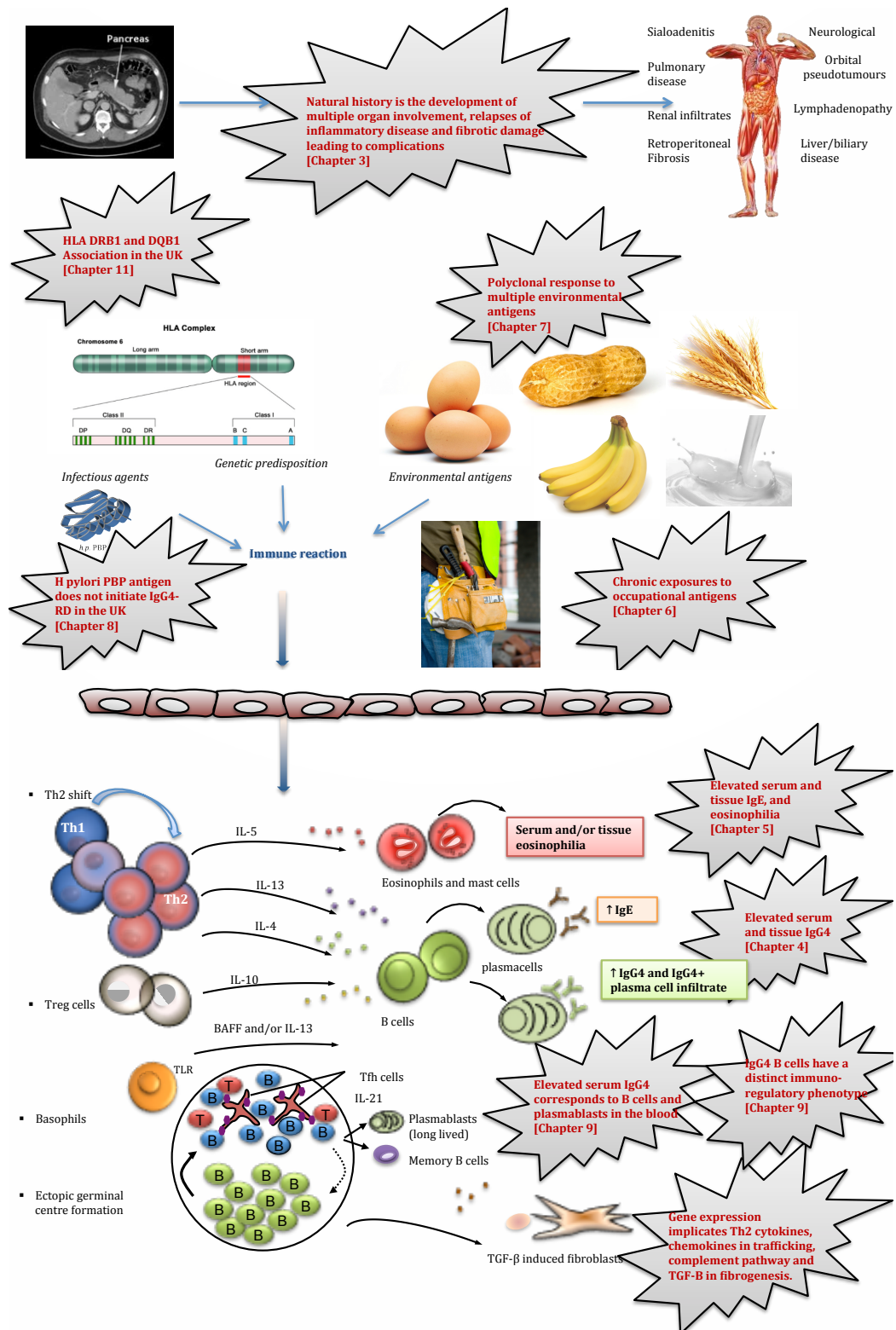


Figure 12: The diagram is a representation of my unifying hypothesis for the natural history and pathogenesis of IgG4-RD. The natural history of the disease is characterized by multiple organ involvement, relapse of inflammatory disease, and the development of complications related to inflammation and fibrosis over time. Chronic exposure to multiple environmental and occupational antigens, which are known to drive an IgG4 response in health, results in the rapid expansion of pre-existing IgG4-switched B cells in genetically susceptible individuals. This leads to an increase in the frequency of IgG4-positive memory B cells and plasmablasts in the blood, which traffic to lymphoid organs to form an inflammatory infiltrate rich in CD4⁺ T lymphocytes, B cells and IgG4 plasmablasts and plasma cells. In the presence of Th2-derived cytokines such as IL4 and IL13 and T regulatory cytokines IL10 and TGF- β , the drive to IgG4-switch, production of fibroblasts and development of fibrosis, respectively, occurs. In the presence of Th2-derived and mast cell-derived cytokines IL4 and IL5, elevated levels of serum IgE, peripheral and tissue eosinophilia are seen, in particular in those with a history of allergy and/or atopy. Ectopic germinal centres in tissues are formed, with T follicular helper cells providing cognate help for B cells and the production of IL21, which drives proliferation and expansion of IgG4-switched cells. Toll-like receptors (TLR) on monocytes and basophils enhance these IgG4 responses, mediated by B cell activating factor (BAFF) and IL13, indicating cross talk between the innate and acquired immune system. Distinct immuno-regulatory functions of the IgG4-switched cells themselves lead to a dampening of immune surveillance, unchecked inflammation and the progression to fibrosis.

Chapter 2 Methods

2.1 CLINICAL COHORT

The clinical cohort was composed of patients with IgG4-RD, disease controls with other inflammatory disorders (such as primary sclerosing cholangitis (PSC), autoimmune hepatitis (AIH) and inflammatory bowel disease (IBD)), and healthy controls.

2.1.1 Patient Identification

Patients with IgG4-RD were identified from 4 sources:

- (1) Hepatology and Gastroenterology outpatient clinics at Oxford NHS Hospital Trust (John Radcliffe and Horton Hospitals);
- (2) Other medical and surgical clinics (including Rheumatology, Respiratory, Renal, Neurology, General surgery, Ear nose and throat) at Oxford NHS Hospital Trust (John Radcliffe, Horton and Churchill Hospitals);
- (3) A histopathology database in the Histopathology Department at the John Radcliffe Hospital, where biopsy and resection specimen reports were recorded (access courtesy of Dr. Lai Mun Wang);
- (4) A clinical immunology database in the Clinical Immunology Department at the Churchill Hospital, where all serum IgG subclasses were recorded (access courtesy of Dr. Ross Sadler).

Patients with PSC, AIH and IBD were identified from 2 sources:

- (1) Hepatology and Gastroenterology outpatient clinics at the John Radcliffe Hospital;

(2) A clinical PSC database (compiled by Dr. Johnny Halliday and myself), AIH database (compiled by Dr. Zoe Raos and myself) and IBD database (compiled by Dr. Katarzyna Orlicka and myself) at the John Radcliffe Hospital, where biochemical, imaging and histology reports were recorded.

Patients with other malignant, inflammatory and autoimmune diseases;

(1) Referred by their consultant specialist to IgG4 outpatient clinics for assessment.

Healthy controls were identified after volunteering for the study, in response to invitation posters displayed on the John Radcliffe Hospital notice boards.

2.1.2 Patient Recruitment

Once a patient or control was identified, they were assessed according to inclusion and exclusion criteria for the study (**Appendix 1**). If they met criteria, they were invited to recruit to the study either (1) directly via outpatient clinic whereby they were approached by one of the named research investigators and given a recruitment pack, or (2) indirectly whereby a recruitment pack was sent via the postal system. The recruitment pack included an invitation letter, information pamphlet, consent form, and a self-addressed envelope with stamp to return to the John Radcliffe Hospital. There was a contact phone number and email address for invited participants to ask additional information regarding the project. Oxfordshire REC-A (10/H0604/51) approved these documents, a copy of which can be found in **Appendix 2**. Once a potential recruit had indicated an interest to participate in the study, informed consent was taken and the consent form was signed.

2.1.3 Diagnostic criteria to define IgG4-RD

The diagnosis of patients with systemic IgG4-RD evolved during the study period. Initially, diagnoses of autoimmune pancreatitis (AIP) and IgG4-related sclerosing cholangitis (IgG4-SC) were made using the Mayo HISORt criteria (Chari et al, Clin Gastroenterol Hepatol 2006) and the International Consensus Diagnostic Criteria (ICDC) (Shimosegawa et al, Pancreas 2011). Patients with type II AIP, which is not IgG4-related and has distinct histopathological characteristics, were excluded (Chari et al, Pancreas 2010). Subsequently, patients with extra-pancreatic disease were diagnosed using the Japanese Comprehensive Diagnostic Criteria for systemic IgG4-RD (JCDC) (Umehara et al, Mod Rheumatol 2012). The Boston Consensus Histopathological Criteria for IgG4-RD were applied to all patients with biopsy and resection specimens available, which were tissue type and organ-specific (Deshpande et al, Mod Pathol 2012).

2.1.4 Study Categories

Once recruited, all patients were reviewed in outpatient clinic and their medical case notes, serological investigations and available histopathology was interrogated. This allowed them to be placed into one of four study categories;

1. Definite IgG4-RD
2. Probable IgG4-RD
3. Not IgG4-RD – not meeting diagnostic criteria but some features consistent
4. Disease controls, such as PSC, AIH and IBD

Definite IgG4-RD

The diagnosis of ‘definite IgG4-RD’ was made if tissue biopsy or resection specimen demonstrated 2 of 3 major histopathology features of the disease (lymphoplasmacytic infiltrate, storiform fibrosis and obliterative phlebitis) and immunostaining confirmed >10 IgG4+ plasma cells per high power field (HPF) on biopsy or >50 IgG4+ plasma cells per HPF on resection, with a ratio of IgG4/IgG of $>40\%$.

Probable IgG4-RD

The diagnosis of ‘probable IgG4-RD’ was made with typical organ involvement, characteristic radiographic appearance of those affected organs (for example, sausage-shaped pancreas of AIP), and radiological response to corticosteroid therapy.

Not IgG4-RD

A patient was defined as ‘not IgG4-RD’ if they did not meet the diagnostic criteria, even if there were some consistent features (for example, response to corticosteroids or an elevated serum IgG4).

Disease Controls

Patients with malignant, inflammatory and autoimmune conditions, where serological evaluation and biopsy specimens excluded IgG4-RD, were defined as ‘disease controls’. The disease controls included patients with pancreatic disease (chronic pancreatitis and pancreatic carcinoma), biliary disease (PSC and cholangiocarcinoma), liver disease (AIH), bowel disease (IBD), renal disease (tubulointerstitial nephritis), and autoimmune conditions (systemic lupus and rheumatoid arthritis).

A diagnosis of PSC was made in accordance with the European Association for the Study of the Liver Guidelines in those with a cholestatic biochemical profile, characteristic cholangiography and/or consistent liver histology when secondary sclerosing cholangitis had been excluded (Beuers et al, J Hepatol 2009). A diagnosis of AIH was made using the International AIH Group's simplified scoring system (Hennes et al, Hepatol 2008), and of overlap syndrome when patients had clinical, laboratory, and histological features of both AIH and one of the cholestatic liver diseases primary biliary cirrhosis (PBC) or PSC. A diagnosis of IBD was based on the European Crohn's and Colitis Organisation guidelines for ulcerative colitis (Dignass et al, JCC 2012) and Crohn's disease (Van Assche et al, JCC 2010).

Acute pancreatitis was classified according to the revised Atlanta classification (Banks et al, Gut 2013) and chronic pancreatitis according to the TIGAR-O system of aetiologies (Etemad et al, Gastroenterol 2001). Pancreatic and/or biliary malignancy suggested by cross-sectional imaging was then confirmed histologically. Tubulointerstitial nephritis was defined in patients with compatible renal profile with supportive histology from renal biopsy. Autoimmune diseases included patients with systemic lupus, coeliac disease, and rheumatoid arthritis who had positive autoantibody profiles and supportive histology where necessary. Lastly, a diagnosis of allergy and atopy was made in accordance with the European Academy of Allergy and Clinical Immunology classification, with clinical symptoms and a positive IgE-specific allergen test.

2.1.5 Data collection and storage

All recruited patients were followed prospectively. Data was collected at presentation and updated at follow-up clinic appointments. Data was stored in a password-protected anonymised excel database. The data collected is shown in **Table 1**.

Table 1: Data collection for the IgG4 study

Date of recruitment
Baseline demographics (age, gender, occupation)
Date of diagnosis
Category of diagnosis: Definite IgG4-RD; probable IgG4-RD; not IgG4-RD; disease control (documented disease and organ involved).
Diagnostic criteria met if IgG4-RD (HISORT, ICDC, JCC, Boston)
Organ involvement
Presence of autoimmune and immune-mediated conditions
Clinical symptoms at presentation (organ-specific and general)
Blood tests before treatment initiated - full blood count; biochemistry (urea, Cr, eGFR, bilirubin, ALP, GGT, ALT, albumin); inflammatory markers (C-reactive protein, erythrocyte sedimentation rate); glucose, amylase, immunology (protein electrophoresis, autoantibodies, rheumatoid factor, serum IgG, IgG1, IgG4, and IgE).
Serum IgG4 level: elevated at presentation or any time during disease course.
Specific blood tests for <i>Helicobacter pylori</i> serology and HLA, at any time point.
Imaging before and after treatment: modality - abdominal US, CT scan of chest/abdomen/pelvis, MRCP, ERCP, EUS; organ involvement; activity of disease. For pancreatic and biliary disease, presence of a pancreatic mass, diffuse pancreatic enlargement, pancreatic duct abnormalities, distal common bile duct stricture, extra-pancreatic cholangitis was documented.
Histology: organ sampled; type of sample including fine-needle aspiration for cytology, biopsy, resection specimen; morphological features including lymphocytes, plasma cells, storiform fibrosis,

obliterative phlebitis, eosinophils; immunohistochemical staining including IgG4 count per 3 HPF, IgG count per 3 HPF (where available).
Treatment given: preparation - prednisolone, azathioprine, mycophenolate, methotrexate, mercaptopurine, rituximab, anti-TNF, budesonide; dose, duration, side effects.
Endoscopic therapy e.g. biliary or pancreatic stenting.
Response to treatment: defined by symptomatic response, biochemical response, serological response, imaging response, removal of stent.
Relapse: when off therapy or during taper, organ involved.
Treatment for relapse: type, duration of therapy, response.
Complications including malignancy (date and type), thrombosis, organ failure, surgery, transplantation, death (date and cause)
Date of last follow-up or death.

Table 1: Data collected in an excel spreadsheet for the IgG4 study.

Abbreviations: IgG4-RD: IgG4-related disease; HISORt: Histology, Imaging, Serum IgG4, Organ involvement, Response to steroid treatment, diagnostic criteria for autoimmune pancreatitis and sclerosing cholangitis; ICDC: International Consensus Diagnostic Criteria for Autoimmune Pancreatitis; JCC: Japanese Consensus Criteria for IgG4-RD; Boston: Boston Consensus Histopathological Criteria for IgG4-RD; ALP: alkaline phosphatase; GGT: gamma-glutamyl transpeptidase; ALT: alanine transaminase; Cr: creatinine; eGFR: estimated glomerular filtration rate; HLA: Human Leukocyte Antigen; AUS: abdominal ultrasound; CT: computerised tomography; MRCP: magnetic resonance scan of pancreas and bile ducts; ERCP: endoscopic retrograde cholangiopancreatogram; EUS: endoscopic ultrasound; HPF: high power field.

2.1.6 Definitions

The definitions of organ involvement, treatment, monitoring, response, relapse, complications and end points for the study are given in **Table 2**.

Table 2: Definitions used for the IgG4 study

Organ involvement: This was defined as single if only one organ system was involved and multiple if more than one organ system was involved (confirmed radiologically and/or histologically) during follow-up. In patients with AIP – a diffuse enlargement or focal mass at the head of the pancreas and a distal common bile duct stricture was defined as one organ.
First-line treatment: In IgG4-RD patients receiving corticosteroids dosing was standardized; prednisolone 0.5 mg/kg (30-40mg) for 2-4 weeks and then reduce the dose by 5 mg per 1-2 weeks, with expected cessation of treatment by 4-6 months.
Monitoring: Patients were assessed at week 0, week 2, week 4, months 3 and 6 after treatment commenced, by myself. Subsequent clinical visits were determined by clinical need. Blood tests including renal function, liver function and serum IgG, IgG4 and IgE levels were performed at each clinic visit. Repeat imaging was performed after 4-8 weeks of corticosteroids, upon completion of medical treatment and as dictated by clinical developments. Discontinuation of medication, due to patient preference, intolerance or other side effects, was recorded.
Treatment response: A reduction in absolute values in liver function tests, reduction in serum IgG4 level, reduction in size or resolution of mass/stricture/inflammatory change on imaging, reduction or resolution of stricture and stent removal at ERCP. There was no absolute number or percentage used to define 'a reduction'.
Disease relapse: This was defined as progression of disease on imaging or deterioration of biochemical parameters (e.g. liver function), after initial treatment had been tapered or discontinued (but not due to stent dysfunction). Sufficient follow-up to determine relapse was defined as a period of greater than 6 months since initial treatment was discontinued or surgery was performed, and the patient was assessed in outpatient clinic.

Treatment for relapse: Relapse was treated with repeat further courses of steroid therapy, and/or additional second line immunosuppressive therapy, at the discretion of the clinicians. As there are currently no international guidelines for the treatment of relapse, our protocol broadly reflected that used in other major US and European centres (Hart et al, Gut 2013).
Disease complications: Non-malignant complications were defined as evidence of end-organ damage or dysfunction developing during the course of the disease. These included pancreatic endocrine and exocrine insufficiency, pancreatic calcification and ductal stones, liver failure and cirrhosis, portal and splenic vein thrombosis. Patients who required repeated or long-term courses of steroids were evaluated for steroid-related complications; clinical assessment, bone densitometry and regular blood glucose measurements were performed.
Malignancy: Defined as any evidence of histologically confirmed malignancy, since diagnosis of IgG4-RD and in the preceding 2 years before diagnosis when symptoms may not have been apparent. For comparison, national age and sex incidence rates for cancer and cancer mortality were obtained from Public Health England Cancer Research UK (www.gov.uk/phe).
Mortality: Date and cause of mortality were confirmed with case records, death certificates and autopsy details when available. Data Age and sex incidence for mortality were obtained from National UK Statistics (2011): (http://www.cancerresearchuk.org/cancer-info/cancerstats/mortality/).

Table 2: Definitions used for the IgG4-study.

Abbreviations: mg/kg: milligrams per kilogram; AIP: autoimmune pancreatitis.

The goals of treatment with corticosteroid therapy are not completely defined in IgG4-RD. In our clinical practice the aims were to (1) improve symptoms, (2) reverse active disease, (3) halt or delay progression of disease including organ failure and additional organ involvement, (4) reduce the need for endoscopic intervention, namely biliary stenting, and (5) achieve long-term maintenance of treatment benefits

2.2 LABAROTORY MEASUREMENTS

2.2.1 Serological testing for IgG and subclasses

Total serum IgG and subclasses (IgG1, IgG2, IgG3 and IgG4) were measured by nephelometry using a BNII analyser (Siemens, Surry, UK) at the Clinical Immunology Department, Churchill Hospital. The normal range for serum immunoglobulin concentrations in the region was determined and validated by Oxford Immunology department, and is closely aligned with national immunoglobulin reference values; IgG 6-16g/l, IgG1 3.2-10.2g/l, IgG2 1.2-6.6g/l, IgG3 0.2-1.9g/l, IgG4 0.1-1.3g/l.

A serum IgG4 level $\geq 1.4\text{g/l}$ was defined as elevated throughout this thesis for consistency, as used in the Mayo HISORt criteria for AIP. For patients with multiple serum immunoglobulin measurements, the first serum IgG4 level recorded was used. The prozone effect (falsely low serum IgG4 values) was accounted for using serial dilutions where necessary (Khosroshahi et al, Arthritis Rheum 2014). Serum values for IgG subclasses were comparable at Oxford, University College London and Sanquin Amsterdam institutes.

2.2.2 Serological testing for IgE

Total IgE was measured by the automated Immunocap method using a Phadia 250 (Phadia, Milton Keynes, UK). For IgE units are expressed as KU/L, equivalent to 1 IU/mL, and equivalent to 2.4ng/mL. An elevated serum IgE was defined as $\geq 120\text{KU/L}$.

2.3 IMAGING

Patients presenting with probable AIP and IgG4-SC underwent a CT pancreas and MRCP, and then an ERCP and EUS as required. At the Oxford site, all patients underwent a CT scan of the chest, abdomen and pelvis to determine evidence of extra-pancreatobiliary involvement. Further investigations were then performed depending on the clinical presentation and biochemistry.

Patients presenting with extra-pancreatic disease all underwent a CT scan of the chest, abdomen and pelvis, and further imaging depending on the organ involved, e.g. MRI head and neck if orbital or salivary gland involvement. PET scanning was performed to look for disease extent and/or response to therapy when the differential of lymphoma or vasculitic disease was questioned, on an individual basis.

2.4 HISTOLOGY SPECIMENS

2.4.1 Evaluation of Morphology

Histopathological evaluation of tissue biopsies and resection specimens was performed by consultant histopathologists - Dr. Adrian Bateman (Southampton General Hospital) and Dr. Lai Mun Wang (John Radcliffe Hospital, Oxford). All specimens were assessed for classical morphological features of IgG4-RD, specifically (1) lymphoplasmacytic infiltrate, (2) storiform fibrosis, (3) obliterative phlebitis, and (4) presence of eosinophils.

2.4.2 Immunostaining for IgG and IgG4

Tissues were immunostained with IgG and IgG4 monoclonal antibody in the histopathology department at the John Radcliffe Hospital. The IgG4 count was reported as the average number of IgG4-positive plasma cells in 3 high-powered fields (HPF). An elevated IgG4 count in a biopsy specimen was defined as >10/HPF and in a resection specimen as >50/HPF, in the pancreas or bile duct. In those with an IgG4 count >10/HPF (biopsy) or >50/HPF (resection), an IgG4 to total IgG ratio was calculated. An elevated IgG4:IgG ratio was defined as >40% (Deshpande et al, Mod Pathol 2012).

2.4.3 Tissue staining for B cells, T cells, macrophages and plasma cells

Tissues were stained for CD4 and CD8 (T cells), CD20 (B cells), CD68 (macrophages) and CD138 (plasma cells), and immunostained for IgE and mast cell tryptase (mast cells), to determine the background inflammatory infiltrate (**Table 3**). In brief, 4 micrometer (μm) paraffin sections were cut from selected blocks and mounted onto coated slides (ThermoFisher Scientific), which were dried for 20 minutes at 60°C. Slides for immunohistochemistry were prepared using the automated Prepstain Detection-system (Dako-Cytomation, Hamburg, Germany). Staining utilised the Dako Medical Systems System with peroxidase and diaminobenzidine (DAB) solution containing 0.05% hydrogen peroxide for visualisation. Interpretation of cellular staining was performed using a multi-headed microscope, to reduce inter-observer variation by Dr Adrian Bateman (consultant histopathologist), Dr Abigail Breach (registrar histopathologist), Toby Mellows (research technician) and myself.

Table 3: Antibodies for cellular staining and immunostaining of tissue specimens in IgG4-RD patients and disease controls.

Antibody	Clone	Supplier	Cat no.	Dilution	Expression
CD3	F7.2.38	Dako	M7254	1:200	T cells
CD4	4B12	Dako	IR649	RTU	CD4+ T cells
CD8	C8/144B	Dako	IR623	RTU	CD8+ T cells
CD20	L26	Dako	M0755	1:250	Mature B cells
CD68	PG.M1	Dako	M0876	1:50	Macrophages
CD138	MI15	Dako	M7228	1:20	Plasma cells
IgE	Polyclonal	Dako	A0094	1:1500	IgE+ve cells
MCT	10D11	Leica Microsystems	NCL-MCTYP-428	1:200	Mast cells

Table 3: This table shows the antibodies used to stain patient and disease control tissue specimens. The clone, supplier, catalogue number, dilution and expected binding of cells subsets are given for each antibody. The antibody retrieval was high for each marker. The visualization system was Envision FLEX. **Abbreviations:** MCT: mast cell tryptase; RTU: undiluted.

2.4.4 Double Immunostaining for IgE, plasma cells and mast cells

Double immunostaining for IgE and CD138 or IgE and mast cell tryptase was performed in order to define IgE-positive cells. Double immunostaining was performed by Toby Mellows (senior research technician) and supervised by Dr. Adrian Bateman at the Histopathology Department at Southampton General Hospital.

2.5 SAMPLE COLLECTION AND PROCESSING

2.5.1 Blood sampling

Blood samples were taken at the time of recruitment. In all patients, baseline bloods were taken if they hadn't been done in the preceding month, or if their clinical condition had changed since they were last taken. These included full blood count, renal and liver function, inflammatory markers, glucose, amylase, protein electrophoresis,

autoantibodies, rheumatoid factor, and serum IgG, IgG1, IgG4 and IgE levels. Blood test measurements were performed at the John Radcliffe biochemistry laboratory and Churchill immunology laboratory. Specific blood tests included *H.pylori* serology and HLA tissue typing, performed at the John Radcliffe microbiology laboratory and Churchill HLA Tissue Typing department, respectively.

In patients who were treatment-naïve (never been exposed to corticosteroid or immunosuppressive therapy) 1 serum tube, 1 EDTA-coated tube, 1 Tempus RNA tube, and 4 full heparinized whole blood tubes were collected. In patients who were treatment-experienced (previous exposure to or currently on corticosteroid or immunosuppressive therapy) all samples except a Tempus RNA tube were collected. The samples were processed and analysed at the Peter Medawar Building laboratory.

2.5.2 Plasma and Serum preparation

EDTA-coated tubes (plasma) and serum-separating tubes (serum) were used to collect 5ml of peripheral whole blood. Samples were centrifuged for 10mins at 200xg within 4 hours of collection. Clear supernatant (plasma or serum) was collected and stored in 2ml vials at -80°C.

2.5.3 Peripheral blood mononuclear cell (PBMC) isolation

Heparinised tubes were used to obtain 50ml peripheral whole blood. Peripheral blood mononuclear cells (PBMCs) were isolated within 4 hours of collection. Whole heparinized blood was separated by density gradient centrifugation using lymphoprep

(Axis-Shield PoC AS, Norway) (centrifuge 2200rpm, 20 minutes, no brakes), after being diluted 1:2 in R-10 (RPMI medium (Gibco®), 10% heat inactivated foetal bovine serum (FCS), 100 U/ml penicillin-streptavidin solution, 2 mM L-glutamine). The mononuclear cell layer was collected and washed twice in R-10 solution (centrifuged 1800rpm, 10minutes, break 9, then 1600rpm, 7minutes break 9). Cells were re-suspended in R-10 and counted using a Sceptre cell counter (Millipore®). On average, 50-100 million lymphocytes were isolated from a given patient.

2.5.4 PBMC freezing and thawing

Cells were frozen (50% FCS, 10% DMSO, 40% RPMI) at approximately 10 million cells per ml, and stored at -80°C for 3 days, then cryopreserved in liquid nitrogen at -180°C. Cells were thawed in a 37°C waterbath. Thawing medium 9ml RPMI solution, 1ml CTL wash, 100 µl DNAase was slowly added (to reduce osmotic load and preserve cell integrity). The sample was centrifuged (1600rpm 7minutes break 9), and supernatant re-suspended in R10 media.

2.5.5 B cell isolation

PBMCs were isolated by density centrifugation on a lymphoprep gradient (Axis-Shield PoC AS, Norway). B cells were purified using CD19 pan B Dynabeads and Detachabead (Invitrogen), according to the manufacturer's instructions. Briefly, Dynabeads were washed twice with PBS supplemented with 0.3% bovine serum albumin (BSA) (Sigma-Aldrich), then 50ul Dynabeads were added per 50 million PBMC's in 0.5ml total volume. The beads and cells were incubated at 4°C 20mins on a

rotator, then bead-bound B cells were separated using a magnet and washed x3 with PBS-BSA 1% to increase purity. The CD19+ B cells were detached from the beads after 60mins incubation with Detachabead reagents on a rotator then washed x3 with PBS-BSA 1%. Purified B cells were re-suspended in B cell medium and counted (Casy Counter, Roche).

2.6 IGE-SPECIFIC IMMUNOCAP

An IgE ImmunoCAP, a solid-phase immunoassay, was used to determine and quantify allergen-specific IgE antibodies in the serum, performed at the clinical immunology department, Churchill Hospital, Oxford, UK. In brief, allergen components were immobilized on a solid substrate in a microarray format, and then incubated with serum samples to detect specific IgE antibodies. Binding of the specific IgE antibodies to the immobilized allergen components was detected by the addition of a secondary fluorescence-labelled anti-human IgE antibody. Images were acquired via a microarray scanner. The ISAC Standardized Units for specific IgE (ISU) were determined and the test results analysed using proprietary software (MIA - Microarray Image Analysis Software). The results are expressed semi-quantitatively in 4 classes (0 = Undetectable or very low, equivalent to $ISU < 0.35$; 1 = Low, $ISU \geq 0.35$ to < 1 ; 2 = Moderate to High, $ISU \geq 1$ to < 15 ; 3 = Very high, $ISU \geq 15$).

IgE-specific allergen testing was performed using allergen mixes including grass pollen mix, mould mix, tree pollen mix and nut mix (Phadia, Thermo Scientific). Each mix contains 8 similar allergens. If a particular allergen was highlighted from the clinical history this was additionally tested, including house dust mite, cat dander, wheat, milk and latex.

2.7 IGG4 RADIO IMMUNOASSAY (RIA)

The radioimmunoassay (RIA) is a sensitive *in vitro* test to determine and quantify antigen-specific antibodies in serum. For the RIA, 250µl of a 2 mg/ml Sepharose suspension (antigen coupled to CNBr-activated Sepharose-4B (Amersham Pharmacia Biotech AB)) was washed (x3) then incubated with 50µl of 1:50 diluted patient or control serum plus 450µl of PBS with human serum albumin, Tween-20 (with mannoside in the case of banana to avoid measurement of non-specific IgG4 due to the presence of lectins in banana), overnight at RT (Calkhoven et al, Clin Exp Allergy 1991, Koshte et al, Int Arch Allergy Immunol 2002). The suspension was washed (x5) with PBS containing 0.005% Tween-20 (PBS-T) to remove unbound IgG4 antibodies and other irrelevant serum components, then 500µl of PBS with 1mg/ml sheep IgG and 3mg/ml human serum albumin was added to 50µl of monoclonal mouse-anti-human IgG4 (¹²⁵I radiolabelled (Sanquin)) and left overnight at RT. After washing 5 times with PBS-T, bound radioactivity was measured. Bound radioactivity was converted to arbitrary units (AU) using a calibration serum with IgG4 to grass pollen set at 1400 AU/ml; one AU is estimated to equal approximately 50 ng IgG4.

The antigens used were egg white and yolk, milk, cat dander and serum, banana, milk, rice and wheat (Sanquin Institute, Amsterdam). These antigens were chosen as they are known to elicit an IgG4 response in the general population. All foods were obtained locally, cat dander from HAL (Haarlem, The Netherlands), and cat serum from a local veterinarian. Antigen extracts (for example, egg white and yolk) were separately coupled to beads, but the beads were combined before testing with serum. The amounts of antigen extracts coupled to dry weight Sepharose are: egg white extract 200 mg coupled to 40 g Sepharose-4B; egg yolk extract 160 mg coupled to 20g Sepharose-4B;

cat dander extract 100 mg coupled to 20g Sepharose-4B; cat serum 5.5 mg coupled to 0.8g Sepharose-4B; banana extract 81 mg coupled to 2g Sepharose-4B; milk extract 609 mg coupled to 10g Sepharose-4B; rice and wheat extracts 240 mg coupled to 6 g of Sepharose-4B.

To compare results, signals were compared to a standard curve (a serially diluted reference serum incubated with 4mg/ml Dactylis Sepharose) and expressed as arbitrary units. Mixtures of chimeric antibodies (anti-Bet v1 and anti-Feld 1) were used to exclude the risk of second-generation IgG4 binding.

2.8 CLONALITY

2.8.1 Serum Electrophoresis

Serum electrophoresis was performed to check for evidence of monoclonality. This was carried out using a Sebia hydrasys focusing system (Sebia Cedex, France). Initial gel electrophoresis of samples was performed using the Sebia Hydragel 54 protein gel kit (Sebia, Cedex, France). Densitometry of electrophoresis gels was analysed using Phoresis (Sebia, Cedex, France). In patients where no monoclonality was detected, individuals were designated as hypogammaglobulinaemic, normal or hypergammaglobulinaemic, determined by the levels of serum IgG and the appearance of the beta/gamma region of densitometry plot.

2.8.2 Serum Immunofixation

In samples with a diffuse increase in immunoglobulins, immunofixation analysis using

IgG, IgA, IgM, kappa light chain and lambda light chain antisera was carried out (Sebia hydrgel IF 2/4 kit (Sebia, Cedex, France)) as a higher sensitivity method to test for the presence of monoclonal bands in the polyclonal gamma region. This method has a lower limit of detection of approximately 0.2g/l of protein.

2.8.3 Tissue Polymerase Chain Reaction

DNA for PCR amplification was extracted from formalin fixed paraffin embedded tissue blocks. Tissue sections (n=6) were cut using a Reichert-Jung microtome block holder and sterile knife (thickness 10 µm) and transferred to a sterile micro centrifuge tube. The DNA from these sections was then extracted using the QIAamp DNA FFPE Tissue kit (Qiagen). In brief, the paraffin wax was dissolved in xylene (Merck) and the tissue digested using proteinase K digestion at 56°C. The lysate was then incubated at 90°C to reverse formalin crosslinking before being added to a QIAamp Mini FFPE column, washed with AW buffer, and then eluted in 100ul of ATE buffer. The DNA was quantified on the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). All DNA was stored at –20°C before PCR amplification.

For the IgH locus; 3 sets of multiplex PCR reactions were set up for the three framework regions (FR1, FR2 & FR3) in conjunction with a consensus JH primer (van Dongen et al, Leukemia 2003). For the Ig kappa light gene rearrangements, 2 multiplex PCR reactions were set up. The KA PCR reaction detected functional IgKappa gene rearrangements and contained Vk primers covering the 7 Vk families and 2 Jk primers covering all the Jk gene segments. The KB multiplex PCR reaction detected non-functional IgKappa gene rearrangements and included all the Vk family primers against

a Kdel primer and an INTRON sequence primer. The Ig lambda gene rearrangements were detected using 2 VL primers and a single consensus JL primer (van Dongen et al, Leukemia 2003).

Each PCR set up included a clonal (monoclonal cell line), a polyclonal and a negative control for each loci tested. The PCR products were run on the ABI 3500xl Genetic analyser, and results analysed with GeneMapper fragment analysis software. Each PCR reaction was interpreted as either polyclonal – showing a typical Gaussian curve of amplified products or clonal showing a single predominant peak, all negative controls showed no amplification.

2.9 PEPTIDE EPITOPES

2.9.1 Linear and overlapping peptide epitopes

Firstly, 3 short linear epitopes, with a 7 amino acid sequence corresponding to those identified by Frulloni et al (N Engl J Med, 2009) for *H. pylori* plasminogen binding peptide (PBP amino acid 298-304: A K E E R R Y), ubiquitin-protein ligase E3 component n-recogin 2 (URB2 amino acid 1186-1192: A K E Q R R Q), and the ‘AIP’ peptide (AIP amino acid 1-7: S K D E R R F) were used. Secondly, the sequence of the *H. pylori* PBP type A peptide 26695 (**Table 4**) was used to create a second set of 111 overlapping fragmented peptides (Mimotope®), consisting of 15-18 amino acids with a 20 amino acid (20 mer) overlap (Jonsson et al, PNAS 2004). The peptides were dissolved in DMSO in a concentration of 40 mg/ml, divided into 8 pools containing 2 µl per peptide (7 pools of 14 peptides and one pool of 13 peptides) to reach a final stock

solution of 300 µg/ml. All 8 pools were combined to make a single ‘total peptide’, and stored at -20°C (40 mg/ml stock).

Table 4: Sequence of *H. pylori* strain plasminogen binding peptide 26695

<i>H. pylori</i> PBP amino acid sequence
MLRLIGLLLSFISLQSASWQEPLRVSIEFVDLPKKIIRFPAHDLQVGEFGFVVTKLSDYEIVNSE
VVIIAVENG VATAKFRAFESMKQRHLPTPRMVARKGDLVYFRQFNNQAFLIAPNDELYEQIRAT
NTDINFISDLLVTFLNGFDPKIANLRKACNVYSGVIYIVTTNTLNILSCESFEILEKRELDTSGVT
KTSTPFFSRVEGIDAGTLGKLFSGSQSKNYFAYYDALVKKEKRKEVRIKKREEKIDSREIKREIKQ
EAIKEPKKANQGTQNAPTL EEK NYQKAERKLDAKEERRYLRDERKKAKATKKAMEFEEREKEH
DERDEQETEGRRKALEMDKGD KKEERV KPKENEREIKQEAIKEPSDGNNATQQGEKQNAPKEN
NAQKEENKPN SKEEKRR LKEEKKKAKAEQ RAREFEQRAREH QERDEKELEERRKALEAGKK

2.10 T CELL ELISPOTS

A T cell ELISpot assay was used to detect IFN-γ and IL4 release according to the manufacturers instructions (Mabtech) from thawed PBMCs rested overnight (**Figure 1**). Positive controls were Concanavalin A (ConA), a lectin extracted from the *Canavalia ensiformis* that induces T cell proliferation and INF-γ release (dissolved in DMSO at 1.2 mg/ml), and *H. pylori* peptide lysate (Invitrogen), containing the most common *H.pylori* strains (3.35 mg/ml stock). Negative controls were DMSO and supplemented RPMI medium without cells. To ensure quality control of the assay a FEC peptide pool (mixed HLA class I restricted peptides from cytomegalovirus, Epstein Barr virus and influenza) was used.

2.10.1 IFN- γ ELISpot

To capture the IFN- γ , 96-well ELISpot plates (Millipore®) were coated with 5 μ g/ml IFN- γ monoclonal antibody solution (Mabtech®) for 15-24 hours at 4°C. The plates were washed with PBS and blocked for 2 hours with R-10 at 37°C. The PBMCs were plated at 2×10^5 cells/well in triplicate in the presence of pooled PBP peptide fragments (8 pools containing 13-14 peptides each), final concentration 3 μ g/ml in each well, and incubated for 15–24 hours at 37°C in a 5% CO₂/95% humidified incubator. After washing 7 times with PBS/Tween 20, a 0.5 μ g/ml solution of biotinylated mouse anti-human IFN- γ antibody (Mabtech®) diluted in sterile 0.5% BSA/PBS was added, and plates incubated at RT for 2-4 hours in the dark. After washing 4 times, a 1:750 alkaline phosphatase-conjugated anti-biotin monoclonal antibody solution (Vector Laboratories®) diluted in sterile 0.5% BSA/PBS was added and incubated at RT for 2 hours in the dark. Plates were washed a further 4 times with PBS/Tween 20 wash buffer and developed using 50 μ l 5-Bromo-4-chloro-3-indolyl phosphate with nitro blue tetrazolium chloride (BCIP/NBT) at RT for 15 minutes, then rinsed again. Plates were left to dry overnight.

Plates were read using a computer-assisted AID ELISpot reader designed to detect spots using predetermined criteria based on size, shape, and colorimetric density, and validated manually. Antigen-specific frequencies of IFN- γ -producing cells were calculated by subtracting the number of detected spots in the absence of antigen from the frequency of spots obtained in the presence of relevant antigen. The total response was calculated by adding the mean response to all peptide pools, and then subtracting the mean background (DMSO) response. Negative control values were always <30 SFU. A positive response was defined as at least 50 SFU/million PBMCs and at least 3

SD above background, based on viral T cell IFN- γ ELISpot protocols (minimum sensitivity: 250 ASC's per 1×10^6 PBMCs).

2.10.2 IL-4 ELISpot

The same protocol was used as for the IFN γ -ELISpot, except that plates were coated with IL4 monoclonal antibody and the development step was longer at 30 minutes.

Figure 1: The IFN- γ and IL4 T cell ELISpot

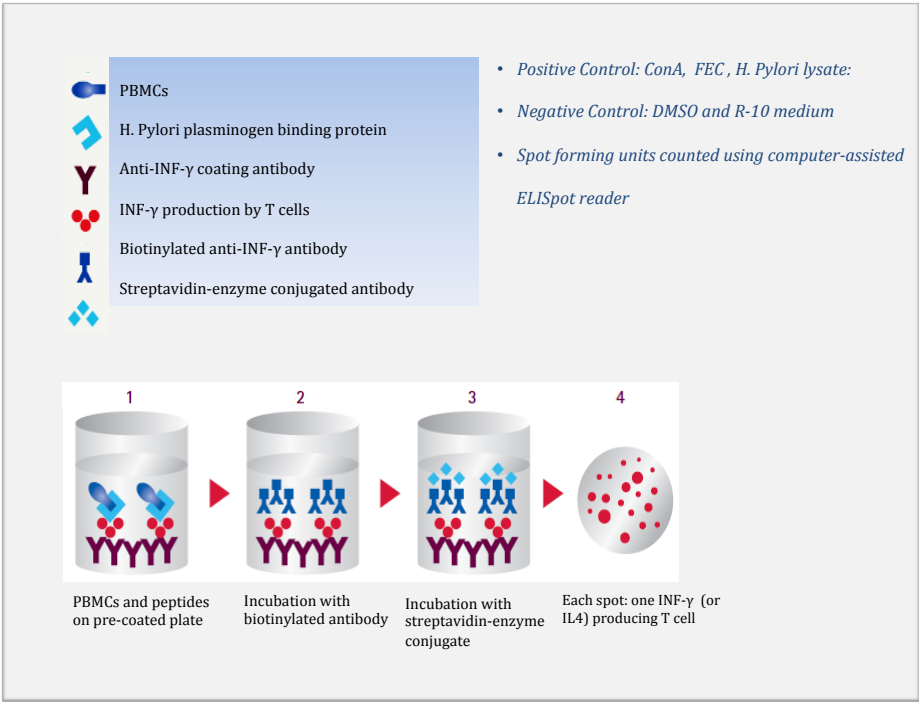


Figure 1: The diagram shows a flow chart of the methodology used for the IFN- γ and IL4 T cell ELISpot. **Abbreviations:** Con A: Concanavalin A; FEC: mixed HLA class I restricted peptides from cytomegalovirus, Epstein Barr virus and influenza.

2.11 B CELL ELISPOT

B cell ELISpots were performed to quantify the number of IgG-producing and IgG4-producing plasma cells derived from the B memory cell compartment. In the ex vivo ELISpot, PBMCs were directly plated without stimulation and antibody-producing plasmablasts were captured (**Figure 2**). In the cultured B cell memory ELISpot, PBMCs were cultured with non-specific stimulants for 7 days to encourage antibody production from memory B cells (**Figure 3**).

Figure 2: *Ev vivo plasmablast ELISpot.*

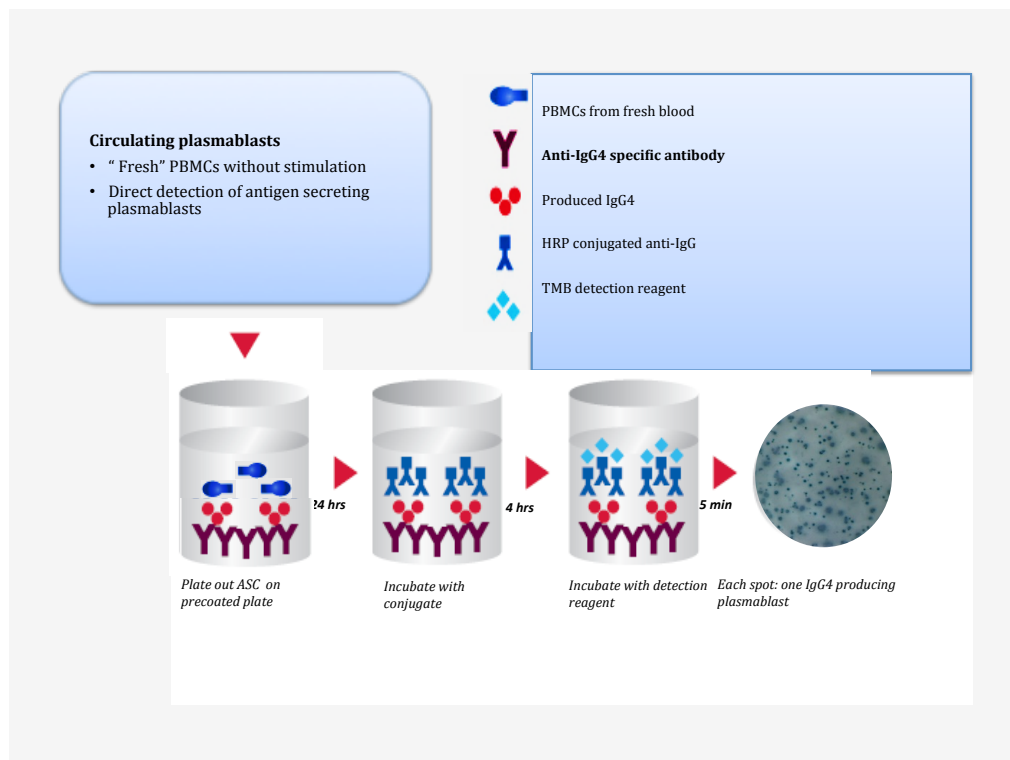


Figure 2: The diagram shows a flow chart of the methodology used for the ex vivo plasma cell/ plasmablast ELISpot. **Abbreviations:** PBMC: peripheral blood mononuclear cells.

The positive control was *H. pylori* peptide lysate (Invitrogen) coated at 10 µg/ml. To validate whether IgG and IgG4 was produced, anti-human IgG4 (Sanquin®) and anti-human total IgG (Sanquin®) were coated at 10 µg/ml. Both IgG4 coating antibody with anti-IgG detection, and IgG coating antibody with anti-IgG4 detection were used and results compared. Negative controls were DMSO and supplemented RPMI medium without cells. For the *H.pylori* lysate, any well with 10 or fewer spots was considered a negative response.

Figure 3: Cultured memory B cell ELISpot

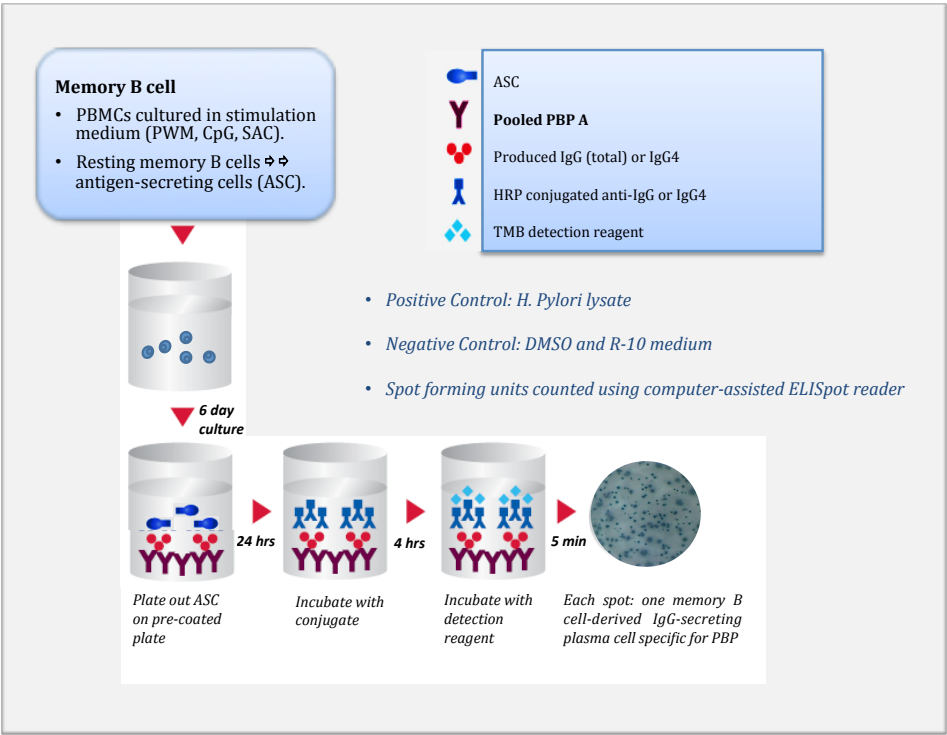


Figure 3: The diagram shows a flow chart of the methodology used for the cultured B cell ELISpot. **Abbreviations:** ASC: Antigen secreting cell; PWM: poke weed mitogen; CpG: CpG2006 oligodeoxynucleotides; SAC: *S. aureus*, Cowan.

2.11.1 Cultured B cell ELISpot

For the cultured B cell memory ELISpot, the protocol was based on a previously described assay (Crotty et al, J Immunol Methods 2004). Thawed PBMCs were plated in 24-well dishes at 2×10^5 cells/well in R-10 supplemented with an optimized mix of polyclonal mitogens: 83 ng/ml of pokeweed mitogen extract (PWM, Sigma-Aldrich®), 2.5 µg/ml of fully methylated CpG (invitrogen®) and a 1:5000 dilution of fixed *S. aureus*, Cowan (SAC, Calbiochem®). Cells were cultured for 6 days to allow the existing quiescent B memory cell population to proliferate and differentiate into antibody-secreting plasma cells (ASCs). After culture, supernatants were plated and preserved at -80°C for cytokine analysis. PBMCs were recovered and washed 3 times in EDTA washing buffer (PBS containing 0.744 g/l EDTA and 0.5% FCS and calibrated at a pH of 7.2-7.4) and re-suspended in R-10 at 2×10^6 cells/ml.

2.11.2 IgG and IgG4 B cell ELISpot

For the B cell ELISpot, 96-well filter plates (MAIPS4510, Millipore®) were coated for 24 hours at RT with *H. pylori* PBP peptide pools, positive and negative controls, and IgG and IgG4 antibodies (Sanquin), at a concentration of 10 µg/ml. The coated plates were washed 3 times in PBS and blocked for 30 minutes with R-10 at 37°C. Serial dilutions (1:10, 1:100 and 1:1000) of the 2×10^6 cells/ml suspension were plated out in triplicate at 100 µl per well on the coated plates and incubated for 20 hours at 37°C in a 5% CO₂/95% humidified incubator. PBMCs were washed 5 times with PBS Tween-20. A 1:2000 solution (0.5 µg/ml) of horseradish peroxidase (HRP) conjugated anti-human IgG4 (Sanquin®) and total IgG (Sanquin®) was added at 50 µl per well to the PBP

peptide pools for 4 hours at RT, and then washed 4 times with PBS Tween-20 and 3 times using deionized H₂O.

ASCs were detected using 50 µl/well of TMB Substrate Reagent (Mabtech®) for 5 mins at RT and the reaction was stopped with tap water. Plates were read using the computer-assisted AID ELISpot reader and validated manually.

2.12 IGG4 AND IGG1 B CELL PURIFICATION AND CULTURE

B cells were purified using anti-CD19 Dynabeads and DETACHaBEAD (Invitrogen, Paisley, UK), according to the manufacturer's instructions. IgG4⁺ and IgG1⁺ memory B-cells were isolated by FACS sorting (BD FACS Aria II) using anti-CD20-PerCP-Cy5, anti-CD27-APC, anti-IgD-PE (BD Bioscience, Franklin Lakes, NJ, US) and anti-IgG4-AF488 or anti-IgG1-Dye488 (Sanquin Blood Supply). Isolated B-cells were sorted 1 cell per well in 96 well flat bottom plates. Cells were cultured for 12 days in RPMI (Gibco) containing 5% FCS (Bodinco.), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 50 µM β-mercaptoethanol (2-ME) (Sigma-Aldrich), 20 µg/ml human apo-transferrin ((Sigma-Aldrich), depleted for human IgG with protein A sepharose (GE healthcare)) 1 ng/ml IL-1β, 50 U/ml IL-2, 0.3ng/ml TNFα, 0.5 µg/ml R848 (Alexis) in the presence of 10.0000 irradiated (50Gy), CD40L-expressing 3T3 cells. After 12 days, supernatants were tested in an IgG subclass ELISA, to determine total IgG production as well as IgG1, IgG4 and IgM production.

2.13 IGG SUBCLASS ELISA

Maxisorp ELISA plates (NUNC, Roskilde, Denmark) were coated overnight with 1 µg/ml anti-IgG or anti-IgG1, anti IgG2, anti-IgG3, anti-IgG4 or anti-IgM (Sanquin Reagents, Amsterdam, The Netherlands) in PBS. After washing 5 times with PBS-Tween, plates were incubated with culture supernatant or serum (diluted appropriately in HPE, Sanquin). Plates were then washed and incubated for 1 hour with HRP-conjugated mouse-anti-human-IgG (0.5 µg/ml in HPE) or HRP-conjugated mouse-anti-human-IgM (0.5 µg/ml in HPE). Plates were washed and developed with 100 µg/ml tetramethylbenzidine in 0.11 M sodium acetate (pH 5.5) containing 0.003% (v/v) H₂O₂. The reaction was stopped with 2M H₂SO₄. Absorption at 450 nm was measured with a multiscan (Multiskan; Titertek, Elfab Oy, Finland). Results were related to a titration curve of purified recombinant IgG1 or IgG4 or a serum pool for IgM in each plate.

2.14 FLOW CYTOMETRY

2.14.1 Labeling of monoclonal antibodies with fluorescent dye

Antibodies (anti-IgG1, anti-IgG2, anti-IgG3 and anti-IgG4 (Sanquin Blood Supply) and purified IgG1-anti-bet v1 and IgG4-anti-bet v1 were labelled with DyLight488 or DyLight649 fluorophore (Pierce/ Thermo Scientific, Rockford, IL, US). Unreacted dye was removed by repeated dilution/concentration using Amicon Centriprep centrifugal filter devices (Millipore, Billerica, MA, US) until no dye could be detected in the filtrate.

2.14.2 Cell lines

CESS human lymphoblast cells expressing IgG1, CRL2632 human lymphoma cells expressing IgG4 and murine EL4B5 cells expressing high CD40L were cultured in Iscove's modified Dulbecco's medium (Lonza, Basel, Switzerland) containing 10% FCS (Bodinco, Alkmaar, The Netherlands), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 50 µM β-mercaptoethanol (2-ME) (Sigma-Aldrich, Steinheim, Germany), 20 µg/ml human apo-transferrin ((Sigma-Aldrich), depleted for human IgG with protein A sepharose (GE healthcare, Buckinghamshire, UK)).

2.14.3 Antibody concentrations

Antibody clones were titrated to assess the optimum antibody concentration for staining within individual panels.

2.14.4 Antibody staining

B cells were washed with PBS supplemented with 0.1% BSA (Sigma-Aldrich) and incubated with monoclonal antibody (mAb) or the appropriate isotype control in the dark at room temperature, for 20 minutes (**Table 5**). Isotype controls were: AF488-conjugated IgG1 isotype control (purified IgG1-anti-bet v1), PE-conjugated IgG2a isotype control (Invitrogen), PE-conjugated IgG1 isotype control (Caltag/Life Technologies), APC-conjugated IgG1 isotype control (MOPC-21), PerCP.Cy5.5-conjugated IgG1 isotype control (MOPC-21). Antibodies were: anti-CD19-PerCP.Cy5.5 (SJ25C1) and anti-CD20-PerCP.Cy5.5 (L27) (BD Biosciences) to identify B cells, anti-IgG1 (MH161-1) and anti-IgG4 (MH164-1) (Sanquin Reagents) labelled with

DyLight488 or DyLight647 (Thermo Scientific) to identify IgG1 and IgG4-positive cells, anti-IgD-PE (IA6-2) and anti-CD27-APC (L128) (BD Biosciences) to identify naïve and memory B cells, anti-CD35-PE (E11) and anti-CD21-APC (B-ly4) to identify Complement receptors 1 and 2, anti-CD24-PE (ML5) anti-CD38-APC (HB-7) as activation markers and to identify transitional cells, anti-CD23-PE (M-L233) anti-CD32-APC (FLI8-26) to identify Fc Epsilon receptor II and Fc Gamma receptor II.

Table 5: Antibody panels for B cell surface staining

Channel	Panel 1	Panel 2	Panel 3	Panel 4	Panel 5	Panel 6	Panel 7	Panel 8
PerCp-Cy5.5	CD20	CD20	CD20	CD20	CD20	CD20	CD20	CD20
APC	CD27	CD27	CD38	CD38	CD21	CD21	CD32	CD32
PE	IgD	IgD	CD24	CD24	CD35	CD35	CD23	CD23
FITC	IgG1	IgG4	IgG1	IgG4	IgG1	IgG4	IgG1	IgG4
Pacific Blue	DAPI	DAPI	DAPI	DAPI	DAPI	DAPI	DAPI	DAPI

Table 5: Antibodies used in the 8 panels used for B cell surface staining, using 5 colour channels on the LSRII Flowcytometer.

PBMC's were washed with PBS supplemented with 0.1% bovine serum albumin (BSA) (Sigma-Aldrich), and incubated with the appropriate mAb or isotype control, in the dark at RT for 30 minutes (**Table 6**). Antibodies were anti-CD19-PerCP.Cy5.5 (SJ25C1) and anti-CD20-PerCP.Cy5.5 (L27) (BD Biosciences) to identify B cells, anti-IgG1 (MH161-1) and anti-IgG4 (MH164-1) (Sanquin Reagents) labelled with DyLight488 or DyLight647 (Thermo Scientific) to identify IgG1 and IgG4-positive cells, anti-CD27-APC (L128) (BD Biosciences) and anti-CD38-APC (HB-7) to identify plasmablasts, HLA-DR (BD Biosciences) to identify plasmablasts, anti-CD4 (BD Biosciences) and anti-CD14 (eBioscience) to identify CD4 T cells and monocytes, respectively.

After staining, antibodies were washed twice with PBS-BSA 0.1% to remove unbound antibody and re-suspended in PBS supplemented with 1% BSA. DAPI (Sigma-Aldrich) was added immediately before analysis to stain for cell viability and to exclude dead cells from analysis.

Table 6: Antibody panels for PBMC surface staining

Channel	Panel 1	Panel 2	Panel 3	Panel 4
PerCp-Cy5.5	CD20	CD20	CD20	CD20
APC	HLA-DR	HLA-DR	HLA-DR	HLA-DR
PE	CD38	CD38	CD138	CD138
FITC	IgG1	IgG4	IgG1	IgG4
PECy7	CD27	CD27	CD27	CD27
APC-Cy7	CD19	CD19	CD19	CD19
Pacific Blue	DAPI	DAPI	DAPI	DAPI
Pacific Orange	CD14	CD14	CD4	CD4

Table 6: Antibodies used in the 4 panels used for PBMC surface staining, using 8 colour channels on the LSRII Flowcytometer.

The cell surface expression and postulated function of each antibody are illustrated in **Table 7.**

Table 7: Antibodies for cell surface and intracellular staining.

Antibody	Cells stained and function
CD20	Expressed on B cells. Retained on mature B cells until plasma cell development.
CD19	Expressed on B cells (CD19 positive) and plasma cells (CD19 low).
IgD	Expressed on naïve B cells, antigen-inexperienced.
CD27	B cell memory, activation and Ig synthesis. TNF-receptor superfamily. Ligand is CD70.
CD38	B cell activation and B regulatory cells. Cyclic ADP ribose hydrolase activation marker.
CD24	Expressed on B regulatory cells. Promotes antigen-dependent proliferation of B-cells and prevents terminal differentiation into antibody-forming cells.
CD21	Complement receptor 2. Classical complement pathway.
CD35	Complement receptor 1. A negative regulator of classical & alternative complement pathways.
CD32	An Fc IIb inhibitory receptor - low affinity for IgG and involved in negative feedback if there is an excess of IgG.
CD23	An FcεRII receptor - Fc epsilon low affinity receptor for IgE.
HLA-DR	Expressed on plasma cells and APCs including B cells, dendritic cells, monocytes and macrophages. Up-regulation of expression on activation. MHC II cell surface receptor.
IgG4	Expressed on IgG4 switched B and/or plasma cells/plasmablasts.
IgG1	Expressed on IgG1 switched B and/or plasma cells/plasmablasts.
CD4	Helper T cell marker. Interacts with MHC class II.
CD14	Expressed on macrophages. Co receptor for LPS detection.
DAPI	Expressed on live cells. 4',6-diamidino-2-phenylindole binds strongly to A-T rich regions in DNA – live cells.

2.14.5 Intracellular staining

Cells were re-suspended in 100ul Fix/Perm buffer (eBioscience) and incubated for 40 minutes in the dark. Cells were washed twice and re-suspended in 50ul Perm buffer.

Intracellular staining antibodies were added and stained in the dark at RT for 45 minutes. Cells were washed twice with PBS and kept on ice for FACS analysis.

2.14.6 Cytokine production

PBMC's were thawed and plated onto 96 well plates at 1×10^6 cells/well in 100ul R10 solution. Cells were stimulated with PMA at 250ng/ml and ionomycin at 500ng/ml, and incubated at 37°C. Brefeldin A at 1ug/ml was added after 1 hour, and then washed twice with 200ul PBS after 5 hours, prior to staining.

IL-10 production by B cells

IL10 cytokine production was measured by intracellular staining after 24 hours of stimulation of CD19+ B cells with CpG (Invitrogen), and stimulation during the last 5 hours with 0.1 µg/ml PMA, 1 µg/ml ionomycin and 10 µg/ml brefeldin A (Sigma-Aldrich). Cells were washed twice with PBS, fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) for 15 minutes, and washed with PBS 1% BSA (Sigma-Aldrich). Cells were permeabilized with 0.5% saponin (Calbiochem, Darmstadt, Germany) in PBS 1% BSA and then incubated with fluorescent antibodies for 30 minutes at room temperature.

2.14.7 FACS Analysis

Expression of cell surface molecules was determined using a Beckton Dickinson LSR II (BD Biosciences) at the Peter Medawar and Sanquin Institute Amsterdam. Data was

acquired using FACS DIVA software (BD Biosciences) and analysed using FlowJo software (v7.6.5, Treestar).

Median fluorescence intensity and percentages of parent populations were calculated (**Figure 4A**). Populations of memory and naive B cells, transitional B cells, and plasmablasts were gated (**Figures 4**).

Figure 4A: Calculating median fluorescence intensity and % of parent population.

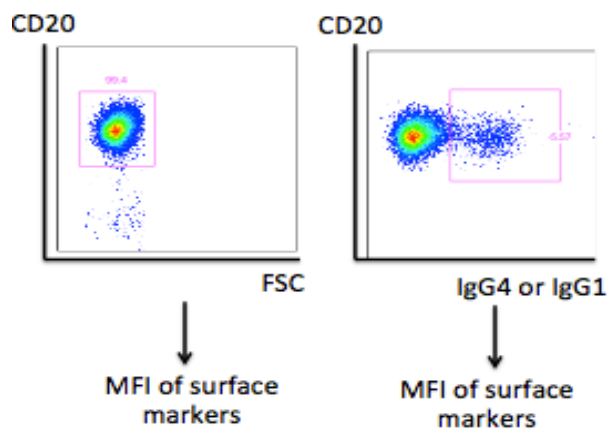


Figure 3: Flow cytometry plots showing determination of median fluorescence intensity (MFI) and percentage (%) of parent population. CD20: B cells, FSC: forward scatter, IgG4 or IgG1: IgG subclass antibody populations.

Figure 4: Gating on memory, naive and transitional B cell populations.

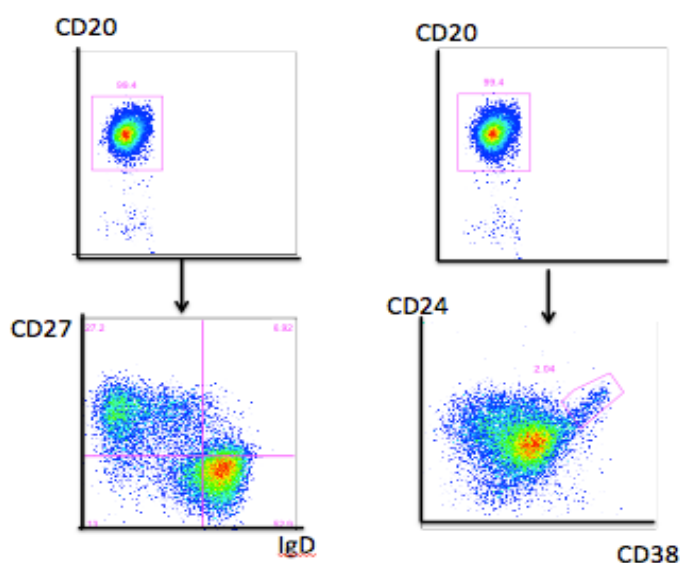


Figure 4: Flow cytometry plots gating strategy to determine B cell memory and transitional cell populations. Naïve b cells: CD20+IgD+; Memory B cells: CD20+CD27+IgD-; Transitional B cells: CD20+CD24+CD38+.

2.15 RT-PCR FOR IL10R AND IL10

RT-PCR was performed to detect expression of IL10 and IL10 receptor. Briefly, RNA was isolated from unstimulated (IL-10R) or stimulated (for IL-10, as above) IgG1+ and IgG4+ B cells and CD27+IgD- memory B cells and reverse transcribed to cDNA using random hexamers in combination with Superscript II and a RNase H-reverse transcriptase kit. TaqMan Gene expression assay kit (Applied Biosystems/Life Technologies) with primers for *18S* rRNA, *IL-10* and *IL-10RA* and *IL-10RB* was used according to the manufacturers protocol. Gene expression was measured in triplicate reactions for each sample. All results were normalized to the internal control *18S* rRNA, and are expressed relative to the expression levels found in total B cells.

2.16 GENE EXPRESSION ANALYSIS

2.16.1 Whole blood RNA Extraction

Whole blood was collected in Tempus RNA tubes (Life Technologies, Applied Biosystems®) and stored at -80°C. RNA was extracted using a Tempus Spin RNA Isolation kit (Applied Biosystems®). In brief, 3 ml of blood was diluted with 1xPBS, vortexed for 30 seconds, and then centrifuged at 300x g for 30 minutes 4°C. The supernatant was discarded and the RNA pellet was re-suspended. The RNA was purified using purification filters, washed x3 with purification washing solution, and then stored in nucleic acid purification elution solution. Micro-centrifugation optimized the purity of the yield. RNA quality check was performed on the Agilent 2100 Bioanalyser platform, providing an integrity number (integrity >6 is sufficient for gene expression analysis), gel images and electropherograms. All samples had integrity scores between 5.7 and 9.5.

2.16.2 Agilent Platform for Whole Genome Oligo-Microarray

Whole human genome oligo-microarray was performed (Miltenyi Biotech). We used a single-colour oligonucleotide array on an Agilent platform to increase flexibility of analysis. Biological replicates were essential for significance and reliability of the observed expression patterns. We controlled for other variables such as patient age and gender and timing of samples by matching to healthy controls. The raw data was then normalized to controls for technical variation between arrays within the study, whilst trying to leave the biological variation untouched. For the microarray, in brief, 100 ng of quality-checked RNA was used to produce Cy-3 labelled cRNA strands, which were

amplified and labelled using the Agilent Low Input Quick Amp Labelling kit. Yields of cRNA and dye-incorporation rates were measured with the ND-1000 Spectrophotometer. The Cy-3 labelled fragmented RNA was hybridized overnight to Agilent platform whole human genome oligo microarrays. Fluorescent signals of the hybridized Agilent Microarrays were detected on an Agilent Microarray Scanner System. The Agilent Feature Extraction Software and the Rosetta Resolver gene expression data analysis system allowed comparison of single intensity profiles in each ratio experiment and was used to create double-log scatter plots. Gene lists were returned as single experiment raw data, normalized data and pre selected candidate genes (Miltényi).

To look at differential gene expression, we used a combination of two approaches – examining each gene individually and examining groups of functionally-related genes to test whether the genes or gene sets show differential expression between samples. We then adjusted for multiple testing by controlling the false discovery rate – the probability that any particular significant finding is a false positive. Once a list of differentially expressed genes was obtained we used functional analysis to interpret the results.

2.16.3 Linear Model for Differential Gene Expression

Differential gene expression was further assessed using the LIMMA package - fitting a linear model to the expression data from each gene and using empirical Bayes shrinkage method to make the analysis stable (Smyth, Statist App Genetics Molec Biol 2004). Normalized data (VSN method) and filtered data (low expressed and low variance

probes were removed from the statistical analysis) were analysed using LIMMA, to select differentially expressed genes between different conditions (single organ versus multiple-organ disease) and between disease patients and healthy controls. Dr Emanuelle Marchi, a postdoctoral bioinformatician, assisted with the LIMMA package analysis.

2.16.4 Software to interpret gene expression profiles and pathway analysis

Gene expression profiles were interpreted using three software packages, to determine potential functional and biological pathways and networks. Firstly the widely available Database for Annotation, Visualisation and Integrated Discovery (DAVID) web-based program (david.abcc.ncifcrf.gov/), secondly the commercially available Ingenuity Pathway Analysis (IPA) (Quiagen), and lastly Metacore (Thomson Reuters) software.

The Database for Annotation, Visualisation and Integrated Discovery (DAVID) is a widely available free-of-charge web-based package that identifies common disease associations, functional gene associations and biological pathways potentially relevant in the given disease (Huagng et al, Genome Biol 2007). Ingenuity Pathway Analysis (IPA) is a commercially available software package that provides a deeper molecular interpretation and shows overlapping molecular functions, pathways and networks, and up/downstream regulators of the disease. Metacore is similar to IPA and was used to confirm the pathway analysis and identify additional genes and biological networks. The use of three tools enabled us to account for differences in databases, statistical tests, definitions/classifications and updates between the software (Tarca et al, PLoSOne 2013).

Analysis was performed in 3 stages: (1) data driven objective – determining the relationship of genes from the microarray, (2) knowledge driven objective – developing a comprehensive pathway knowledge base for areas of interest e.g. cell types and disease, (3) repeat of data-driven analysis after generating new hypotheses.

2.16.5 Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) of entire gene sets or pathways was then used to detect subtle changes that individual gene expression may have missed (by combining identification of differential expression and functional interpretation into a single step). GSEA enabled us to (1) assess co-expression of gene sets sharing common biological functions, chromosomal location or regulation, and (2) cross-compare expression profiles to publicly available data sets (Subramanian et al, PNAS 2005). For the latter, we cross-referenced known immunological and oncological signatures (represented by gene sets stored in GSEA databases) with our gene set enrichment conditions (IgG4-RD versus healthy controls, single organ versus systemic IgG4-RD). Gene set enrichment (GSE) plots and heat maps were created.

2.16.6 Public repository data

Furthermore, enrichment of our gene expression signatures were compared with selected experiments available in public repository microarray databases - Array Express www.ebi.ac.uk/arrayexpress/ and Gene Expression Omnibus (GEO) www.ncbi.nlm.nih.gov/geo/ (Rustici G, et al Nucleic Acids Res 2013; Edgar et al, Nucleic Acids Res 2002). Advanced search terms: '*Homo sapiens*', '*agilent whole*

genome oligomicroarray', *RNA assay*', were used. Key words were: '*autoimmune pancreatitis*', '*chronic pancreatitis*', '*pancreatic cancer*', '*systemic lupus*'. Enrichment of genes up-regulated (or down-regulated) uniquely in multi-organ IgG4-RD were compared with experiments in systemic lupus (accession number GSE-10325); single-organ pancreatic disease with pancreatic adenocarcinoma (accession number E-GEOD-49641) and chronic pancreatitis (accession number E-EMBL-6); all AIP patients compared with a tissue microarray (pancreatic tissue) in chronic pancreatitis and AIP (accession number E-MEXP-804).

2.16.7 Deconvolution Algorithm

A deconvolution algorithm with csSAM package was applied to infer the contribution of each cell type in whole blood to the general expression profile (Shen-Orr et al, Nature Methods, 2010; Abbas et al, PLoSOne 2009). This used an R package for cell-type specific differential expression of microarray experiments in heterogeneous samples, using statistical analysis for microarrays. It utilized the relative contribution of the full blood count (specifically white count), in the disease cohort and controls.

2.16.8 Principle Component Analysis

Principle component analysis (PCA) - a statistical procedure to calculate the co-variance of a set of variables – was used to identify the principle directions in which the data varied (Ringner, Nature Biotech 2008). The PCA showed similarities (or dissimilarities) between sample expression profiles, using the set of differentially expressed genes between the two sample groups (all IgG4-RD and all healthy controls). The cohorts

were compared using 590 significantly different genes between the two groups. These were illustrated on a 2 dimensional PCA plot.

2.16.9 Dendrogram and Heat Map

A dendrogram with gene expression heat map was produced showing clustering of samples based on expression of activity of differentially expressed genes between disease and control samples. Gene probes were collapsed and intensities were centralised by row to highlight differences in gene expression. Euclidian distance was used as a measure of similarity. Dr Emanuelle Marchi (postdoctoral bioinformatician) assisted with the GSEA, deconvolution algorithm, PCA and dendrogram with gene expression heat map.

2.16.10 Taqman RT-PCR

Gene expression was investigated at the RNA level by Taqman real-time polymerase chain reaction (RT-PCR). In brief, RNA was extracted using a Tempus Spin RNA Isolation kit (Applied Biosystems®), as described above. cDNA was synthesized from 200ng total RNA with an oligo-dT primer (Invitrogen®) and Superscript III reverse transcriptase (Invitrogen®). RT-PCR measurements were performed at 50°C on a Lightcycler (Roche®). Housekeeping genes GAPDH and HPRT1 were tested for stability; HPRT1 was used. Values were quantified using the comparative cycle threshold (CT) method, and samples were normalized to HPRT1 expression.

2.16.11 Luminex Cytokine Array

Quantitative multiplexed immunoassays based on Luminex technology were performed using a Procarta immunoassay kit (polystyrene beads) from Affymetrix. Analytes tested were IL4, IL5, IL6, IL8, IL10, IL13, IL17A, IL21, BAFF and TGF β . For the TGF β assay, 40 μ l samples were acidified with 20 μ l of 1NHCL, incubated 10mins at RT, then neutralised with 8 μ l 1.2N NaOH/0.5M HEPES to covert the complexed form of TGF β to its active form, then processed as a single plex assay. For the luminex assay, two 96-well filter plates were prepared by incubating with 150 μ l of Reading buffer for 5mins at RT. 50 μ l of antibody polystyrene beads were added to each well, then washed once.

A 4-fold serial dilution 8-point standard curve was created using antigen standards, and plasma samples were serially diluted to determine the optimum dilution for each analyte. 50 μ l of standards, samples and blanks (culture medium) in duplicate were added and incubated on a shaker at 700rpm 60min at RT, then washed x3. 25 μ l of detection antibodies were added and incubated on a shaker at 700rpm 30min at RT, then washed x3. 50 μ l of SAPE solution was added and incubated on a shaker at 700rpm 30min at RT, then washed x3. Lastly, 120 μ l of Reading buffer was added and incubated on a shaker at 700rpm 5min at RT.

The plate was analysed on the calibrated Luminex instrument, detecting both low and high RP1 target values. The concentrations of the samples were calculated by plotting expected concentrations of standards against the MFI generated by each standard.

2.17 HUMAN LEUCOCYTE ANTIGEN SEROTYPING

HLA serotyping class I and II was performed in the Immunotyping Laboratory, Churchill Hospital, Oxford, UK. Low-resolution typing for the HLA-A, -B, -C, -DR, and -DQ genes was performed by means of the polymerase chain reaction sequence (PCR) sequence specific oligonucleotide (SSO) method.

Alleles at HLA-DRB1 (0101, 1501, 1502, 1602, 0401, 0403, 0405, 0406, 0410, 1101, 1201, 1202, 1301, 1302, 1401, 1403, 1405, 1406, 0802, 0803, 0901, 1001), -DQB1 (0501, 0502, 0503, 0601, 0602, 0603, 0604, 0609, 0301, 0302, 0303, 0401, 0402), and -DPB1 (0201, 0202, 0301, 0401, 0402, 0501, 0901, 1301, 1401, 1901) were identified by the polymerase chain reaction restriction fragment length polymorphism method, to resolve major allele groups to 4 digits, with some degeneracy. In brief, the genomic DNA was amplified using PCR then incubated with a panel of oligonucleotide probes, which have distinctive reactivity with different HLA-types. Phenotype frequencies were calculated by direct counting for each HLA allele.

2.17.1 DNA Extraction

DNA was purified from whole blood using the Qiagen Gentra Puregene kit (Qiagen). Briefly, 3ml of whole blood and 9ml RBC lysis solution were mixed (inversion), incubated for 5 minutes, and centrifuged (2000xg 2 minutes) to pellet white blood cells. The supernatant was discarded, the pellet re-suspended by vortex, and 3ml Cell Lysis Solution was added and mixed by vortex. 1ml Protein Precipitation Solution was added, vortexed and centrifuged (2000xg 5 minutes), and the supernatant was poured into a new tube containing 3ml isopropanol, which was mixed (inversion) until the DNA

threads were visible. The sample was centrifuged (2000xg 3 minutes) and supernatant discarded. 3ml of 70% ETOH was added, the sample mixed (inversion) to wash the DNA, centrifuged (2000xg 1minute) and supernatant discarded. 300ul DNA Hydration solution was added, the solution vortexed, and incubated for 1 hour 65⁰C to dissolve the DNA. Samples were incubated at RT overnight with gentle agitation.

2.18 STATISTICS

Data were analysed using Graphpad prism software (v6.0). A non-Gaussian distribution was assumed and thus non-parametric tests were used, unless otherwise indicated. Values are presented as medians and range. A one-way Kruskal-Wallis test with post hoc Dunn's test, or ANOVA using log-transformed data, was used for multiple comparisons between groups, a two-tailed Mann-Whitney for non-paired analyses, a Wilcoxon signed-rank test for paired comparisons, and Chi squared with Yates' correction or Fisher's Exact for categorical variables. Spearman's rank correlation with 95% confidence intervals and Gaussian approximation was calculated for serum immunoglobulin levels. Receiver operator characteristic (ROC) curves were calculated for sensitivity and specificity. A P-value of <0.05 was considered statistically significant.

Multivariate analysis was used to determine predictors of disease relapse, after univariate analysis using a two-tailed Fisher's exact test. The Poisson distribution was used to determine if the number of cancer cases, cancer associated mortality and all cause mortality in the IgG4-RD patients observed significantly differed from that expected by using rates from PHE cancer research UK registry and national statistics UK (taking account of the number of patients that fell into each of the sex/age

categorisations and the duration of follow-up of each patient). The incidence ratio and 95% confidence intervals were calculated as previously described (Rothman et al, Modern Epidemiology, 2012). Cancer and cancer mortality statistics were calculated using the R programming language (www.R-project.org) by Dr. Jacob Hurst. A p value of <0.05 was considered statistically significant.

Chapter 3 Natural History and Outcome in IgG4-RD

Extracts from Chapter 3 have been published:

- M Huggett*, **EL Culver***, M Kumar, et al. IgG4-related autoimmune pancreatitis and cholangitis is associated with extra-pancreatic organ failure, malignancy and mortality in a prospective UK cohort. *Am J Gastro* 2014;109;1675-1683.

*denotes joint first authorship.

Appendix 3 details relevant publications, which are included in this Chapter:

- P Hart, T Kamisawa, W Brugge, **EL Culver**, et al. Long-term outcomes of autoimmune pancreatitis: a multicentre, international analysis. *GUT* 2013;62:1771-1776.

3.1 HYPOTHESIS

The natural history of IgG4-RD involves sequential organ involvement, relapse after medical therapy, and the development of complications including organ dysfunction and failure in the longer term.

3.2 INTRODUCTION

IgG4-RD was historically viewed as a corticosteroid-responsive condition with limited long-term sequel. A large multinational retrospective analysis of AIP therapy and relapse was recently published (Hart et al, Gut 2013), which showed variations in strategies for maintenance treatment and relapse rates between Asia and the West. There have also been no such evaluations of treatment and relapse rates in patients with extra-

pancreatic disease, and no prospective randomised controlled studies of optimal treatment regimens. There is limited short-term follow-up of patients in most documented series.

The necessity and urgency of treatment often depends on the involvement of vital organs and risk of organ dysfunction or failure, although this risk is not currently defined. The majority of patients have a rapid response to corticosteroids (Kamisawa et al, GUT 2009), most dramatic in those with early inflammatory rather than fibrotic disease. Relapse after immunosuppressive therapy occurs frequently, estimated as 30-50% in retrospective series of AIP (Sandanayake et al, Clin Gastro Hep 2009). Second line immunosuppressive agents and B cell depletion therapies have been used to treat relapsing disease with varying success (Hart et al, Gut, 2013; Carruthers et al, Ann Rheum Dis 2015). The risk of malignancy in IgG4-RD has received recent attention, but remains largely undefined (Shiokawa et al, Am J Gastroenterol 2013; Yamamoto et al, Mod Rheumatol 2012).

This chapter describes the cohort of prospectively recruited patients with IgG4-RD, concentrating particularly on those with pancreatic (AIP) and biliary (IgG4-SC) manifestations of the disease. There is particular emphasis on the natural history of disease and long-term outcome, including organ involvement, treatment response, evidence of relapse, second line immunosuppressive medication, organ dysfunction and failure, malignancy and mortality.

3.3 SPECIFIC METHODS

Methods detailing patient identification, data storage, diagnostic criteria, definitions (organ involvement, first-line treatment, treatment response, monitoring, disease relapse, treatment of relapse, complications, malignancy, mortality), serological measurements, tissue immunostaining and statistics are described in **Chapter 2**.

3.3.1 Patient recruitment

Data were collected from two UK tertiary referral centres, University College Hospital, London (UCH) and The John Radcliffe Hospital, Oxford (JRH). These are currently the largest units treating patients with IgG4-RD in the UK. Patients referred from February 2004 (UCH) or February 2005 (JRH) until February 2013, were included in the analysis.

3.3.2 Diagnostic criteria for AIP and IgG4-SC

Using the Mayo HISORt criteria (Chari et al, Clin Gastroenterol Hepatol 2006) and the International Consensus Diagnostic Criteria (ICDC) (Shimosegawa et al, Pancreas 2011), 115 patients were defined as having type I AIP and/or IgG4-SC.

3.3.3 Sample size and Power calculations

Sample size and power calculations for this Chapter were performed using an online calculator (<http://powerandsamplesize.com/Calculators/>). To calculate predictors of relapse a test of proportions (2 sample, 1 sided) was used, assuming a confidence level of 95%. To achieve a power of 80% to 90%, a sample size of 31 to 43 persons, would be required, where the proportion of IgG4-related SC was predicted to be 60% and the

expected difference between the two groups (relapse and non-relapse) was predicted at 30%. To calculate malignancy, a test of proportions (1 sample, 1 sided) was used, assuming a confidence level of 95%. To achieve a power of 80%, a sample size of 236 persons, would be required, where the observed incidence equals 3% and the standard population incidence equals 1%. In the latter case, incidence rates of all malignancy were compared to age and gender matched national population statistics (Public Health England cancer research UK), based on predicted 80% male and 60-year median age in the IgG4-RD cohort.

3.3.4 Statistical analysis

Statistical analysis for disease relapse was univariate using a two-tailed Fisher's exact test. The Poisson distribution was used to determine if the number of cancer cases, cancer associated mortality and all cause mortality in the IgG4-RD patients observed significantly differed from that expected by using rates from Public Health England cancer research UK registry and national statistics UK (taking account of the number of patients that fell into each of the sex/age categorisations and the duration of follow-up of each patient). The incidence ratio and 95% confidence intervals were calculated as previously described (Rothman et al, Modern Epidemiology, 2012). Cancer and cancer mortality statistics were calculated using the R programming language (www.R-project.org) with assistance from Dr. Jacob Hurst. A P-value of <0.05 was considered significant.

3.4 RESULTS

3.4.1 Demographics

A total of 115 patients with AIP/IgG4-SC were included; 85 male (74%), median age 61 years (range 16-85), with a median follow-up from diagnosis of 32.5 months (range 0.8-107). At least 12 months follow-up data was available for 88 patients (77% of the cohort).

3.4.2 Clinical presentation

Eighty-five patients (74%) were jaundiced at presentation. Forty-two patients (37%) described a history of abdominal pain but there were only 4 (3%) confirmed cases of IgG4-RD presenting as acute pancreatitis. Twenty-four patients (21%) had undergone surgery for suspected pancreaticobiliary cancer. Of these, 17 had a resection (14 pancreatic and 3 hilar) with the aim of achieving a surgical cure; in these patients IgG4-RD was subsequently diagnosed on assessment of resected tissue. The other 7 patients underwent biliary bypass for presumed unresectable adenocarcinoma.

3.4.3 Organ manifestations of IgG4-RD

The prevalence of organ manifestations in the cohort is shown in **Figure 1**.

Figure 1: Prevalence of organ manifestations in the prospective cohort.

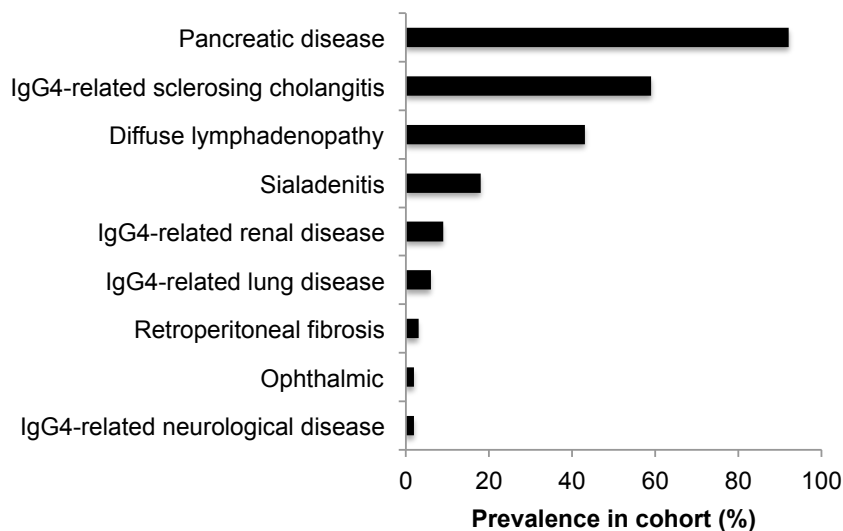


Figure 1: The bar chart shows the prevalence of organ manifestations in the 115 patients in the prospective IgG4-RD cohort. The x-axis displays the % of patients in the cohort with individual organ manifestations. The y-axis list the organs involved in the disease. Patients can have more than one organ involved.

3.4.4 Pancreatic disease (AIP)

Overall, 106 patients (92%) had pancreatic disease, of whom 60 (56%) also had IgG4-SC; 9 patients in the cohort (8%) had IgG4-SC only with no involvement of the intra-pancreatic portion of the CBD or the pancreatic parenchyma. Ninety-eight patients (85%) had pancreatic abnormalities on imaging; of these 50 (43%) had a diffusely enlarged ‘sausage-shaped’ pancreas, and 62 (54%) had imaging evidence of a discrete pancreatic mass, with or without diffuse enlargement. Eighty-three patients (72%) had evidence of pancreatic duct abnormalities (strictures, side branch dilatation, diffusely abnormal ducts) on imaging, but only 9% had pancreatic calcification or ductal stones. Sixty-nine percent had a low common bile duct (CBD) stricture, usually in association with an inflammatory mass in the head of pancreas. Of the patients who did not have a low CBD stricture, 50% had proximal biliary strictures.

3.4.5 Biliary disease (IgG4-SC)

Extra-pancreatic disease was common and occurred in 84 patients in the cohort (73%). Biliary involvement was the most frequent feature, occurring in 68 patients (59%). IgG4-SC was defined as patients who had evidence of biliary stricturing disease proximal to the intra-pancreatic bile duct.

3.4.6 Extra-pancreaticobiliary disease

Forty-one patients (36%) had disease beyond the biliary tree or pancreas. Diffuse lymphadenopathy with enlarged intra-abdominal, chest or peripheral nodes (e.g. axillary or iliac) was the most common manifestation in 43% of patients. IgG4-related sialadenitis was seen in 18%, renal infiltrates or masses in 9%, pulmonary disease with nodules, interstitial lung disease or pulmonary fibrosis in 6%, retroperitoneal fibrosis in 3%, ocular manifestations with proptosis and retro-orbital disease in 2%, and neurological sequelae including hypopituitarism caused by a pituitary mass and a rapidly progressive autoimmune encephalitis in 2%.

3.4.7 Autoimmune disease

Other autoimmune diseases were seen in this patient group. The most frequent of these was inflammatory bowel disease (IBD), which occurred in 11 patients (10%): ulcerative colitis in 8, Crohn's disease in 1 and IBD-unclassified in 2. Thyroid disease affected 7% of patients. Four patients had evidence of an inflammatory arthropathy, 2 suffered from polymyalgia rheumatic, and vitiligo, coeliac disease, psoriasis and alopecia affected 1 patient each.

Figure 2: Prevalence of autoimmune disease in the prospective cohort.

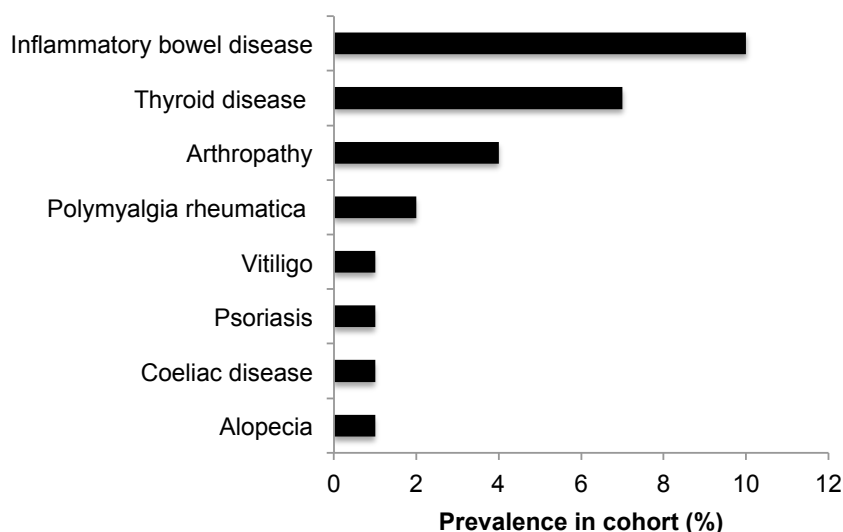


Figure 2: The bar chart shows the prevalence of autoimmune disease in the 115 patients in the IgG4-RD cohort. The x-axis displays the % of patients in the cohort with co-existent autoimmune disease. The y-axis lists the autoimmune diseases that co-exist with the disease. Patients can have more than one autoimmune disease.

3.4.8 Serum IgG4 concentrations

Serum IgG4 concentrations were available for 112 patients (3 patients had serum IgG but not IgG4 subclass measured, and the diagnosis was made based on imaging and histology). Serum IgG4 was raised (>1.4 g/L) in 70% at diagnosis (in those with available serum values; $n=105$), and in 77% of patients during follow-up. In those with a raised serum IgG4 at diagnosis and available levels following treatment, 55 patients (96%) had a fall in IgG4 with treatment, but levels returned to the normal range (<1.4 g/l) in only 21 patients (37%).

3.4.9 Tissue IgG4 immunostaining

Histology samples were obtained in 101 patients (88%) from a variety of sites including pancreas ($n=36$), ampulla ($n=46$), biliary tree ($n=23$), liver ($n=22$), kidney ($n=3$) and

peripheral lymph nodes (n=10). Forty-one patients had biopsies taken from more than one site and 38 patients had non-diagnostic biopsies from at least one site.

Tissue IgG4 was raised (>10 IgG4+ve plasma cells per high power field) in 75% of tissue biopsies that were stained for IgG4. Tissue IgG4 was raised (>50 IgG4+ve plasma cells per high power field) in 11 of 16 patients who had surgical resections and IgG4 staining performed.

3.4.10 Initial treatment and response

A total of 98 patients (87%) received oral corticosteroids for a median duration of 5.5 (range 1-73) months. Fourteen patients (14% of those treated) received less than 3-months, 38 patients (39%) received 3 to 6 months, 30 patients (31%) received 6 to 12 months, and 16 patients (16%) received over 12 months of corticosteroids. Starting doses were 30mg to 40mg of prednisolone in the majority, at the discretion of the treating clinician. A lower dose of 20mg prednisolone (8 patients) was used where patients had known diabetes or were deemed more likely to get steroid-induced complications. The remainder were not treated medically due to retrospective diagnosis following resection of an affected organ and no subsequent objective disease activity.

There was a 97% response rate to steroids, as defined by improvement in radiological findings and liver function tests. Sixty-one patients (53%) required biliary stenting with a mean of 2 stents in patients with available data (range 1-10 stents). It was possible to remove biliary stents in 77% of patients treated de novo during the first course of oral steroids.

3.4.11 Disease relapse and predictors of relapse

Fifty-eight patients (50%) relapsed post steroids, at a median time of 4.6 (0-64) months after stopping the first course of steroids, with 2 of these patients relapsing whilst taking steroids. In this prospective cohort of patients from Oxford and UCL, univariate analysis showed that extra-pancreatic disease predicted relapse ($P<0.01$). When IgG4-SC was assessed independently, the presence of IgG4-SC predicted relapse ($P<0.01$), but other organ involvement in the absence of IgG4-SC did not ($P=0.53$).

3.4.12 Treatment for disease relapse and response

Second-line immunosuppression was given to 66% of patients who relapsed, with the remainder being treated solely with a second course of oral corticosteroids (**Table 1**). The usual approach was to treat with a further course of steroids and add in azathioprine (to a dose of 2 mg/kg/day), which was started in 41 patients (36% of the cohort and 71% of those who relapsed). Of these, 8 relapsed and 13 were intolerant of Azathioprine.

Alternative immunosuppression in the event of azathioprine intolerance or side effects included 5 patients who received mycophenolate, 4 methotrexate and 2 who received mercaptopurine (**Table 1**). These agents were tolerated well. Two patients who were intolerant of azathioprine were treated with long-term corticosteroids. In those 8 whom relapsed on azathioprine, corticosteroids were re-started in all with an increased dose in azathioprine (4), or change in immunosuppression (4). Twenty-four patients remained on immunomodulatory monotherapy, without requirement for additional corticosteroids.

Table 1: Treatment for disease relapse in the IgG4-RD cohort.

Treatment	Agent used for treatment of relapse	% of 58 patients
1. Steroids alone	Prednisolone 0.5 mg/kg for 2-4 weeks then taper by 5 mg/1-2 weeks	20/58 (34)
2. Steroids+ IMT	Prednisolone 0.5 mg/kg for 2-4 weeks then taper by 5 mg/week	44/58 (76)
IMT 1 st line	Azathioprine 2 mg/kg	41/58 (71)
IMT 2 nd line if Azathioprine intolerant (13 patients)	Mycophenolate 500 mg-1000 mg bd	5/13 (38)
	Methotrexate 15 mg weekly	4/13 (31)
	Mercaptopurine 50mg od	2/13 (15)
	Low dose prednisolone 5 mg od	2/13 (15)
If relapse on IMT (8 patients)	Prednisolone	8/8 (100)
	+ increase Azathioprine dose or	4/8 (50)
	+ change IMT	4/8 (50)
If relapse despite steroids+IMT and/or intolerant of IMT	Rituximab 1gram, 2 infusions 14 days apart	1/2 (50)
	Cyclophosphamide 6 infusions	1/2 (50)

Table 1: The table shows the treatment algorithm for patients who relapsed after initial therapy, and the proportion of patients that received each treatment.

Abbreviations: IMT: immunosuppressive therapy; mg: milligrams; kg: kilograms; od: once daily; bd: twice daily.

Rituximab (a humanised chimeric anti-CD20 monoclonal antibody) was given to 1 patient in this cohort; with AIP, IgG4-SC and renal lesions refractory to high dose corticosteroids and intolerant of azathioprine and mercaptopurine. There was evidence of clinical, biochemical and radiological response at 3 months, but no further data is available at the time of writing. Cyclophosphamide (a nitrogen mustard, alkylating agent from the oxazophosphorine group) was given to a second patient with orbital and salivary gland disease, whilst awaiting rituximab, with clinical and radiological resolution of her orbital pseudotumour and glandular enlargement after 6 cycles. A further 4 patients are scheduled to receive rituximab.

3.4.13 Organ dysfunction in patients with IgG4-RD

With regard to pancreatic complications, exocrine insufficiency occurred in 53% and endocrine insufficiency manifesting as diabetes in 37% of patients. Exocrine insufficiency was determined by measurement of faecal elastase (defined as <200ug in a solid stool sample; measured in 114 patients). In 89% of diabetic patients, the diabetes was pre-existent before steroid therapy. In addition, 9% of patients had pancreatic calcification or ductal stones (**Table 2**).

In terms of liver-related complications, 6 patients (5%) developed liver cirrhosis during follow-up. Cirrhosis was diagnosed on liver biopsy in 2 patients (**Figure 3**), and clinically in 4 patients (patients with signs of synthetic dysfunction and/or hepatic decompensation with consistent radiology). One patient underwent a successful liver transplantation, with no evidence of disease recurrence at 2 years. Furthermore, 12% of patients developed biochemical renal impairment, ranging from stage 2 to 4 kidney disease. Portal and/or splenic vein thrombosis occurred in 9%, but with no evidence of variceal bleeding in any patients (**Table 2**).

Table 2: Non-malignant complications in the IgG4-RD cohort.

Non-Malignant Complications	% of 115 patients
Liver	
Cirrhosis and liver failure	5%
Liver Transplant	0.9%
Pancreas	
Exocrine insufficiency	53%
Endocrine insufficiency (Diabetes)	37%
Pancreatic calcification and stones	9%
Renal	
Impaired renal function – stage 2-4 CKD	12%
Vascular	
Portal and splenic vein thrombosis	9%

Table 2: This table shows the non-malignant complications in the 115 patients in the IgG4-RD cohort. The percentage of patients suffering from each complication is given. Individual patients can suffer from more than one complication.

Figure 3: Morphology and IgG4 immunostaining of a cirrhotic liver.

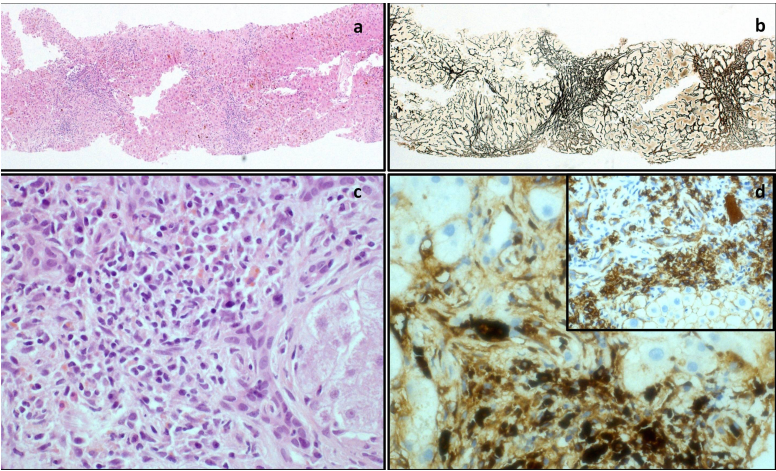


Image 1: Liver biopsy specimen in a patient with AIP and IgG4-SC, who developed liver cirrhosis. Panel (a) H&E x40 magnification, showing a lymphoplasmacytic infiltrate with areas of fibrosis (b) Reticulin x40 magnification, showing thick fibrous bands with nodule formation. (c) H&E x400 magnification, showing the inflammatory cell infiltrate rich in lymphocytes and plasma cells. (d) IgG4-positive immunostaining x400 magnification, inset CD138 plasma cell staining x200 magnification, showing large number of plasma cells expressing IgG4.

3.4.14 Malignancy in patients with IgG4-RD

In total 13 patients (11%) were diagnosed with a malignancy within 3 years prior to the diagnosis of IgG4-RD (n=4), concurrently (n=2), or during follow up (n=7) (**Table 3**).

Three cancers were confirmed to originate from the pancreas or biliary tree (2 cholangiocarcinomas and 1 pancreatic adenocarcinoma). The patients with concurrent pancreatic adenocarcinoma and cholangiocarcinoma had an elevated serum IgG4 (4 times ULN), diagnostic histological features for IgG4-RD, with diffuse disease in the resection specimens, and the patient with pancreatic adenocarcinoma had parotid IgG4-RD which relapsed post surgery. The other patient with cholangiocarcinoma had diagnostic features of IgG4-RD, which pre-dated the cancer diagnosis by 2 years.

In total, 2 patients developed colorectal cancer, 3 prostate, 2 transitional cell bladder tumours, 1 patient had a testicular cancer, 1 lung cancer, and 1 cancer of unknown primary. In addition to these cancers, one patient was found to have a low-grade pancreatic intraepithelial neoplasia (PanIN) lesion of the pancreas after Whipple's resection, and another had a small gastrointestinal stromal tumour within the duodenum.

Using public data from Cancer Research UK, the expected age and sex-matched number of cancer cases to occur within the follow up period was 4.5 cases. The actual number to have occurred was 9 cases. Within this cohort there was an increased incidence ratio of 2.25 (CI 1.23-3.94, P=0.02) for all cancers compared to the general population.

Table 3: Temporal relationship between the diagnosis of IgG4-RD and the diagnosis of malignancy.

Malignancy diagnosis	Time in relation to IgG4-RD diagnosis
Prior to diagnosis	
Prostate cancer	-3 years
Testicular leiomyosarcoma	-3 years
Transitional cell tumour of the bladder	-2 years
Prostate cancer	-1 year
Concurrent diagnoses (in resection specimen)	
Pancreatic cancer	
Cholangiocarcinoma	
After diagnosis	
Transitional cell tumour of the bladder	1 year
Colorectal cancer	1.5 years
Colorectal cancer	2 years
Cholangiocarcinoma	2 years
Lung cancer	4 years
Metastatic cancer (unknown primary)	5 years
Prostate	10 years

Table 3: The table shows the time of malignancy diagnosis in relation to IgG4-RD diagnosis, in years, where minus is before diagnosis.

3.4.15 Mortality

Eleven patients (10%) in our cohort died during follow-up (**Table 4**). Four died from cancer- 1 from a cholangiocarcinoma, 1 from transitional cell tumour of the bladder, 1 from lung cancer, and 1 from cancer of unknown primary. One patient developed rapidly progressive biliary stricturing disease with progressive jaundice, and died from cholangitis before liver transplantation. One patient, with biopsy-proven multi-organ IgG4-RD, developed a rapidly progressive encephalitic illness and a diagnosis of an autoimmune encephalitis was made on the basis of characteristic features of encephalitis on gadolinium MRI and cerebrospinal fluid analysis. This patient died despite treatment with high dose steroids and immunosuppression. Two patients died of pulmonary

fibrosis, one of pulmonary embolism and one of pneumonia. One patient died of post-operative complications of a Whipple's resection performed for presumed cancer (the resection sample showing AIP).

Using National UK mortality statistics the expected age and sex-matched number of deaths to occur within the follow up period was 5.4 and 11 were observed in this cohort. There was an increased incidence ratio of all cause mortality of 2.07 (CI 1.07-3.55, $P=0.02$). The cancer-related mortality was not increased compared to the national statistics (expected 2.1 deaths and observed 4 deaths; incidence ratio 1.95, CI 0.6-4.51; $P=0.17$).

Table 4: Causes of death in the IgG4-RD cohort.

Cause of death	Time after diagnosis of IgG4-RD
Pulmonary fibrosis	6 months
Post-op death	1.5 years
TCC bladder	1.5 years
Pneumonia	2 years
End stage liver disease	2.5 years
Cholangiocarcinoma	2.5 years
Autoimmune encephalitis	3 years
Lung cancer	4 years
Pulmonary embolus	5 years
Metastatic cancer (unknown primary)	5 years
Pulmonary fibrosis	9 years

Table 4: This table shows the causes of death in the 11 patients who died during follow-up, with a time course after diagnosis of IgG4-RD in years.

3.5 DISCUSSION

A developing consensus on the clinical characteristics, diagnostic features and pathology of this disease has led to important advances, including recognition that AIP is one component of a multi-system IgG4-RD (Kamisawa et al, *Am J Gastroenterol* 2003; Kamisawa et al, *Clin Gastroenterol Hepatol* 2008), and that this is a global disease (Varadarajulu et al, *Gastroenterol* 2003; Chari et al, *Pancreas* 2009; Kamisawa et al, *Pancreas* 2011). Early studies of IgG4-RD focused on the predictable and gratifying clinical and radiological response to an initial course of steroids, and for this reason IgG4-RD is commonly perceived to run a benign disease course. However, disease relapse following an initial course of steroids has been reported (Sandanayake et al, *Clin Gastro Hep* 2009; Chari et al, *Gastroenterol* 2008), and relapse frequently arises in organs other than the pancreas. Furthermore, long-term clinical outcome data in those both receiving, and not receiving, treatment remains limited (Kamisawa et al, *Gut* 2009; Takuma et al, *Eur J Gastroenterol Hepatol* 2011), and the true morbidity and mortality associated with IgG4-RD is unclear.

In this study we describe the largest prospective cohort of IgG4-RD reported from Europe. We found that the pattern of clinical presentation, response to steroids, and subsequent disease relapse (50%) was similar to that reported in studies from Japan and the USA (Hart et al, *Gut* 2013; Ghazale et al, *Gastroenterol* 2008). Extra-pancreatic disease was particularly common, occurring in 73% of patients. This was most frequently proximal biliary disease, although this may reflect the hepatobiliary specialisation in both UK centres. Although serum IgG4 was previously believed to be highly sensitive and specific for AIP (Hamano et al, *NEJM* 2001), our combined data show that raised serum IgG4 was present in only 70% of patients at diagnosis. This

reinforces data from other studies that serum IgG4 cannot be used in isolation to make a firm diagnosis, or differentiate from other diseases (Ghazale et al, *Am J Gastroenterol* 2007; Kamisawa et al, *J Gastroenterol* 2011). This is further explored in **Chapter 4**.

The number of surgical operations carried out in this cohort for suspected pancreaticobiliary cancer was high (21%). The majority of resections were performed pre 2008 when there was less awareness of AIP and IgG4-SC, however it still highlights the fact that IgG4-RD is difficult to distinguish from resectable cancers. All patients with suspected pancreaticobiliary cancer are now discussed in a joint hepatopancreaticobiliary multidisciplinary meeting with medical, surgical, radiology and histopathology input in an attempt to reduce the number of misdiagnosed patients. Serum IgG4 levels are routinely measured if there is clinical suspicion, with the caveat that 30% of AIP/ IgG4-SC patients will have a normal serum IgG4 at diagnosis and that elevated levels are not specific for IgG4-RD. A level of 4 times the upper limit (5.6g/l) has been reported to be highly specific to differentiate IgG4-SC from cholangiocarcinoma (Oseini et al, *Hepatology* 2011) and PSC (*Boostr & *Culver et al, *Hepatology* 2014). Histological evidence of disease from the pancreas or other involved organs is important, where possible. All biopsies should be reviewed for the key morphological features and immunostaining for IgG4 performed (Deshpande et al, *Modern Pathol* 2012; Culver & Bateman, *J Clin Path* 2012). Some physicians advocate a short (2 weeks) course of corticosteroid therapy to aid the diagnosis (Moon et al, *Gut* 2008). However, this approach should be applied with caution, since a steroid-related improvement in symptoms, a fall in serum IgG4, reduction in liver function tests and reduction of a mass on imaging may all occur in the presence of malignancy or other inflammatory diseases (**Chapter 4**).

We found that patients with extra-pancreatic disease, specifically IgG4-SC, were more likely to relapse and had a more complicated course. This supports the finding of other studies of IgG4-RD, showing that proximal/intrahepatic biliary disease strongly predicted relapse after an initial favourable steroid response (Ghazale et al, *Gastroenterol* 2007; Hart et al, *Gut* 2013). The high relapse rate at 50% after corticosteroid therapy and the need for re-treatment or prolonged therapy, especially with IgG4-SC, enforces the urgent need for international prospective studies to evaluate rational treatment strategies, including the early use of second line treatment regimens and B cell depletion therapy to prevent relapse and long term complications of disease.

In our patient cohort, long-term follow-up of up to 11 years (median 3 years) revealed a significant number of cases of organ dysfunction, with an associated morbidity and mortality. This occurred despite close clinical follow up and treatment of clinically active IgG4-RD. Particular sites of organ dysfunction included the pancreas, liver, and kidneys. Pancreatic exocrine insufficiency occurred in > 50% of cases, and this included patients who had an excellent clinical and radiological response to steroid therapy, and without disease relapse. This finding is similar to other studies, and reinforces the extent to which inflammation and subsequent fibrosclerosis can be a rapid process in IgG4-RD. Although steroid therapy undoubtedly has a dramatic and widely reported effect in resolving the pancreatic mass in IgG4-RD, it remains uncertain the extent to which steroids impact on preserving pancreatic structure and function (Hirano et al, *Gut* 2007; Uchida et al, *J Gastroenterol* 2009; Maruyama et al, *J Gastroenterol* 2012). Pancreatic exocrine failure can, of course, be managed relatively straightforwardly with enzyme supplementation, however we found a number of cases of other organ dysfunction and

failure that were irreversible. This included cerebral involvement (including one patient with pituitary failure due to a mass), fatal pulmonary fibrosis, stage 4 chronic kidney disease requiring renal dialysis, and liver cirrhosis resulting in liver transplantation or death.

Many of our cohort (59%) had IgG4-SC, 6 of whom went on to develop liver cirrhosis. Whilst there can be diagnostic difficulty between IgG4-SC and PSC (Webster et al, J Hepatol 2009) each of these patients fulfilled HISORt and ICDC criteria for AIP and IgG4-SC, and all of them had organ involvement outside the biliary tree and liver. There is on-going interest concerning the observation that patients with PSC who have an elevated IgG4 in serum or tissue have elevated parameters of liver disease severity and a more rapid progression to transplantation (Mendes et al, Am J Gastroenterol 2006). Indeed, there are considerable clinical and radiographic similarities between PSC and IgG4-SC (Kalaitzakis et al, Clin Gastroenterol Hepatol 2011), although the treatment strategies for the two conditions are currently different (Culver et al, Aliment Pharm Therap 2011). However, the extent of pathogenic overlap between the 2 conditions remains uncertain (Zen et al, Semin Diagn Pathol 2012; Zen et al, Hepatology 2014).

The fundamental question of whether IgG4-RD is associated with cancer (Ghazale et al, Pancreas 2007), and an overall reduced life expectancy, remains unanswered. When comparing this cohort with expected age and sex-matched number of cancer cases within the follow up period from public health data from Cancer Research UK, there was an increased incidence ratio of 2.25 (CI 1.23-3.94, P=0.02) for all cancers compared to the general population. In particular the number of pancreaticobiliary

cancers in this cohort (3/115 patients) was higher than expected for the age-standardised incidence in the UK of 3 per 100,000 for pancreatic carcinoma and 0.5-1 per 100,000 for intra-hepatic or extra-hepatic cholangiocarcinoma, respectively. A recent retrospective analysis of pancreatic resection specimens showed that 82% (23 cases) of AIP showed grade I PanIN and 25% (7 cases) grade 2 PanIN. In addition, 2.4% (2 of 84) of AIP patients developed pancreatic carcinoma, 6 and 10 years after diagnosis, raising further concerns for an elevated risk of malignancy in AIP (Gupta et al, *Pancreas* 2013). In another series, 1 of 21 (4.8%) AIP patients developed pancreatic cancer after 50 months of follow up (Uchida et al, *J Gastroenterol* 2009). Furthermore, in a large international retrospective study of 978 patients with AIP type 1, pancreaticobiliary cancers were reported in 8 (0.8%) patients (of which 5 were pancreatic and 3 cholangiocarcinoma) (Hart et al, *Gut* 2013).

Similarly, there are recent reports of extra-pancreatic and biliary malignancy in patients with AIP. In one multicentre retrospective study, 15 of 108 (13.9%) AIP patients developed cancer during a median of 3.3 years follow-up, with the majority occurring within the first year after diagnosis (Shiokawa et al, *Am J Gastroenterol* 2013). In our prospective study the development of any cancer in 13 patients (11%) following, or within three years of, the diagnosis of AIP/IgG4-RD, was higher than we expected. We also noted the presence of IgG4-positive plasma cells in patients with pancreatic carcinoma and cholangiocarcinoma, without other histological or radiological evidence to support IgG4-RD. Indeed, the finding of an intense IgG4+ve infiltrate in the cancer stroma of 6 of 8 patients studied by Shiokawa et al, prior to steroid therapy, and an absence of AIP disease relapse in any of these 6 after successful cancer therapy, led the authors to suggest the possibility of AIP/IgG4-RD being a paraneoplastic phenomenon

in some patients (Shiokawa et al, *Am J Gastroenterol* 2013). This may be the case in 2 of our patients where there were histological features of IgG4-RD in resection specimens from pancreaticobiliary cancers.

The aetiopathogenesis of cancer in IgG4-RD merits on-going attention. The concept of inflammation leading to malignancy is well recognised and has been described in a variety of disease states such as hepatocellular cancer and liver inflammation secondary to viral hepatitis, colonic cancer in inflammatory bowel disease, and cholangiocarcinoma on a background of PSC. Therefore, in IgG4-RD it is plausible that malignancy develops in a setting of chronic inflammation. Indeed, chronic pancreaticobiliary inflammation may be linked to the development of cancer in 3 of our patients with pancreaticobiliary malignancy. However, a recent publication has identified a potential cellular mechanism whereby IgG4 antibodies are specifically generated in response to malignant disease (Karogiannis et al, *J Clin Invest* 2013); in human melanoma patients, tumour-associated B cells are polarised to produce IgG4, stimulated by a Th2 (IL-10 secreting) tumour microenvironment. Strikingly, antigen-specific and nonspecific IgG4 inhibited IgG1-mediated tumoricidal functions, mediated through reduction of FcγRI activation, both in vitro and in a human melanoma xenograft mouse model. Higher serum IgG4 levels were associated with decreased patient survival. This study provides evidence for an alternative hypothesis linking malignancy to IgG4-RD whereby the generation of IgG4 antibodies, in the context of malignancy, may restrict effector cell function against tumours and represents a mechanism of tumour-induced immune escape.

Analysis of this prospective cohort has a number of limitations. Although the cohort size and study follow-up period are relatively large for this rare disease, these values are still numerically small and there is therefore the possibility of underestimating the risk of organ dysfunction and malignancy (especially if pancreaticobiliary cancers are a consequence of chronic inflammation, as in alcohol-related chronic pancreatitis). Such studies are also at significant risk of ascertainment bias as the rate of malignancy may be artificially elevated by earlier discovery of cancer in patients having scans for evaluation of IgG4-RD. Furthermore, conclusions drawn from a comparison with national statistics should be treated with caution as they are not adjusted for other risk factors such as smoking, alcohol intake and obesity. A diseased control cohort would have been preferable in this respect and would potentially eliminate some of these variables and may help to explain the discrepancy between this analysis and a previous US study showing similar 5-year survival to the general population (Sah et al, *Gastroenterology* 2010).

3.6 SUMMARY

Our data adds to a growing body of evidence showing that IgG4-RD may run a clinical course that is associated with a significant morbidity and mortality. Importantly, we have shown that patients with proximal/intra-hepatic biliary disease have a higher relapse rate and a more complicated course. Detailed clinical evaluation for evidence of organ dysfunction and associated malignancy is required both at first presentation and during long-term follow up in all of these patients. Ultimately, prospective studies of natural history and novel therapeutic agents, in very carefully defined patient cohorts, will be required to establish the true clinical course of the disease.

Chapter 4 Serum IgG4 in diagnosis and outcome in IgG4-RD.

Extracts from Chapter 4 have been published in abstract format:

- **EL Culver**, R Sadler, B Ferry, E Barnes. Elevated serum IgG4 in multiple hepatobiliary pathologies. J Hepatology (abstract) April 2012.

Appendix 3 details relevant publications, which are referenced in this Chapter:

- K Bostra*, **EL Culver***, L Maillette de Buy Wenniger*, et al. Serum IgG4 and IgG1 for distinguishing IgG4-Associated Cholangitis from Primary Sclerosing Cholangitis. Hepatology 2014;59(5):1954-1963.
- LM Hubers*, ME Doorenspleet*, **EL Culver**, et al. IgG4+ B cell receptor clones in peripheral blood distinguishes IgG4-associated cholangitis/autoimmune pancreatitis from PSC. J Hepatology (abstract) April 2015. Oral presentation at EASL Vienna.

4.1 HYPOTHESIS

Elevated serum IgG4 levels are useful in differentiating IgG4-RD from other malignant, inflammatory and immune diseases, and in determining the risk of relapse, multi-organ involvement and a worse outcome.

4.2 INTRODUCTION

IgG4 is the least prevalent of the four IgG subclasses, representing 3-6% of total IgG in the serum of healthy adults. However, it can account for up to 80% of total IgG after

chronic exposure to antigen (Aalberse et al, J Immunol 1983). In the case of IgG4-RD, it can be elevated up to 50 times the upper limit (Stone et al, N Eng J Med 2012). The reason and role for this elevated IgG4 is uncertain. Levels of IgG4 have been suggested to be important in the diagnosis of IgG4-RD, reflected by its inclusion in diagnostic criteria for the disease, however the upper limit of normal for serum IgG4 varies in these criteria (range 1.3-1.4g/l) depending on the assay used for measurement and regional differences (Chari et al, J Gastroenterol 2007; Okazaki et al, J Gastroenterol 2006; Otsuki et al, J Gastroenterol 2008; Shimosegawa et al, Pancreas 2011). Studies reviewing routine serum IgG4 level requests in France and the US have shown that raised serum IgG4 can be seen in a variety of other conditions (Ebbo et al, Int J Rheumatol 2012; Ryu et al, Int J Rheumatol 2012; Carruthers et al, Ann Rheum Dis 2015). There have also been inconsistent reports on the value of serum IgG4 levels in monitoring response to treatment, determining risk of relapse and predicting organ involvement (Ghazale et al, Am J Gastroenterol 2007; Sah et al, Curr Opin Rheumatol 2011; Carruthers et al, Ann Rheum Dis 2015). All of these studies have been retrospective, and most focused on the pancreatic manifestation of the disease.

In this Chapter, we investigated consecutive serum IgG4 measurements performed at the Oxford University Hospitals Trust over a six-year period to ascertain the variety of conditions that were associated with a raised serum IgG4 level. We subsequently considered all patients who had serum IgG4 concentrations measured to discriminate between a diagnosis of IgG4-RD and a non-IgG4-RD inflammatory, autoimmune or malignant condition. Those with an elevated serum IgG4 were recruited into a longitudinal follow-up study to determine a final clinical diagnosis. The utility of serum IgG4 measurements in diagnosis, monitoring response to corticosteroid therapy, organ

involvement, and disease relapse in the prospective cohort of IgG4-RD patients was then explored.

4.3 SPECIFIC METHODS

Methods detailing patient identification, data storage, diagnostic criteria, definitions, serological measurements and tissue immunostaining are described in **Chapter 2**.

4.3.1 Review of serum immunoglobulin measurements

A review of all serum IgG4 subclass concentrations measured in the clinical immunology department, Churchill Hospital, Oxford, UK from January 2007 until December 2012 (6 years) was performed. The indication for IgG4 subclass measurement and the clinical department requesting the test were recorded. A serum IgG4 level $\geq 1.4\text{g/l}$ was defined as elevated throughout this chapter and thesis.

4.3.2 Patient identification and recruitment

We identified those patients in whom the serum IgG4 concentration was measured to discriminate between a diagnosis of IgG4-RD and a non-IgG4-RD inflammatory, autoimmune or malignant condition. All had retrospective evaluation of clinical details, radiological investigations and histopathology. Those patients with an elevated serum IgG4 (225 patients) were recruited into a prospective longitudinal follow-up study to determine final diagnosis and outcome. These patients were reviewed in the IgG4 outpatient clinic, and were carefully evaluated with attention to clinical presentation, serological values (by Dr. Ross Sadler), cross sectional imaging for evidence of pancreatic or other organ involvement (by Dr Helen Bungay), review of morphology

and IgG4 immunostaining of liver, bile duct, pancreatic, colonic and other biopsies, where available - 182 specimens in total (by Dr. Lai Mun Wang). The ‘final diagnosis’ was determined by at least two members of the clinical IgG4 team (myself and either Dr. Roger Chapman, Dr. Tony Ellis, or Dr. Jane Collier, Dr. Joel David).

4.3.3 Diagnostic criteria

The diagnostic criteria for IgG4-RD and other inflammatory, autoimmune and malignant diseases are described in **Chapter 2**. As the Mayo HISORt criteria for AIP and IgG4-SC, and the Japanese Consensus Diagnostic Criteria for IgG4-RD, included different cut-offs for elevated serum IgG4 level in their algorithms, the Boston histology-based diagnostic criteria were applied to those with biopsy/resection specimens (Deshpande et al, Mod Pathol 2012). In accordance with these criteria, 58 patients were defined as having IgG4-RD and 167 patients with an elevated serum IgG4 who did not meet IgG4-RD criteria were identified.

4.3.4 Statistical analysis

A two-tailed Mann-Whitney test and a one-way Kruskal-Wallis test with post hoc Dunn’s test were used to compare individual and multiple groups, respectively. A chi-squared test with Yates’ correction was performed for gender differences. Spearman’s rank correlation with 95% confidence intervals and Gaussian approximation was calculated for serum immunoglobulin levels. Receiver operator characteristic (ROC) curves were calculated for sensitivity and specificity. Statistics were calculated using Graphpad Prism v6.0. A P-value of <0.05 was considered statistically significant.

4.4 RESULTS

4.4.1 Serum IgG4 measurements

A flow diagram showing serum IgG4 measurements is shown in **Figure 1**. Over a 6-year period, 2067 blood samples from 1510 patients had serum IgG4 subclass levels measured. Of these, 610 samples from 243 unique patients had an elevated serum IgG4 ($>1.4\text{g/l}$). The serum IgG4 was elevated in 16.1% (243/1510) of the patients in whom IgG4 was requested.

Figure 1: Flow diagram of serum IgG4 measurements in Oxford.

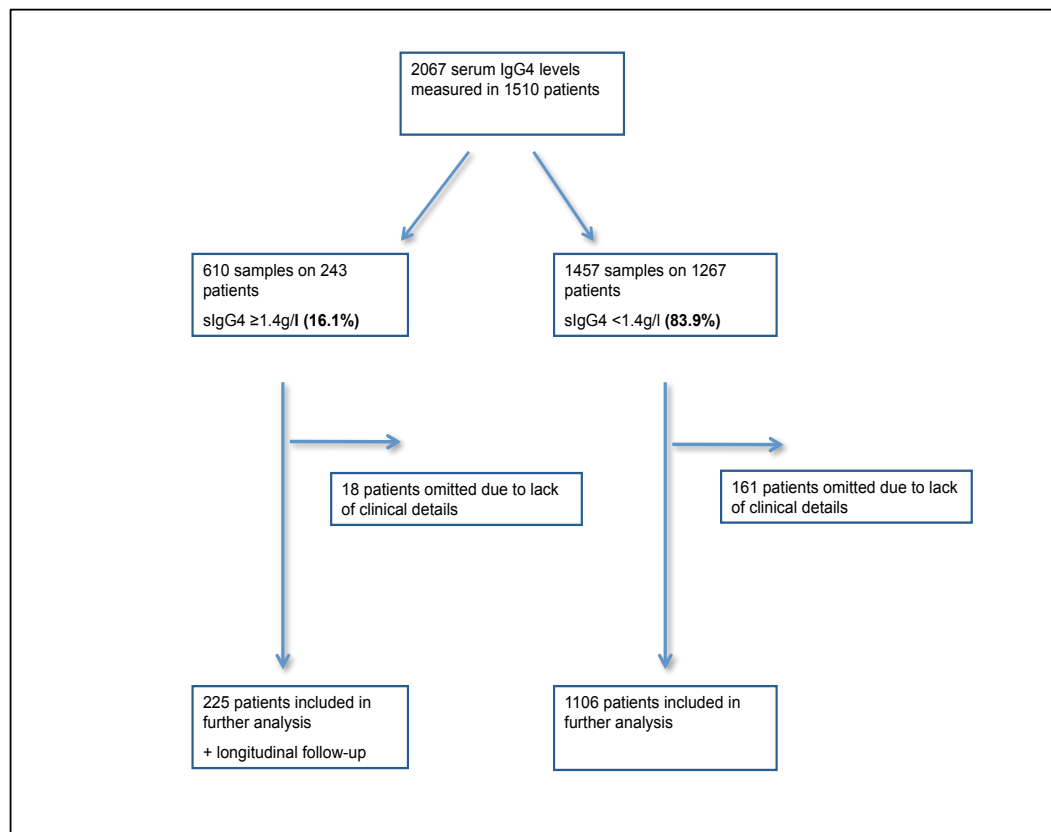


Figure 1: A flow diagram illustrating the number of serum IgG4 subclass measurements performed at the clinical immunology department, Churchill Hospital, Oxford, UK over a six-year period from January 2008 until December 2013.

4.4.2 Demographics

The demographics of patients with a normal and elevated serum IgG4 are shown in **Table 1**. Overall, there was a male predominance in the 243 patients with an elevated serum IgG4 (67.9%) and a female predominance in the 1267 patients with normal serum IgG4 group (54.5%) ($p<0.0001$). A higher median age was observed in patients with an elevated (59.4 years) compared to normal (55.0 years) serum IgG4 ($p=0.035$).

4.4.3 Serum Immunoglobulin concentrations

Serum total IgG and IgG1 subclass were higher in the elevated serum IgG4 group, compared to the normal IgG4 group ($p<0.0001$) (**Table 1**).

Table 1: Demographics and immunoglobulin concentrations in patients with an elevated and normal serum IgG4.

	High serum IgG4	Normal serum IgG4	P value	
Samples	610	1457		
Unique no. of patients	243	1267		
Gender M/F absolute (%)	165/78 (M 67.9%; F 32.1%)	577/690 (M 45.5%; F 54.5%)	<0.0001	****
Age median (range) (years)	59.4 (3-93)	55.0 (1-97)	0.035	*
Serum IgG median (range) (g/l)	14.6(4.13-47.3)	11(0.76-43.20)	<0.0001	****
Serum IgG4 median (range) (g/l)	2.24(1.37-54.1)	0.35(0-1.39)	<0.0001	****
Serum IgG1 median (range) (g/l)	8.56(3.59-32.2)	7.19(0.5-66.0)	<0.0001	****

Table 1: The demographics and serum immunoglobulin levels of patients with a normal and elevated serum IgG4. Mann Whitney test of continuous variables, where p values; * $p<0.05$, ** $P<0.0001$. Fishers Exact test of categorical variables, where p values; **** $P<0.0001$. Abbreviations: M; male, F; female; g/l: grams/litre.**

4.4.4 Clinical indication for serum IgG4 measurements

From 1510 patients, sufficient clinical details were available in 1331 patients (225 patients with an elevated serum IgG4 and 1106 patients with a normal serum IgG4). The indications for serum IgG4 measurement in these patients are shown in **Table 2**. The main indication for serum IgG4 measurements was to distinguish between IgG4-RD and other diseases (85.6%; 1140/1331 patients). Only those patients who had serum IgG4 measured when considering a diagnosis of IgG4-RD (214 with elevated serum IgG4 and 926 patients with normal IgG4 levels) are considered throughout the rest of this Chapter.

Table 2: Indication for serum IgG4 measurements

Indication for serum IgG4	Serum high IgG4	Serum norm IgG4
Distinguish between IgG4-RD and inflammatory, autoimmune or other disease mimic (%)	155 (68.9%)	839 (75.8%)
Distinguish between IgG4-RD and malignancy (%)	59 (26.2%)	87 (7.9%)
Immunodeficiency: recurrent or persistent infections (%)	6 (2.7%)	130 (11.8%)
Part of an autoimmune work-up including for Addison's, myasthenia gravis, vasculitis, rheumatoid, lupus (%)	5 (2.2%)	50 (4.5%)
Total patients (%)	225	1106

Table 2: This table shows the main indications for measurement of serum IgG4 levels at the clinical immunology department, Churchill Hospital, Oxford. Absolute numbers and percentages are given.

4.4.5 Final diagnosis and disease conditions

The final diagnosis in the 1140 patients in whom serum IgG4 was measured to distinguish between IgG4-RD and other diseases (IgG4-RD versus other inflammatory and autoimmune diseases (994 patients) or malignancy (146 patients) from **Table 2**) is shown in **Table 3**. The most common conditions in which serum IgG4 was measured were pancreatitis and primary sclerosing cholangitis (to differentiate from AIP and IgG4-SC). In fact, only 5.1% (58/1140) of patients who had serum IgG4 measured had

a final diagnosis of IgG4-RD. Overall, an elevated serum IgG4 was seen in 18.7% (214/1140) of patients in whom it was measured to differentiate IgG4-RD from non-IgG4-RD. Of these, serum IgG4 is elevated in 15.3% of non-IgG4-RD diagnoses versus 82.8% of those confirmed with IgG4-RD ($p<0.0001$).

Table 3: Final diagnosis in patients who had serum IgG4 measured.

Clinical diagnosis	Absolute number and (%) of patients in each group	Absolute number and (%) of patients with an elevated serum IgG4
IgG4-RD criteria met	58 (5.1)	48/58 (82.8)
IgG4-RD criteria not met	1082 (94.9)	166/1082 (15.3)
Malignancy	115	28/115 (24.3)
PSC	234	36/234 (15.4)
Pancreatitis	279	31/279 (11.1)
Cirrhosis	30	12/30 (40.0)
Autoimmune hepatitis	57	7/57 (12.3)
Inflammatory bowel disease	32	5/32 (15.6)
Autoimmune disease	57	14/57 (24.6)
Hepatitis	151	12/151 (8.0)
Biliary disease	77	9/77 (11.7)
Gallstones	11	6/11 (54.5)
Overlap PSC/AIH	9	3/9 (33.3)
PBC	28	1/28 (3.6)
Drug-induced	2	2/2 (100)
Total	1140	214/1140 (18.7)

Table 3: The table shows the final diagnosis attributed to the patients in whom serum IgG4 was measured to distinguish between IgG4-RD and other diseases. The proportion (absolute number, percentage) of patients in each group, and with an elevated serum IgG4 in each disease category, is given. **Abbreviations:** PSC; primary sclerosing cholangitis, PBC; primary biliary cirrhosis; AIH; autoimmune hepatitis..

4.4.6 ROC curves for a diagnosis of IgG4-RD

Using the final diagnosis attributed to the 1140 patients, the sensitivity, specificity and predictive values of serum IgG4 to distinguish IgG4-RD from other disease conditions were determined. The serum IgG4 was higher in those patients meeting criteria for IgG4-RD (median 1.74g/l, range 0-54.1g/l) than in those with non-IgG4-RD diagnoses (median 0.42g/l, range 0-15.0g/l) ($p<0.0001$) (**Figure 2A**).

At a serum IgG4 cut-off of 1.4g/l (upper limit of normal), the sensitivity was 82.8% (48/58) specificity was 84.7% (916/1082), positive predictive value (PPV) was 22.4% (48/214) and negative predictive value (NPV) was 98.9% (916/926), with an area under the curve (AUC) of 0.90, to diagnose IgG4-RD (**Figure 2B**).

Figure 2: The use of serum IgG4 levels to diagnose IgG4-RD.

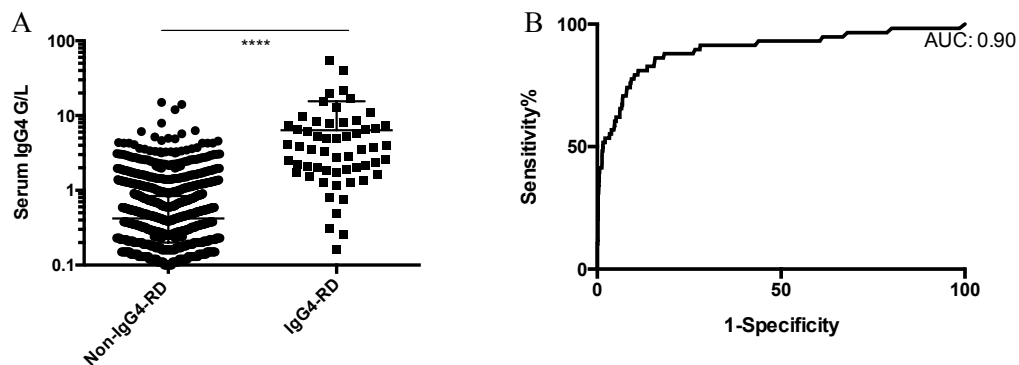


Figure 2: The utility of serum IgG4 to diagnose IgG4-RD. **(A)** The dot plot shows the serum IgG4 levels in non-IgG4-RD and IgG4-RD diagnoses. On the y-axis is Log 10 scale serum IgG4 in grams per litre (G/L). On the x-axis are patients with non-IgG4-RD diagnoses and IgG4-RD diagnoses. The error bars represent median and inter-quartile range. Mann-Whitney test; NS (not significant) $\geq p0.05$, $*p<0.05$, $**p<0.005$, $***p<0.005$, $****P<0.0001$. **(B)** The receiver operator characteristic (ROC) curve shows the sensitivity (%) and 1 minus specificity (%) of serum IgG4 for the diagnosis of IgG4-RD. The area under the curve (AUC) is 0.9.

As there were many patients with elevated serum IgG4 who did not meet the diagnostic criteria for IgG4-RD, a higher IgG4 cut-off to diagnose the disease was evaluated. Increasing the IgG4 to 2.8g/l (twice the upper limit), the sensitivity was 56.9% (33/58) specificity was 96.2% (1041/1082), PPV was 44.6% (33/74) and NPV was 97.7% (1041/1066) to diagnose IgG4-RD. Furthermore, increasing the IgG4 to 5.6g/l (four times the upper limit), the sensitivity was 36.2% (21/58), specificity was 99.5% (1077/1082), PPV was 80.8% (21/26) and NPV was 96.7% (1077/1114).

Although 41 patients (3.7%) with non-IgG4-RD diagnoses had a serum IgG4 above 2.8g/l, only 5 patients (0.5%) had a serum IgG4 above 5.6g/l. However, this did include 2 patients with cancer (1 resectable CCA and 1 metastatic with unknown primary).

4.4.7 Clinical diagnosis in patients with elevated serum IgG4

Of the 214 patients with an elevated serum IgG4, only 22.4% (48/214) of patients met IgG4-RD criteria. However, IgG4-RD was the most frequent diagnosis made in the high serum IgG4 group (**Table 4**).

Amongst other non-IgG4-RD diagnosis, PSC accounted for 16.8%, pancreatitis 14.5% and malignancy 13.1%. The patients who met IgG4-RD criteria were older (65.5 years) than those with non-IgG4-RD diagnoses (57.4 years) ($p=0.0006$). Both groups had a male preponderance ($p=0.14$).

Table 4: Final diagnosis in patients with an elevated serum IgG4.

Clinical diagnosis (elevated serum IgG4)	Absolute number	% of patients	Male	Female	Age median (range) years
IgG4-RD criteria met	48	22.4	37	11	65.5 (24-83.8)
IgG4-RD criteria not met	166	77.6	109	57	57.4 (2.9-93.0)
Malignancy	28	13.1	12	16	72.1 (42.6-87.7)
PSC	36	16.8	26	10	51.9 (12.4-84.8)
Pancreatitis	31	14.5	25	6	45.9 (15.6-83.0)
Cirrhosis	12	5.6	8	4	60.4 (46.2-84.4)
Autoimmune hepatitis	7	3.3	1	6	62.7 (24.4-73.1)
Inflammatory bowel disease	5	2.3	4	1	30.1 (19.1-71.1)
Autoimmune disease	14	6.5	9	5	57.6 (8.6-75.0)
Hepatitis	12	5.6	10	2	52.8 (22.4-65.6)
Biliary disease	9	4.2	6	3	53.0 (29.2-75.0)
Gallstones	6	2.8	4	2	66.2 (19.4-79.8)
Overlap PSC/AIH	3	1.4	2	1	61.0 (11-64)
PBC	1	0.5	0	1	64
Drug-induced	2	0.9	2	0	49.9 (34.4-65.3)
Total	214	100	146	68	

Table 4: The table shows the final diagnosis in patients with an elevated serum IgG4 recruited into a prospective longitudinal study at the Oxford Radcliffe Hospital Trust. **Abbreviations:** PSC; primary sclerosing cholangitis, PBC; primary biliary cirrhosis; AIH; autoimmune hepatitis.

4.4.8 Serum immunoglobulins levels and ratios in IgG4-RD and non-IgG4-RD patients with an elevated serum IgG4

Serum immunoglobulin levels and ratios in the IgG4-RD patients and non-IgG4-RD patients with an elevated serum IgG4 were then evaluated, as shown in **Table 5** and **Figure 2**. Serum total IgG and IgG4 were higher in the IgG4-RD patients (IgG median 16.5g/l, range 8.9-42.3g/l; IgG4 median 4.5g/l, range 1.48-54.1g/l) than in those with non-IgG4-RD diagnoses (IgG median 14.2g/l, range 4.13-47.3; IgG4 median 2.06g/l, range 1.40-15.0g/l) (IgG $p=0.043$ and IgG4 $p<0.0001$). There was no difference in IgG1 levels between the two groups ($p=0.201$.)

Table 5: Serum Immunoglobulin levels and ratios in IgG4-RD and non-IgG4-RD patients with an elevated serum IgG4.

Immunoglobulin serum levels	IgG4-RD	Non-IgG4-RD	p value	
IgG g/l median (range)	16.50 (8.90-42.30)	14.20 (4.13-47.30)	0.043	*
IgG1 g/l median (range)	8.40 (4.68-32.20)	8.62 (3.59-20.10)	0.201	NS
IgG4 g/l median (range)	4.51 (1.48-54.10)	2.06 (1.40-15.00)	<0.0001	****
Ratio IgG1/IgG4 median (range)	2.18 (0.29-6.55)	4.04 (0.29-10.93)	<0.0001	****
Ratio IgG/IgG4 median (range)	3.78 (1.04-12.08)	6.48 (0.72-16.18)	<0.0001	****
Ratio IgG4/IgG1 median (range)	0.46 (0.15-3.40)	0.25 (0.09-3.44)	<0.0001	****
Ratio IgG4/IgG median (range)	0.27 (0.08-0.96)	0.15 (0.06-1.38)	<0.0001	****

Table 5: Serum immunoglobulin levels and ratios in IgG4-RD patients and non-IgG4-RD patients with elevated serum IgG4. Mann-Whitney test *p* values; NS (not significant) $\geq p0.05$, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.005$, **** $P < 0.0001$. **Abbreviations:** g/l: grams/litre.

Serum IgG/IgG4 and IgG1/IgG4 ratios were lower in patients with IgG4-RD (IgG/IgG4 median 3.78, range 1.04-12.08; IgG1/IgG4 median 2.18, range 0.29-6.55) than non-IgG4-RD diagnoses (IgG/IgG4 median 6.48, range 0.72-16.18; IgG1/IgG4 median 4.04, range 0.29-10.93) (both ratios $p < 0.0001$).

In those patients with an elevated IgG4, serum electrophoresis demonstrated a polyclonal pattern of increased γ -globulin, with no evidence of monoclonal bands (shown in **Chapter 7**).

Figure 3: Serum immunoglobulin values and ratios in IgG4-RD and non-IgG4-RD patients with an elevated serum IgG4 level

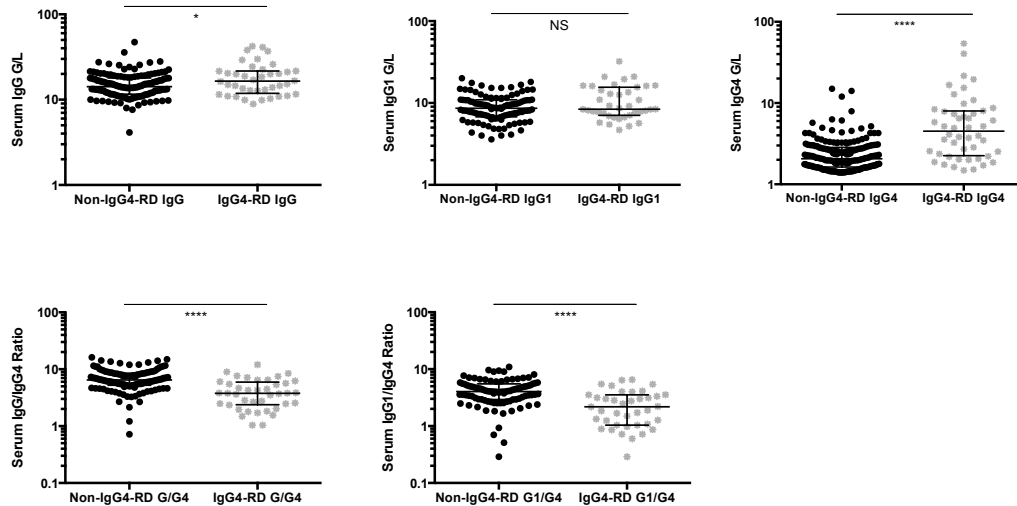


Figure 3: The dot plot shows serum immunoglobulin levels and ratios in IgG4-RD patients and non-IgG4-RD patients with an elevated serum IgG4 level. On the y-axis is Log 10 scale serum IgG, IgG1 and IgG4 in grams per litre (G/L), IgG1/IgG4 and IgG/IgG4 ratio. On the x-axis are patients with non-IgG4-RD diagnoses and IgG4-RD diagnoses. The error bars represent median and inter-quartile range. Mann-Whitney test; NS (not significant) $\geq p0.05$, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$.

4.4.9 IgG4-RD patients

From 1140 patients in whom serum IgG4 was measured to distinguish between IgG4-RD and other diseases, 58 patients met probable or definite IgG4-RD diagnostic criteria. The clinical characteristics and serum immunoglobulin values in patients with IgG4-RD are shown in **Table 6** and **Figure 4**.

The majority of IgG4-RD patients were male (77.6%) in the 6th decade of life (median 64.3 years, range 24-84 years). Most patients (48/58, 82.8%) had an elevated serum IgG4 level. In the 10 patients with a normal serum IgG4 level, there was lower total IgG ($p < 0.001$), IgG1 ($p = 0.015$), IgG/IgG4 ratio ($p = 0.0004$) and IgG1/IgG4 ratio ($p = 0.038$) compared to the 48 patients with an elevated serum IgG4 (**Figure 4**).

Table 6: Clinical characteristics and immunoglobulin levels in IgG4-RD.

	All IgG4-RD	IgG4-RD with normal IgG4	IgG4-RD with high IgG4	P value	
Patient number	58	10	48		
Age median(range)	64.3 (24-83.8)	62.2 (27-80.5)	65.5 (24-83.8)	0.2732	NS
Gender Male(Female)	43(15)	6(4)	37(11)	0.2651	NS
Organs Single(Multiple)	34(24)	9(1)	25(23)	0.0354	*
Relapse yes(no)	30(22) +	3(7)	27(15) +	0.0753	NS
Serum IgG median(range)	14.60 (8.09-42.30)	9.44 (8.09-12.70)	16.5 (8.97-42.30)	<0.0001	****
Serum IgG1 median(range)	8.23 (4.46-32.2)	6.06 (4.46-8.98)	8.4 (4.68-32.20)	0.0153	*
Serum IgG4 median(range)	3.52 (0.0-54.10)	0.62 (0.0-1.27)	4.51 (1.48-54.10)	<0.0001	****
Serum IgG/IgG4 ratio median(range)	4.06 (0.0-50.56)	16.5 (0.0-50.56)	3.78 (1.04-12.08)	0.0004	***
Serum IgG1/IgG4 ratio median(range)	2.47 (0.0-27.88)	7.35 (0.0-27.88)	2.18 (0.29-6.55)	0.038	*

Table 6: The clinical characteristics and serum immunoglobulin values in patients with IgG4-RD. Mann U Whitney test of age and serum immunoglobulin values, *p* values as per figure 3. Fishers Exact test of gender, organ involvement and relapse; NS (not significant) $\geq p0.05$, * $p<0.05$, ** $p<0.005$, *** $p<0.005$, **** $P<0.0001$. **Abbreviations:** G/L: grams/litre

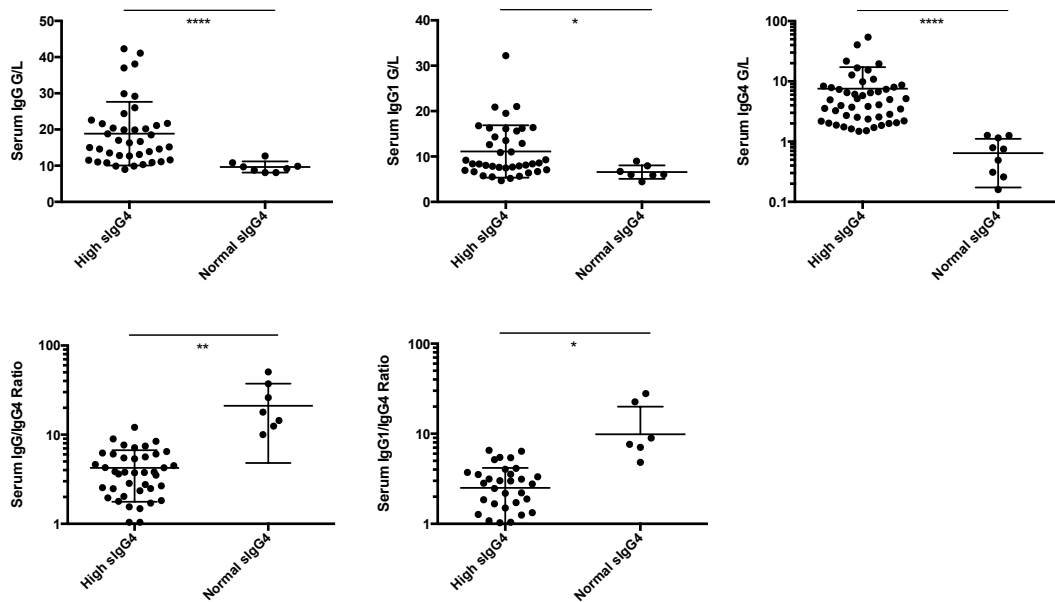
Figure 4: Serum immunoglobulin values in IgG4-RD patients.

Figure 4: The dot plots show serum immunoglobulin values in IgG4-RD patients. . On the y-axis is linear scale serum IgG and IgG1, and Log 10 scale IgG4 in grams per litre (G/L) IgG1/IgG4 and IgG/IgG4 ratio. The x-axis shows IgG4-RD patients with a high serum IgG4 (>1.4g/l) and a normal serum IgG4 ($\leq$ 1.4g/l). The error bars and Mann-Whitney p values are as per figure 3.

In IgG4-RD patients, there was a positive correlation between serum IgG4 levels and serum IgG (rank 0.80, <0.0001) and IgG1 (rank 0.51, $p=0.0003$) levels. There was a negative correlation between serum IgG4 levels and IgG/IgG4 ratio (rank -0.83, $p<0.0001$) and IgG1/IgG4 ratio (rank -0.69, $p<0.0001$) (**Figure 5**).

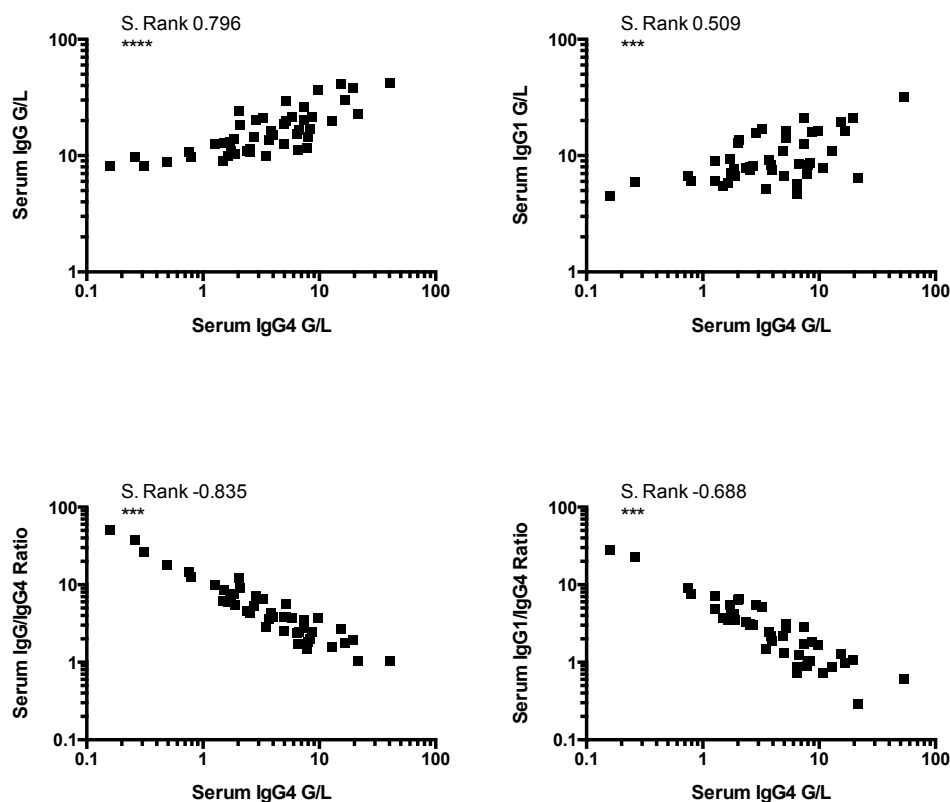
Figure 5: Correlation of serum immunoglobulin levels in IgG4-RD.

Figure 5: The correlation plots show the relationship of serum IgG4 and serum IgG and IgG1 levels, and IgG1/IgG4 and IgG/IgG4 ratios in patients with IgG4-RD. The x and y-axes are as per figure 3. Spearman's rank correlation; p values * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

4.4.10 Serum IgG4 and organ involvement in IgG4-RD

The relationship of serum IgG4 and organ involvement in patients with IgG4-RD was evaluated, followed up for a median of 30.7 months (range 1.3 to 73.5 months) shown in **Figure 6**. Twenty-five patients (43.1%) had multiple organ involvement. The serum IgG4 level in patients with multi-organ disease (median 6.141g/l, range 0.31-54.1g/l) was higher compared with those with single-organ disease (median 2.12g/l, range 0-21.7g/l) ($p=0.0001$). At diagnosis, a serum IgG4 of $>1.4\text{g/l}$ ($p=0.0354$) and $>2.8\text{g/l}$ ($p=0.0004$) predicted risk of multiple rather than single organ involvement at follow-up

(Figure 6). Nine of ten patients with normal serum IgG4 had single organ involvement. Follow-up was comparable in the two groups (multiple-organ disease median 30.9 months, range 1.5 to 73.5 months; single organ disease median 28.8 months, range 1.3 to 68.6 months) ($p=0.6801$).

Figure 6: The relationship between serum IgG4 and organ involvement.

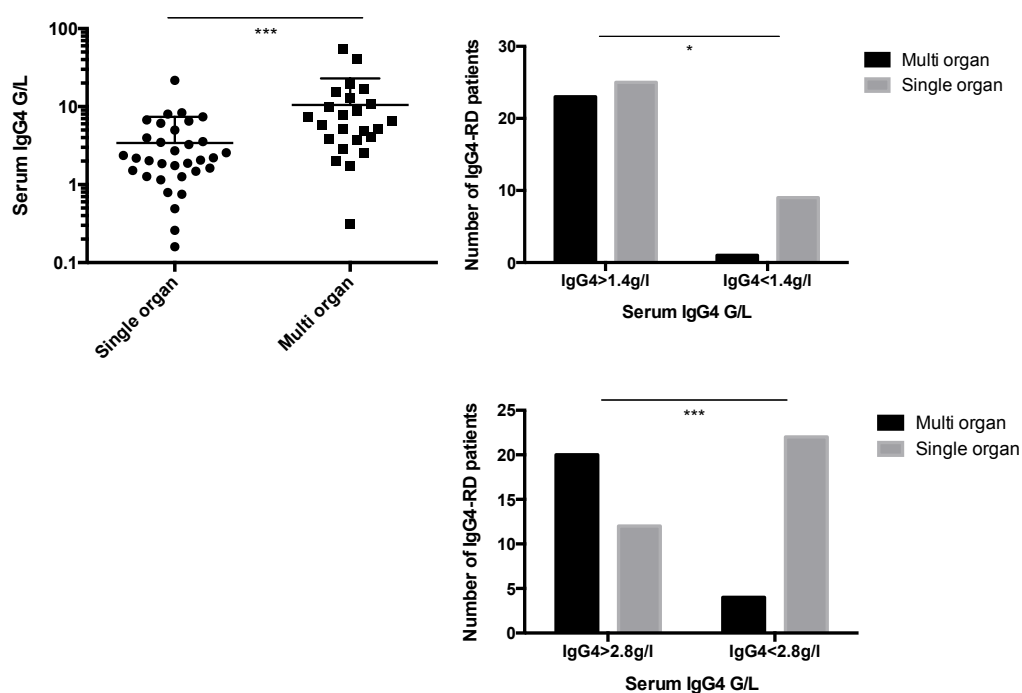


Figure 6: The dot plot shows the relationship of serum IgG4 and organ involvement in patients with IgG4-RD. On the x-axis are patients with single or multiple-organ disease. Y-axis, error bars and Mann-Whitney p values as per figure 2. The bar charts show the number of IgG4-RD patients who have single or multiple organ IgG4-RD. Fishers Exact; NS (not significant) $\geq p0.05$, $*p<0.05$, $**p<0.005$, $***p<0.005$, $****P<0.0001$.

4.4.11 Serum IgG4 levels and treatment in IgG4-RD

The relationship of serum immunoglobulin levels and corticosteroid therapy was next examined. Forty-six patients (79.3%) received corticosteroid therapy. There was a decrease in serum total IgG and IgG4, but not IgG1, during corticosteroid treatment (Figure 7 upper panel).

Serum IgG4 levels fell within 4 weeks of initiation ($n=7$, $p=0.084$), and were significantly lower compared with pre-treatment levels at 8 weeks ($n=11$, $p=0.033$) and 12 weeks ($n=15$, $p=0.018$) (**Figure 7 lower panel**). The steepest slope of decline was between 0 and 8 weeks at the highest dose of steroids, 20-40mg prednisolone/day, and fluctuated whilst at lower doses 5-10mg prednisolone/day.

Figure 7: Immunoglobulin levels in response to corticosteroid therapy in IgG4-RD.

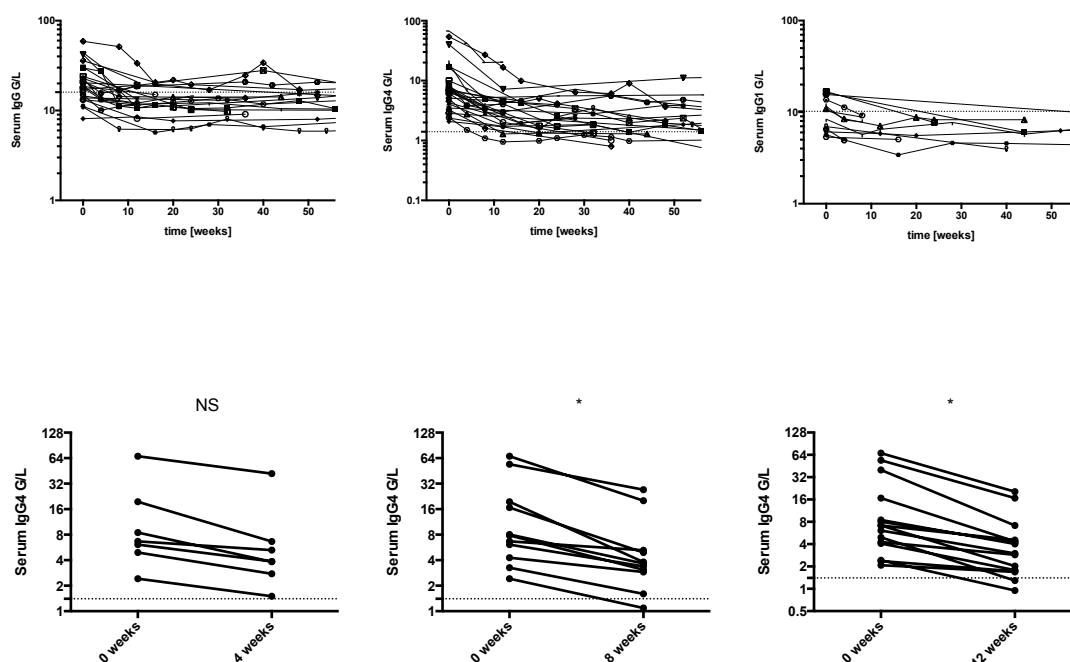


Figure 7: (Upper panel) The graphs show serum immunoglobulin levels in relationship to length of time on corticosteroid therapy in IgG4-RD patients. The x-axis plots corticosteroid therapy over time in weeks, where 0 weeks is before initiation of steroids. The Y-axis is per figure 3. The dashed lines represent the upper limit of normal for serum IgG (16g/l), IgG1 (10.2g/l) and IgG4 (1.4g/l) levels. **(Lower panel)** Serum IgG4 concentrations in IgG4-RD patients before and at 4 weeks, 8 weeks, and 12 weeks of corticosteroid therapy. On the y-axis is Log 2 serum IgG4 concentrations KU/L (international units). On the y-axis are time points: before corticosteroids (0 weeks) and 4, 8, 12 weeks into corticosteroid treatment (x weeks). Dashed line is the upper limit of IgG4. Two-tailed paired t-test p values, where NS $p \geq 0.05$, * $p < 0.05$.

However, a fall in serum IgG4 was not specific to IgG4-RD and other non-IgG4-RD disease conditions when treated with steroids (such as PSC-IBD and AIH) similarly

showed a decrease in serum IgG4 (**Figure 8**). Serum IgG4 levels also were significantly lower compared with pre-treatment levels at 12 weeks (n=5, p=0.036).

Figure 8: IgG4 levels in response to corticosteroids in non-IgG4-RD patients.

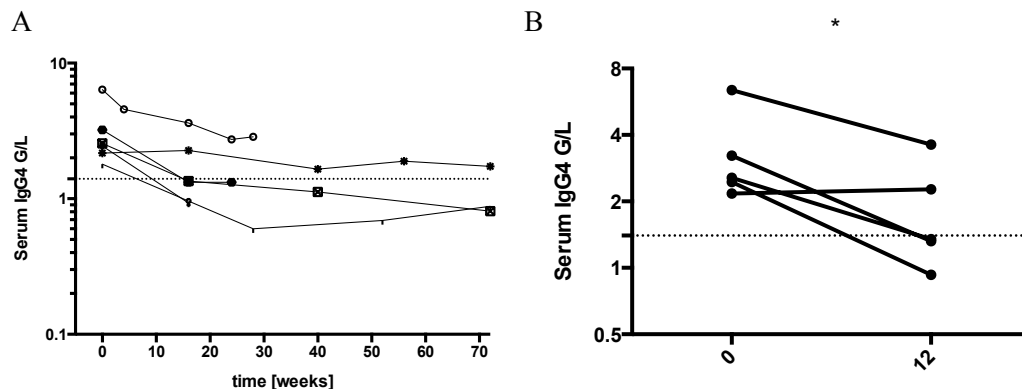


Figure 8: (A) The graph shows serum IgG4 levels in relationship to length of time on corticosteroid therapy in non-IgG4-RD patients (6 patients) with an elevated serum IgG4 at diagnosis. The x and y-axes and dashed-line are as per figure 7. **(B)** Serum IgG4 concentrations in non-IgG4-RD patients before and at 12 weeks during corticosteroid therapy. The x and y-axes and dashed-line are as per figure 7.

4.4.12 Serum IgG4 levels and risk of relapse in IgG4-RD

The relationship of relapse to serum IgG4 in the IgG4-RD cohort was next investigated. There was sufficient follow-up data (>6 months since treatment commenced and assessed in outpatient clinic) in 52 of 58 patients with IgG4-RD after treatment; 30 of which relapsed at least once after treatment (57.7%). There was a significantly higher serum IgG4 levels at diagnosis in those who relapsed after corticosteroid therapy and/or surgical intervention (median 5.05g/l, range 0.0-54.10) compared to those that did not relapse (median 1.95, range 0.16-16.70) (p=0.0099) (**Figure 9**). There was a significant difference in risk of relapse in IgG4-RD with a serum IgG4 >2.8g/l (p=0.0108) but not with a serum IgG4 >1.4g/l (p=0.0753) (**Figure 9**). There was no association with

normalization of elevated serum IgG4 during or after treatment and risk of relapse (p=0.516).

Figure 9: Relapse and serum IgG4 levels in IgG4-RD

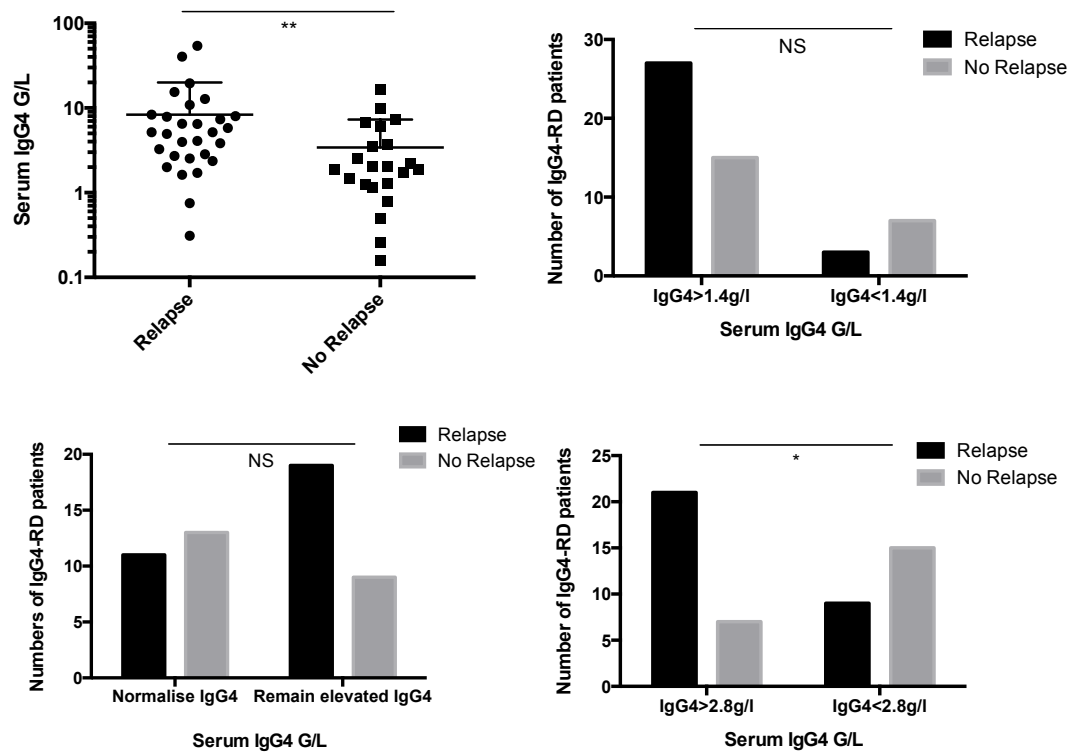


Figure 9: The dot plot shows the relationship of serum IgG4 and relapse in patients with IgG4-RD. On the x-axis are patients who have or have not relapsed. Y-axis, error bars and Mann-Whitney p values as per figure 2. The bar charts show the number of IgG4-RD patients who have relapsed or not relapsed. Fishers Exact test as per figure 6.

4.4.13 Serum IgG4, malignancy and mortality in IgG4-RD

Five of 58 (8.6%) IgG4-RD patients developed malignancy during follow-up (median 13.5 months, range 2.5 to 42.1 months from IgG4-RD diagnosis to cancer diagnosis). All cancers were histologically confirmed; prostate adenocarcinoma (2), transitional cell carcinoma (1), pancreatic adenocarcinoma (1) and cholangiocarcinoma (1). All 5

patients had an elevated serum IgG4 ($>2.8\text{g/l}$) at IgG4-RD diagnosis (median IgG4 5.16g/l , range $2.85\text{-}9.84\text{g/l}$). However, the serum IgG4 level did not predict risk of malignancy ($p=0.277$).

Six of 58 (10.3%) IgG4-RD patients died during follow-up (median 13.0 months, range 1.5 to 51.5 months from IgG4-RD diagnosis to date of death). Cause of mortality included pneumonia (2), end-stage pulmonary fibrosis (2), post-operative Whipple's surgery for suspected malignancy (1) and metastatic transitional cell bladder carcinoma (1). All 6 patients had an elevated serum IgG4 ($>1.4\text{g/l}$) at IgG4-RD diagnosis (median IgG4 2.71g/l , range $1.86\text{-}9.84\text{g/l}$). The level of serum IgG4 was not predictive of mortality ($p=0.99$).

4.5 DISCUSSION

Using consecutive serum IgG4 measurements performed in a large regional diagnostic laboratory, we evaluated conditions associated with an elevated serum IgG4, investigated the diagnostic utility of IgG4 in differentiating patients with IgG4-RD from other disease conditions, and examined the role of IgG4 in a prospective cohort of IgG4-RD patients. Overall, IgG4-RD diagnostic criteria were met in only 5.1% (58/1140) of patients who had serum IgG4 measured for the purpose of discriminating IgG4-RD from other disease conditions. This highlights the clinical challenge in differentiating IgG4-RD from conditions that mimic it, with the majority of IgG4 measurements being performed for non-IgG4-RD conditions. The high volume of serum IgG4 requests for this purpose (85.6%; 1140/1331 patients) also suggests an increasing awareness of the disease; historically, the main indication for serum IgG subclass measurements was to investigate immunodeficiency, chronic and recurrent

infections (Ebbo et al, Int J Rheumatol 2012; Maguire et al, Ann Clin Biochem 2002), which was the case in only 9% of our cohort. Practice patterns with regard to test ordering during the period of this study may have influenced these results, in particular a referral bias from the gastroenterology and hepatology departments may account for an over-representation of hepatobiliary diseases.

Importantly, IgG4-RD diagnostic criteria were met in only 22.4% (48/214) of patients found to have an elevated serum IgG4. This is particularly noteworthy given the importance placed on a serum IgG4 in diagnosis of the disease, where an elevated serum IgG4 is more likely to identify non-IgG4-RD conditions (in 77.6% of cases), and places more emphasis on the need for accurate interpretation of these levels in the context of disease. Earlier French and US retrospective studies support these findings, where recurrent infection, systemic autoimmune conditions and pancreatobiliary disease, were prominent among the diagnoses of subjects with elevated serum IgG4 levels (Ebbo et al, Int J Rheumatol 2012; Carruthers et al, Ann Rheum Dis 2015). A male predominance in the group with higher serum IgG4 levels and a female predominance in the group with normal levels supports known gender differences in healthy adults (French et al, Clin Exp Immunol 1984), although the reason for this is currently unexplained. This gender balance was observed in almost every disease condition in which an elevated serum IgG4 was found. Given that IgG4 is the predominant subclass in situations of chronic exposure and tolerance, it is possible that the older male distribution in IgG4-RD is explained by a long-standing history to certain environmental or occupational antigens, to which IgG4-RD patients have an aberrant response. This concept is explored further in **Chapter 6**.

The diagnostic utility of serum IgG4 in retrospective single-organ AIP cohorts has been well described. The original landmark study in Japan suggested that a serum IgG4 level of 1.35g/l had a sensitivity and specificity of 97% to distinguish AIP from pancreatic cancer (Hamano et al, *N Engl J Med* 2001). A subsequent meta-analysis of 7 retrospective studies reported sensitivities of 67-96% and specificities of 73–100%, with an AUC of 0.92, to differentiate AIP from pancreatic cancer (Morselli-Lebate et al, *J Gastroenterol Hepatol* 2009). More recently, a retrospective US study reported that a serum IgG4 of 1.35g/l had a sensitivity of 90% and specificity of 60% to diagnose multi-systemic IgG4-RD and differentiate it from other disease conditions (Carruthers et al, *Ann Rheum Dis* 2014). Our results suggest a serum IgG4 of 1.4g/l has a sensitivity of 82.8%, specificity of 82.1%, PPV of 22.4% and NPV of 98.7%, with an AUC of 0.9, to differentiate patients with multi-system IgG4-RD from inflammatory, autoimmune and malignant conditions, followed up prospectively to confirm the diagnosis. Doubling the cut-off value for serum IgG4 (2.8g/l) improved the overall test characteristics, with an increased specificity of 95.6% and PPV of 44.6%, but a fall in sensitivity to 56.9% and NPV to 97.3%. Although it is possible that a diagnosis of IgG4-RD may have been under-recognised by the absence of histology in some patients, anticipated to lower the specificity and the PPV of elevated serum IgG4, it remains an unsuitable single marker for diagnosis, whereby IgG4 elevations in patients with low pre-test probability of disease are likely to be false-positives.

Failure to understand the test characteristics of serum assays for IgG4 and to employ them effectively in clinical practice can lead to over-diagnosis of IgG4-RD and delays in diagnosis of important malignant and autoimmune conditions. The optimal use of serum IgG4 concentrations in this setting is as an initial diagnostic test that offers

important support of an IgG4-RD diagnosis if elevated ($>2.8\text{g/l}$), as well as a significant argument against the diagnosis if normal. The identification and validation of additional diagnostic markers, such as the IgG/IgG4 mRNA ratio by quantitative PCR (**Appendix 3**), a serum IgG4:IgG1 ratio (**Appendix 3**), and presence of IgG4 plasmablasts (**Chapter 9**), thus remains an important objective.

The use of serum IgG4 in determining organ involvement, treatment response and disease relapse seems more promising. In our cohort, 43% of patients had multiple-organ disease, with a serum IgG4 of 1.4g/l and 2.8g/l at diagnosis predictive of multi rather than single organ involvement. Similarly, 57.7% had at least one episode of disease relapse, with a serum IgG4 of 2.8g/l at diagnosis predictive of this. However, normalization of serum IgG4, as suggested by others (Frulloni et al, *Am J Gastroenterol* 2009), did not predict relapse. Given that the majority of patients who had multi-organ involvement had eventual disease relapse, the link between these factors requires further exploration.

Corticosteroids are the first-line treatment for IgG4-RD patients with inflammatory disease. We have observed a fall in serum IgG4 whilst on steroid treatment, with significant decline at 8 and 12 weeks of therapy. In some centres, a steroid trial has been used to help differentiate AIP from pancreatic cancer in cases of diagnostic difficulty (Moon et al, *Gut* 2008). However we, and others, have shown that serum IgG4 will also fall in non-IgG4-RD conditions (Nishino et al, *Intern Med* 2006; Gardner et al, *Am J Gastroenterol* 2009). Hence, reduction in serum IgG4 levels following initiation of steroid therapy cannot be used to distinguish IgG4-RD from other conditions.

Using this data, we recommend that IgG4-RD patients with a serum IgG4 of 2.8g/l at diagnosis, should have contrast-enhanced imaging in search for evidence of subclinical organ involvement at diagnosis and follow up, as this will guide need for and urgency of treatment. There should also be early consideration of second line therapies, such as immunosuppressive therapy, or prolonged low dose corticosteroid, in these patients in whom relapse risk is high. Conversely, normal serum IgG4 often suggests single organ involvement in whom relapse risk is low, and these patients need less stringent follow-up, possibly with annual outpatient appointments at the referral centre.

4.6 SUMMARY

To our knowledge, this is the first study where patients with an elevated serum IgG4 were followed up prospectively to determine final diagnosis, and IgG4-RD patients were recruited into a longitudinal study to specifically evaluate serum IgG4 levels in relation to organ involvement, response to treatment and relapse. This is also the largest study in the UK analysing patients with systemic IgG4-RD. The clinical indication for serum IgG4 measurements has changed and interpreting an elevated serum IgG4 requires knowledge of the clinical, radiological and histopathological scenario. In those patients where there is diagnostic doubt, a multidisciplinary meeting is the appropriate arena to come to a decision and guide management decisions. An elevated serum IgG4 >2.8g/l at diagnosis may guide further imaging to search for subclinical organ involvement and early consideration of prolonged corticosteroid or second line treatments to prevent relapse. However, serum IgG4 cannot be used in isolation and other measures including the role of IgG4-positive plasmablasts and B cells in disease particularly in their relation to rituximab, requires careful attention.

Chapter 5 Serum IgE and Atopy in IgG4-RD.

Extracts from Chapter 5 have been presented in abstract format:

- **EL Culver**, R Sadler, D Simpson, F Highfield, S Yates, RW Chapman, E Barnes, B Ferry. Elevated serum IgG4 and IgE in IgG4-RD and other disease mimics. Poster at International Symposium IgG4-RD, Honolulu Hawaii, Feb 2014.

5.1 HYPOTHESES

1. There is an increased prevalence of allergy and/or atopy, elevated IgE levels and eosinophilia in IgG4-RD patients compared with the general population.
2. Elevated serum IgE levels are helpful in differentiating IgG4-RD from other malignant, inflammatory and immune diseases, and in determining relapse, multi-organ involvement and a worse outcome.
3. Tissue infiltration with IgE-positive cells support a role for IgE-mediated immune responses in the pathogenesis of IgG4-RD.

5.2 INTRODUCTION

An elevated serum IgG4 and abundance of IgG4-positive plasma cells infiltrating the involved organs of IgG4-RD patients is well described (Deshpande et al, Mod Pathol 2012). However, elevated serum total IgE concentrations, which were first reported in a series of AIP patients in Japan (Umemura et al, Hepatol 2007), have received much less attention. Retrospective studies suggest a prevalence of 34 to 86% of elevated serum IgE levels in AIP patients (Kamisawa et al, Euro J Gastro Hep 2009; Hirano et al,

World J Gastro 2010). Furthermore, a clinical history of allergy has also been reported in 40 to 80% of AIP patients (Umemura et al, Hepatol 2007; Kamisawa et al, Eur J Gastro Hep 2009). This is particularly interesting as high levels of antigen-specific IgE are traditionally found in allergic disease, where it binds to both allergens and Fc receptors on mast cells resulting in degranulation (Oxdemir et al, Clin Exp Allergy 2011.) Moreover, both a peripheral and tissue eosinophilia has been described in AIP (Sah et al, Am J Gastro 2010), which is traditionally seen in the context of atopic and allergic conditions.

The increase in IgE and history of allergy is perhaps unsurprising, given the well-known links between IgG4 and IgE. IgG4 responses are often associated with IgE-mediated allergy, but IgG4 and IgE responses are distinct. Both IgG4 and IgE are induced by the Th2 cytokines IL-4 and/or IL-13 (Punnonen et al, Proc Natl Acad Sci 1993), however production of IgE antibodies often occurs well before IgG4 antibodies appear e.g. in novice beekeepers (Aalberse et al, J Immunol 1983). It is also common to find IgG4 antibodies in the absence of IgE antibodies, a process called the modified Th2 response (Platts-Mills et al, Lancet 2001). An important regulatory component in the modified Th2 response is IL-10. Under the influence of this cytokine, the switch to IgG4 is promoted, whilst the switch to IgE is inhibited (Jeaninin et al, J Immunol 1998). IgG4 responses require frequent and/or high antigen exposure and are observed in situations associated with tolerance induction, such as during allergen immunotherapy. This can result in an increase of IgG4 10 to 100 fold, along with a gradual decline in anti-allergen IgE antibodies and symptoms of allergic rhinitis and asthma. Th2-cell-dominant immune responses are reportedly increased in the peripheral blood and tissue of IgG4-RD patients, and abundant regulatory cells producing IL10 and TGF-Beta infiltrate

affected organs (Zen et al, Hepatol 2007; Okazaki et al, Gastroenterol 2000; Tsubio et al, Arthritis Res Ther 2013). Excessive Th2 cell and Treg cytokine responses are thought to potentiate IgG4 production, inflammation and fibrosis, although the origin of these cytokine imbalances remains unclear.

In this Chapter, we set out to establish the prevalence of serum IgE elevation, peripheral blood eosinophilia, and a clinical history of allergy and/or atopy in our prospective cohort of IgG4-RD patients. In those atopic individuals, we used an immunoblot assay to measure IgE-specific response to a number of well-defined allergens, to assess if a particular allergen or group of allergens predominated in the disease. Given the challenges in diagnosing patients with IgG4-RD, we sought to determine the utility of serum IgE in differentiating patients with an elevated serum IgG4 who had IgG4-RD from non-IgG4-RD diagnoses. We also explored the relationship of serum IgE to organ involvement, clinical relapse and corticosteroid use in those with IgG4-RD. Lastly, we sought to detect the presence of tissue eosinophils and IgE-positive cells in affected organs, and determine which cell type expressed IgE.

5.3 SPECIFIC METHODS

Methods detailing patient identification, data storage, diagnostic criteria, definitions, serological measurements and tissue immunostaining are described in **Chapter 2**.

5.3.1 Patient and control identification and recruitment

For this Chapter, patients and controls were recruited from the John Radcliffe Hospital, Oxford, UK, between March 2011 and March 2014.

5.3.2 Diagnostic criteria

The diagnostic criteria for IgG4-RD and other inflammatory, autoimmune and malignant diseases are described in **Chapter 2**. Using these criteria, 48 patients were defined as having IgG4-RD and 42 patients were defined as ‘disease controls’ who did not meet IgG4-RD criteria (25 PSC, 3 autoimmune disease (1 sarcoid, 1 hypereosinophilic syndrome, 1 coeliac), 4 hepatitis (1 autoimmune, 2 viral, 1 alcoholic), 3 chronic cholecystitis, 4 cirrhosis (3 alcoholic, 1 cryptogenic), 2 infections (2 recurrent pneumonia and pleural effusion) and 1 chronic pancreatitis. Fifty-one healthy controls, with no known immune or inflammatory disease, were gender-matched to IgG4-RD patients and disease controls. Atopy was defined according to the European Academy of Allergy and Clinical Immunology classification, essentially allergic-type symptoms and an IgE-mediated protein response.

5.3.3 Peripheral Eosinophilia

Peripheral blood eosinophil count was measured at diagnosis (before steroid therapy) at the haematology diagnostic laboratory, John Radcliffe Hospital, Oxford. An elevated eosinophil count was defined as >500 cells/ul (count >0.5).

5.3.4 Serum IgE

Serum IgE was measured at diagnosis, during steroid treatment, at remission and at time of relapse at the clinical immunology department, Churchill Hospital, Oxford. Total IgE was measured by the automated Immunocap method using a Phadia 250 (Phadia, Milton Keynes, UK). For IgE units are expressed as KU/L, equivalent to 1 IU/mL, and equivalent to 2.4ng/mL. An elevated serum IgE is defined as ≥ 120 KU/L.

5.3.5 IgE-specific allergen testing

An IgE ImmunoCAP was used to determine and quantify allergen-specific IgE antibodies in the serum, performed at the clinical immunology department, Churchill Hospital, Oxford (**Chapter 2**). A set of allergen mixes were tested including grass pollen mix, mould mix, tree pollen mix and nut mix (Phadia, Thermo Scientific), each mix containing 8 similar allergens. In those patients with a clinical history of a specific allergy, additional allergen testing was performed to confirm this.

5.3.6 Tissue Eosinophilia

The number of eosinophils considered 'physiologically normal' in a tissue sample depends on the organ sampled and the size of high power field used. There are currently no guidelines to define these cut-offs, except in the context of eosinophilic oesophagitis where >15 eosinophils per high power field in oesophageal squamous mucosa is considered diagnostic with a compatible clinical situation (Hurrell et al, Adv Anat Pathol 2011). On an H&E specimen, the number of eosinophils was graded as 'none' (0), 'occasional' (<10), 'moderate' (10-25) or 'marked' (>25) by the reporting pathologist.

5.3.7 Tissue staining for B cells, T cells, macrophages and plasma cells

Five IgG4-RD and 5 disease control biopsy specimens were stained for CD4 and CD8 (T cells), CD20 (B cells) and CD68 (macrophages) to determine the background inflammatory infiltrate (**Chapter 2**).

5.3.8 Double immunostaining for IgE and mast cells and/or plasma cells

Twelve IgG4-RD specimens (9 resection, 3 biopsies) and 6 disease control specimens (5 resection, 1 biopsy) were immunostained for IgE, mast cell tryptase (mast cells), and CD138 (plasma cells) (**Chapter 2 and Table 1**). Double immunostaining for IgE and CD138 or IgE and mast cell tryptase was performed in order to define IgE-positive cells. Tissue staining was supervised by Dr. Adrian Bateman, at the Histopathology Department, Southampton General Hospital.

Table 1: Tissue specimens from IgG4-RD patients and disease controls.

IgG4-RD patient samples	
Resection	1. Retroperitoneal mass 2. Lymph node and mesenteric mass 3. Pancreas: whipples 4. Common bile duct 5. Salivary gland 6. Pancreas and duodenum: whipples 7. Pancreas and duodenum: whipples 8. Pancreas and duodenum: whipples
Biopsy	1. Salivary gland 2. Buccal mucosa 3. Renal 4. Liver including portal tracts
Disease control samples	
Resection	1. Lymph node Reactive from malignant squamous carcinoma 2. Gallbladder Chronic cholecystitis 3. Pancreas Alcoholic chronic pancreatitis 4. Common bile duct Secondary sclerosing cholangitis 5. Pancreas Non-alcoholic chronic pancreatitis
Biopsy	1. Nasal polyp Chronic sinusitis

Table 1: Tissue specimens for cellular staining in IgG4-RD patients and disease controls with inflammatory conditions. Sections were identified (by Lai Mun Wang, Consultant Histopathologist, John Radcliffe Hospital, Oxford, UK) which represented areas of inflammatory infiltrate in both the patient and disease control samples.

5.4 RESULTS

5.4.1 Demographics

The majority of IgG4-RD patients were male (69%) in the 6th decade of life (median 63.0 years, range 30-82 years). The healthy controls were gender matched (58.9% male; (p=0.403)) with a younger median age of 42.8 years, range 20-78 years (p<0.0001).

5.4.2 Prevalence of serum IgG4 and IgE elevation in IgG4-RD

The serum IgG, IgG1, IgG4 and IgE levels were all higher in IgG4-RD patients than in healthy controls (p<0.0001). There was no difference in the IgE/IgG4 ratio between the two groups (**Table 2 & Figure 1**). Elevated serum IgG4 concentrations were demonstrated in 81.3% (39/48), and elevated IgE concentrations in 54.2% (26/48) of the cohort, both more prevalent compared with the healthy control group (p<0.0001) (**Table 2**).

Table 2: Clinical characteristics and laboratory measurements in IgG4-RD patients and healthy controls.

	IgG4-RD patients n=48	Healthy controls n=51	P values
IgG median (range)G/L	14.7 (6.36-59.1)	10.75 (6.33-16.0)	<0.0001 ****
IgG1 median (range)G/L	8.38 (3.94-32.2)	6.65 (3.41-10.6)	<0.0001 ****
IgG4 median (range)G/L	3.57 (0.0-54.1)	0.57 (0.0-2.4)	<0.0001 ****
IgE median (range)G/L	142.0 (0.0-2024)	25.1 (1.99-491.0)	<0.0001 ****
IgE/IgG4 ratio median (range)	69.19 (0-705.1)	57.93 (0-2728)	0.6491 NS
Eosinophils median (range)	0.44 (0.0-5.05)	0.16 (0.05-0.78)	<0.0001 ****
Clinical history of Atopy/Allergy (%)	30/48 (62.5)	6/35 (17.1)	<0.0001 ****
High serum IgG4 >1.4G/l (%)	39/48 (81.3)	3/51 (5.9)	<0.0001 ****
High serum IgE >125KU/L (%)	26/48 (54.2)	8/51 (15.7)	0.0001 ***
Serum Eosinophilia >0.5 (%)	18/48 (37.5)	3/35 (8.6)	0.0042 **

Table 2: Clinical characteristics and laboratory measurements of IgG4-RD patients, taken at diagnosis, and healthy controls. P values were calculated by using Mann-Whitney for comparison between 2 groups and Fishers Exact test for categorical variables, where NS $p \geq 0.05$, ** $p = 0.01$, * $p = 0.001$, **** $p < 0.0001$ Abbreviations: G/L: grams per litre; KU/L: equivalent to 1 international unit per millilitre.**

Figure 1: Serum IgG4, IgE and IgE/IgG4 ratio in IgG4-RD and healthy controls.

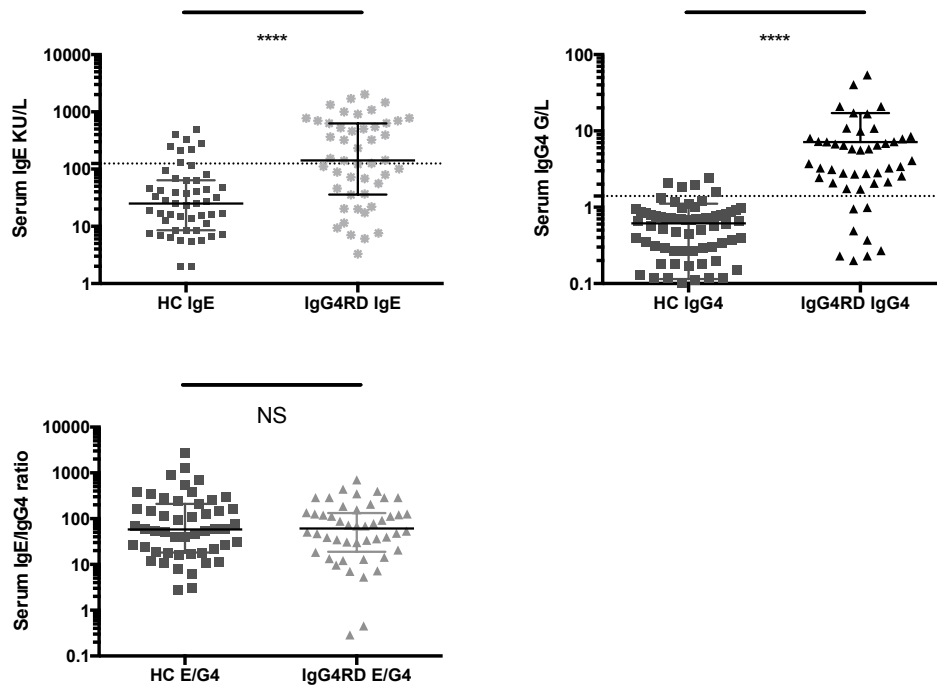


Figure 1: Dot plots showing serum IgG4 and IgE concentrations and the IgE/IgG4 ratio in IgG4-RD patients and healthy controls. The IgG4-RD patients and healthy controls (HC) are on the x-axis. The y-axis shows the Log 10 scale serum IgE concentration in KU/L (international unit), Log 10 scale serum IgG4 in G/L (grams per litre), Log 10 scale serum IgE/IgG4 ratio. Dashed line is the serum IgE upper limit of normal 120KU/L and serum IgG4 upper limit of normal 1.4G/L. Mann-Whitney p values, as per Table 3 where NS $p \geq 0.05$, **** $p < 0.0001$.

In the 9 IgG4-RD patients with a normal serum IgG4 at diagnosis, only 1 had an elevated serum IgE level ($p=0.017$) (**Figure 2**).

Figure 2: Serum IgE in IgG4-RD patients with high and normal serum IgG4.

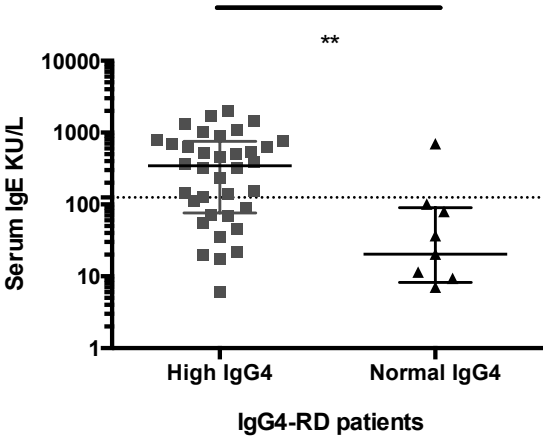


Figure 2: Dot plot showing serum IgE concentration in IgG4-RD patients with a high and normal serum IgG4 level. The y-axis shows the Log 10 scale serum IgE concentration in KU/L (international units). Dashed line is the serum IgE upper limit of normal 120KU/L. Mann-Whitney p values, as per Table 3 where ** $p < 0.01$.

There was a positive correlation between serum IgE and IgG4 (Sp.Rank 0.46, CI 0.182-0.656; $p=0.001$), and serum IgE and IgG (Sp.Rank 0.32, CI 0.028-0.558; $p=0.028$) levels (Figure 3).

Figure 3: Correlation of serum IgE with serum IgG4, IgG and IgG1 in IgG4-RD.

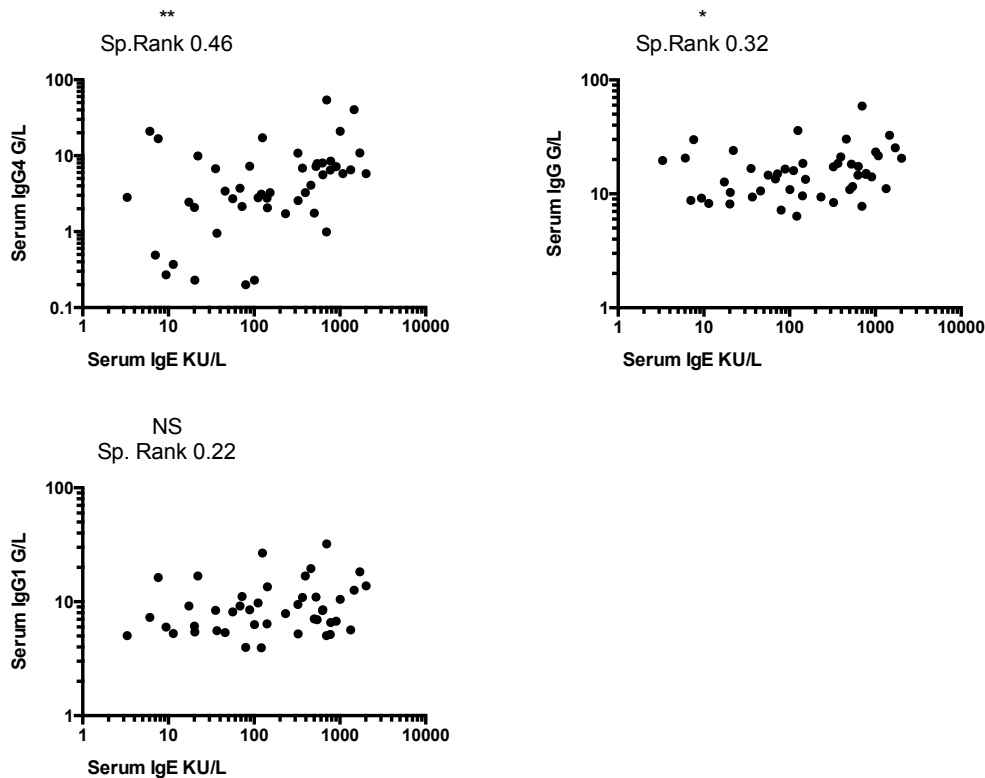


Figure 3: Correlation plots showing serum IgE concentration plotted against serum IgG4, IgG4 and IgG concentrations in IgG4-RD patients. Log 10 scale serum IgE KU/L (international units) on the x-axis and Log 10 scale serum IgG4, IgG1 and IgG G/L (grams per litre) on the y-axis. Spearman's Rank (Sp. Rank) correlation co-efficient and p values are expressed as NS≥0.05, *P<0.05, **P<0.01.

5.4.3 Prevalence of peripheral eosinophilia in IgG4-RD

Peripheral blood eosinophilia was present in 37.5% of patients (median of 0.44, range 0.0-5.05) versus 8.6% of healthy controls (median 0.16, range 0.05-0.78) (p=0.004) (**Table 2**). There was a positive correlation between eosinophil count and serum IgE (Sp.Rank 0.45, CI 0.178-0.654; p=0.001) and IgG4 (Sp.Rank 0.34, CI 0.049-0.572; p=0.019) levels (**Figure 4**).

Figure 4: The relationship of peripheral blood eosinophil count with serum IgE and IgG4 in IgG4-RD.

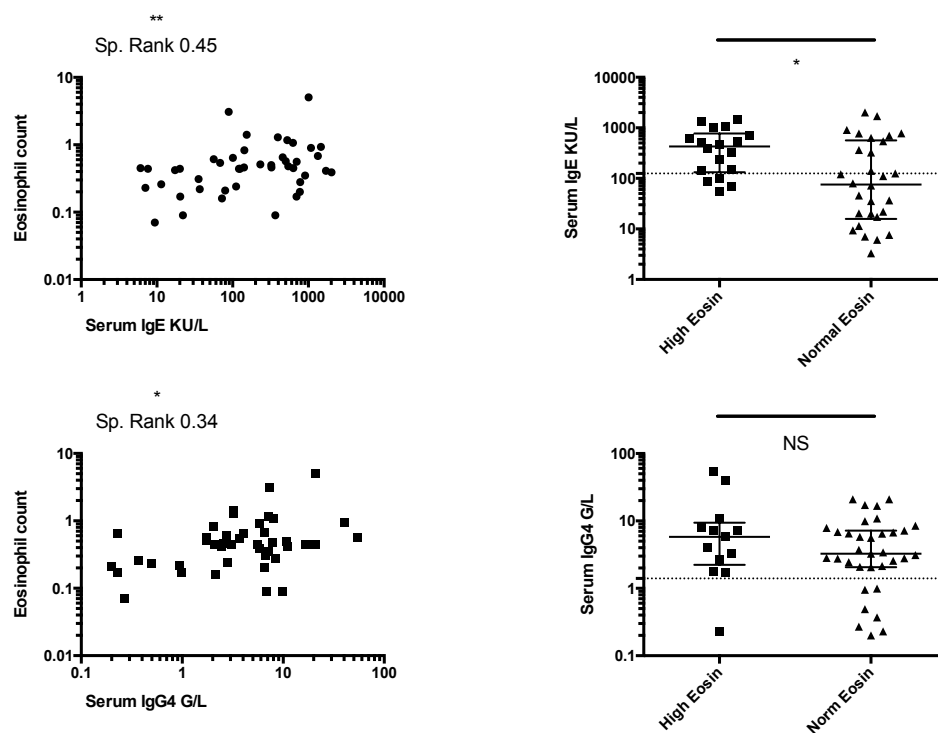


Figure 4: Correlation plots showing eosinophil count plotted against serum IgE and IgG4 concentrations. On the y-axis is Log 10 scale eosinophil count (cells/ul). On the x-axis is Log 10 serum IgE KU/L (international units) and Log 10 scale serum IgG4 G/L (grams per litre). Spearman's Rank (Sp. Rank) correlation co-efficient and p values are expressed as NS ≥ 0.05, *P < 0.05, **P < 0.01. Dot plots of Log 10 scale serum IgE and IgG4 concentrations in IgG4-RD patients with a high (upper limit is count 0.5) or normal eosinophil count. Dashed line is the serum IgE upper limit of normal 120 KU/L and serum IgG4 upper limit of normal 1.4 G/L. Mann-Whitney p values, where NS p ≥ 0.05, * p < 0.05.

5.4.4 Clinical History of Allergy and/or Atopy in IgG4-RD

A clinical history of allergy and/or atopy was identified in 62.5% of patients, compared with the healthy control cohort of 17% (p < 0.001), and national UK statistics of 20% (<http://www.allergyuk.org/allergy-statistics/allergy-statistics>). There was a higher serum IgE level in patients with a combined history of allergy or atopy than in those without (p = 0.031) (**Figure 5**). No patient had a history or evidence of parasitic infection.

Figure 5: Clinical history of allergy and/or atopy and serum IgE levels in IgG4-RD.

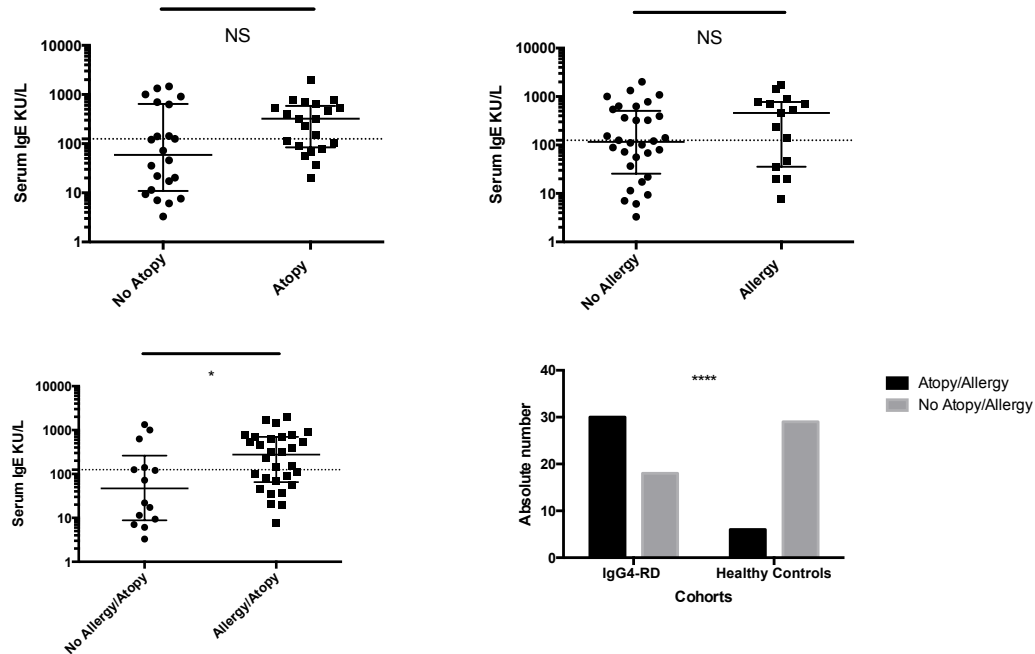


Figure 5: Dot plots showing serum IgE concentration in IgG4-RD patients with and without a history of allergy, atopy or both. The y-axis shows the Log 10 scale serum IgE concentration in KU/L (international units) and the x-axis divides patients into those with/without a clinical history of allergy, atopy or both allergy and atopy. Dashed line is the serum IgE upper limit of normal 120KU/L. Mann-Whitney p values, as per Table 3 where NS $p \geq 0.05$, * $p < 0.05$. Bottom right bar graph shows the absolute number of IgG4-RD patients and healthy controls with a clinical history and allergy and/or atopy. Fishers exact p values, **** $p < 0.0001$.

5.4.5 IgE-specific allergen response

A true definition of atopy merits a clinical history of allergic symptoms in addition to evidence of an IgE-mediated protein response. By testing for evidence of an IgE-mediated allergen-specific response in symptomatic individuals, 39.6% (19/48) of IgG4-RD patients were defined as ‘atopic’.

In total, 61.5% (16/26) of IgG4-RD patients with an elevated serum IgE level had a positive IgE-specific allergen response and 40.9% (9/22) of patients with a normal IgE had a positive IgE-allergen specific response ($p=0.246$), independent of a clinical history of atopy and/or allergy.

We then tested 16 patients with an elevated IgE and 17 patients with a normal IgE level to four broad allergen panels (grass, mould, tree and nut mixes; each containing 8 allergens). Three quarters of those with an elevated IgE level (12/16) were positive to at least one of these panels (7 to 1, 2 to 2 and 3 to 3 panels), whereas 23.5% of those with a normal IgE (4/17) were positive to a single grass allergen panel and none to other panels ($p=0.005$), as shown in **Table 3**.

Table 3: Serum IgE and allergen-specific IgE responses to four allergen panels.

Serum IgE	Grass mix	Mould mix	Tree mix	Nut mix	Total
Elevated IgE	8/16 (50%)	2/16 (12.5%)	3/16 (18.75%)	7/16 (43.75%)	12/16 (75%)
Normal IgE	4/17 (23.5%)	0/17 (0%)	0/17 (0%)	0/17 (0%)	4/17 (23.5%)

Table 3: The table shows the number and percentage of positive responses to allergen specific IgE from four different allergen panel mixes, each panel containing 8 individual allergens. The groups are divided into patients with a normal and elevated IgE level ($>120\text{KU/L}$)

5.4.6 Serum IgE to differentiate IgG4-RD from non-IgG4-RD diagnoses.

Serum IgE levels were measured in patients with an elevated serum IgG4 in both patients with IgG4-RD and a group of non-IgG4-RD patients who had other inflammatory, autoimmune and infective diseases, to investigate if IgE levels may help to discriminate the two groups. The groups were well matched; median age IgG4-RD 63 years versus non-IgG4-RD 58 years ($p=0.212$), and male gender IgG4-RD 68.8% versus non IgG4-RD 69.0% ($p=1.0$).

Considering those IgG4-RD patients with an elevated serum IgG4 only (n=39), the serum IgE concentrations were significantly higher (median 323KU/L, range 3.31-2024KU/L) than in the non-IgG4-RD diagnoses with an elevated serum IgG4 (n=42) (median 55.7KU/L, range 5.11-4457KU/L) ($p=0.0034$) (**Figure 6A**). Including all patients with IgG4-RD regardless of IgG4 level (normal n=9), the serum IgE was persistently higher (median 142KU/L, range 0-2024KU/L) compared with the non-IgG4-RD diagnoses with an elevated IgG4 ($p=0.047$).

The ability of serum IgE to distinguish IgG4-RD from non-IgG4-RD diagnoses, both with an elevated serum IgG4, is shown on the ROC curve; area under the curve (AUC) was 0.69 ($p=0.004$, 95% CI 0.57-0.81) (**Figure 6B**).

Figure 6: Dot plot (A) and ROC curve (B) of serum IgE levels in IgG4-RD patients and non-IgG4-RD disease controls.

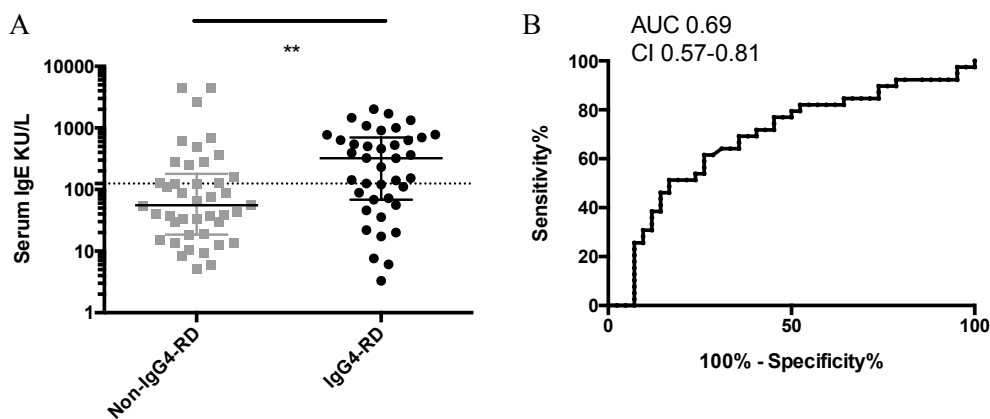


Figure 6: (A) Dot plot showing serum IgE concentration in IgG4-RD patients and non-IgG4-RD disease controls with an elevated serum IgG4. The y-axis shows the Log 10 scale serum IgE concentration in KU/L (international units) and the x-axis divides patients into those meeting criteria for IgG4-RD and those with non-IgG4-RD diagnoses. Dashed line is the serum IgE upper limit of normal 120KU/L. Mann-Whitney p values * $p<0.05$. **(B)** ROC curve shows the sensitivity and specificity of IgE

in distinguishing IgG4-RD from non-IgG4-RD diagnoses, with an elevated serum IgG4. The y-axis is sensitivity in %, the x-axis is 1-specificity in %. The area under the ROC curve (AUC) and confidence intervals (CI) are given.

At a serum IgE cut-off of 120KU/L (reference upper limit of normal), the sensitivity was 66.7%, specificity was 64.3%, NPV was 67.5%, and PPV was 63.4%, with a likelihood ratio of 1.8, to distinguish IgG4-RD from non-IgG4-RD diagnoses with an elevated serum IgG4. At a serum IgE of 480KU/L (4 times the upper limit) the sensitivity fell to 35.9%, specificity increased to 85.7%, PPV was 70% and NPV was 59%, with a likelihood ratio of 3.23. At a serum IgE of 689KU/L (5.7 times the upper limit), the sensitivity dropped to 25.7%, specificity was 92.9%, with a maximum likelihood ratio of 3.59.

5.4.7 Relationship of serum IgE to corticosteroid use in IgG4-RD.

In this cohort of 48 patients, 79.2% (38/48) received corticosteroid therapy, 14.6% (10/48) underwent a Whipples or other resection, and 6.2% (3/48) had conservative management due to severe diabetes (1), end-stage cirrhosis (1), and patient preference (1). In the group who received corticosteroid therapy, serum IgE levels fell within 2 weeks of initiation, and were significantly lower compared to pre treatment levels at 12 weeks ($p=0.0009$) (**Figure 7A**). Over a longer duration of follow-up serum IgE levels often plateaued or increased, corresponding to a time when corticosteroids were discontinued and patients were in clinical remission (**Figure 7B**).

Figure 7: Serum IgE and relationship to corticosteroid therapy in IgG4-RD.

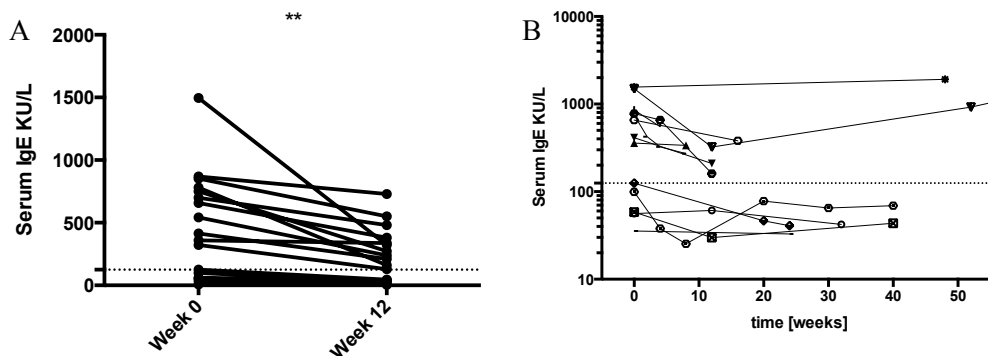


Figure 7: (A) Serum IgE concentrations in IgG4-RD patients before and at 12 weeks of corticosteroid therapy. On the y-axis is linear serum IgE concentrations KU/L (international units). On the x-axis are time points: before corticosteroids (0 weeks) and 12 weeks into corticosteroid treatment (12 weeks). Dashed line is the upper limit of IgE 120KU/L. Two-tailed paired t-test p values, where $**p<0.01$ **(B)** Serum IgE concentrations in IgG4-RD patients over 12 months of corticosteroid therapy. The x-axis shows Log 10 IgE concentrations KU/L and the y-axis is time in weeks on corticosteroid therapy. The dashed line is the same as in (A).

5.4.8 Relationship of serum IgE and disease relapse in IgG4-RD.

In the cohort of 48 patients, 43 had sufficient follow-up after initial treatment (>6 months) to determine evidence of relapse. At least one episode of biochemical and/or radiological relapse was recorded in 53.5% (23/43) of patients. Serum IgE level was higher in those IgG4-RD patients who relapsed than those who did not ($p=0.001$) (**Figure 8A and B**). However, an IgE level of 120KU/L at diagnosis (institution upper limit) did not predict relapse itself.

5.4.9 Relationship of serum IgE and organ involvement in IgG4-RD.

Multiple-organ involvement was documented in 50% (24/48) of patients. The IgE level was higher in IgG4-RD patients who had multiple rather than single organ involvement, although this did not reach statistical significance ($p=0.065$) (**Figure 8C**).

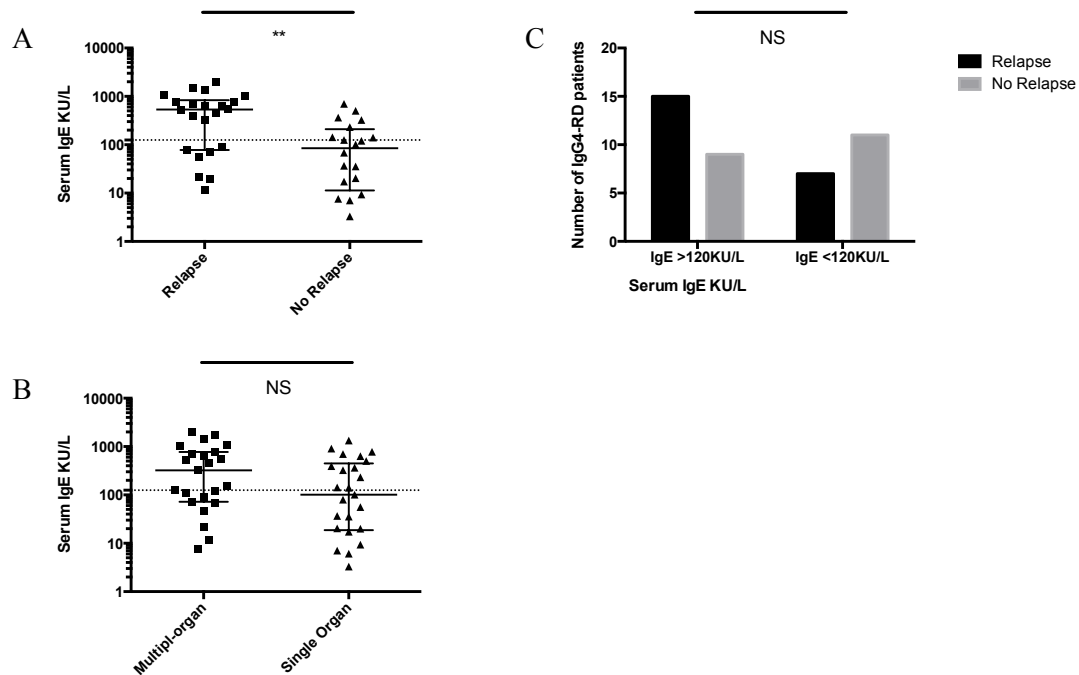
Figure 8: Relationship of serum IgE to relapse and organ involvement in IgG4-RD.

Figure 8: (A) Dot plot showing serum IgE concentrations and evidence of disease relapse. The y-axis shows the Log 10 scale serum IgE concentration in KU/L (international units) and the x-axis divides patients into those with and without evidence of biochemical and radiological relapse. Dashed line is the serum IgE upper limit of normal 120KU/L. Mann-Whitney p values NS $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$. **(B)** The bar chart shows the number of IgG4-RD patients with a high serum IgE (>120 KU/L) or a normal IgE (≤ 120 KU/L). Fishers exact p value NS $p \geq 0.05$. **(C)** Dot plot showing serum IgE concentrations and organ involvement. The x-axis divides patients into those with multiple and single organ involvement. The y-axis, dashed line and Mann Whitney p values as of (A).

5.4.10 Tissue morphology and cellular infiltrate in IgG4-RD.

Histological specimens were available from 77.1% (37/48) of IgG4-RD patients for morphological review - 15 resection and 32 biopsy specimens (some patients had both biopsy and resection specimens). The inflammatory infiltrate was composed of lymphocytes and plasma cells, often with germinal centre formation and lymphoid aggregates (**Image 1**). In the pancreas and salivary gland there was evidence of a storiform pattern of fibrosis and an obliterative phlebitis. However, not all organs had

this ‘classical’ morphology, for example the pleural biopsies had non-storiform pattern of fibrosis and lymph nodes had a reactive follicular hyperplasia with no fibrosis.

Image 1: The cellular infiltrate in a patient with IgG4-RD.

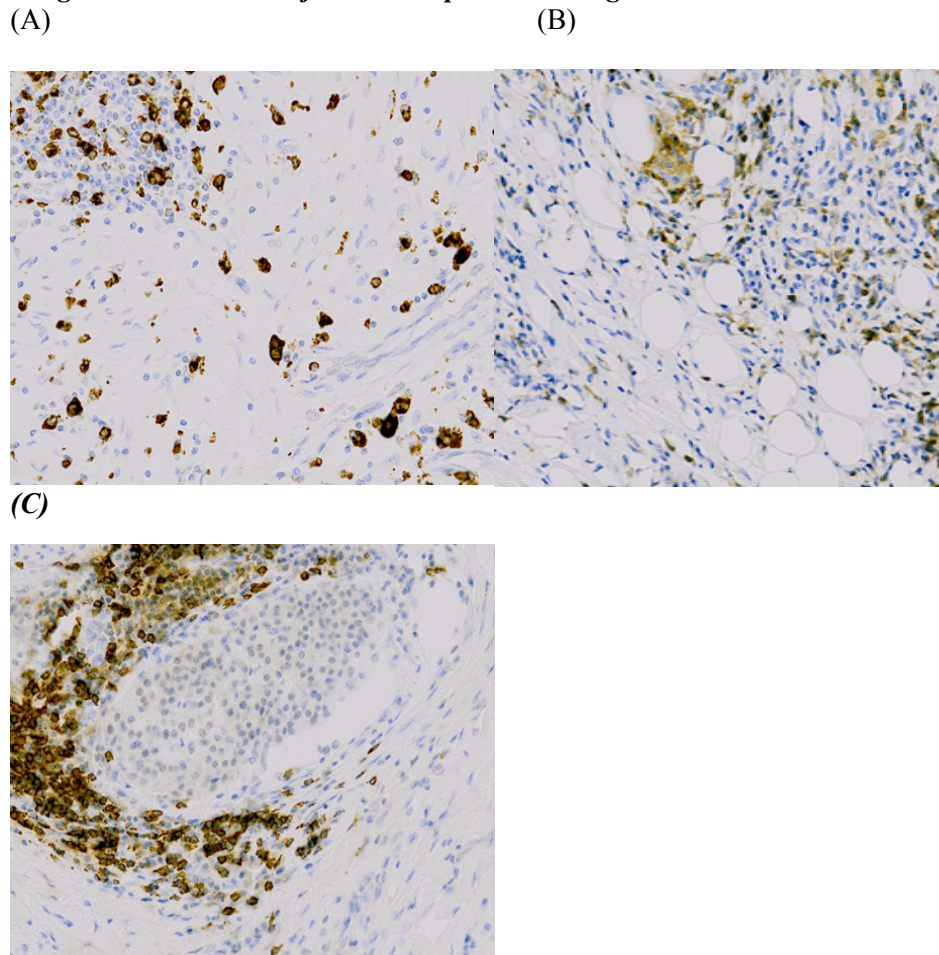


Image 1: The cellular infiltrate in a patient with IgG4-mesenteric fibrosclerosis consists of (A) CD138 plasma cells (B) CD4 T cells, (C) CD20 B cells. The CD138 staining is diffuse throughout the inflammatory infiltrate. The CD4 staining is localised within the germinal centre, lymphoid aggregates and mantle zone and CD20 staining is predominantly around the germinal centres.

On routine staining of 5 IgG4-RD and 5 disease control tissue samples, the inflammatory infiltrate in IgG4-RD consisted of predominantly CD4+ T cells (**Image 1B**), CD20+ B cells (**Image 1C**) and CD138+ plasma cells (**Image 1A**), with few CD8+ T cells or CD68+ macrophages.

5.4.11 Tissue eosinophils in IgG4-RD.

The number of tissue eosinophils in specimens was graded as ‘moderate’ (10-25 eosinophils/HPF) or ‘marked’ (>25 eosinophils/HPF) in 27% (10/37) of tissue specimens (**Image 2**). The number of eosinophils considered ‘physiologically normal’ varies between organs, and unlike in the oesophagus, there is no absolute value that defines the difference between healthy and disease. However, the grading reflected an increase in number compared to what would be expected in the tissue examined, as defined by the reporting pathologist (Dr Adrian Bateman).

Image 2: *Eosinophil granulocytes on an H&E stained section in IgG4-RD.*

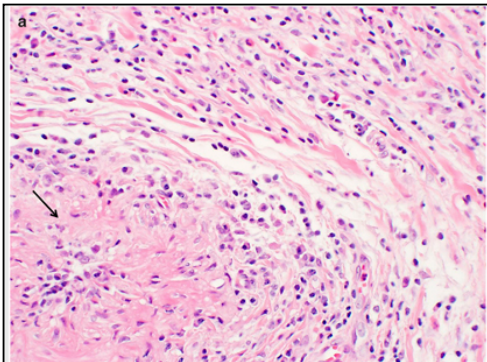


Image 2: *The H&E section shows evidence of ‘marked’ eosinophils in a lymphocytic (CD4+ T cells and CD20+ B cells) and plasma cell-rich (IgG4-positive CD138+ plasma cells) infiltrate. The eosinophils are brick red after staining with eosin (arrow), which stains the cellular cytoplasm (x100 magnification).*

5.4.12 Tissue IgE-positive cells in involved organs.

IgE immunohistochemistry was performed in 8 IgG4-RD and 6 disease control tissue specimens. The IgE-positive cell count in IgG4-RD tissues (median 10 cells/HPF, range 1-30 cells/HPF) was higher than disease control tissues (median 4 cells/HPF, range 0-10

cells/HPF) ($p=0.150$). Using the diagnostic cut off for IgG4-positive plasma cells in biopsy specimens in IgG4-RD (IgG4 count >10 /HPF), the IgE-positive cell count was >10 /HPF in 50% of samples stained (4/8) (**Table 4**).

The IgE-positive cell staining was both nuclear and surface (**Image 3**). IgE-positive cells were localised to the mantle zone of the germinal centre, within lymphoid aggregates, and also scattered throughout the inflammatory infiltrate (**Image 4**). In some samples, IgE-positive cells were in close proximity to IgG4-positive plasma cells.

Image 3: IgE immunostaining of pancreatic tissue in IgG4-RD.

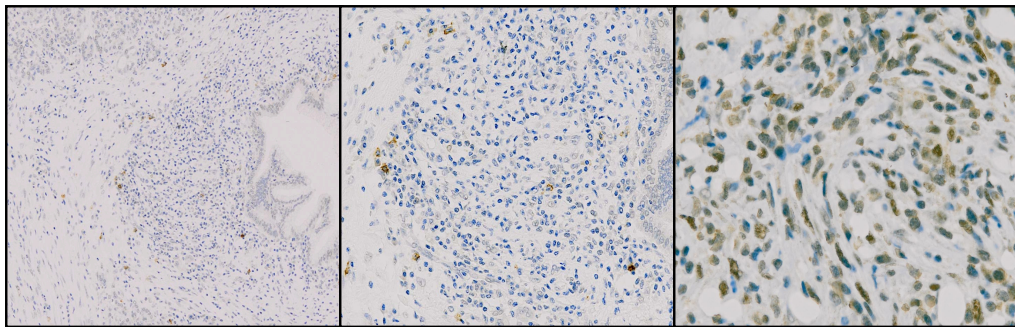


Image 3: IgE immunostaining at x50 and x100 and x200 magnification in pancreatic tissue of AIP patients. Cytoplasmic membrane labelling of IgE positive cells are shown in brown (DAB-positive).

Image 4: Distribution of IgE immunostaining in IgG4-RD and disease controls.

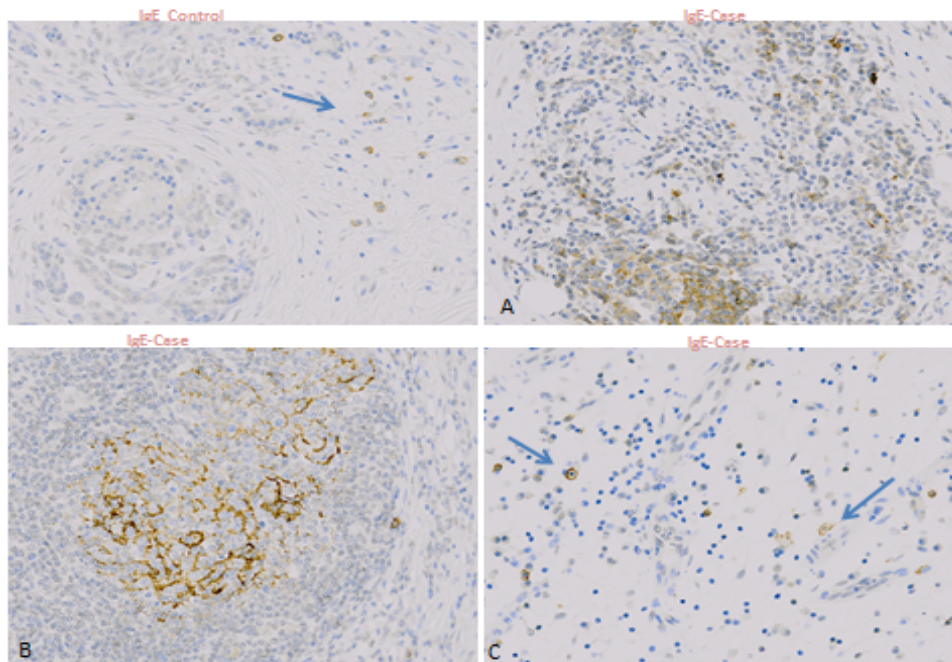


Image 4: Distribution of IgE immunostaining in IgG4-RD (cases A, B, C) and a disease control with chronic pancreatitis (control). There are a few scattered IgE-positive plasma cells (blue arrows) within the epithelium of the control pancreatic tissue. There are numerous IgE-positive cells localized within the germinal centre (A and B) of a patient with AIP. There are scattered IgE-positive cells in the salivary gland tissue (blue arrows) of a patient with IgG4-sialoadenitis (C).

Table 4: IgE immunostaining in IgG4-RD and disease controls.

Disease	Sample type	Specimen type	Mean of 3 IgE+ve cells/HPF	Description of IgE+ve cell staining
IgG4-RD	Mesenteric mass in IgG4-FS	R	7	Scattered IgE+ve cells, diffuse peripheral and central GC staining, cells in areas of fibrosis.
	Whipples in AIP	R	18	Numerous scattered IgE+ve cells with nuclear staining
	CBD in AIP and IgG4-SC	R	7	Scattered IgE+ve cells only
	Submandibular gland in IgG4-SA	R	12	Scattered IgE+ve cells and diffuse GC staining
	Whipples in AIP	R	19	Cell surface and nuclear staining. PC in close proximity.
	Whipples in AIP	R	1	Scattered IgE+ve cells only
	Pancreas and CBD in AIP	R	16	Scattered diffuse IgE+ve cells, ductal inflammation, duodenum many IgE+ve cells
	Liver in IgG4-SC	B	2	Scattered IgE+ve cells around portal tracts
Disease Controls	Reactive lymph node	R	1	Paracortex and medulla staining
	Gallbladder in chronic cholecystitis	R	3	Scattered IgE+ve cells only
	Pancreas in chronic pancreatitis	R	10	IgE+ve cells accumulate in lymphoid aggregates
	CBD and Liver specimen in Secondary sclerosing cholangitis	R	8	IgE+ve cells accumulate in lymphoid aggregates, and in periductal fibrosis
	Pancreas in chronic pancreatitis	R	0	No IgE+ve cells
	Nasal polyp	B	5	IgE+ve cells accumulate in lymphoid aggregates and in GC.

Table 4: The specimen, mean IgE count and cell distribution in tissue sections from IgG4-RD patients and disease controls. **Abbreviations:** GC: germinal centres; PC: plasma cells; CBD: common bile duct; IgG4-SA: IgG4-related sialadenitis; IgG4-FS: IgG4-mesenteric fibrosclerosis; R: resection; B: biopsy.

Morphologically the IgE positive staining cells did not look like plasma cells (no large irregular eccentric nuclei and peri-nuclear cytoplasm). Double immunostaining of IgE-

positive cells with mast cell tryptase and CD138+ plasma cells revealed these IgE-positive cells were in fact mast cells (**Image 5**). In 3 IgG4-RD patients with the highest mean count of IgE-positive mast cells, we performed a retrospective serum mast cell tryptase, which was in the normal range.

Image 5: Double immunostain of IgE and CD138 plasma cells in IgG4-RD.

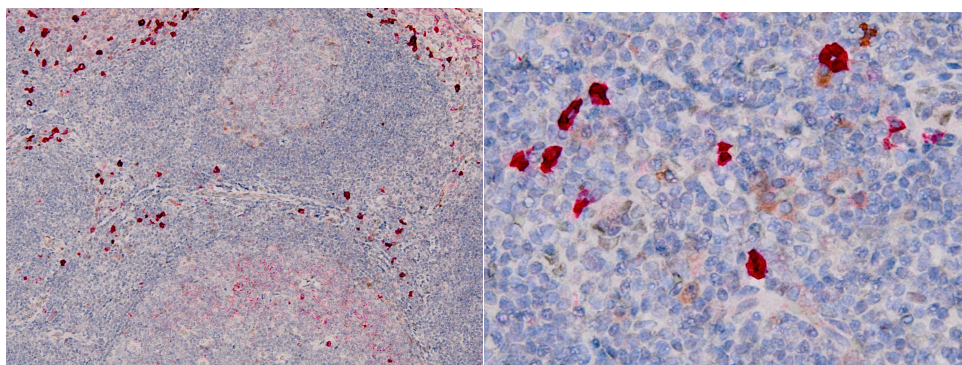


Image 5: Double-immunostaining of CD138 (red) and IgE (brown) in lymphoid tissue. The bright red plasma cells and light brown IgE-positive cells are distinct, morphologically and immunohistochemically, on the x100 (left) and x400 (right) magnification. There was no separation of staining for mast cell tryptase and IgE.

5.5 DISCUSSION

An elevated serum IgG4 and abundance of IgG4 positive plasma cells infiltrating the involved organs of IgG4-RD patients is well described (Deshpande et al, Mod Pathol 2012). However, an elevated serum IgE and the presence of IgE-positive mast cells in involved tissues are novel findings, providing new insights for pathogenic mechanisms of disease. Moreover, the utility of serum IgE in diagnosis and prognostication has been largely unexplored.

The aetiology of the elevated IgG4 and IgE levels in IgG4-RD is unknown, although the strong links between these antibodies are well described. Both IgG4 and IgE share certain requirements for induction, such as the Th2 cytokines IL4 and IL13, but respond differently to regulatory IL10, which promotes the production of IgG4-switched cells and down regulates IgE (Jeanin et al, J Immunol 1998). Furthermore, IL5 and IL6 enhance IL4-dependent IgE synthesis (Pene et al, Euro J Immunol 1988; Vercelli et al, Eur J Immunol 1989), whilst IL13 induces and IL7 potentiates IL4-independent IgG4 and IgE synthesis (Punnonen et al, Proc Natl Acad Sci USA 1993; Jeaninin et al, Blood 1998). The origin of the IgG4 and IgE cells is similarly uncertain. It is plausible that IgE-producing memory B cells and plasma cells may arise directly through a germinal centre IgE-intermediate cell (Talay et al, Nature Immunol 2012; Wang et al, Scand J Immunol 2011) or indirectly from class switching of IgG4 or IgG1 B cells (Aalberse et al, JACI 2004). Both situations could lead to elevated IgE and IgG4 concentrations in IgG4-RD, the former by sharing of Th2 conditions and the latter in a higher frequency switch from IgG4 cells.

In line with this, studies have shown that Th2-dominant (IL4, IL5, IL13) and T regulatory immune responses (IL10 and TGF-beta) are more activated in IgG4-RD in both the peripheral blood and affected tissues of patients (Zen et al, Hepatology 2007; Tanaka et al, Arthritis Rheum 2012; Nakashima et al, Clin Nephrol 2010). The Th2 cytokines IL4 and IL5 also control the accumulation of eosinophils. Hence, in IgG4-RD the IL4 and IL13 could drive IgE production, the IL4 and IL5 eosinophil accumulation, IL10 drive IgG4 production, and TGF-beta drive the process of interstitial fibrosis. The inflammatory milieu itself may indeed sustain the IgE elevation and eosinophilia observed, independent of any antigen trigger.

The presence of tissue IgE-positive mast cells in the affected organs points to the role of an IgE-mediated response. Mast cells are involved in a variety of immune responses including host defence, immune regulation, allergy, chronic inflammation, and autoimmune disease (Gri et al, Front Immunol 2012). IgE is a key stimulator of mast cells. In response to IgE stimulation, mast cells secrete mediators including Th2 and regulatory cytokines. Non-specific polyclonal IgE alone can also induce cytokine secretion, independent of any antigen (Kashiwakura et al, Allergol Int 2009). Chronic elevation of antigen-independent non-specific IgE up regulates the high-affinity IgE receptor, Fc Epsilon Receptor I (FcεRI), on mast cells, which inhibits mast cell apoptosis and promotes cytokine production (Bax et al, Front Immunol 2012). The number of FcεRI receptors on mast cells increases in the presence of elevated serum IgE levels. Indeed, this may explain the predominantly surface IgE-positive mast cell staining seen in our tissue specimens, due to very high levels of membrane-associated IgE. Alternatively, antigen cross-linking could lead to the aggregation and internalisation of FcεRI (Burton et al, Immunol Rev 2011). Therefore, IgE may also be detected in the nucleus of mast cells as a result of the internalization and endocytosis of FcεRI (Fattakhova et al, Mol Immunol, 2009). Mast cells might also contribute to fibrosis (Tsuneyama et al, J Pathol, 1999), supported by our observation of IgE-positive mast cell infiltration in an IgG4-related fibrosclerotic mesenteric mass and in the periductal and ductal fibrosis of a sclerosing cholangitis disease control (**Table 4**).

Our data supports an increased prevalence of atopy in IgG4-RD (39.6%); defined as the production of IgE against environmental antigens such as house dust mite, grass pollen, animal proteins that lead to disease including asthma, rhinitis and atopic dermatitis. A clinical history of allergy/atopy was recorded in 62.5% of IgG4-RD patients compared

with healthy controls (17%) and national population statistics (20%). The frequent clinical history of allergy is supported by data in Japanese AIP patients (Kamisawa et al, *Euro J Gastro Hep* 2009), but not by other groups (Hirano et al, *World J Gastro* 2010; Van Toorenenbergen et al, *Scand J Immunol* 2010; Della Torre et al, *Allergy* 2014), none of whom measured IgE-specific protein responses to define atopy. The concentration of total serum IgE was higher in those atopic individuals than those deemed non-atopic. This data is reinforced by a recent US study showing elevated IgE levels and peripheral blood eosinophilia predominantly seen in atopic IgG4-RD patients (Della Torre et al, *Allergy* 2014) with such atopic patients expressing up regulated Th2 cytokines in the peripheral blood, in contrast to those who were non-atopic (Mattoo et al, *Allergy* 2014). The finding of patients with an elevated total IgE concentration and no clinical allergic/atopic history in our cohort may be explained by the ability of IgG4 to block IgE-mediated immune function, as one of its important anti-inflammatory responses, (Aalbersee et al, *J Immunol* 1983; Aalberse et al, *Clin Exp Allergy* 2009), hence reducing allergic manifestations. This data highlights the potential importance of IgE-mediated responses in the pathogenic mechanism of IgG4-RD.

Serum IgE concentrations may also be a useful adjunct in confirming diagnosis and predicting clinical relapse or multi-organ involvement. In the original landmark study of serum IgG4 levels in AIP, Hamano et al found no significant difference in total IgE levels in patients compared with 20 healthy controls (Hamano et al, *N Engl J Med* 2001). However, subsequent retrospective studies reported an elevated serum IgE in 34-86% of AIP patients (Umemura et al, *Hepatology* 2007; Kamisawa et al, *Euro J Gastro Hep* 2009; Hirano et al, *World J Gastro* 2010). We demonstrated an elevated serum IgE in 56.8% of patients at diagnosis, and a positive correlation of IgE levels with serum IgG4 and eosinophil count. The utility of serum IgE in differentiating AIP from

pancreatic cancer was suggested by a Dutch study, reporting that an IgE level of 138kU/L had a sensitivity of 77% and specificity of 100%, with an AUC of 0.923 ($p=0.0003$) to distinguish the two conditions, although there were only 15 patients in each group (Van Toorenenbergen et al, Scand J Immunol 2010). Our data support the utility of serum IgE at 480KU/L (4 times the upper limit) as an adjunct in those with an elevated serum IgG4, in differentiating IgG4-RD patients from non-IgG4-RD patients, with a specificity of 85.7% and sensitivity of 35.9%. An elevated IgE concentration at diagnosis was also a marker of relapse, with a non-significant trend to identify those with multiple organ involvement. Taken together, serum IgE serves as a useful adjunct to IgG4 levels when trying to establish diagnosis in those patients with an elevated serum IgG4 and predicting risk of relapse in IgG4-RD.

Corticosteroids are currently the first line medication for IgG4-RD. In our study, both serum IgG4 (**Chapter 4**) and IgE concentrations decreased whilst on corticosteroid therapy in the first 12 weeks, with a parallel fall in disease activity in the patients. This finding was supported by an earlier observation in a retrospective cohort of AIP patients (Umemura et al, Hepatology 2007), but not by others (Hirano et al, World J Gastro 2010). Rituximab, an anti-CD20 B cell depletion therapy used in refractory disease, similarly results in a decrease in serum IgG4 and IgE levels with regression of active disease; in fact the concentration of IgE showed a steeper decline than serum IgG4 levels (Khosroshahi et al, Med Baltimore 2012). In contrast to this, total serum IgE levels do not reflect disease activity in most allergic diseases; IgE levels did not fall with corticosteroid therapy in atopic dermatitis (Gunnar et al, Br J Derm 1970) and serum IgE changes are not related to severity of asthma or allergic rhinitis (Kumar et al, JACI 1971). However, in allergic bronchopulmonary aspergillosis, IgE is a useful

marker for therapeutic monitoring (Ricketti et al, JACI 1984). This suggests a complex interplay between allergic disease and total elevated IgE in IgG4-RD.

Interestingly, a recent case report documented the successful use of an antihistamine agent (epinastine hydrochloride) alone resulting in regression of IgG4-related dacryoadenitis (Ohshima et al, J Clin Exp Hematop 2013). In vitro, antihistamines inhibit mediator release from mast cells. Furthermore, an in vitro study of human conjunctival mast cells revealed that epinastine inhibited mast cell secretion of cytokines, including IL10 (Cuss et al, Clin Exp Allergy 1999). This is an interesting potential therapeutic target, although it remains unclear why antihistamines inhibit cytokine secretion - H1 antagonism seems unrelated. Another possible therapeutic target is the prostaglandin D2 receptor, CRTH2 (chemoattractant receptor homologous molecule expressed on Th2 cells), important in allergic inflammation. It is expressed on Th2 cells and innate cells such as eosinophils and responds to mast cell derived factors (Nagata et al, J Immunol and FEBS lett 1999). The frequency of CRTh2 cells in CD4+ T cells was reportedly increased in patients with IgG4-RD, specifically IgG4-siloadenitis, with a non-significant correlation of these cells with the serum IgE and peripheral blood eosinophils (Saito et al, Int Arch Allergy Immunol 2012). We also report the upregulation of PGD2 and CRTh2 in gene expression analysis of IgG4-RD patients (**Chapter 10**). Furthermore, blockade of CRTh2 can reduce allergic inflammation in rodent models of antigen-induced airway inflammation, allergic rhinitis, atopic dermatitis, and hyper-responsiveness in asthma. These agents may be important in IgG4-RD patients with a history of allergy, elevated IgE, and eosinophilia.

5.6 SUMMARY

The data from this chapter support an increased prevalence of atopy/allergy, elevation of serum IgE, peripheral and tissue eosinophilia, and abundant IgE-positive mast cells in IgG4-RD patients. This antigen-independent non-specific IgE may bind to FcεRI on mast cells to promote the production of Th2 (IL4) and regulatory (IL10 and TGFβ1) cytokines, which induce the distinctive histological features of IgG4-RD. Mast-derived IL10, an anti-inflammatory cytokine that can suppress acquired or innate immune responses, may also reduce allergic inflammation in the affected organs (Grimbaldeston et al, Nat Immunol 2007). In addition, IL4 and IL13 can induce IgE production, which in turn stimulates mast cells. It is plausible that corticosteroids and rituximab may exert their effects through inhibition of IgE-producing B cells and plasma cells. This hypothesis requires further substantiation.

Chapter 6 Occupational exposures

Extracts from Chapter 6 have been published:

LJ Maillette de Buy Wenniger*, EL Culver*, U Beuers. Exposure to occupational antigens might predispose to IgG4-related disease. *Hepatology* 2014;60(4):1453-1454.

*denotes joint first authorship.

6.1 HYPOTHESIS

IgG4-RD patients have an increased risk of chronic exposure to occupational agents compared to other fibro-inflammatory disorders and the general population.

6.2 INTRODUCTION

The biological implications of an elevated serum IgG4 in IgG4-RD are unexplained. IgG4 represents the smallest fraction of total IgG in serum and is regarded as a regulatory antibody (van der Neut Kolfchoten et al, *Science* 2007). It has been consistently reported to be upregulated in chronic immune stimulation, as illustrated by elevation of bee-venom-specific IgG4 in beekeepers (Garcia-Robaina JC, et al, *Clin Exp Allergy* 1997). It is plausible therefore that chronic antigenic stimulation may underlie the elevated levels of serum IgG4 in IgG4-RD. Based on clinical experience in our IgG4-RD cohort, we postulated that chronic exposure to occupational antigens in these patients may be an aetiological mechanism.

This chapter describes the lifetime occupational exposures of IgG4-RD patients in two independent European cohorts (Oxford, UK and Amsterdam, The Netherlands). National statistics from both countries and disease controls were used for comparison.

6.3 SPECIFIC METHODS

Methods detailing patient identification, data storage, diagnostic criteria, definitions, serological measurements and statistics are described in **Chapter 2**.

6.3.1 Occupational history

A detailed employment and occupational exposure history was taken in 44 IgG4-RD patients, with predominant biliary (IgG4-SC) and pancreatic (AIP) involvement, and in 82 PSC patients, from Oxford, UK. The PSC group, a male-predominant disease with similar clinical and imaging characteristics to IgG4-SC, were further divided into those with an elevated serum IgG4 but no other organ involvement or histological evidence to support IgG4-SC (28 patients), and those with a normal serum IgG4 (55 patients).

A detailed employment and occupational exposure history was similarly taken in 25 IgG4- SC patients and 21 PSC patients from Amsterdam, The Netherlands (by Lucas Maillette de Buy Wenniger, PhD student).

6.3.2 Occupational exposure categories

The major occupational categories were defined using the International Standard Classification of Occupations (ISCO-08) system (**Table 1**);

<http://www.ilo.org/public/english/bureau/stat/isco/>.

Table 1: ISCO-08 Major Categories.

1. Managers
2. Professionals
3. Technicians and associated professionals
4. Clerical support workers
5. Service and sales workers
6. Skilled agricultural, forestry and fishery workers
7. Craft and related trade workers
8. Plant and machine operators, and assemblers
9. Elementary occupations (e.g. selling goods, cleaning)
10. Armed forces occupations

6.3.3 Occupational exposure questionnaire

Using a questionnaire a detailed employment history and occupational exposure was ascertained from patients and controls in Oxford and Amsterdam. In Oxford, a research nurse (Faye Highfield) who was blinded to the clinical question contacted each patient individually (either in the outpatient clinic or over the telephone) to clarify exposures.

6.3.4 National statistics

National statistics were gathered from the online UK Labour Force Survey and the UK Census for employment from 1981 in the UK. Dutch occupational labour statistics were gathered from 1980 (Praag CS, et al, SUN 1999). This year range was chosen as both groups would have been in employment at this time and the national data was readily accessible.

6.4 RESULTS

6.4.1 Demographics and characteristics

There was an older male predominance in both the IgG4-RD and PSC patient groups. In the IgG4-RD cohort, 75% (33/44) of patients were male, with a median age of 64 years (range 28 to 84 years). All had pancreatic and/or biliary disease, with other systemic organ manifestations (**Table 2**). Patients in the PSC disease control group with an elevated serum IgG4 were gender matched, 71% (20/28) of patients were male ($p=0.78$) with a younger median age of 54 years (range 23 years to 80 years) ($p=0.013$) (**Table 3**).

6.4.2 Occupational categories in IgG4-RD and PSC cohorts

The main occupation held by patients in the IgG4-RD and PSC high IgG4 patient cohorts in the UK are shown in **Figure 1**. The majority of the IgG4-RD patients were employed as ISCO-08 category 8: plant and machine operators and assemblers (29.6%), as compared to 7.2% of PSC high IgG4 patients ($p=0.035$), and 14% of the Oxfordshire population ($p=0.017$) (**Figure 2**). In contrast, the majority of the PSC high IgG4 patients were ISCO-08 category 2: professionals (21.4%) or category 4: clerical support and office workers (21.4%), compared to 13.6% ($p=1.0$) and 9.1% ($p=0.17$) in the IgG4-RD cohort, and 17% ($p=0.39$) and 4% ($p=0.007$) in the Oxfordshire population, respectively (**Figure 2**).

Figure 1: The ISCO-08 in IgG4-RD and PSC high IgG4 patients.

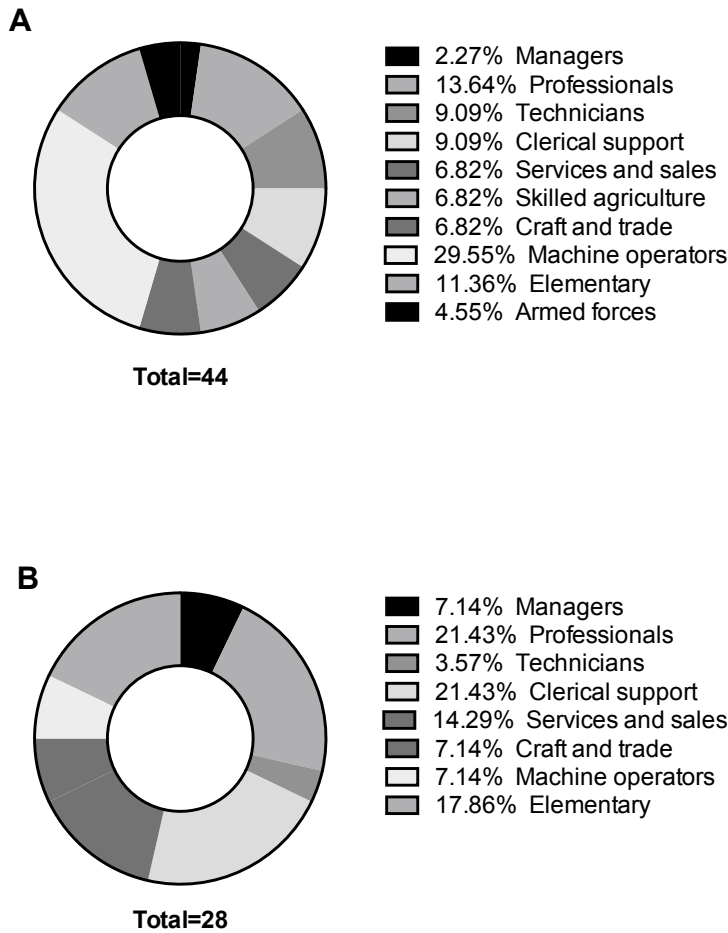


Figure 1: Donut plots showing the International Standard Classification of Occupations (ISCO-08) major category in (A) IgG4-RD patients and (B) PSC high IgG4 patients. The percentage of patients in each category is listed on the right of the plots and the total number of patients in each cohort is given at the bottom of the plot.

Figure 2: The distribution of occupations in the IgG4-RD and PSC patient cohorts and the Oxfordshire population statistics.

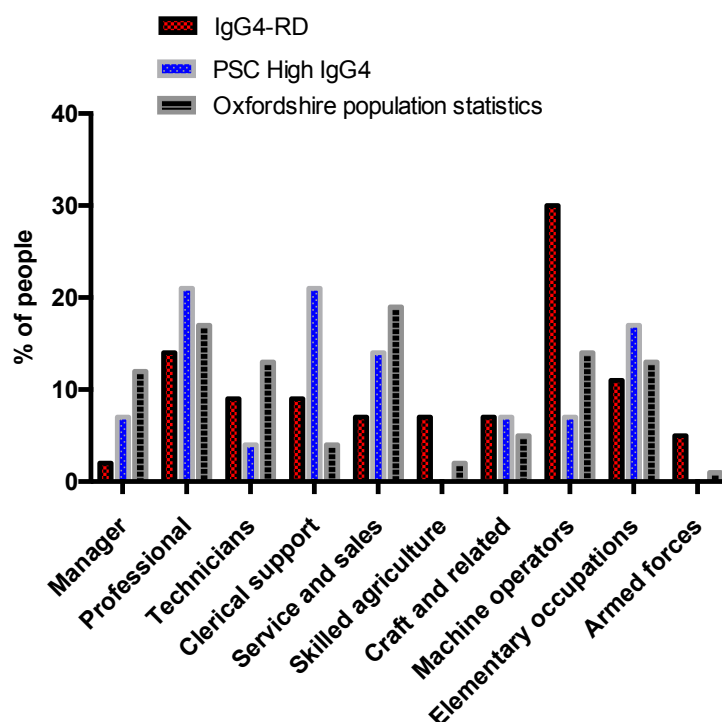


Figure 2: Bar chart showing the International Standard Classification of Occupations (ISCO-08) major category in the three groups: IgG4-RD patients, PSC high IgG4 patients, Oxfordshire population statistics. On the y-axis is the percentage of people in each category, and on the x-axis are the ISCO-08 major categories. Above the bar chart is a colour-coded key for the bars.

6.4.3 Occupational exposures in IgG4-RD and PSC cohorts

The occupation and exposure questionnaire was taken in 44 patients with IgG4-RD, with 61% of these describing ‘blue-collar’ professions during their lifetime and recalling regular (>1 year) exposure to potentially harmful compounds (**Table 2**). Intensive and prolonged exposure to solvents, industrial dusts, pesticides or industrial oils or polymers was reported by 52% of IgG4-RD patients. In the disease control cohort of 28 PSC patients with an elevated serum IgG4 level, 22% described blue-collar

work (IgG4-RD versus PSC high IgG4 patients $p<0.0001$) (**Table 3**). Among the 55 PSC patients with a normal serum IgG4, 7% reported any (often incidental) exposure to these compounds (IgG4-RD versus PSC normal IgG4 patients $p<0.0001$; PSC high IgG4 versus PSC normal IgG4 patients $p=0.004$).

Using the Labour Force Survey statistics for the UK (1981), 38% of those employed met the blue-collar work criteria (IgG4-RD versus UK national statistics $p=0.002$), and this was much lower at 28% in the South East of England from where our patient cohorts reside (IgG4-RD versus UK South East statistics $p<0.0001$; PSC high IgG4 versus UK South East statistics $p=0.42$; PSC normal IgG4 versus UK South East statistics $p<0.0001$).

Table 2: Occupation and Exposure history in the IgG4-RD patients

No.	Age years	Gender	Organs involved	Main Occupation (>1year)	Regular exposures (>1 year)
1	59.71	M	IRC	Electrician	Lead, solvents, solder, nickel, possible asbestos, epoxy resins, man-made fibres
2	78.06	M	IRC, ILD, AIP, L	Manager of a builders merchant, retail	No known hazards/exposure.
3	81.95	M	IRC, AIP	Accountant (for oil company), academia	No known hazards/exposure. Travel to Middle East and Africa
4	75.89	F	IRC, cirrhosis	Telephonist, Ammunition depot, Dairy worker, Laundry	Chemicals, gunpowder, metal (copper-tin, lead, mercury), detergents, bleach
5	80.41	M	AIP, IRC	Analytic chemist	Plutonium and other heavy metals
6	58.84	M	AIP, IRC	Technical consultant in timber research, construction	Chromium arsenate wood preservative, wood dust
7	59.82	M	IRC, AIP	Tax man, Retail (office work)	No known hazards/exposure.
8	50.59	M	IRC	Financial adviser, Carpet fitter, Solider	Glues, solvents, Mustard gas
9	66.76	M	AIP, IRC	Mechanical engineer and factory worker	Asbestos-related pleural plaques, metals, solvents, fuels
10	53.75	M	AIP	Professor - earth science and engineering	Organic chemicals (possible)
11	36.78	F	IRC, ILD	Solicitor, child care	No known hazards/exposure.
12	57.01	M	IRC, AIP	Factory machine worker, delivery man	Heavy metals, solvents, possible asbestos,
13	66.52	M	AIP, IRC	Finance office worker	No known hazards/exposure.
14	83.09	M	AIP, IRC	Gas engineer	Compressed gas, solvents
15	62.46	M	AIP, IRC	Caretaker, Motor engineer/ Mechanic	Heavy metals (brake fluids, detergents, lubricants, paint remover, thinners), lead, solvents, detergents
16	64.87	M	IRC, MP	Farmer - livestock	Organophosphates, sheep dip chemicals, pesticides

17	76.71	M	AIP, IRC, Renal	Accounting, Marketing (office based)	No known hazards/exposure.
18	81.06	M	IRC, AIP, O, P	Plastics company - sales, Author	No known hazards/exposure.
19	56.98	F	AIP, IRC	Administrative assistant, factory worker	Acids (used to clean needles), organophosphates, metals
20	80.30	F	IRC	Dinner lady	No known hazards/exposure.
21	30.56	M	AIP, IRC	Communications and Information Technology	No known hazards/exposure.
22	50.86	M	AIP	Mechanic (garage)	Industrial chemicals including heavy metals (brake fluids, detergents, lubricants, paint remover, thinners), lead, solvents
23	68.28	F	AIP, IRC	Office based - bank, teacher, publishing	No known hazards
24	59.95	M	AIP, IRC	Environmental health officer - residential inspections, Engineering	No known hazards
25	68.12	M	SA, AIP, ILD, IRC	Engineer and boiler stoker in Royal Navy	Asbestos, fuels, coal
26	54.78	M	IRC	Nuclear waste, waste management, PhD	Hydrocarbons, fuels, waste effluents
27	58.86	M	TIN, IRC, AIP	Sheep Farmer	Organophosphates, sheep dip chemicals, pesticides
28	66.09	M	AIP	Taxi driver, factory work making metal parts for cars, bus driver.	Metals, solvents
29	63.49	M	IRC, AIP	Investment banker, pharmaceutical company business	Worldwide travel but no known hazards/ exposure
30	70.87	M	IRC	Factory engineer - metal moulds and plastics, Shipping and navy	Heavy metals, solvents, plastics
31	64.29	M	IRC, AIP	Telecom engineer, Science technician, ship worker	Various chemical exposure, asbestos, lead, oils, epoxy resins
32	64.56	M	IRC, AIP	Sheet metal worker	Metals, solvents, fuel, benzene, asbestos, welding,

					MMMF dust
33	76.08	M	IRC, AIP, TIN	Mechanic	Heavy metals (brake fluids, detergents, lubricants, paint remover, thinners), lead, solvents
34	65.14	M	TIN, IRC	Courier	No known hazards/exposure.
35	69.05	M	AIP, IRC	Electrical trade, Gardener, Pigeon breeder	Lead, UVPC, solvents, solder, nickel, asbestos, epoxy resins, man-made fibres, keeps pigeons and chickens, pesticides
36	84.38	F	AIP, IRC	Clerk in radiology dept., office based work	No known hazards/exposure.
37	71.25	M	IRC, SA, ILD, AIP	Coffee grinding and production	Caffeine, chemicals
38	29.38	M	IRC	Nursing home worker/carer	No known hazards/exposure.
39	27.63	F	AIP, IRC	Window cleaner, car washing	Bleach and detergents
40	61.79	F	AIP	Accounts, office based work	No known hazards/exposure.
41	41.67	M	AIP, IRC	Warehouse worker, assembly line	Plastics, metals
42	81.28	F	IRC, ILD, MP, L	Caretaker, Soldiers barracks cleaning acid tanks, Retail	Acid and chemical (hazardous), detergents, stripping liquids
43	60.32	F	IRC, AIP	Computer Programmer	No known hazards/exposure.
44	76.64	M	AIP, LN, Renal, SA	Livestock land holder, farmer sheep and cattle	Organophosphate, sheep dip chemicals

Table 2 - Abbreviations: M; male; F: female; AIP: autoimmune pancreatitis type I; IRC: IgG4-related sclerosing cholangitis, SA: IgG4-related sialadenitis; MP: mesenteric panniculitis; ILD: IgG4-related interstitial lung disease; L: generalised lymphadenopathy; TIN: IgG4-related tubulointerstitial nephritis; Renal: IgG4-related renal lesions.

Table 3: Occupation and exposure history in PSC-high IgG4 patients

N	Age years	Gender	Disease	Liver Biopsy	Main Occupation (>1 year)	Regular exposures (> 1year)
1	63.10	M	PSC+CD	Y (OLT)	Stone mason (10 years). Retail/sales/management	Stone, dust.
2	57.56	M	PSC	Y	Insurance broker, office based	No known hazards/exposure.
3	62.01	M	PSC+UC	Y	Sales Manager	No known hazards/exposure.
4	52.95	M	PSC+UC	Y	Mental Health Nurse	No known hazards/exposure.
5	69.40	M	PSC	Y	Police Officer	No known hazards/exposure.
6	55.32	F	PSC	Y	Nursery nurse/nanny	No known hazards/exposure.
7	39.62	F	PSC	Y	Outdoor course instructor	No known hazards/exposure.
8	35.01	M	PSC+UC	Y (OLT)	Admin/office work. Painting and decorating as a teenager.	No known hazards/exposure.
9	48.78	M	PSC+CD	Y	Construction and refuse collectors	Refuse waste, metals, possible asbestos exposure.
10	58.33	M	PSC+UC	Y (OLT)	Design engineer, badminton coach	No known hazards/exposure.
11	55.67	M	PSC	Y	Taxi driver, retired	No known hazards/exposure.
12	24.71	M	PSC+UC	N	Engineer	No known hazards/exposure.
13	66.88	F	PSC+UC	Y	Dancer, hairdresser, offices and shops	No known hazards/exposure.
14	75.81	F	PSC	N	Housewife	No known hazards/exposure.
15	33.83	M	PSC+UC	Y	Police Officer	No known hazards/exposure.
16	67.25	M	PSC+CD	Y	Office based administrator, previously on car factory assembly line.	Metal exposure on assembly line.
17	75.19	F	PSC+UC	Y	Receptionist	No known hazards/exposure.
18	22.54	M	PSC+UC	Y	Retail warehouse manager, office-based.	No known hazards/exposure.
19	24.24	M	PSC	Y	Finance, accounts assistant, office	No known hazards/exposure.
20	79.94	F	PSC+UC	N	Office administration	No known hazards/exposure.
21	79.42	M	PSC	Y	Civil servant, department of health spokesman	No known hazards/exposure.
22	46.31	M	PSC	Y	Unknown	
23	52.87	M	PSC+UC	N	JCB driver	Concrete, fuel.
24	22.92	F	PSC+UC	N	Customer services	No known hazards/exposure.

25	38.49	M	PSC+UC	N	Temp office worker, fast food restaurant, teaching abroad	No known hazards/exposure.
26	73.71	M	PSC+UC	N	Forklift driver, caretaker at a factory using solvents, car assembly plant	Concrete, solvents, metals
27	74.54	M	PSC+UC	Y	Construction, metal worker, factory	Metals, solvents, asbestos exposure
28	51.04	F	PSC	Y	Sudanese, political asylum seeker, housewife	No known hazards/exposure.

Table 3 - Abbreviations: *M*; male; *F*: female; *PSC*: primary sclerosing cholangitis; *UC*: ulcerative colitis; *CD*: Crohn's disease; *Y*: yes; *N*: no.

6.4.4 Validation cohort of Dutch IgG4-RD and PSC patients

In a cohort of 25 patients with AIP and IgG4-SC in Amsterdam, 88% had a history of blue-collar work of at least one year, but often of a whole career (**Table 4**). Chronic exposure to solvents, industrial and metal dusts, pigments and oils used in the automotive industry were among the most frequent potential occupational hazards. In comparison, amongst a disease control cohort of 21 patients with PSC, only 14% reported a history of working in a blue-collar profession (AIP and IgG4-SC versus PSC $p < 0.0001$).

Dutch occupational labour statistics from 1980 (Praag CS, et al, SUN 1999) reported 47% of the employed population worked in blue-collar occupations (AIP and IgG4-SC versus Dutch national statistics $p < 0.0001$; PSC versus Dutch national statistics $p < 0.0001$).

Table 4: Occupation and exposure in the Dutch cohort with AIP and IgG4-SC.

Job history of 25 patients from the Amsterdam cohort (≥ 1 year)	Recalled regular occupational exposures (≥ 1 year)
1. Musician, painter, metal worker, carpenter	Car paint, metal, solvents, pigments
2. Carpenter	Sawdust, wood, chipboard, solvents
3. Glass worker, project manager at multinational	Glass dust, glass components, lead, barium, cobalt, nickel, lead, silica, industrial dust, building sites
4. Plasterer	Chalk dust, sawdust, wood, chip wood, solvents
5. Industrial warehouse forklift driver	Unknown (deceased)
6. Industrial fuel/waste oil laboratory, skipper	Crude oil, ship waste oil, solvents, chemicals
7. Miner, tiler, bath superintendent	Silica dust, mine dust, asbestos, solvents, glue, chlorine
8. Metal worker, textile worker	Metal dust, solvents, textiles, pigments, paints
9. Shipping	Solvents, asbestos, crude oil
10. Painter, army officer, flight arrangements, tomato farmer	Paint, pigments, solvents, kerosene, pesticides, friction plate dust
11. Painter	Paint, pigments, solvents, dust
12. Small machine factory owner	Car paint, metal dust, solvents, asbestos, oils
13. Builder, plumber	Plumbing materials, dust, sawdust, clipboard, glue, lead
14. Self-employed ophthalmician	Lens glass dust, lens plastic dust, acetone
15. Carpenter	Sawdust, clipboard, glue, solvents
16. Bricklayer, industrial cleaner of house walls	Silica dust, concrete dust, brick dust, solvents, asbestos
17. Mud worker, shipping, mud industry manager	Solvents, oil products, dust
18. Builder, painter	Solvents, sawdust, clipboard, paints
19. Car industry worker	Solvents, oil products
20. Historian, rebuilt 3 houses in last 20 years	Sawdust, silica dust, solvents, paint
21. Builder, wall miller	Silica dust, sawdust, dust, solvents
22. Hospital cleaner	Cleaning products
23. Teacher	No known exposures
24. Nurse	No known exposures
25. Unknown (deceased)	Unknown (deceased)

6.4.5 DISCUSSION

The observed high rate of chronic occupational exposure in two independent cohorts of IgG4-RD patients suggests that chronic exposure to occupational antigens may play a role in the initiation and/or maintenance of IgG4-RD in susceptible individuals. This is supported by findings of clonal expansions of IgG4-switched cells in the blood and tissues of patients with IgG4-SC by our Dutch collaborators (Maillette de Buy Wenniger LJ et al, *Hepatology* 2013), and more recently oligoclonal expansion of plasma cells in the blood of IgG4-RD patients in the US (Mamoon V et al, *JACI* 2014).

Interestingly, environmental risk factors have also been described in pemphigus foliaceus, an epidermal organ-specific autoimmune disease mediated by IgG4 autoantibodies. Chronic exposure to black fly antigens - from simuliidae bites - in peasants who lived and worked on farms in endemic states in Brazil was deemed a significant environmental trigger (odds ratio of 4.7, $P < 0.0001$), the authors suggesting that IgG4 antibodies against desmoglein 1 was initiated by exposure to the black fly antigens, which then cross-reacted with epidermal antigens and caused acantholysis and clinical expression of pemphigus (Lombardi et al, *J Invest Dermatol* 1992). Furthermore, the prevalence of desmoglein 1 antibodies was reportedly higher in endemic areas of pemphigus foliaceus, with the proportion of 'normal subjects' with positive tests increasing with closer proximity to this area, suggesting an environmental trigger with restricted geographical distribution (Warren et al, *N Engl J Med* 2000).

Theoretically, occupational exposure to carcinogenic agents, such as asbestos, mineral oils, solar radiation, silica, diesel engine exhaust, coal tars and pitches, paints and welding effluents, can predispose to cancer – in particular mesothelioma, sinonasal,

lung, nasopharynx, non-melanoma skin cancer, bladder and breast (Kauppinen T et al, *Occup Environ Med* 2000). Overall, 5.3% of all deaths by cancer (8010 cancer deaths) and 13598 cancer registrations could be attributed to occupational carcinogens or circumstances in the UK in 2004 and 2005 (Labour Force Statistics UK and the CARcinogen Exposure (CAREX) database). Approx. 56% of cancer registrations in men occurred in the construction industry and 54% in women were due to shift (night) work. Furthermore, neoplasms of B cell lineage (chronic lymphocytic leukaemia, IgG myeloma and IgA myeloma) have all been detected in patients with heavy occupational exposure to asbestos dust (Kagan et al, A, *J Med* 1979). Given the increased risk of all cancers (OR 2.25) that our group has described in IgG4-RD compared to the age and gender-matched UK population (**Chapter 3**), it is plausible that occupational exposure may be important in IgG4-RD. A specific occupational antigen has not yet been identified.

6.5 SUMMARY

This chapter describes our observation of occupational exposure in two independent cohorts of IgG4-RD patients in Oxford and Amsterdam. The majority of these patients had had a blue-collar occupation with prolonged exposures to potentially hazardous antigens. This chronic antigenic load could be one of the factors contributing to the development of IgG4-RD. This idea is supported by the production of IgG4 antibodies in the context of chronic antigenic stimulation, for example in bee-keepers. In order to investigate the relationship of occupational exposure further, an international collaboration has been developed and a robust occupational survey led from Amsterdam, involving specialists in occupational medicine. This survey will be extended to multiple European centres and involve a large control cohort of both

healthy individuals and disease controls that are age and gender matched. An international registry of IgG4-RD is being led by our group to record the incidence of malignancy and relationship to potential occupational and other carcinogens (EASL Registry Grant for IgG4-RD awarded 2015).

Chapter 7 Polyclonal response to multiple antigens.

Extracts from Chapter 7 have been published:

EL Culver, E Vermeulen, M Makuch, A van Leeuwen, R Sadler, T Cargill, P Klenerman, R Aalberse, SM van Ham, E Barnes, T Rispens. Increased IgG4 responses to multiple food and animal antigens indicate a polyclonal expansion and differentiation of pre existing B cells in IgG4-related disease. *Annals Rheum Dis* 2015;74(5):944-947.

7.1 HYPOTHESIS

Elevated serum IgG4 levels in IgG4-RD are the result of a generalised increase in IgG4 antibodies against multiple commonly observed antigens that are known to elicit an IgG4 antibody response in the general population.

7.2 INTRODUCTION

An immune-mediated pathogenesis in IgG4-RD is supported by a HLA class II association, serological abnormalities (presence of autoantibodies and elevated levels of IgG4), histological infiltrate of lymphocytes and abundant IgG4-positive plasma cells, and a dramatic response to corticosteroid therapy (Kawa et al, *Gastroenterology* 2002; Hammano et al, *N Engl J Med* 2011, Stone et al, *N Engl J Med* 2012)]. Antibodies against a range of auto-antigens have been proposed including anti-nuclear antigens, lactoferrin, carbonic anhydrase II and IV, pancreatic secretory inhibitor, trypsinogens, and an unknown 13.kDa protein (Lohr et al, *Am J Gastroenterol* 2010; Asada et al, *Pancreas* 2006). However, none has been consistently found in the disease, and the fact

that they are of the IgG1 and not the IgG4 subclass makes their overall significance unclear.

A role for *Helicobacter pylori* plasminogen binding peptide, through a process of antibody cross-reactivity with pancreatic ubiquitin-protein ligase E3 component n-recogin 2 (molecular mimicry) in genetically predisposed individuals, has been suggested in AIP (Frulloni et al, N Engl J Med 2009), and is discussed further in **Chapter 8**. Furthermore, next generation sequencing of whole blood in IgG4-related SC patients highlighted highly abundant IgG4-positive clones in the B cell repertoire, with the authors concluding that specific B cell responses are pivotal to disease pathogenesis (Maillette de Buy Wenniger et al, Hepatology 2013).

Our alternative hypothesis is that the elevated IgG4 may not be triggered by specific (auto)-antigens, but be an indirect consequence of the expansion of pre-existing IgG4-switched B cells. In this case, one would expect to find a more generalised increase in IgG4 antibodies against different commonly observed antigens that are known to elicit an IgG4 antibody response in the general population. We tested this hypothesis by (1) investigating the level and persistence of the IgG4 response to a variety of known IgG4-inducing non-infectious environmental antigens, (2) performing serum electrophoresis and immunofixation to look for evidence of clonal bands, and (3) performing tissue polymerase chain reaction for B cell receptor clonality. We analysed patients with IgG4-RD, patients with PSC and elevated IgG4 (a subset of PSC patients who have an elevated serum IgG4 level but no histological or radiological evidence of IgG4-RD), and healthy controls in a UK cohort.

7.3 SPECIFIC METHODS

Methods detailing patient identification, data storage, diagnostic criteria, definitions, serological measurements, tissue immunostaining, blood sampling and processing, radioimmunoassay, electrophoresis, tissue PCR and statistics are described in **Chapter 2**.

7.3.1 Diagnostic criteria

Patients with IgG4-RD who were referred to Oxford from February 2005 to February 2013 and followed prospectively were recruited. A total of 60 patients with IgG4-RD met diagnostic criteria, of whom 53 patients (88.3%) had an elevated serum IgG4. Of these 60 patients, 24 patients who had serology collected at presentation and 4-8 weeks after starting corticosteroid treatment were included. Patients with primary sclerosing cholangitis (PSC) referred to Oxford from January 2002 to February 2013 and followed prospectively were recruited. A total of 220 patients met PSC criteria, of whom 33 patients (15%) had an elevated serum IgG4. Of these 33 PSC patients with an elevated IgG4, 9 patients who had serology collected at presentation and had never had immunosuppressive therapies were included. A total 50 healthy controls (with no history of autoimmune disease) were recruited, of whom 2 controls (4%) had an elevated serum IgG4. Of these 50 controls, 18 who were gender-matched to the patient cohort and had serum collected were included.

The European Academy of Allergy and Clinical Immunology classification was used to record a history of allergy or atopy.

7.3.2 Serum samples for Radio Immunoassay and Electrophoresis

Serum from 24 patients (23 with elevated serum IgG4 and 1 with normal serum IgG4) was collected at disease presentation (14 patients treatment-naïve) and at 4-8 weeks after starting high dose (30-40 mg prednisolone) corticosteroids (10 patients treatment-experienced). There were 4 paired treatment samples. Serum from 9 PSC patients with elevated serum IgG4 were collected at recruitment, with no concurrent corticosteroid or immunosuppressive medication, imaging evidence to confirm no extra-biliary involvement except inflammatory bowel disease, and no evidence on liver biopsy of IgG4-SC morphology. Serum from 18 healthy controls with normal serum IgG4 and no history of autoimmune disease, corticosteroid or immunosuppressive therapies were included.

The radio immunoassay (RIA) is a sensitive *in vitro* test to determine and quantify antigen-specific antibodies in serum, as described in **Chapter 2**. Serum electrophoresis was performed in all patients and controls to check for evidence of monoclonality. In samples with a diffuse increase in immunoglobulins, immunofixation analysis was performed as a higher sensitivity method to test for the presence of monoclonal bands in the polyclonal gamma region, as described in **Chapter 2**.

7.3.3 Tissue samples for Polymerase Chain Reaction

Tissue specimens from 5 patients with IgG4-RD (5 resection specimens; pancreas (2), bile duct and pancreas (1), parotid gland (1), submandibular gland (1), lymph node (1)) were collected at disease presentation and used for clonality assessment. DNA for PCR amplification was extracted from formalin fixed paraffin embedded tissue blocks and

multiplex primers were used to determine B cell IgG heavy and light chain clonality, as described in **Chapter 2**.

7.3.4 Statistical analysis

A two-tailed Mann-Whitney test and an ANOVA using log-transformed data were used to compare IgG4-specific antigen responses in IgG4-RD patients, PSC patients and healthy controls. A two-tailed Mann-Whitney was used to compare responses in treatment-naïve and treatment-experienced IgG4-RD patients. Spearman's Rank correlation plots were used for serum immunoglobulin levels and IgG4-specific antigen responses. Statistics were calculated using Graphpad Prism v6.0 and a p-value of <0.05 was considered statistically significant.

7.4 RESULTS

7.4.1 Characteristics of the cohort

The demographics, clinical characteristics and serum immunoglobulin values of patients and controls are shown in **Table 1**. There was an older male predominance in both the IgG4-RD and PSC high IgG4 patient groups. The majority (83%) of the IgG4-RD patients had pancreatic (AIP) and/or biliary (IRC) involvement, with 71% having other systemic organ involvement. As expected, the concentration of serum total IgG, IgG4, IgE, and to a lesser extent IgG1, was higher in IgG4-RD patients versus healthy controls, as well as in the PSC-high IgG4 patients versus healthy controls (**Figure 1**).

Table 1: Clinical characteristics and serum immunoglobulin values.

	IgG4-RD patients	PSC high IgG4	Healthy controls	P values (ANOVA)	IgG4-RD vs PSC (KW)	IgG4-RD vs HC (KW)	PSC vs HC (KW)
Absolute number	24 14 naïve 10 experienced	8	18				
Age years median (range)	65 (32-84)	65 (35-76)	48.5 (24-67)	0.001	0.7085	0.0007	0.0905
Gender (male) %	87.5	62.5	55.6	0.608			
Organs involved	83.3	0	0				
AIP/IRC %	70.8	0	0				
Systemic OOI %							
Allergy or atopy %	41.67	25.0	37.5	0.073			
Serum IgG G/L median (range)	19.55 (8.24-59.1)	14.15 (11.6-20.1)	10.22 (8.3-14.5)	<0.0001	0.0727	<0.0001	0.1054
Serum IgG1 G/L median (range)	8.27 (5.25-32.2)	8.26 (6.94-14.6)	5.98 (4.37-7.31)	0.0104	0.9512	0.0126	0.0622
Serum IgG4 G/L median (range)	8.22 (0.37-54.1)	2.33 (1.4-3.53)	0.31 (0.04-0.9)	<0.0001	0.0186	<0.0001	0.0002
Serum IgE G/L median (range)	397.5 (5.9-1006.0)	57.1 (29.6-125)	39.65 (7.75-300)	0.0025	0.1148	0.0019	0.5994

Table 1: The table shows clinical characteristics and serum immunoglobulin levels in IgG4-RD patients, PSC patients and healthy controls. ANOVA with multiple comparisons *p* values; NS $p \geq 0.05$, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$. **Abbreviations:** IgG4-RD: IgG4-related disease; PSC: primary sclerosing cholangitis; AIP: autoimmune pancreatitis; IRC: IgG4-related sclerosing cholangitis; OOI: other organ involvement; HC: healthy controls; ; vs: versus; naïve: treatment-naïve; experienced: treatment experienced after 4-6 weeks of corticosteroids; KW: Kruskal-Wallis test with multiple comparisons; N/A: not applicable.

Figure 1: Serum IgG, IgG1, IgG4 and IgE levels in treatment-naïve IgG4-RD patients, PSC high IgG4 patients and healthy controls.

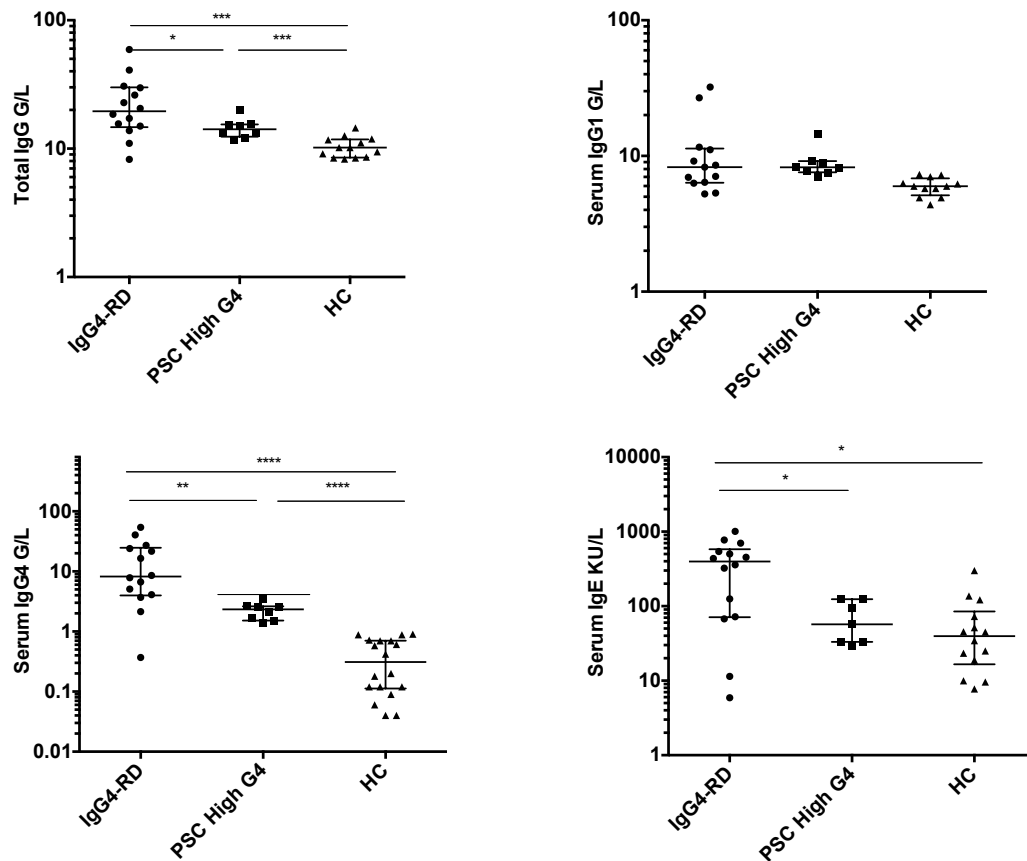


Figure 1: The dot plots show serum IgG, IgG1, IgG4 and IgE levels in treatment-naïve IgG4-RD patients, PSC high IgG4 patients and healthy controls. On the X-axis is IgG4-RD – IgG4-RD patients, PSC High G4 –PSC patients with elevated serum IgG4 levels, HC – healthy controls with normal serum IgG4. On the Y-axis is serum immunoglobulin levels, log 10 scale, IgG, IgG1 and IgG4 in grams per litre and IgE in international units KU/L. Error bars represent median and interquartile range. Mann-Whitney test p values * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

7.4.2 Antigen specific responses

We analysed the IgG4 response to proteins from egg, milk, peanut, banana, rice, wheat, and cat. In line with our hypothesis, the response to egg ($p=0.004$), milk ($p=0.04$), peanut ($p=0.0003$), cat dander and serum ($p=0.012$), rice and wheat ($p=0.006$) antigens was found to be significantly higher in IgG4-RD patients than in healthy controls, and the response to egg ($p=0.03$), cat dander and serum ($p=0.04$), and rice and wheat ($p=0.01$) antigens was higher in PSC-high IgG4 patients than in healthy controls (Figure 2).

Figure 2: IgG4 antigen-specific responses in treatment-naïve IgG4-RD patients, PSC high IgG4 patients, and healthy controls

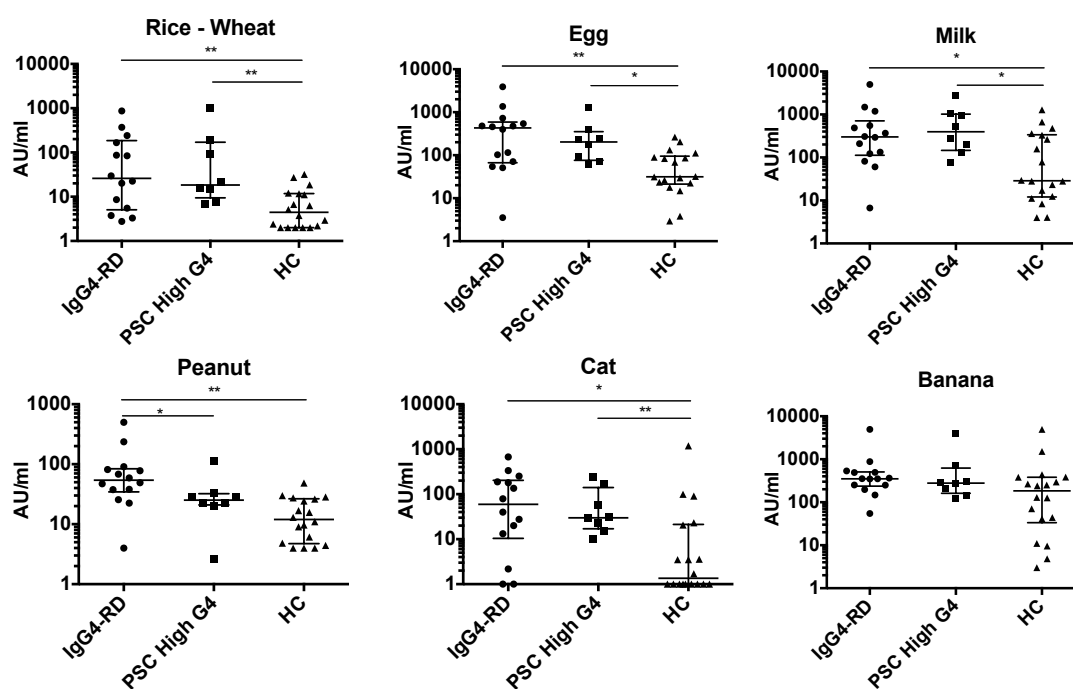


Figure 2: The dot plots show IgG4 antigen-specific responses in treatment-naïve IgG4-RD patients, PSC high IgG4 patients, and healthy controls. X-axis labels as in Figure 1. Antigens were rice and wheat, egg, milk, peanut, cat dander and serum, banana. On the Y-axis is IgG4 specific antigen response, log 10 scale, in arbitrary units per ml. Error bars represent median and interquartile range. Mann-Whitney test p values * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

We further examined the correlation of serum immunoglobulin levels with antigen specific responses. In IgG4-RD patients there was a positive correlation between serum IgG4 levels, and IgG4 responses to banana (Rank 0.38, 95% CI -0.002-0.67, $p=0.045$), peanut (Rank 0.49, 95% CI 0.14-0.74, $p=0.007$), cat dander and serum (Rank 0.61, 95% CI 0.29-0.80, $p=0.0006$), rice and wheat (Rank 0.38, 95% CI -0.002-0.67, $p=0.045$) antigens (**Figure 3**). In contrast, total serum IgE levels did not correlate with antigen-specific responses (not shown).

Figure 3: Correlation plots of serum IgG4 and IgG4-specific antigen responses in treatment-naïve and experienced IgG4-RD patients

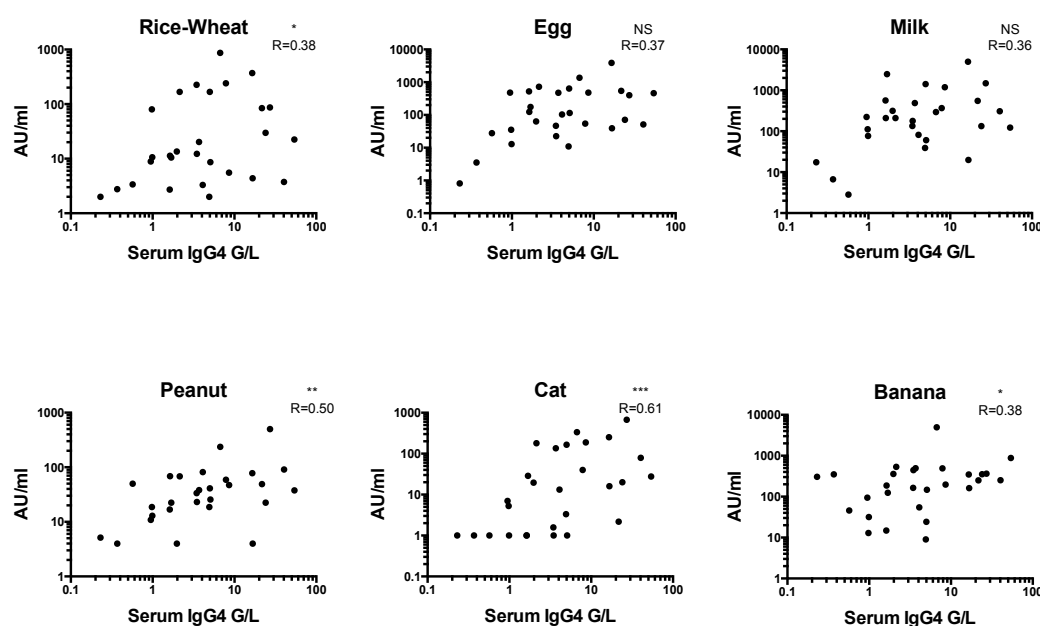


Figure 3: Correlation plots of serum IgG4 and IgG4-specific antigen responses in treatment-naïve and experienced IgG4-RD patients. Antigens were rice and wheat, egg, milk, peanut, cat dander and serum, banana. On the X-axis is serum IgG4, log₁₀ scale, in grams per litre. On the Y axis is IgG4 specific antigen response, log₁₀ scale, in arbitrary units per ml. Spearman's rank correlation R values and p values * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

7.4.3 Treatment-naïve and experienced IgG4-RD patients

We also tested the differences in immunoglobulin levels and IgG4-specific antigen responses in 14 treatment-naïve and 10 IgG4-RD patients receiving corticosteroid therapy (treatment-experienced). Levels of antibodies to banana ($p=0.001$), egg ($p=0.039$), peanut ($p=0.003$), and cat ($p=0.006$) antigens were significantly lower in treatment-experienced compared with naïve patients (Figure 4), as were serum total IgG ($p=0.017$) and IgG4 ($p=0.001$) (Figure 5).

Figure 4: IgG4 antigen-specific responses IgG4-RD patients.

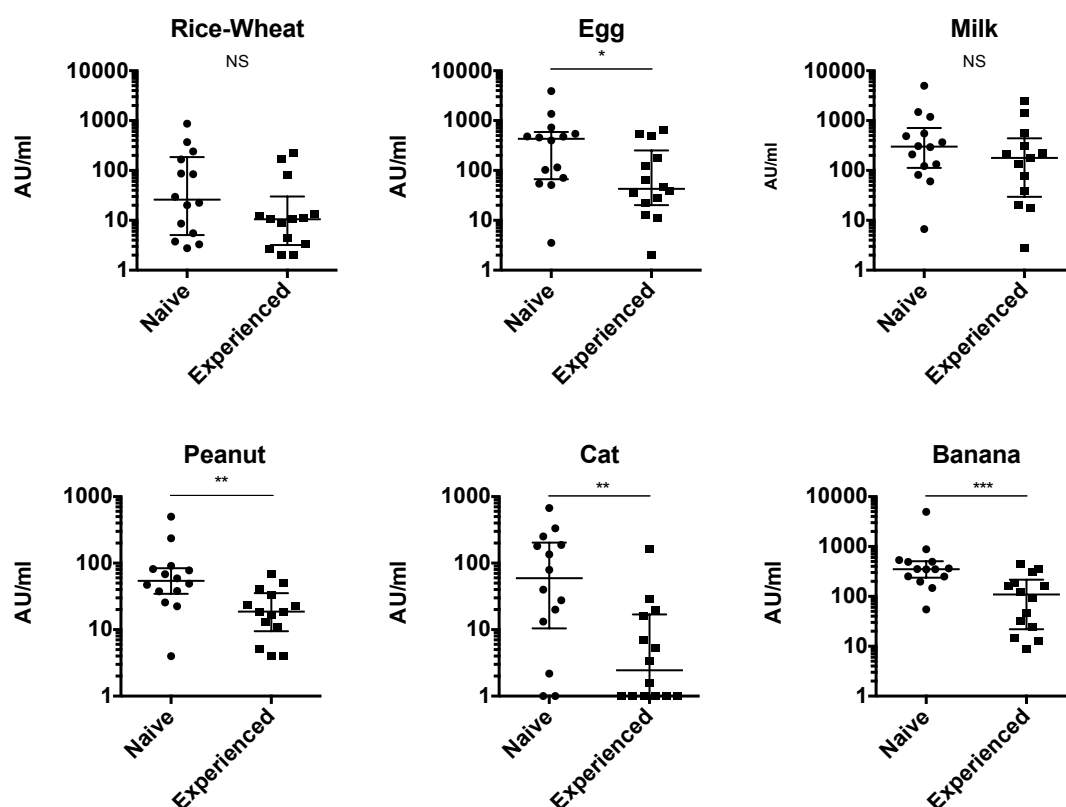


Figure 4: The dot plots show the IgG4 antigen-specific responses in treatment-naïve and treatment-experienced (on corticosteroids) IgG4-RD patients. Antigens were rice and wheat, egg, milk, peanut, cat dander and serum, banana. Units as in Figure 1. Error bars represent median and interquartile range. Mann-Whitney * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.

Figure 5: Serum IgG, IgG1, IgG4, IgE levels and IgG4/IgE ratio in IgG4-RD.

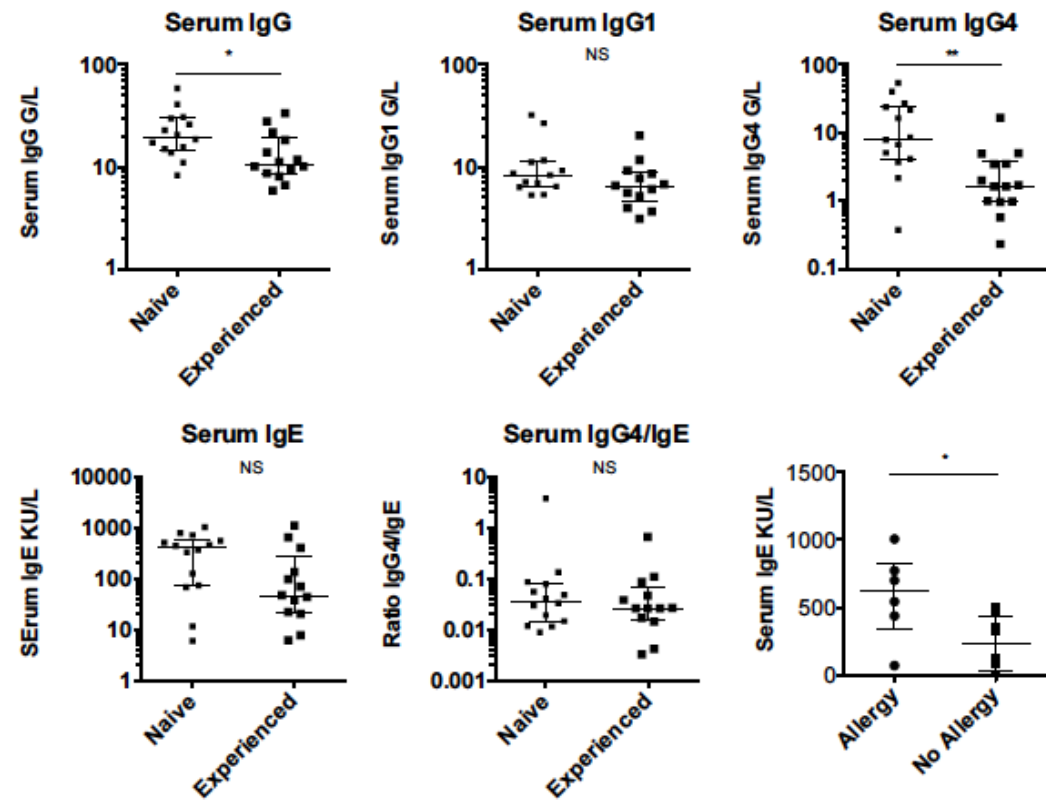


Figure 5: The dot plots show the serum IgG, IgG1, IgG4, IgE levels and IgG4/IgE ratio in treatment-naïve and treatment-experienced (on corticosteroids) IgG4-RD patients. The last dot plot shows the serum IgE level in patients with and without a history of allergy. Units as in Figure S1. Error bars and Mann-Whitney as per Figure 4.

7.4.4 Serum Electrophoresis

We analysed serum electrophoresis data for evidence of monoclonality in patients and controls, but no monoclonal bands in the polyclonal gamma region was observed (**Figure 6A**). All individuals showed polyclonality; 11 of 14 (78.6%) IgG4-RD patients, 3 of 9 (33.3%) PSC high IgG4 patients, and 0 of 18 healthy controls showed hypergammaglobulinaemia, and none showed a hypogammaglobulinaemic profile. Further immunofixation analysis of patient samples with a diffuse increase in immunoglobulins, using IgG, IgA, IgM, kappa light chain and lambda light chain antisera, confirmed no monoclonal bands in the polyclonal gamma region (**Figure 6B**).

Figure 6: Serum electrophoresis in a patient with multiple myeloma, IgG4-RD and a healthy control, and serum immunofixation in a patient with IgG4-RD.

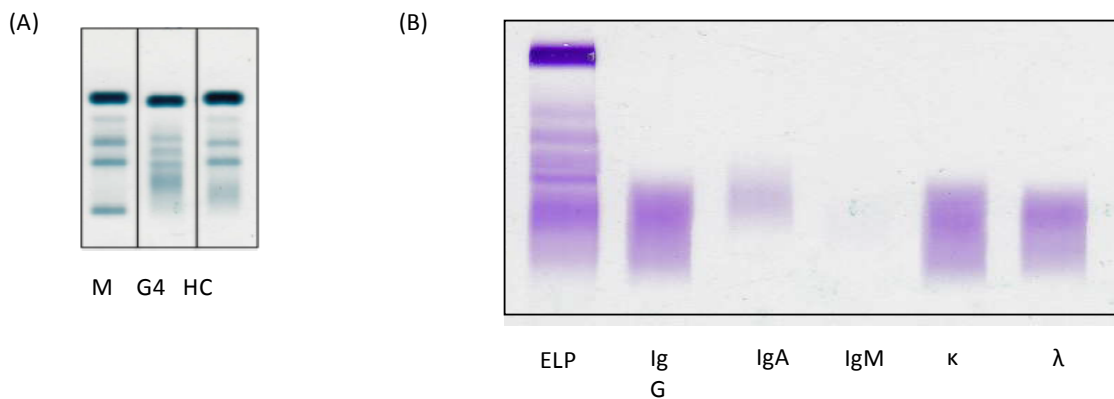


Figure 6: A. Representative serum protein electrophoresis gel in a patient with multiple myeloma (M), patient with IgG4-RD (G4) and a healthy control (HC). B. Representative negative serum protein immunofixation using antisera directed at heavy chains of IgG, IgA and IgM plus Kappa (κ) and Lambda (λ) light chain in an IgG4-RD patient who displayed hypergammaglobulinaemia. ELP is the original standard gel control.

7.4.5 Tissue PCR for clonality

Lastly, we performed PCR on extracted DNA from IgG4-RD patient tissue samples, using multiplex primers for B cell immunoglobulin heavy and light chains. There was no evidence of clonality (**Table 2**). All 6 IgG4-RD patients had polyclonal heavy and light chain rearrangement, with a gaussian distribution (**Figure 7**).

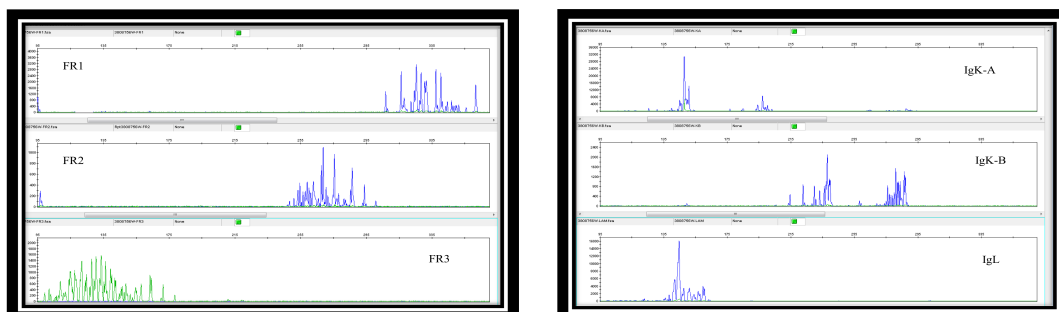
Table 2: Tissue PCR for B cell clonality in IgG4-RD patients

Tissue	IgH VH FR1JH	IgH VH FR2JH	IgH VH FR3JH	IgL K-A	IgL K-B	IgL-L
Pancreas	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Polyclonal
Submandibular gland	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Polyclonal
Ampulla and Pancreas	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Polyclonal
Pancreas and bile duct	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Polyclonal
Parotid gland	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Polyclonal
Lymph node	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Polyclonal	Polyclonal

Table 2: The table shows the results of tissue polymerase chain reaction (PCR) for B cell clonality using multiplex primers for variable immunoglobulin heavy (VH) region FR1-3 and immunoglobulin light (L) chain kappa (K) and lamda (L) rearrangements.

Figure 7: Gene profile scan of Ig heavy and light chains in an IgG4-RD patient.

A



B

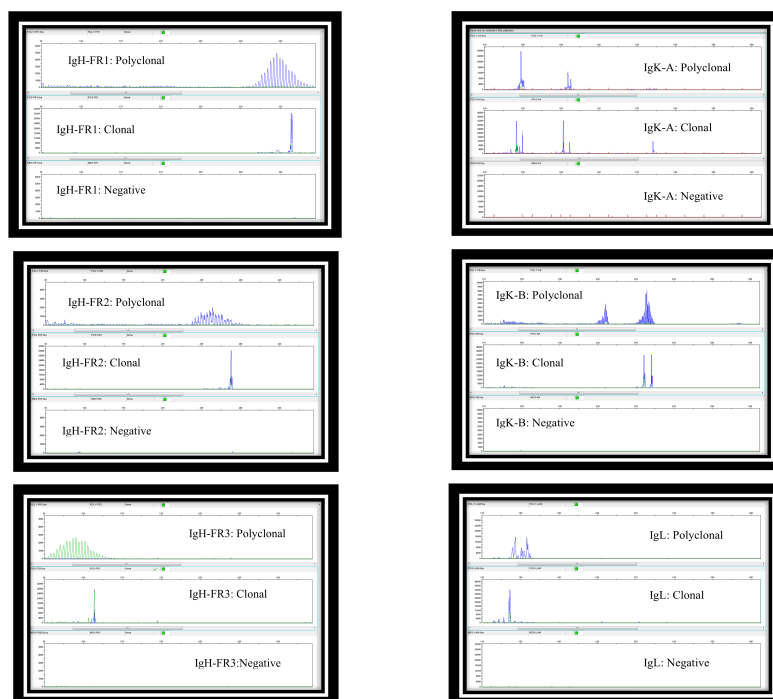


Figure 7: (A) Representative gene profile scan of Immunoglobulin heavy (FR1, FR2, FR3) and light chains (Kappa A and B, Lamda) in an IgG4-RD patient. (B) Representative control scans showing a polyclonal distribution, clonal distribution and negative scan for heavy chain region 1, 2 and 3, kappa and lambda chains are shown for comparison.

7.5 DISCUSSION

A single antigen responsible for driving IgG4-RD has yet to be found. Importantly, our data show that IgG4-RD is associated with an elevated IgG4 response to multiple diverse antigens. These results suggest that the elevated levels of IgG4 in IgG4-RD patients and PSC-high IgG4 patients may be the result primarily of a polyclonal expansion of many IgG4 B cells irrespective of their specificity. This would imply that factors other than antigen induce an expansion and differentiation of IgG4 B cells. However it does not exclude the possibility that either a single antigen (self or exogenous) serves as the initial trigger or that the polyclonal IgG4 plays an active role in the disease process.

The elevated levels of IgG4 in IgG4-RD patients, targeting multiple antigens that are known to be associated with an IgG4 B cell response, make it plausible that pre-existing subsets of IgG4 B cells expand and differentiate in the course of developing IgG4-RD. Whilst the clonality of the IgG4 response makes it unlikely that these B cells initiate IgG4-RD, it does not preclude it to be an important contributing factor to maintenance of disease progression once established. The IgG4-switched B cells may be selectively triggered to expand and differentiate into plasma cells. This may involve IL21 driving proliferation and expansion of IgG4-switched cells, and the up-regulation of activation-induced cytidine deaminase (AICDA), B lymphocyte induced maturation protein 1 (BLIMP1) and Xbox protein 1 (XBP1), all of which have been shown to be up regulated in patients with various organ manifestations of IgG4-RD (Kuchen et al, J Immunol 2007; Tsuboi et al, Arthritis Res Ther 2012). Alternatively, there may be factors that enhance class switch to IgG4 such as IL4 and IL13 driving class switch of both IgG4 and IgE, and IL10-producing T and B regulatory cells driving a switch more

specifically to the IgG4 subclass (Jeannin et al, J Immunol 1998; Miyoshi et al, Pancreas 2008). Further research is necessary to determine if the high frequencies of IgG4 plasma cells are predominantly the result of disease-associated class switch or disease-associated expansion and terminal differentiation of IgG4 B cells.

The fall in IgG4-specific antigen responses after corticosteroids may be explained by suppressed proliferation of expanded IgG4-switched B cells or suppressed differentiation into IgG4 plasmablasts. Corticosteroids interfere with the production of cytokines critical for T cell proliferation, and the binding of interleukins to B cells suppressing proliferation and antibody production. The steroid effect highlights the rapid turn over of the IgG4-producing plasma cells, as opposed to production by long-lived plasma cells in the bone marrow.

Evidence for polyclonality in IgG4-RD is supported by the diversity of antigen specificity, and by serum electrophoresis and immunofixation analysis where no monoclonal bands in the polyclonal gamma region was observed (**Figure 6**). However, this is complicated by the ability of the bispecific IgG4 antibody to undergo Fab-arm exchange (known to take place *in vivo*), which limits the potential to detect an oligoclonal response where multiple clones have comparable abundance. In model systems using chimeric antibodies, Fab arm exchange can affect the electrophoretic mobility of IgG4, although if >50% of the IgG4 would be monoclonal then >25% would still remain monoclonal after fab-arm exchange. Recently, oligoclonal somatically hypermutated plasmablast populations were demonstrated by next generation sequencing in patients with active IgG4-RD (Mattoo et al, J Allergy Clin Immunol 2014). Further evidence for the absence of a clonal population of B cells is

provided by PCR using multiplex primers in the involved tissue of patients with IgG4-RD. This method is used routinely in the detection of clonal proliferations in classical and B-cell Non-Hodgkin lymphomas, detecting even small numbers of malignant cells (detection rate 79%) (van Dongen et al, Leukemia 2003; Hebeda et al, J Hematop 2009).

Mechanisms responsible for driving IgG4-RD (e.g. Th2 cytokines) may also increase IgE B cell expansion in certain individuals. In this instance one may expect an increased predisposition to allergies in later years of life. However, the frequency of allergy/atopy was not significantly different between patient and control groups (**Table 1**). Furthermore, there was no significant differences between total and antigen-specific IgG4 responses in patient and control groups (data not shown). However, serum IgE levels in IgG4-RD patients with allergy/atopy were higher than in those without ($p=0.0255$) (**Figure 5**); consistent with Della Torre et al (Allergy, 2014). Therefore, it is plausible that elevated serum IgG4, resulting from polyclonal expansion of many IgG4 B cells irrespective of antigen specificity, may be linked to IgE B cell expansion in a subset of allergic/atopic individuals with IgG4-RD.

7.6 SUMMARY

In summary, this is the first study to show a polyclonal IgG4 response to multiple non-infectious environmental antigens in IgG4-RD. Multiple different antigen responses are higher in treatment-naïve IgG4-RD patients compared to those on corticosteroids. The elevated IgG4 levels may reflect aberrant immunological regulation of the overall IgG4 response in the disease.

Chapter 8 *Helicobacter pylori* as a microbial antigen.

8.1 HYPOTHESIS

There is no role for *Helicobacter pylori* plasminogen binding peptide as a single antigen initiating IgG4-RD in genetically susceptible individuals.

8.2 INTRODUCTION

A role for antigen presentation in the aetiology of IgG4-RD is supported by the HLA class-II DR and DQ association in AIP, an increase in IgG4 class-switched cells, and the discovery of oligoclonal B cells and plasma cells in the blood and tissue (Kawa et al, Gastroenterol 2002; Maillette de Buy Wenniger et al, Hepatology 2013; Mattoo et al, J Allerg Clin Immunol 2014). One such antigen hypothesised to be causally related to AIP was *Helicobacter pylori* (*H. pylori*).

H. pylori is a gram-negative spiral flagellated bacterium, with a circular chromosome comprising more than 1400 genes, which colonises the gastric mucosa of adults, and is responsible for the majority of duodenal and gastric ulcers (Marshall & Warren, Lancet 1984). A high prevalence (34.8%) of gastric ulcers has been reported in AIP patients, with close proximity of the ulceration to inflammatory sites at the ampulla, common bile duct and head of the pancreas (Shinji et al, Gastrointest Endoc 2004). *H. pylori* has also been associated with other autoimmune conditions, such as autoimmune sialadenitis, primary biliary cirrhosis, PSC, autoimmune hepatitis, and hepatitis C virus-

related liver disease (Nilsson et al, J Clin Microbiol 2000; Nilsson et al, J Physiol Pharmacol 1999; Leong et al, Aliment Pharmacol Ther 2002; Dore et al, Dig Dis Sci 2002). This led to the hypothesis that molecular mimicry between *H. pylori* and the host might trigger AIP through induction of auto-reactivity and apoptosis of pancreatic tissue in genetically predisposed individuals (Kountouras et al, J Cell Mol Med 2005).

Further support for this hypothesis came from *in silico* protein analysis, in which protein sequences of potential pancreatic auto-antigens human carbonic anhydrase II (CA-II) and lactoferrin (LF) were compared to all known *H. pylori* proteins (Guarneri et al, J Cell Mol Med 2005). There was significant homology between CA-II and the *H. pylori* α -carbonic anhydrase (α -CA), an enzyme fundamental to the bacterium's survival and proliferation in the acidic gastric environment. Moreover, the homologous segments contained the binding motif of HLA DRB1*0405, the susceptibility allele for AIP reported in the Japanese population (Kawa et al, Gastroenterol 2002). Increased serum IgG4 concentrations were also shown to strongly correlate with elevated CA-II antibody levels in AIP patients (Aparisi et al, Gut 2005). This strengthened the argument that the host immune system could develop a response against α -CA in genetically predisposed individuals, which could in turn cross-react with the pancreatic auto-antigen CA-II.

In a landmark paper, Frulloni *et al.* provided further evidence for a role of a different *H.pylori* antigen in AIP (Frulloni et al, N Engl J Med 2009). After screening an *E. coli*-based random dodecamer peptide library with pooled immunoglobulin purified from AIP patients, they mapped potentially relevant peptides using a dissociation-enhanced lanthanide fluorescence immunoassay (DELFI) method

and found a peptide epitope recognized by 18 of 20 AIP patients. Comparison with all known bacterial protein sequences revealed that this epitope contained a high degree of homology with an amino acid sequence of the *H. pylori* plasminogen-binding protein type A (PBP). The majority (95%; 19/20) of AIP patients had serological IgG antibodies against PBP, whilst 10% (4/40) of pancreatic cancer patients, and none of those with alcohol-induced chronic pancreatitis, other autoimmune disease, or healthy controls had antibodies. They validated this in a second Italian AIP cohort, yielding similar results (Frulloni et al, N Engl J Med 2009). The PBP was compared with all known human pancreatic proteins, and showed homology with the ubiquitin-protein ligase E3 component n-recognin 2 (UBR2), an enzyme expressed in acinar cells of the pancreas. Reactivity against this protein was detected in 83% (22/35) serum specimens from AIP patients, and not in patients with other pancreatic diseases. This led to the theory that in AIP there is autoimmunity against pancreatic acinar cells, via molecular mimicry with *H. pylori* PBP. However evidence to support this conclusion has never been shown.

In this chapter, the presence and role of *H.pylori* as a microbial antigen in a prospective IgG4-RD cohort is investigated. Patients with autoimmune disease and an elevated serum IgG4 and healthy donors are used as control groups. Firstly, clinical history and endoscopic evidence of gastric inflammation and peptic ulceration is established. Secondly, the prevalence of anti-*H.pylori* IgG positivity in serum and *H.pylori* organisms in gastric tissue specimens is determined. Lastly, evidence of *H.pylori*-specific T cells producing Th1 and/or Th2 cytokines, and *H.pylori*-specific B cell IgG and IgG4 immunological memory is investigated.

8.3 SPECIFIC METHODS

Methods detailing patient identification, data storage, diagnostic criteria, definitions, serological measurements, tissue immunostaining, blood sampling and preparation, PBMC isolation, peptides, T and B cell ELISpot assays and statistics are described in **Chapter 2**.

8.3.1 Patient and control recruitment

For this chapter, 55 patients with IgG4-RD who were referred to Oxford from February 2005 to February 2013 and followed prospectively were included. Fifty non-IgG4-RD patients with inflammatory or autoimmune conditions and an elevated serum IgG4 level, who were identified from March 2010 until February 2013 and followed up prospectively were included (27 PSC with (20) and without (7) IBD, 5 pancreatitis, 4 cholestasis, 3 rheumatoid arthritis, 3 cryptogenic cirrhosis, 2 biliary stricture, 2 vasculitis, 2 coeliac, 2 interstitial lung disease and bronchiectasis, 1 sjogren's, 1 hypereosinophilic syndrome). The IgG4-RD patients and disease controls were matched for gender, age and serum IgG4 levels. A cohort of 24 healthy donors was included.

8.3.2 Clinical history of peptic ulceration and *H.pylori* infection

A routine set of questions was asked to each patient and control in outpatient clinic, with details confirmed over the telephone, by GP and hospital records, and by retrieving endoscopy and histology reports. A history of indigestion, dyspepsia, prior or current peptic ulceration, previous upper endoscopy and outcome, known *H.pylori* infection and whether eradication was administered, were specifically recorded.

8.3.3 *Helicobacter* Serology

Serum samples in 55 IgG4-RD patients, 52 non-IgG4-RD disease controls and 24 healthy controls were tested for serum anti-*H.pylori* IgG (Immulite 2000, Siemens) using a commercial kit validated at the John Radcliffe Microbiology department.

8.3.4 *Helicobacter* on H&E gastric biopsies

Formalin fixed and paraffin embedded gastric biopsy samples from 17 IgG4-RD patients and 16 non-IgG4-RD disease controls were assessed for evidence of *H.pylori* bacteria on routine H&E stain, where a minimum of 20 HPF were examined (reported sensitivity 91% and specificity 100%). In areas where there was active gastritis, an immunohistochemical stain for *H.pylori* bacteria was also used (B-0471, dilution 1:200; DAKO, Carpinteria, CA) to increase detection rates (100% specificity and sensitivity if combined (Smith et al, Am J Clinical Pathol 2012)). Morphological assessment for the presence of plasma cells, lymphocytes and neutrophils was made.

Gastric and duodenal biopsy samples in 17 patients with IgG4-RD and 16 non-IgG4-RD disease controls were assessed for the triad of morphological characteristics of IgG4-RD and IgG4 immunostaining performed, as described in **Chapter 2**.

8.3.5 Peptide Epitopes

Linear and overlapping peptide epitopes used are described in **Chapter 2** and **Appendix 4**.

8.3.6 T cell and B cell ELISpots

The protocol for IFN- γ (Th1) and IL4 (Th2) T cell ELISpots, Ex-vivo and Cultured memory B cell ELISpots are described in **Chapter 2**.

8.3.7 Statistical analysis

A non-parametric Kruskal-Wallis test was used for multiple comparisons between groups, Mann-Whitney for 2 group comparisons, and Chi squared or Fishers Exact for categorical variables. Graphpad prism (v6.0) was used and p values <0.05 were considered to be statistically significant.

8.4 RESULTS

8.4.1 Clinical characteristics

In the IgG4-RD cohort, the majority of patients had an elevated serum IgG4 level (83.6%), pancreatic and/or biliary disease (85%; 47/55) and extra-pancreatic systemic manifestations (89%; 49/55). The demographics and characteristics of the IgG4-RD, disease control and healthy donor cohort are shown in **Table 1**. The three groups were well matched in terms of gender, with the exception of a younger healthy donor cohort (p=0.027). The serum IgG4 level was higher in both IgG4-RD patients and disease controls with a higher serum IgG4 than in healthy donor cohort (p<0.0001).

Table 1: Characteristics and *H.pylori* status of IgG4-RD patients, non-IgG4-RD disease controls with an elevated serum IgG4 and healthy donors.

	IgG4-RD patients	Disease Controls	Healthy controls	P value IgG4-RD v DC	P value IgG4-RD v HC	P value DC v HC
Gender: Absolute M/F (% of male)	39/55 (71)	36/50 (72)	19/25 (76)	1.00 NS	0.788 NS	0.787 NS
Age, median (range) years	63.0 (30-82)	61 (19-89)	35 (23-60)	0.49 NS	0.0247 *	0.0247 *
High serum IgG4 (>1.4g/l): Absolute (%)	46/55 (83.6)	42/50 (82.0)	1/25 (4.0)	1.00 NS	<0.0001 ****	<0.0001 ****
Autoimmune disease: Absolute (%)	15/55 (27.3)	35/50 (70.0)	0/25	<0.0001 ****	0.0038 ***	<0.0001 ****
HP serology +ve: Absolute (%)	13/55 (23.6)	16/52 (30.7)	5/25 (20.0)	0.386 NS	0.781 NS	0.414 NS
History of indigestion or dyspepsia: Absolute (%)	19/54 (35.2)	17/50 (34.0)	2/25 (8.0)	1.00 NS	0.0131 *	0.0225 *
Peptic ulcer: Absolute (%)	4/54 (7.4)	6/50 (12.0)	0/25	0.515 NS	0.302 NS	0.169 NS
GU: Absolute (%)	1/4	2/6	N/A	N/A		
DU: Absolute (%)	3/4	4/6	N/A	N/A		
UGIE: Absolute (%)	26/54 (48.1)	31/50 (62.1)	0/25	0.17 NS	<0.0001 ****	<0.0001 ****
Gastritis: Absolute (%)	15/26 (57.7)	16/30 (53.3)	N/A	0.793 NS		
Duodenitis (*including 3 whipples specimens): Absolute (%)	5/29 (17.2)	1/30 (3.3)	N/A	0.103 NS		
HP biopsy +ve: Absolute (%)	4/17 (23.5)	2/16 (12.5)	N/A	0.656 NS		
Duodenum IgG4 cells/HPF	7-54/HPF	0-5/HPF	N/A	<0.0001 ****		
Stomach IgG4 cells/HPF	0-5/HPF	0-5/HPF	N/A	1.00 NS		

Table 1: The table shows the demographics and clinical characteristics in IgG4-RD, disease controls and healthy donors. **Abbreviations:** M: male; F: female; HP: *H.pylori*; GU: gastric ulcer; DU: duodenal ulcer; UGIE: upper gastrointestinal endoscopy; HPF: high power field; +ve: positive; DC: disease controls; HC: healthy controls. P values were calculated using Fisher's exact and Kruskal-Wallis test with multiple comparisons; NS $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

8.4.2 Autoimmune diseases

The proportion of patients with co-existent autoimmune disease was higher in the disease controls with an elevated serum IgG4 than the IgG4-RD patients ($p<0.0001$), and in both groups compared with healthy controls (IgG4-RD v HC $p=0.0386$; DC v HC $p<0.0001$).

8.4.3 Indigestion and peptic ulceration

A history of dyspepsia/indigestion and peptic ulceration was elicited in 35.2% (19/54) and 7.4% (4/54) IgG4-RD patients, respectively. There was no difference in the prevalence of dyspepsia/indigestion ($p=1.0$) or ulceration ($p=0.515$) between IgG4-RD patients and disease controls. Unsurprisingly, a clinical history of dyspepsia/indigestion was less frequent (8%; 2/25) in the younger healthy control cohort than either the IgG4-RD patients ($p=0.013$) or disease controls ($p=0.023$) (Table 1).

8.4.4 Upper endoscopy, biopsies and IgG4 immunostaining

Similar numbers of IgG4-RD patients (48%; 26/54) and disease controls (62%; 31/50) had an upper endoscopy with biopsies ($p=0.172$). The main indications for endoscopy in IgG4-RD patients were to sample the duodenum/ampulla in patients with a head of pancreas mass or duodenal stricture (34.6%; 9/26), investigation of weight loss (19.2%; 5/26), or abdominal pain (19.2%; 5/26).

In IgG4-RD patients, biopsies of the gastric mucosa confirmed gastritis in 57.7% (15/26) and helicobacter colonization in 23.5% (4/17), which was similar to 53.3% (p=0.793) and 12.5% (p=0.656), respectively in disease controls (**Table 1**).

In the 4 IgG4-RD patients with *H.pylori*-associated gastritis, tissue IgG4 immunostaining was performed. IgG4-positive plasma cells were scarce - no patient had an elevated gastric tissue IgG4 count, median 2cells/HPF (range 1-4 cells/HPF). There was a chronic active gastritis with lymphocytes, neutrophils, macrophages and mast cells. There was no evidence of a storiform fibrosis, phlebitis or eosinophils characteristic of IgG4-RD. The *H.pylori* bacteria were amongst the chronic inflammatory infiltrate but did not co-localise with IgG4-positive cells (**Image 1**). Interestingly, there was an elevated IgG4-positive plasma cell count (>10/HPF) in 3 of 5 duodenal specimens of IgG4-RD patients with duodenitis (**Image 2**); all 3 had AIP.

Image 1: Gastric tissue biopsies in a patient with AIP (1) IgG4-positive plasma cells in gastric tissue (2) *H.pylori*-associated chronic active gastritis.

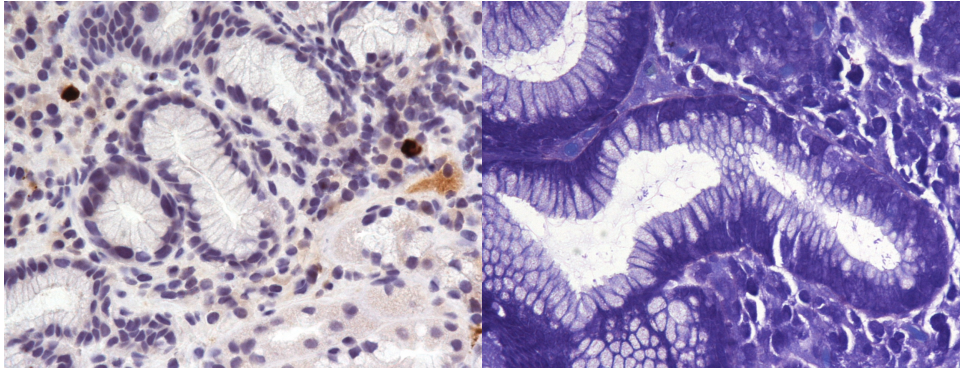


Image 1: Left (1): DAB-positive (brown) IgG4-positive plasma cells in gastric tissue. Right (2): *H.pylori*-associated gastritis with lymphocytes and neutrophils.

Image 2: IgG4-positive plasma cells in a duodenal biopsy in a patient with AIP.

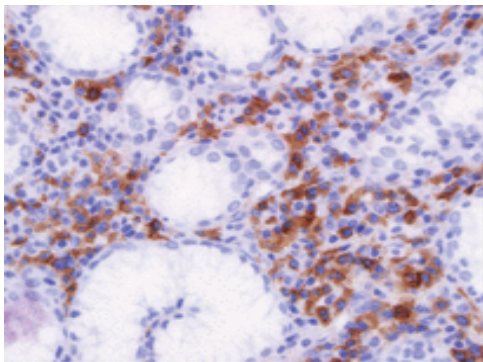


Image 2: The image shows DAB-positive (brown) IgG4-positive plasma cells in duodenal tissue of a patient with AIP (IgG4 cells >10 per high power field).

8.4.5 Anti-*H.pylori* IgG serology

Patients were tested for serological evidence of *H.pylori* exposure. In total, 23.6% (13/55) of IgG4-RD patients, 30.7% (16/52) of disease controls with an elevated serum IgG4, and 16.6% (4/24) of healthy controls were anti-*H.pylori* IgG serology positive (p=NS).

8.4.6 *H.pylori* lysate

We first tested the utility of *H.pylori* lysate as a positive control for *H.pylori* IgG serology positive patients. In the 20 samples tested using this lysate (11 anti-*H.pylori* IgG positive and 9 anti-*H.pylori* IgG negative), it had a sensitivity of 90% and specificity of 89% (**Table 2**). Using the B cell ELISpot there was a more positive response in those who were anti-*H.pylori* IgG positive than negative ($P < 0.0001$) (**Figure 1**).

Table 2: The test characteristics of *H.pylori* lysate to confirm anti-*H.pylori* IgG.

	Anti- <i>H.pylori</i> IgG positive	Anti- <i>H.pylori</i> IgG negative	
Positive lysate	10	1	Positive predictive value: 91%
Negative lysate	1	8	Negative predictive value: 89%
	Sensitivity: 90%	Specificity: 89%	Total number = 20

Table 2: The table shows the sensitivity, specificity, positive and negative predictive value of *H.pylori* lysate to confirm anti-*H.pylori* IgG results.

Figure 1: *H.pylori* lysate response in patients/controls with positive and negative anti-*H.pylori* IgG serology.

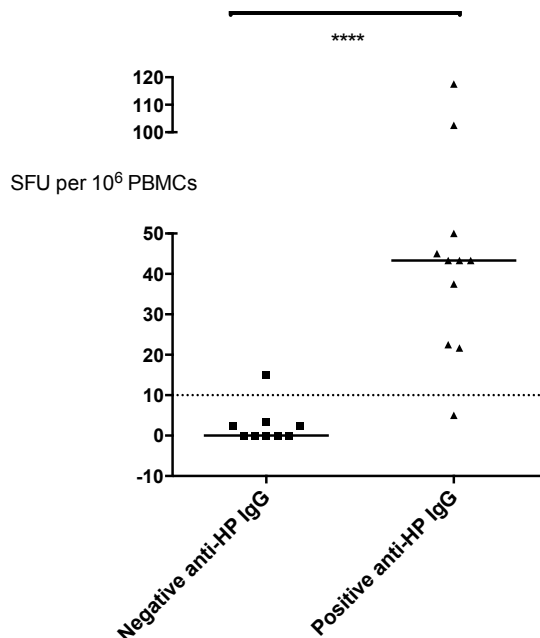


Figure 1: A dot plot showing *H. pylori* lysate response. On the x-axis are two groups of patients with positive and negative anti-*H. P* (*H. pylori*) IgG serology. On the y-axis is the number of spot forming units (SFU) per 10^6 PBMCs incubated with *H. pylori* lysate. Median values are represented by a solid line for both groups. The cut-off for a positive (>10 SFU) *H. pylori* lysate response are represented by a dashed line. Mann-Whitney *p* value **** <0.0001 .

8.4.7 IFN- γ and IL4 T cell responses to PBP

Using the ELISpot system, a technique for sensitive quantification of cytokine-secreting cells at the single-cell level, we first assessed T cell **IFN- γ** and IL4 responses to *H.pylori*, using overlapping peptides derived from PBP. There was a non-significant increased total T-cell response to *H.pylori* PBP in those who were anti-*H.pylori* IgG positive compared with anti-*H.pylori* IgG negative (p=0.08) (**Figure 2 Left**). There was a significant difference in total **IFN- γ** response between the 3 groups (ANOVA p=0.021), and in individual comparison between IgG4-RD

patients and healthy controls ($p=0.002$) (**Figure 2 Middle**). In the anti-*H.pylori* IgG positive group, there was no difference between IgG4-RD patients, disease controls with an elevated serum IgG4 level and healthy controls (**Figure 2 Right**).

Figure 2: Total IFN- γ T cell response in IgG4-RD, disease and healthy controls

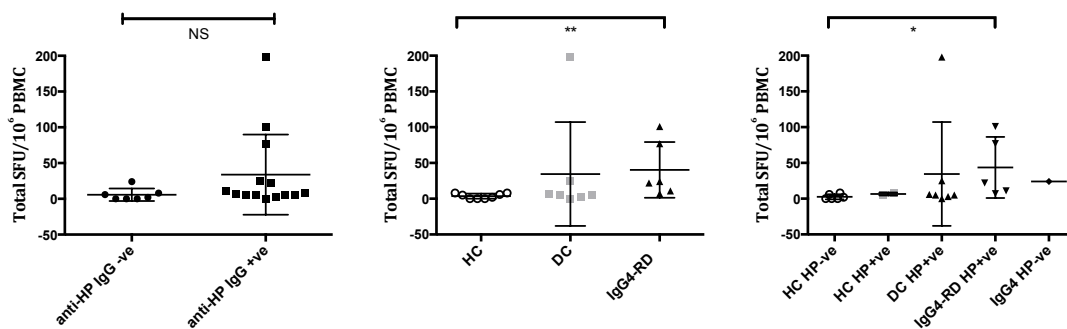


Figure 2: The dot plots show (**Left**) the total IFN- γ T cell response in anti-*H.pylori* IgG positive and negative patients and controls, where the x-axis is anti-*H.pylori* IgG positive and negative status, and the y-axis is total spot forming units per million of PBMCs. Error bars show the median and interquartile range. Mann-Whitney p values where NS ≥ 0.05 , * <0.05 , ** <0.01 . (**Middle**) the total IFN- γ T cell response in IgG4-RD patients (IgG4-RD), disease controls with an elevated serum IgG4 (DC) and healthy controls (HC). The x and y-axes and Mann-Whitney p values as for the Left plot. (**Right**) the total IFN- γ T cell response in HP (anti-*H.pylori* IgG) positive and negative IgG4-RD patients, disease (DC) and healthy controls (HC). The x and y-axes as for the Left plot. One-way ANOVA $p>0.05$ NS, $p<0.05$ *, $p<0.01$ **.

The IFN- γ T cell response to individual PBP peptide pools, are shown in **Figure 3:** peptide pool 6 containing the PBP sequence of interest (Frulloni et al, N Engl J Med 2009).

Figure 3: *IFN- γ T cell response to each *H.pylori* PBP peptide pool in IgG4-RD, disease controls and healthy controls.*

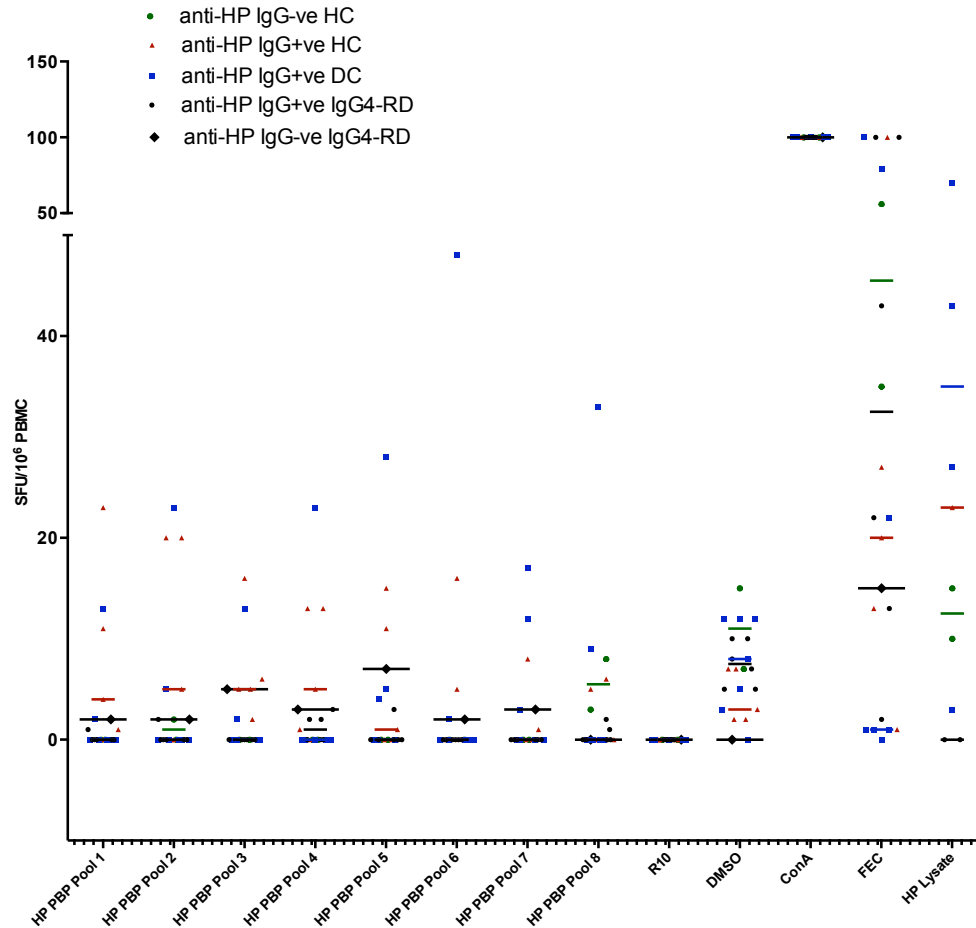


Figure 3: *The dot plot shows the mean IFN- γ T cell response to individual peptide pools in IgG4-RD patients, disease and healthy controls. The X-axis shows *H. pylori* PBP peptide pools 1 to 8, positive controls (Con A, FEC and HP lysate) and negative controls (R10-medium and DMSO). The Y-axis is the mean spot forming units per million of PBMCs. The thick black line is the median IFN- γ T cell response. **Abbreviations:** Con A: Concanavalin A; FEC: mixed HLA class I restricted peptides from cytomegalovirus, Epstein Barr virus and influenza; HP lysate: *H.pylori*.*

One individual with the highest response to peptide pool 6 (48 SFU/10⁶ PBMCs) – a disease control with elevated serum IgG4 and anti-*H.pylori* IgG positive - was further mapped down to the individual peptide sequences in pool 6. There was no increased response to the peptide sequence of interest (A K E E R R Y) on individual peptide mapping in triplicate.

8.4.8 IgG and IgG4 B cell memory responses to PBP

To determine whether IgG4-RD patients that were exposed to *H. pylori* infection have a memory B cell mediated antibody response against *H. pylori* PBP peptide, we performed a 6-day cultured memory B cell ELISpot to detect human antigen-specific B memory cells non-specifically stimulated to differentiate into antibody-producing plasma cells. There were non-significant differences between the IgG response in anti-*H. pylori* positive and negative individuals (**Figure 4 Left**), IgG4-RD patients and disease controls with an elevated serum IgG4 (**Figure 4 Middle**), and group comparison of IgG4-RD patients and disease controls that were anti-*H. pylori* IgG positive or negative (**Figure 4 Right**). There were also non-significant IgG (and IgG4) responses to grouped PBP peptide pools (**Figure 5**).

Figure 4: Total IgG response in IgG4-RD, disease and healthy controls.

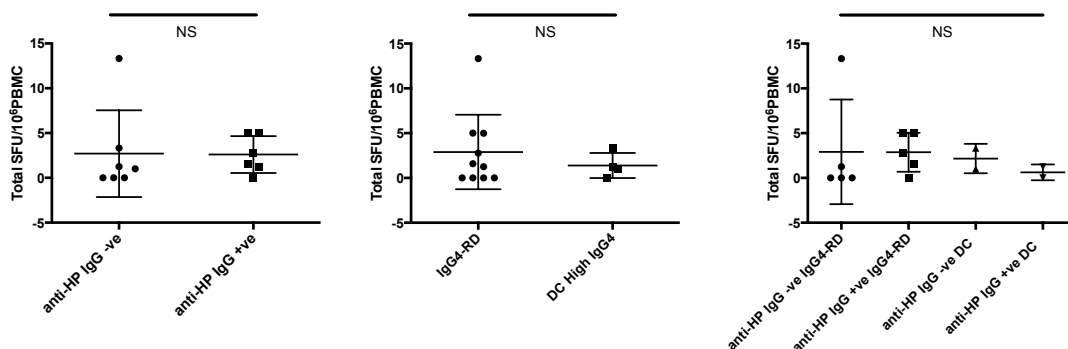
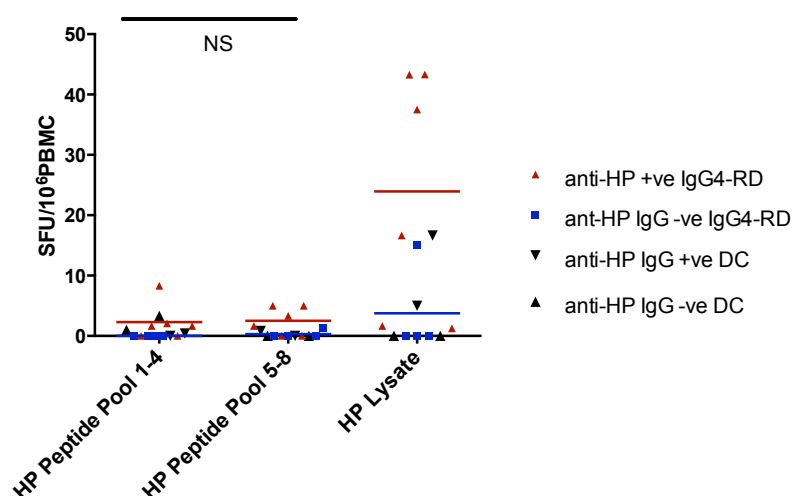


Figure 4: The dot plots show **(Left)** the total IgG response in anti-*H. pylori* IgG positive and negative patients and controls, where the x-axis is anti-*H. pylori* IgG positive and negative status, and the y-axis is total spot forming units per million of PBMCs. Errors bars show the median and interquartile range. Mann-Whitney *p* values, where NS ≥ 0.05 . **(Middle)** the total IgG response in IgG4-RD patients (IgG4-RD) and disease controls with an elevated serum IgG4 (DC). The x and y-axes and Mann-Whitney *p* values, as for A. **(Right)** the IgG response in HP (anti-*H. pylori* IgG) positive and negative IgG4-RD patients and disease controls (DC). The x and y-axes as for A. One-way ANOVA *p* values, where NS ≥ 0.05 .

Figure 5: *IgG response to PBP peptide pools in IgG4-RD and disease controls.***Figure 7:** *The dot plot shows the mean IgG response to grouped peptide pools in IgG4-RD patients and disease controls. The X-axis shows *H. pylori* PBP peptide pools 1 to 4 and 5 to 8, and *H. pylori* lysate as the *H. pylori* serology control. The Y-axis is the mean spot forming units per million of PBMCs. Mann-Whitney *p* values where NS ≥ 0.05 .*

8.5 DISCUSSION

In this chapter, the presence and role of *H. pylori* as a microbial antigen in IgG4-RD was investigated. On the basis of clinical history and endoscopic evidence, peptic ulceration was documented in only 7.2% of IgG4-RD patients, predominantly duodenal ulcers, which was much lower compared to the 22.5 and 34.8% of gastric ulcers described in Chinese and Japanese AIP patients, respectively (Shinji et al, Gastrointest Endosc, 2004; Chang et al, Pancreas, 2009). The single patient with a gastric ulcer in our cohort had ulceration at the gastric antrum, which was not linear in appearance, unlike those by Shinji et al in AIP patients (Shinji et al, Gastrointest Endosc, 2004).

The proportion of patients with serological exposure to *H.pylori* was only 23.6% in our cohort, considerably lower than described in the Italian AIP cohort (84%) but similar to that documented in a Chinese AIP cohort (27.5%). This is likely to reflect the over-representation of type II AIP (a discrete pathological entity from the IgG4-RD spectrum) included in the Italian study, supported by the low rate of elevated serum IgG4 levels in their cohort at 50% compared to 82% in our type I AIP/IgG4-RD cohort. It may also reflect a higher prevalence of *H.pylori* colonisation in Italy compared to the UK. Given the link between *H.pylori* and autoimmune conditions, it was unsurprising to see a higher proportion of disease controls (30.7%) with positive *H.pylori* serology, compared with IgG4-RD patients (23.6%) and healthy controls (20%) in our cohort (Leong et al, *Aliment Pharmacol Ther* 2002; Dore et al, *Dig Dis Sci* 2002).

In a susceptible host, *H.pylori* infection determines chronic active gastritis, gastric and duodenal ulceration, gastric cancer and MALT-lymphoma (Warren, *Gastroenterol Clin North Am*, 2000). *Helicobacter*-associated gastritis was detected in 23.5% (4 of 17 with gastric biopsies) of IgG4-RD patients, similar to the 20% described in a Chinese AIP cohort (Chang et al, *Pancreas*, 2009). This *H.pylori*-associated chronic active gastritis was characterised by an infiltration of the gastric epithelium and lamina propria by B and T lymphocytes, plasma cells, neutrophils, macrophages and mast cells. There were few IgG4-positive plasma cells (<10/HPF) present, and they were not seen to co-localise with *H.pylori* bacteria. This is unsurprising, given the proposed role for *H. pylori* antigen in triggering AIP via molecular mimicry - a mechanism by which antibodies directed against a foreign epitope become auto-reactive

due to cross-reactivity between foreign peptide and structures of the host (self-antigens) that share structural homology (Guarneri et al, J Cell Mol Med 2005). In this instance, *H.pylori* would share one or more epitopes with self-antigens causing failure to recognise these self-antigens, loss of immunological tolerance and bystander activation of immune cells with auto-aggressive potential, leading to autoimmunity. Furthermore, a study that sought to identify conserved sequences of *H.pylori* in pancreatic tissue and pancreatic juice using nested PCR, found no evidence of *H.pylori* DNA in AIP, chronic pancreatitis or ductal pancreatic adenocarcinoma patients (Jesnowski et al, Pancreatology 2010), suggesting direct infection by the microbial agent unlikely in pathogenesis.

Proposed theories regarding the initiation of the immune response in IgG4-RD suggest triggering by auto-antigens that may activate antigen-presenting cells in genetically predisposed individuals. Frulloni et al showed sequence homology between a ~55kD surface protein of *H. pylori* PBP type A and a protein expressed in acinar cells of the pancreas, UBR2 (Frulloni et al, N Engl J Med 2009). AIP patients had antibodies directed against a 7 amino acid linear peptide sequence of *H.pylori* PBP, which could cross-react with a linear peptide sequence of UBR2, at least *ex vivo*. To verify these findings, we assessed the antigen-specific IFN- γ and IL4-secreting T cell responses and B cell (IgG and IgG4) immunological memory to linear and overlapping peptides derived from *H.pylori* PBP. If patients with AIP have serum anti-PBP antibodies, one would expect immunological memory upon re-exposure to PBP, however this was not the case. There appeared to be an increased immunogenicity to *H pylori* peptides and lysate, with increased IFN γ T cell responses in those patients and controls

that were anti-*H. Pylori* IgG positive compared to those who were negative. The one patient with a positive serology and negative lysate test may have been infected with a less common bacterial strain. There was a significant difference in total IFN- γ response between the IgG4-RD patients and healthy controls. However, there were no differences in B cell memory IgG or IgG4 production between IgG4-RD patients, disease controls with elevated IgG4 and healthy controls.

There are several possibilities for the differences between our results and that of Frulloni *et al.* Firstly, it is likely that the majority of the Italian cohort of AIP patients had type II disease, which is a separate pathological (and immunological) entity to type I AIP as part of the IgG4-RD spectrum, and the antibodies directed against PBP may only be found in type II disease. Secondly, there is a higher prevalence of *H.pylori* exposure reported in Italy and Japan compared to the UK, with different pathogenic strains which could express altered forms of PBP on the bacterium's surface. PBP itself is not specific to *H.pylori*, and is also expressed by other enterobacteria (Holtet *et al*, Prot Sci 1997), none of which have been further examined. Thirdly, the use of fragmented overlapping peptides containing the linear peptide sequences may have resulted in suboptimal epitope presentation, as epitopes situated in the middle of longer peptides may not be detected (Rika *et al*, AIDS 2004). There may have also been insufficient binding to epitopes due to the loss of the three-dimensional conformational structure of an intact protein when using the overlapping peptides. Lastly, genetic susceptibility is partly determined by HLA expression, and whilst Kawa *et al* showed significant homology between CA-II and *H.pylori* α -CA, where the homologous segments contained the binding motif of HLA DRB1*0405 locus,

which confers susceptibility in Japanese AIP patients, this HLA is not predominant in the UK IgG4-RD cohort (discussed in **Chapter 11**).

8.6 SUMMARY

Our data shows that the prevalence of peptic ulceration and *H.pylori* infection in IgG4-RD is similar to those with other autoimmune and inflammatory conditions, and there is a lack of immunological memory to *H.pylori* PBP in patients with IgG4-RD. This does not support *H.pylori* PBP as the initiating antigen in our cohort of patients, although it does not exclude a role of other microbial antigens in disease.

Chapter 9 IgG1 and IgG4 B cells in health and IgG4-RD.

Extracts from Chapter 9 have been published:

LC Lighaam, E Vermeulen, T Bleker, KJ Meijlink, R Aalberse, E Barnes, **EL Culver**, Marieke van Ham, T Rispens. Phenotypic differences between IgG4⁺ and IgG1⁺ B cells point to distinct regulation of the IgG4 response. *J Allerg Clin Immunol* 2014;133(1):267-270.

9.1 HYPOTHESES

1. IgG1⁺ and IgG4⁺ B cells can be identified in the blood of healthy donors and IgG4-RD patients.
2. IgG1⁺ and IgG4⁺ B cells have a distinct phenotype and function in health and in disease states with an elevated serum IgG4, such as IgG4-RD.
3. Elevated serum IgG4 levels in IgG4-RD represent antigen-experienced switched IgG4⁺ B cells and circulating IgG4⁺ plasmablasts in the blood.
4. B cells have distinct immune and regulatory functions in IgG4-RD, as determined by expression of complement receptors, Fc receptors, transitional cell and regulatory cell markers.

9.2 INTRODUCTION

IgG4 antibodies are thought to provide humoral immune tolerance in the context of chronic antigenic exposure. Specific IgG4 antibodies are observed during repeated antigenic stimulation, such as immune therapy or treatment with therapeutic proteins e.g. factor VIII and adalimumab (Aalberse et al, *Clin Exp Allergy* 2009), and as a result

of asymptomatic helminth infections (Adjobimey et al, *Ann Trop Med Parasitol* 2010). Similarly, IgG4-RD is characterised by the accumulation of IgG4 antibodies, with elevated serum levels of IgG4 and tissue-rich IgG4+ plasma cells forming tumour-like masses (Stone et al, *N Engl J Med* 2012). In all of these situations, it remains unclear which factors drive the development and maintenance of the IgG4 response. In particular, the characteristics of B cells that produce these antibodies and how the delay in IgG4 production is regulated are unknown.

The Th2 cytokines IL-4 and IL-13 are deemed necessary to induce isotype switching to IgG4. The tolerance-associated cytokine IL-10 may preferentially promote class switch toward IgG4 over IgE (Jeannin et al, *J Immunol* 1998). IL-10 production becomes prominent only in those situations in which immune tolerance is required. Interestingly, in both immune therapy and helminth infections, IgG4 serum levels correlate with immune tolerance, which can at least in part be explained by the poor immune-activating properties of IgG4 antibodies (Aalberse et al, *Clin Exp Allergy* 2009). A regulatory role for IL-10 in the induction of the tolerogenic IgG4 response would thus coordinate cellular and humoral tolerance. Indeed, sorted IL-10-producing B cells produce large amounts of IgG4 after culture (van de Veen et al, *JACI* 2013). It is not known whether established IgG4 B cells produce IL-10 to promote tolerance in an autocrine or paracrine fashion. B-cell intrinsic factors may also play a role in the slow appearance of IgG4 antibodies, such as the possibility of indirect isotype switch, or a differential readiness of IgG4 and IgG1 memory B cells to differentiate into antibody-secreting cells.

The need to understand how IgG4 B-cell formation is regulated and related to immune

tolerance is hampered by complete lack of information on the phenotype and function of IgG4 B cells. Only recently have attempts to positively isolate and characterize IgG4 B cells been successful (Lighaam et al, JACI 2014). Furthermore, although elevated levels of serum IgG4 and abundant tissue IgG4 plasma cells in IgG-RD are reported, it is unknown whether these IgG4 cells circulate in the blood itself or localise in the tissue. Understanding the phenotype and function of IgG4 B cells in both health and disease states where there is an excess of IgG4 antibodies may help to answer some of these questions.

This chapter sought to identify phenotypic and functional differences between IgG1 and IgG4 B cells in health and IgG4-RD. Recently established protocols for the isolation and sorting of IgG4⁺ B cells using flow cytometry, by our collaborators at the Sanquin Institute in Amsterdam, were used to characterise IgG1 and IgG4 B cells in the blood of healthy donors (Lighaam et al, JACI 2014) and were then applied to patients with IgG4-RD. Furthermore, protocols were developed for a cultured memory B cell ELISpot expressing IgG1 and IgG4 antigen-experienced memory B cells, and an ex vivo B cell ELISpots expressing IgG1 and IgG4 antibody-producing plasmablasts, to investigate B cells in the active disease phase.

9.3 SPECIFIC METHODS

9.3.1 Diagnosis of IgG4-RD

The diagnosis was made in accordance with the HISORt criteria for patients with AIP and IgG4-SC and the Japanese Consensus Criteria for IgG4-RD as described in **Chapter 2**. The Boston Consensus Histopathological Criteria for IgG4-RD was applied to all 12 IgG4-RD patients with biopsy and/or surgical resection specimens.

9.3.2 Monoclonal antibody labelling and Cell lines

Antibodies (anti-IgG1, anti-IgG2, anti-IgG3 and anti-IgG4) were labelled with DyLight488 or DyLight649 fluorophores. CESS human lymphoblast cells expressing IgG1, CRL2632 human lymphoma cells expressing IgG4 and murine EL4B5 cells expressing high CD40L were cultured as described in **Chapter 2**.

9.3.3 B cell purification, isolation and culture

Peripheral blood was taken from 12 patients with active IgG4-RD and 22 healthy controls. PBMC isolation, B cell purification, cell sorting and culture are described in **Chapter 2**.

9.3.4 IgG subclass ELISA

Anti-IgG and subclass (anti-IgG1, anti IgG2, anti-IgG3, anti-IgG4) ELISA's are described in **Chapter 2**.

9.3.5 FACS analysis of IgG1 and IgG4 specific B cells

Expression of surface and intracellular antibodies, use of the LSRII and Flowjo software for analysis are described in **Chapter 2**.

9.3.6 IL10 intracellular staining and RT-PCR

IL10 cytokine production was measured by intracellular staining after stimulation, and IL10 and IL10R expression were measured by Taqman RT-PCR, as described in **Chapter 2**.

9.3.7 B cell ELISpots

IgG1 and IgG4 plasmablasts were measured by Ex vivo ELISpot and memory IgG and IgG4 B cells were measured by cultured ELISpots, as described in **Chapter 2**.

9.3.8 Statistical analysis

Continuous variables were evaluated using an unpaired 2-sample Mann-Whitney for single comparisons, and by Kruskal-Wallis for multiple comparisons, for non-parametric measures. Paired 2-sided Wilcoxon Rank tests were calculated for matched samples. Statistical analysis was performed using GraphPad Prism (version 6.0). P values were considered significant: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

9.4 RESULTS

9.4.1 Testing an IgG4 antibody clone for FACS

Three anti-IgG4 clones were first tested for their ability to stain a B-cell-line-expressing IgG4 (CRL2632) by our collaborators at the Sanquin Institute, Amsterdam (by Laura Lighaam, PhD student). Clones HP6025 and MH164.4 poorly recognised the membrane-bound form of IgG4, as shown in **Figure 1**. These antibodies bound to the CH3 domain of IgG4, which may be less accessible in its membrane-bound form. In contrast, clone MH164.1 bound to the membrane-bound form of IgG4 on the CRL2632 cell line (**Figure 1**). This clone is directed against the more membrane-distal CH2 domain. No cross reactivity to other IgG subclasses was observed using ELISA, and no binding to membrane-bound IgG1 was demonstrated (**Figure 2**).

Figure 1: Ability of different anti-IgG4 clones to stain membrane bound IgG4.

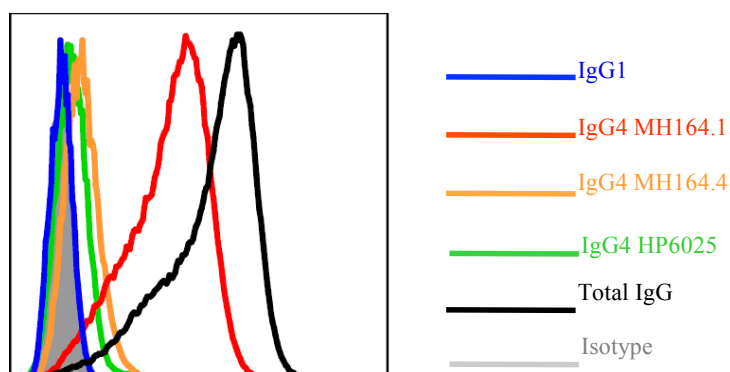


Figure 1: Histograms showing the binding of different anti-IgG4 antibodies to the IgG4-expressing cell line CRL2632. Colour coding of lines to represent IgG1, three clones of IgG4 MH164.1, MH164.4 and HP6025, total IgG and an isotype control.

Figure 2: Anti-IgG4 clone MH164.1 specifically stains IgG4⁺ B cells.

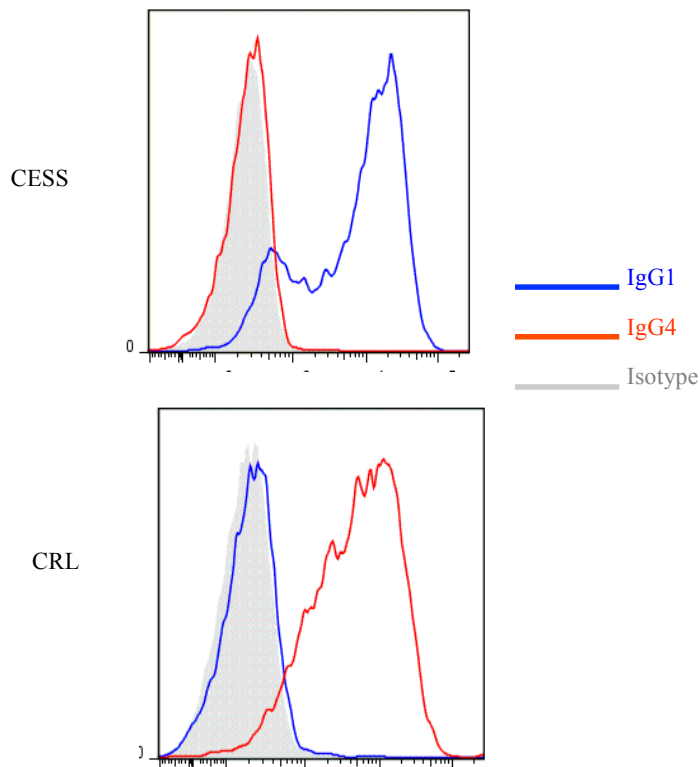


Figure 2: Histograms showing anti-IgG4 clone MH164.1 staining IgG4⁺ B cells. Upper panel: staining for IgG1, blue: CESS (expresses IgG1). Lower panel: staining for IgG4, red: CRL-2632 (expresses IgG4). Grey is the isotype control.

Using anti-IgG4 clone MH164.1, IgG4⁺ B cells were identified from primary B-cell isolates (using CD19⁺ dynabead selection) from healthy donors. Representative staining of a healthy donor is shown in **Figure 3**. The IgG4⁺ B cells were a small but distinct population of cells (frequency 0.3% to 5%) with a B cell memory phenotype, predominantly IgD negative and CD27 positive, similar to IgG1⁺ B cells (**Figure 3**).

Figure 3: *IgG4+ and IgG1+ B cells with a memory phenotype*

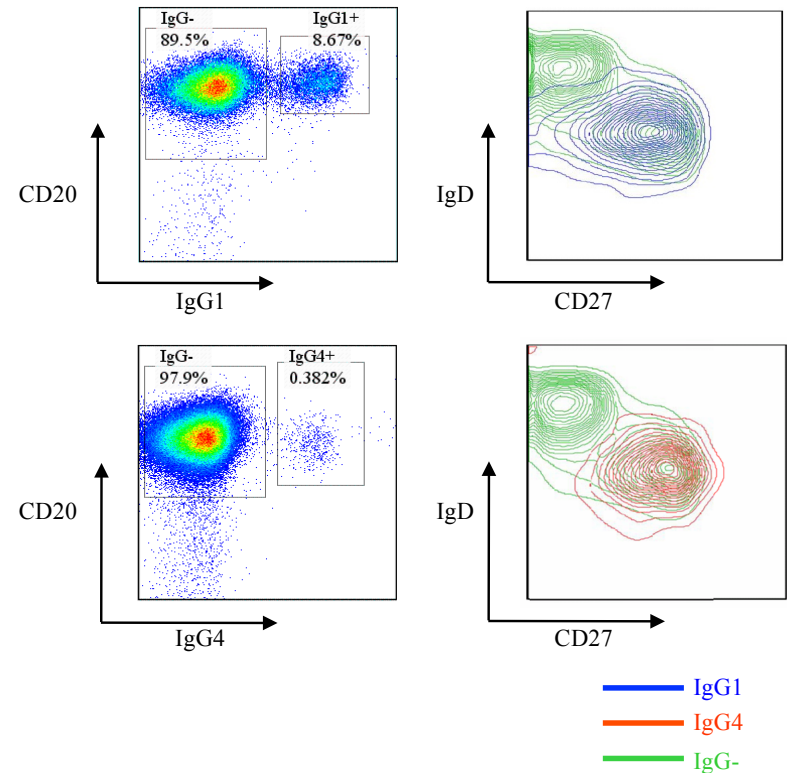


Figure 3: Fluorescent activated cell (FAC) plots showing IgG1 (upper panels) and IgG4 (lower panels) staining on B cells isolated with anti-CD19 magnetic beads from peripheral blood mononuclear cells (PBMCs) of a healthy donor. Cells were co-stained for CD20, IgD, and CD27. FAC plots; left: y-axis CD20 B cells, x-axis IgG1+ or IgG4+ cells, right: gated on CD19+ sorted B cells. y-axis IgD naive marker, x-axis CD27 memory marker. Blue: IgG4, red: IgG4, green: IgG-ve cells.

In single-cell cultures, cells sorted for IgG4 produced exclusively IgG4 antibodies, demonstrating that sorting was subclass specific, shown in **Figure 4**. The percentage of sorted cells that produced antibodies was higher for IgG1-sorted cells (83%) than for IgG4+-sorted (71%) cells, suggesting that different culture conditions may be needed for IgG4+ cells (data not shown).

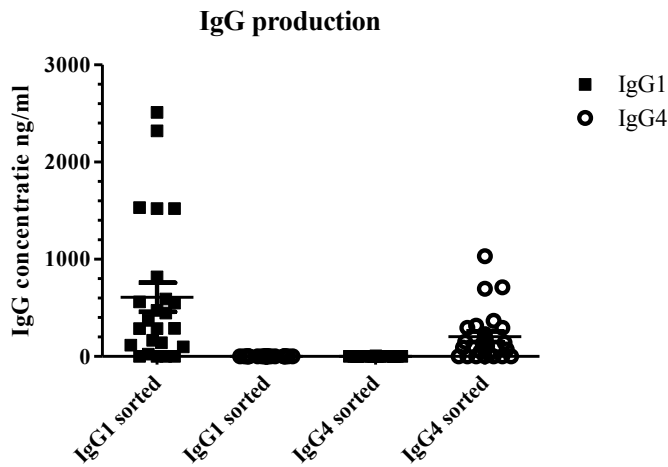
Figure 4: Antibody production by sorted single B cells

Figure 4: Dot plots showing antibody production by sorted single B cells. Displayed are 24 samples per condition, assay cut-off 6 ng/mL. Cell lines CRL (expresses IgG1), CRL-2632 (expresses IgG4). On the x-axis are IgG1+ and IgG4+ sorted cells, and the y-axis is IgG concentration in ng/ml. Symbols: open circles - IgG1 antibodies, closed squares - IgG4 antibodies.

Combined, these data show that the anti-IgG4 clone MH164.1 can be used to specifically identify and isolate IgG4 memory B cells using flow cytometry.

9.4.2 Relationship between surface IgG staining and IgG subclasses

Next, the amount of circulating IgG1 and IgG4 B cells in the blood were compared with their plasma serum levels. There was an overall correlation between the relative abundance of all IgG subclasses for serum IgG and circulating IgG B cells in healthy donors, as shown in **Figure 5**.

Figure 5: IgG subclasses and circulating IgG subclass-specific B cells

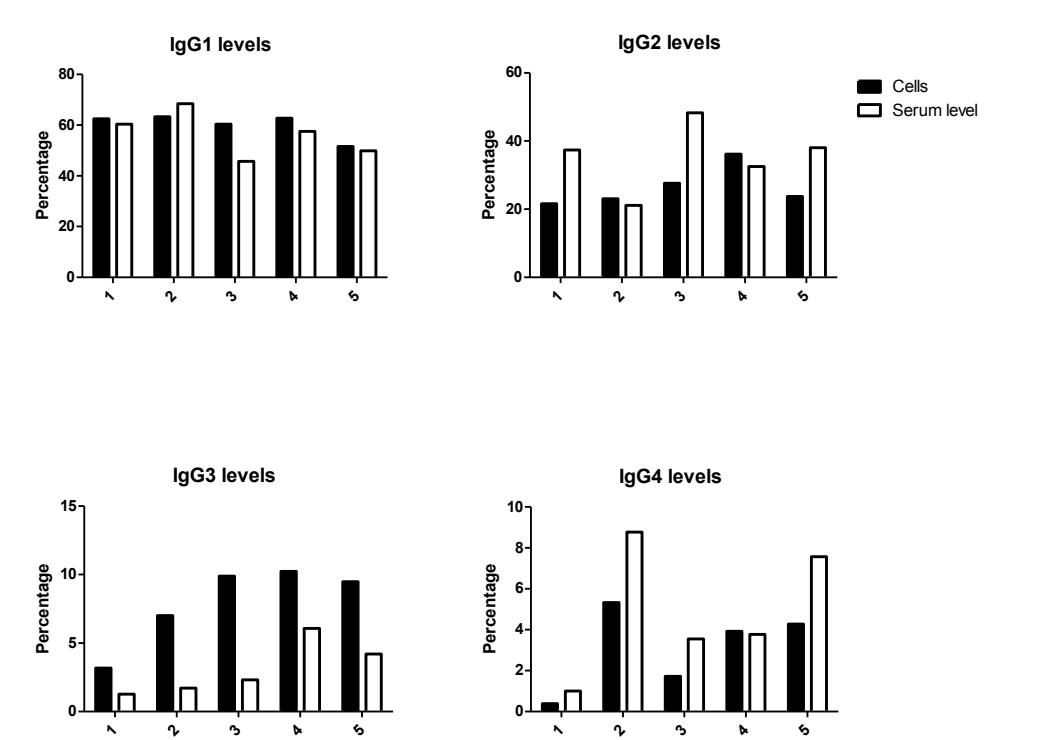


Figure 5: Bar graphs showing antibody levels that correlate to B-cell frequency expressing IgG isotypes. Serum and EDTA blood were obtained from 5 healthy donors (numbered 1- 5 on the x-axis). In serum, levels of IgG subclasses were measured by using ELISA. On the y-axis the percentages of subclass antibodies relative to total IgG is shown (black bars) and in the same donors, the percentage of B cells expressing IgG subclasses 1 to 4 relative to cells expressing total IgG is shown (white bars).

IgG1+ B cells were most abundant (50% to 65%), followed by IgG2+ (20% to 35%), IgG3+ (3% to 10%) and IgG4+ (0.3% to 5%). In the serum, a similar pattern was found, IgG1 being most abundant (4.2-7.5 mg/mL; 50% to 70%) followed by IgG2 (2.3-6.4 mg/mL; 20% to 40%), IgG3 (0.1-0.8 mg/mL; 1.2% to 6%), and IgG4 (0.1-1.0 mg/mL; 1% to 9%). These data suggest that the number of subclass-specific memory B cells correlates with the level of IgG subclass-specific antibody secreting cells.

9.4.3 Phenotype of IgG1 and IgG4 B cells in healthy donors

To determine the phenotype of IgG1 and IgG4 B cells (isolated from PBMC's by CD19 dynabeads and stained with surface IgG1 or IgG4 antibody), the surface expression of memory markers, complement receptors, Fc receptors, transitional cell and/or regulatory markers in healthy donors were assessed.

Memory and naïve B cell markers in healthy donors

IgG1 and IgG4 B cells expressed similar amounts of surface CD27 (switched memory cells) and IgD (naïve cells) (**Figure 6**). Both IgG1 and IgG4 B cells have a switched memory phenotype (**Figure 3**), suggesting that they have encountered antigen.

Figure 6: Memory marker expression on IgG1 and IgG4 cells.

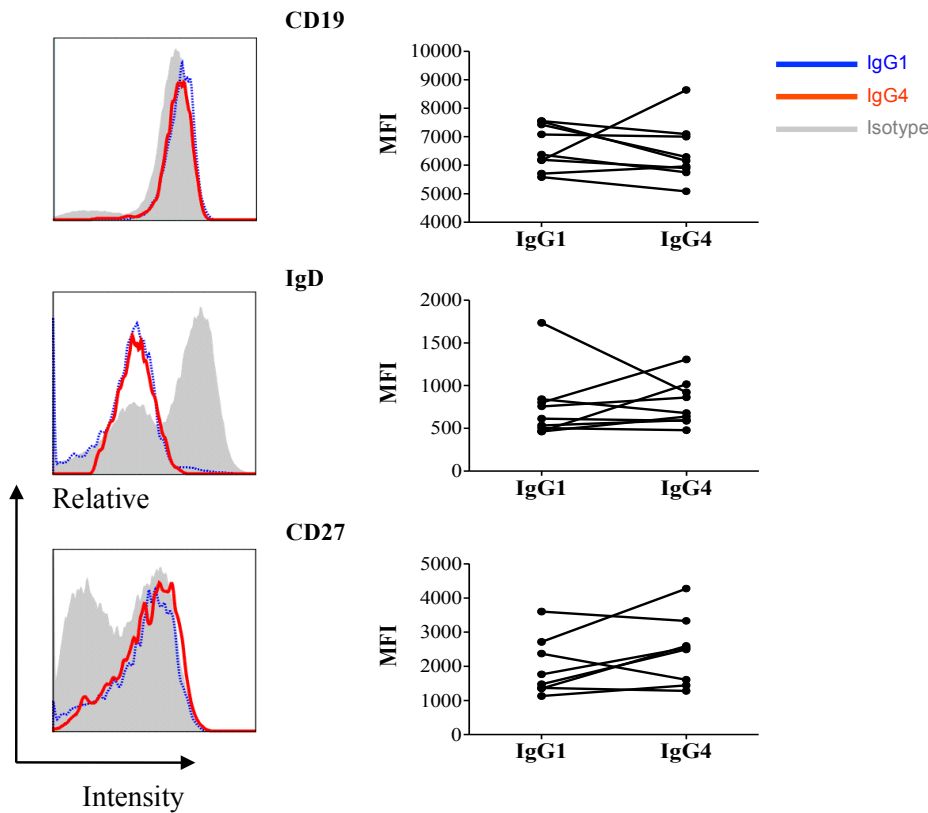
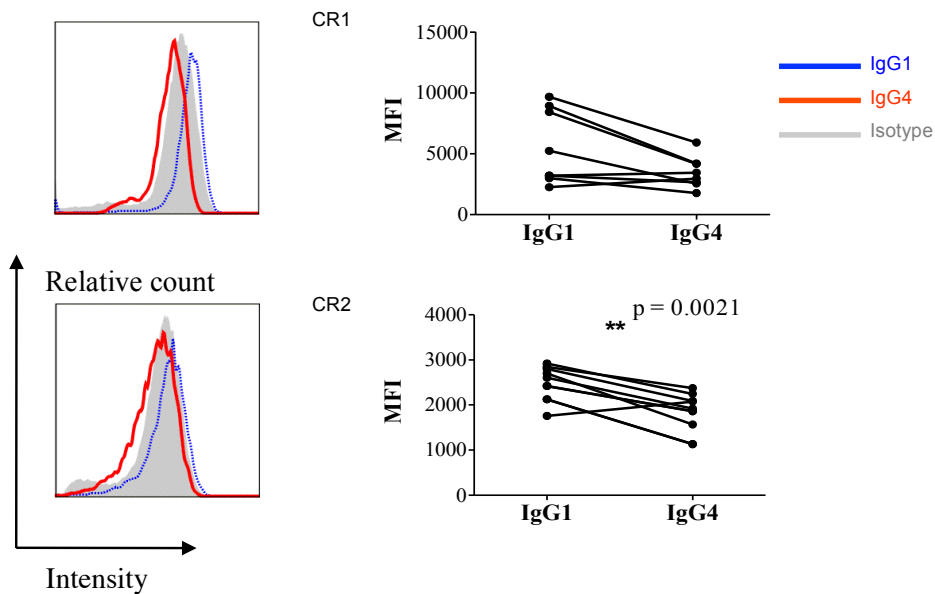


Figure 6: Histograms (left) and paired frequency plots (right) showing surface expression of CD19, CD27 and IgD in CD20+IgG1+ and CD20+IgG4+ B cells. Left: Histograms for a representative experiment. The x-axis displays fluorescence intensity and the y-axis relative frequency. Blue: IgG1, red: IgG4, grey: isotype control. Right: Median fluorescence intensities (MFI) from 8 healthy donors for each surface expression marker. The x-axis displays IgG1+ and IgG4+ B cells and y-axis charts MFI. Wilcoxon Rank paired test, where $p > 0.05$ is NS.

Complement receptors

During an on going immune response, immune complexes can exert regulatory functions via complement receptors. The expression of complement receptors (CR) involved in the classical and alternative complement pathway, namely CR1 and CR2, were compared on IgG1 and IgG4 B cells. Notably, IgG4 B cells expressed significantly less CR2 (CD21) and less CR1 (CD35) compared with IgG1 B cells, as shown in **Figure 7**, suggesting a decreased sensitivity to complement components.

Figure 7: Complement receptor expression on IgG1 and IgG4 cells.**Figure 7:** Histograms (left) and paired frequency plots (right) showing surface expression of complement receptors CD21 (CR2) and CD35 (CR1) in CD20+IgG1+ and CD20+IgG4+ B cells. Left and right panels and axes as per **Figure 6**. Wilcoxon Rank paired test, where $*p < 0.05$, $**p < 0.01$.

Fc receptors

Fc receptors bind the Fc fractions of antibody both freely circulating and within antibody-containing immune complexes. The expression of an inhibitory receptor FcγRIIb and a low-affinity receptor for IgE FcεRI, on IgG1 and IgG4 B cells were compared. Notably, there was a higher expression of the FcεRI (CD23) on IgG4 B cells compared with IgG1 cells, but no difference in expression of FcγRIIb (CD32) (**Figure 8**).

Figure 8: Fc receptor expression on IgG1 and IgG4 cells.

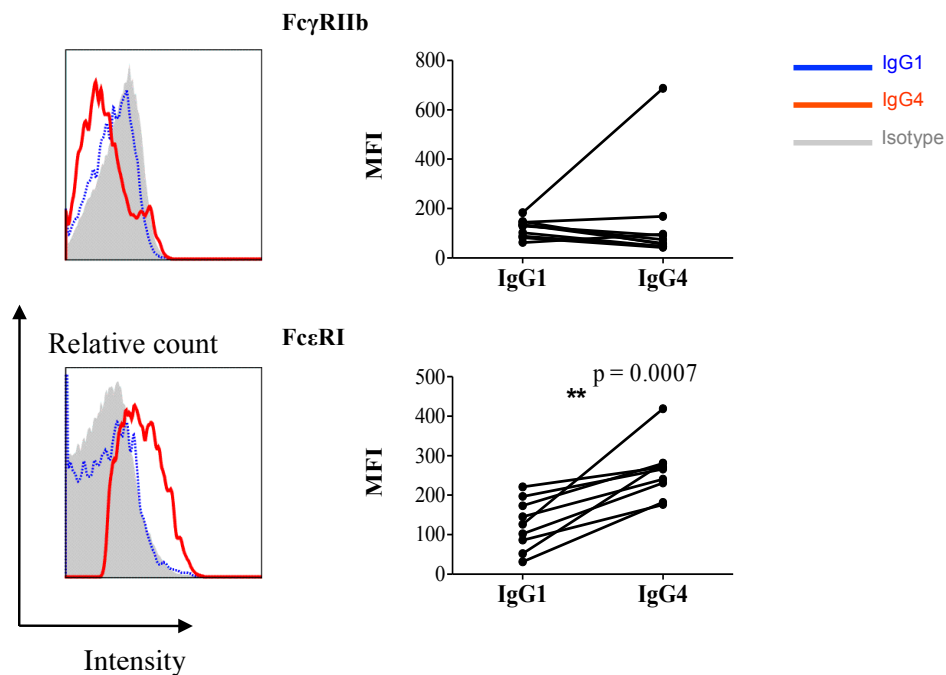
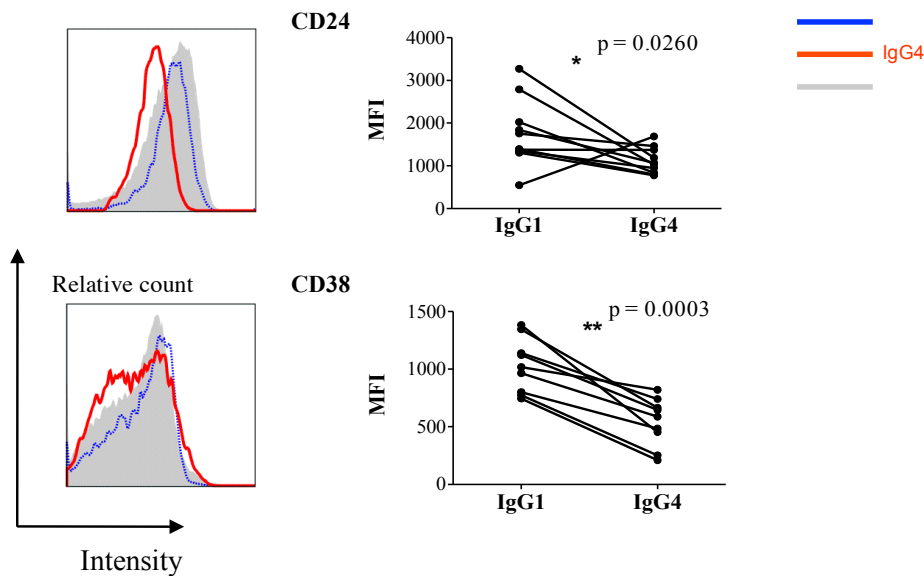


Figure 8: Histograms (left) and paired frequency plots (right) showing surface expression of Fc receptors CD23 (FcεRI) and CD32 (FcγRIIb) in CD20+IgG1+ and CD20+IgG4+ B cells. Left and right panels and axes as per **Figure 6**. Wilcoxon Rank paired test values as per **Figure 7**.

Transitional cells and/or regulatory B cells

The expression of CD24 and CD38 are described to be present on human transitional cells and regulatory B cells (Mauri et al, Annu Rev Immunol 2012). IgG4 production is increased in cultures of isolated IL-10-producing regulatory B cells (van de Veen et al, JACI 2013). To investigate whether IgG4 B cells had a transitional and/or regulatory phenotype, surface staining with CD24 and CD38 antibodies, B cell stimulation and IL10 intracellular staining was performed. The expression of both CD24 and CD38 were lower on IgG4 than on IgG1 B cells (**Figure 9**), suggesting that IgG4 B cells did not have a typical transitional cell phenotype.

Figure 9: Transitional cell markers on IgG1 & IgG4 cells.**Figure 9:** Histograms (left) and paired frequency plots (right) showing surface expression of CD24 and CD38 in CD20+IgG1+ and CD20+IgG4+ B cells. Left and right panels and axes as per **Figure 6**. Wilcoxon Rank paired test values as **Figure 7**.

Stimulation (with CpG) of isolated B cells from healthy donors demonstrated that IgG4+ B cells do not have increased IL-10 production compared with IgG1+ B cells (**Figure 10**). Overall, 0.2% to 8.8% of the cells were IL-10 positive relative to CD20+ B cells, while 0.6% to 4.4% of CD20+IgG1+ cells and 3.5% to 8.9% of CD20+IgG4+ cells were positive for IL-10 (n = 5). This was further supported by IL10 qPCR (data not shown).

There were also no differences in expression of IL-10 receptors (ILRA or ILRB) between IgG4 and IgG1 B cells (**Figure 11**), assessed by using RT-PCR on mRNA isolated from IgG1+ or IgG4+ sorted B cells. This suggests that the responsiveness toward IL-10 is similar for both cell types.

Taken together, these data suggest that IgG4 B cells are also not “classical” regulatory B cells in either phenotype or function.

Figure 10: IL10 production from IgG1 and IgG4 B cells.

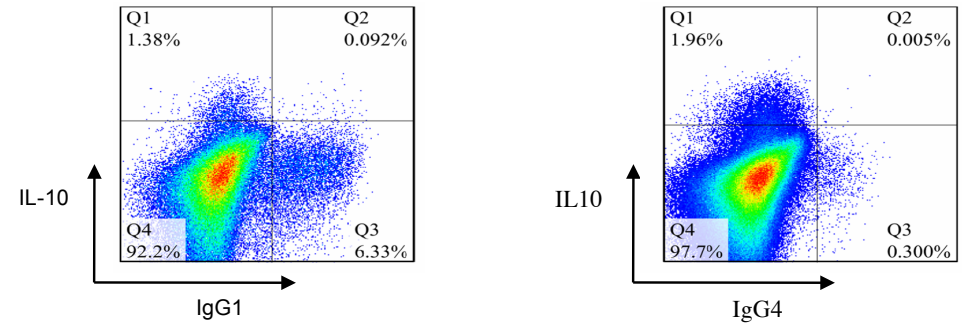


Figure 10: Fluorescent-activated cell dot plots showing a representative experiment of IL10 production from IgG1 and IgG4 B cells. B cells were isolated by CD19+ dynabeads and stimulated with CpG. IL-10 production was analysed by using intracellular staining on flow cytometry. Cells were first gated on CD20 positivity.

Figure 11: IL10 receptor expression on IgG1 and IgG4 cells.

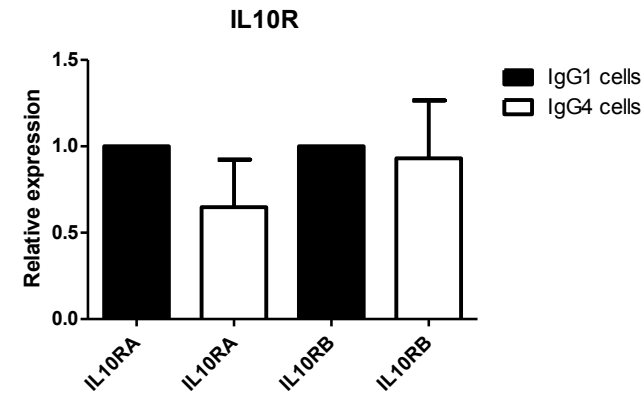


Figure 11: Bar charts showing IL-10 receptor A and B expression on IgG1 and IgG4 cells. IL10R expression was assessed by using RT-PCR on mRNA isolated from IgG1+ or IgG4+ sorted B cells (n=5). Expression is shown relative to 18S (control).

9.4.4 Frequency of IgG1 and IgG4 B cells in IgG4-RD.

Our data suggest that an elevated serum IgG4 is found in almost 80% of patients with IgG4-RD (**Chapter 3**). Whether these represent IgG4+ memory B cells and/or antibody-producing plasmablasts in the blood is unknown. To investigate this, the proportion of CD19+CD20+ B cells and CD19+CD20-CD27++CD38++ plasmablasts in the blood of IgG4-RD patients compared with healthy controls was determined.

IgG1 and IgG4 B cells in IgG4-RD

The percentage of CD19+CD20+ B cells isolated from PBMCs in patients with active IgG4-RD was not significantly different to healthy donors ($p=0.094$) (**Figure 12**).

Figure 12: Percentage of CD20+ B cells from PBMCs

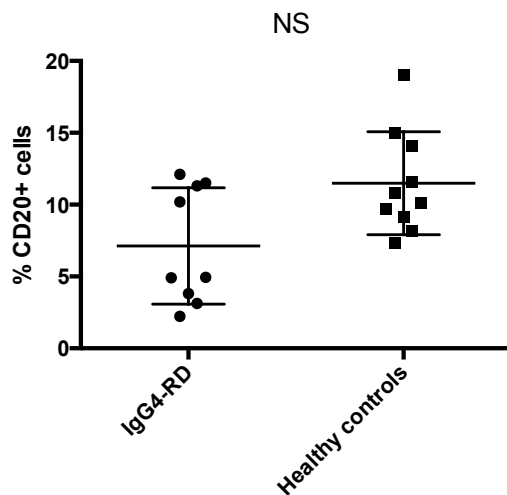


Figure 12: The dot plot shows the percentage of PBMCs that are CD20+ B cells. The x-axis displays the 2 cohorts, and the y-axis displays the percentage of PBMCs that were CD19+CD20+. Mann-Whitney values, where $p>0.05$ is NS..

However, the proportion of those CD19+CD20+ B cells that were IgG1+ (median 7.77% versus 3.37%; $p=0.0187$) and IgG4+ (median 4.11% versus 0.69%; $p<0.0001$) was higher in IgG4-RD patients compared with healthy controls (**Figure 13**).

Figure 13: *IgG1+ and IgG4+ B cells in IgG4-RD and healthy controls.*

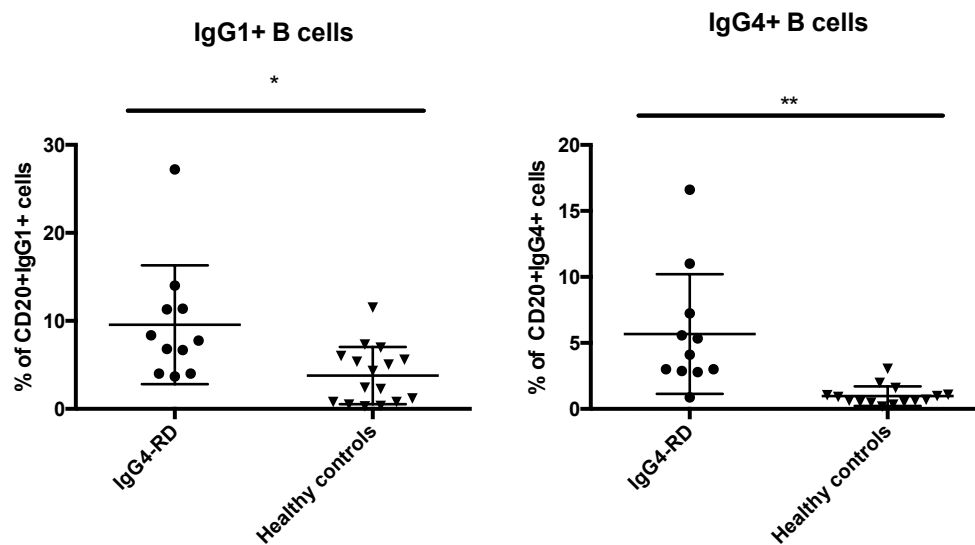


Figure 13: *Dot plots showing the percentage of CD20+ B cells that are IgG1+ and IgG4+ in IgG4-RD patients and healthy controls. The x-axis displays the 2 cohorts, and the y-axis displays the percentage CD20+ IgG1 or IgG4 population. Mann-Whitney values, where * $p<0.05$, ** $p<0.01$.*

Furthermore, there was a correlation between serum IgG4 levels and the abundance of IgG4+CD20+ B cells determined by flow cytometry (**Figure 14**) and cultured B cell memory ELISpot (**Figure 15**) in IgG4-RD patients and healthy controls, suggesting that the elevated serum IgG4 may be in part the result of an expanded population of circulating IgG4+ B memory cells.

Figure 14: Serum IgG4 and IgG4+ B cells on flow cytometry.

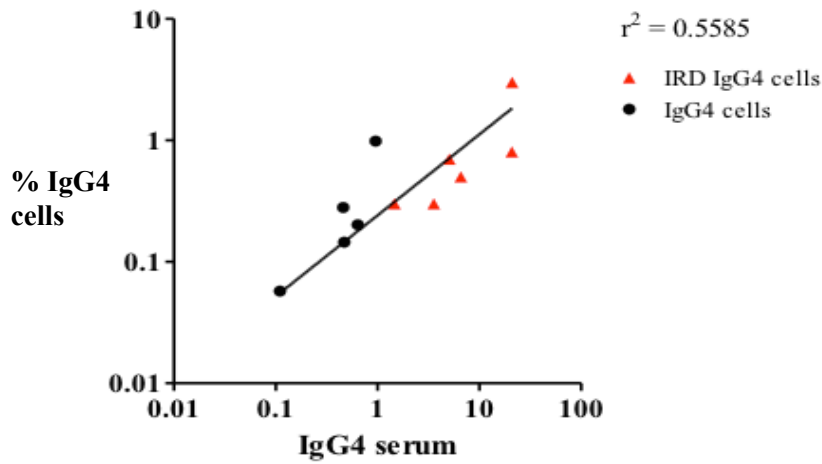


Figure 14: Correlation plot showing IgG4 serum levels and number of B cells that express surface IgG4 using flow cytometry, of healthy controls (black circles) and patients with IgG4-RD (IRD red triangles), expressed as a percentage of total IgG. The x-axis displays Log 10 serum IgG4 levels in grams/litre; the y-axis shows Log 10 the number of B cells expressing IgG4 as a percentage of total IgG cells. Spearman's rank coefficient is calculated with an r^2 value. Non-linear best fit line.

Figure 15: Serum IgG4 and IgG4+ B cells on cultured B cell ELISpot.

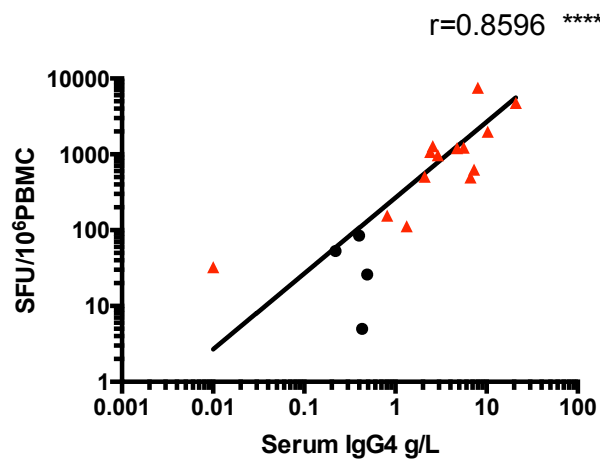


Figure 15: Correlation plot showing serum IgG4 levels and number of IgG4+ spot forming units (SFU) per 10^6 PBMCs using a cultured B cell memory ELISpot, in healthy controls (black circles) and patients with IgG4-RD (red triangles). The x-axis displays Log 10 serum IgG4 levels in grams/litre; the y-axis shows Log 10 number of IgG4+ spot forming units (SFU) per 10^6 PBMCs. Spearman's rank coefficient r value and p value, where **** $p < 0.0001$. Non-linear best fit line.

IgG1 and IgG4 Plasmablasts in IgG4-RD

The percentage of CD19+CD27++CD38++ plasmablasts in the blood of patients with active IgG4-RD was not significantly different to that of healthy controls ($p=0.792$). The plasmablasts were negative for CD20 and expressed high levels of HLA-DR, whereas IgD expression was low. However, the proportion of these plasmablasts that were IgG4+, but not IgG1+, was higher in IgG4-RD patients compared with healthy controls (Figure 16).

Figure 16: IgG1+ & IgG4+ plasmablasts in IgG4-RD and healthy controls

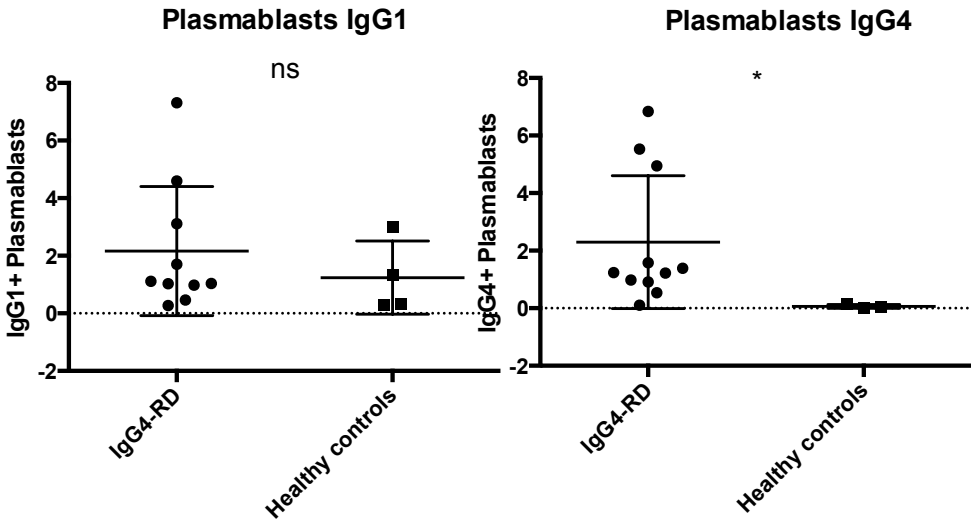


Figure 16: Dot plot showing the percentage of IgG1+ and IgG4+ CD19+CD20-CD27+CD38hi plasmablasts isolated from PBMC's in IgG4-RD patients and healthy controls. The x-axis displays the 2 cohorts, and the y-axis displays the percentage of IgG1 or IgG4 cells of the parent plasmablast population. Mann-Whitney values, where * $p<0.05$.

This was supported by ex vivo B cell ELISpot, where antibody-producing IgG4+ plasmablasts were seen in higher numbers in active IgG4-RD patients than in healthy controls (Figure 17).

Figure 17: IgG4+ plasmablasts in IgG4-RD and healthy controls (ex vivo)

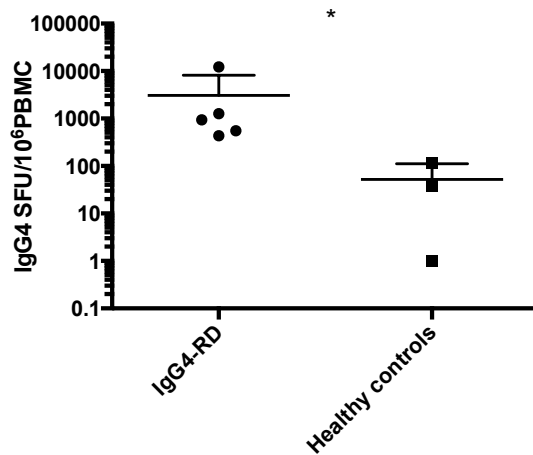


Figure 17: Dot plot showing the percentage of IgG4+ plasmablasts on ex vivo B cell ELISpots in IgG4-RD patients (n=5) and healthy controls (n=3). Y-axis is IgG4 spot forming units (SFU) per 10⁶ PBMCs. Mann-Whitney values, where * $p < 0.05$.

Similar to the IgG4+ memory B cells, the circulating IgG4+ plasmablasts correlated to serum IgG4 levels in IgG4-RD and healthy controls (**Figure 18**), suggesting that the elevated serum IgG4 is also in part reflecting an IgG4+ plasmablast population.

Figure 18: Serum IgG4 and IgG4+ plasmablasts on ex vivo ELISpot

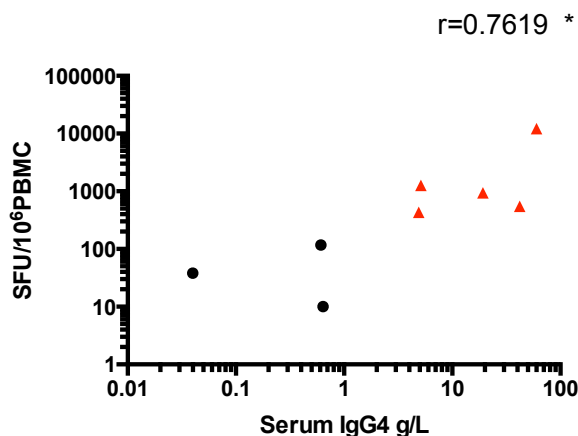


Figure 18: Correlation plot showing serum IgG4 levels and number of IgG4 antibody producing plasmablasts using an ex vivo B cell ELISpot, in healthy controls (black circles) and patients with IgG4-RD (red triangles). Axes as per **Figure 15**. Spearman's rank coefficient r value and p value, where * $p < 0.05$.

Similarly, in involved tissues of IgG4-RD patients, immunohistochemical analysis reveals the presence of CD20+ B-cell follicles surrounded by CD19+CD20-IgG4+ cells that co-stain for CD138 (plasma cells/plasmablasts), as seen in **Figure 19**. These cells had the typical morphology of a plasma cell – oval-shaped cells with a large eccentric nucleus and distinct peri-nuclear zone.

Figure 19: *CD138+ IgG4+ plasmablasts/plasma cells in IgG4-RD tissue.*

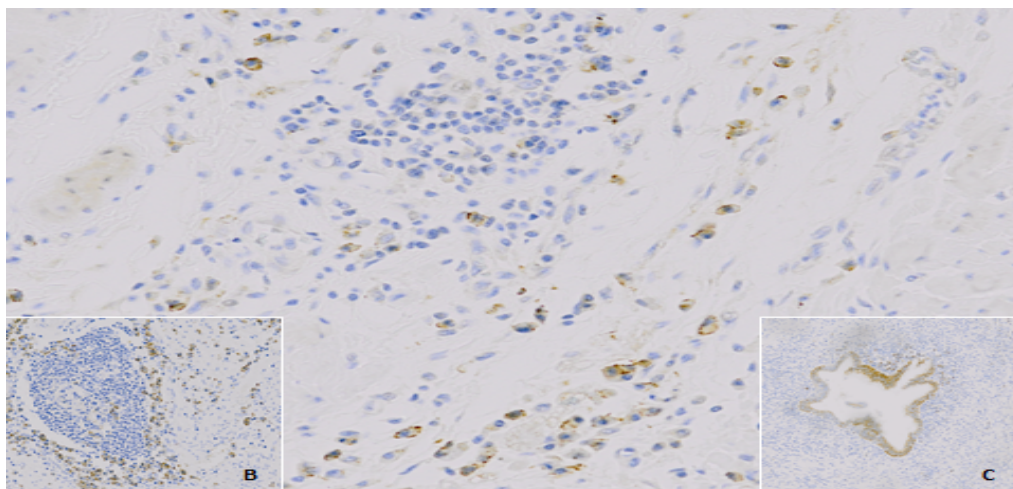


Figure 19: *Tissue sections in IgG4-RD organs (x40 magnification), CD138+ IgG4+ cells stain DAB-positive (brown). Main section: CD138+ plasma cells localized to the germinal centre of salivary gland tissue in IgG4-related sialadenitis; inset B: patchy distribution of CD138+ plasma cells in a pancreatic section from a Whipples resection in AIP; inset C: tight collection of CD138+ plasma cells in the epithelium of bile duct tissue in IgG4-related SC.*

Together, this suggests that in IgG4-RD, the elevated serum IgG4 levels are the result of IgG4+ plasmablasts and IgG4+ memory B cells in the blood, although only the IgG4+ plasma cells/plasmablasts are prominent in the tissue of involved organs.

9.4.5 Phenotype of IgG1 and IgG4 B cells in IgG4-RD patients.

Given the increase in IgG1+ and IgG4+ B cells in IgG4-RD compared with healthy controls (**Figure 13**), the surface phenotype of these cells was next explored. A second cohort set of healthy controls were utilised for this analysis, to validate the original findings and ensure experimental reproducibility.

Memory and naïve B cells in IgG4-RD and controls.

In both IgG4-RD patients and healthy controls the majority of B cells were naïve CD27-IgD+ (median 64.1% versus 72%; $p=0.765$) with a smaller proportion of switched memory CD27+IgD- (median 10.7% versus 4.52%; $p=0.146$) B cells (**Figure 20**).

Figure 20: Memory and naïve B cell populations in IgG4-RD and controls.

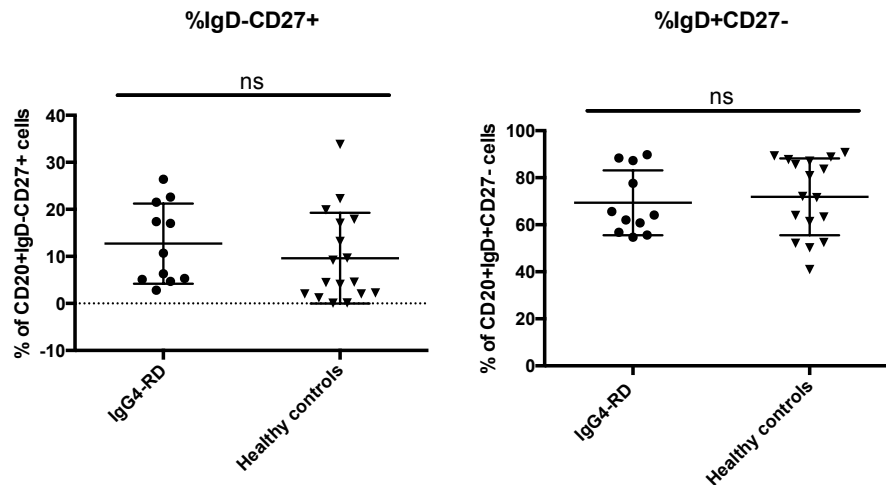


Figure 20: Dot plots showing the percentage of memory B cells (left panel) and naïve B cells (right panel) in IgG4-RD patients and healthy controls. The x-axis displays the 2 cohorts, and the y-axis displays the percentage of CD20+ cells that are memory - defined as CD20+CD27+IgD- (left panel) and naïve - defined as CD20+CD27-IgD+ (right panel). Mann-Whitney values, where NS is $p>0.05$.

IgG1+ and IgG4+ B cells expressed similar surface CD27 (memory) and IgD (naïve) markers in IgG4-RD and healthy control populations (**Figure 21**). The IgG1+ and IgG4+ B cells were a memory phenotype, predominantly CD20+CD27+IgD-.

Figure 21: Memory and naïve markers on IgG1+ and IgG4+ B cells in IgG4-RD patients and healthy controls.

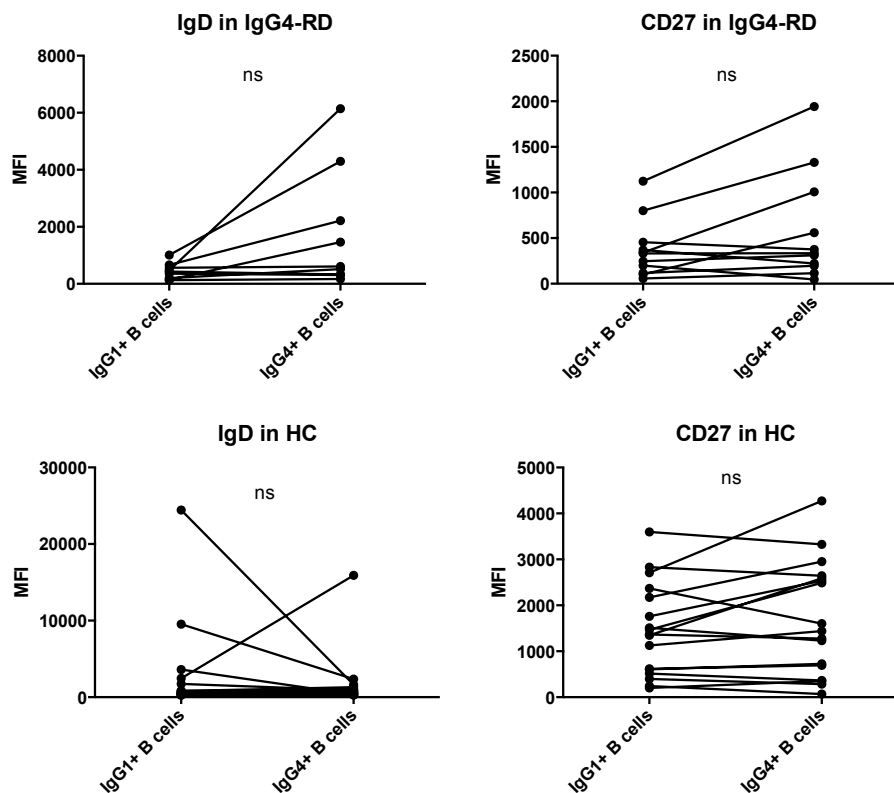


Figure 21: Paired frequency plots showing surface expression of IgD and CD27 in CD20+IgG1+ and CD20+IgG4+ B cells in IgG4-RD patients (IgG4RD) and healthy controls (HC). Mean fluorescence intensities (MFI) are calculated for each surface expression marker; y-axis is MFI and x-axis is IgG1+ and IgG4+ B cells. Wilcoxon Rank paired test, where NS $p > 0.05$, * $p < 0.05$, ** $p < 0.001$, * $p < 0.001$, **** $p < 0.0001$.**

To account for the differential expression of CD27 and IgD on IgG1 and IgG4 B cells, a ratio of the mean fluorescence intensity was calculated. Overall, IgG4-RD patients had less naïve IgD+ B cells than healthy controls ($p = 0.008$) (**Figure 22**).

Figure 22: Memory and naïve B cell populations and ratio of IgG1:IgG4 B cells in IgG4-RD and healthy controls.

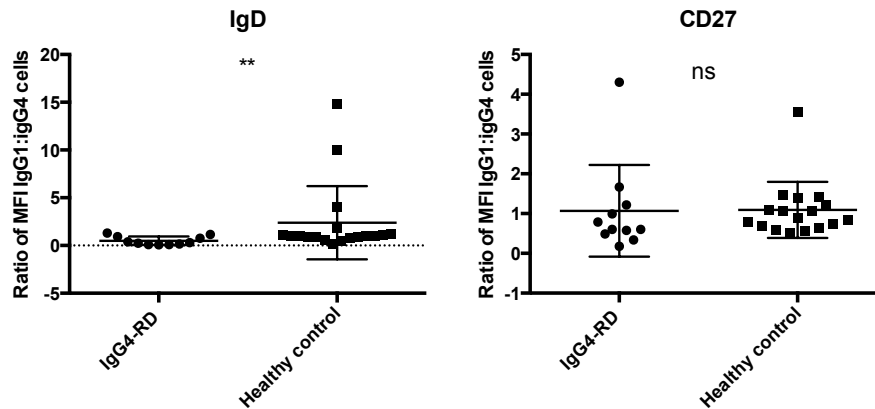


Figure 22: Ratio of memory and naïve B cell markers on IgG1:IgG4 B cells in IgG4-RD and healthy controls. On the x-axis are the 2 cohorts, and y-axis is the ratio of IgG1 to IgG4 cells measured by mean fluorescence intensity (MFI). Mann-Whitney values, where NS is $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Complement receptors in IgG4-RD and healthy controls.

IgG4-RD patients and healthy controls expressed similar surface complement receptors, CR2 (CD21) and CR2 (CD35) (**Figure 23**).

Figure 23: Complement receptors on B cells in IgG4-RD and controls.

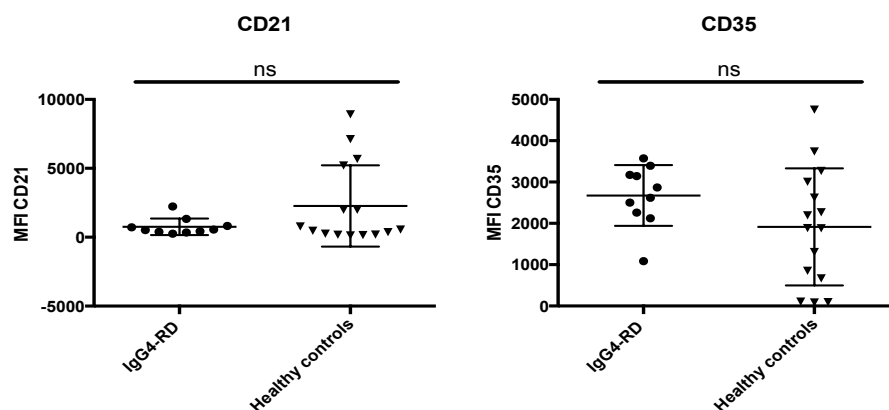


Figure 23: Dot plots showing mean fluorescence intensity (MFI) of CD21 and CD35 on CD19+CD20+ B cells in IgG4-RD patients and healthy controls. On the x-axis are the 2 cohorts, and on the y-axis is the MFI. Mann-Whitney values, where NS is $p > 0.05$, $*p < 0.05$, $**p < 0.01$.

IgG4 B cells in IgG4-RD patients and healthy controls express lower CR1 (CD35) compared to IgG1+ cells, whereas IgG4 B cells in healthy controls, but not IgG4-RD, express lower CR2 (CD21) than IgG1 cells (**Figure 24**).

Figure 24: Complement receptors on IgG1+ and IgG4+ B cells in IgG4-RD and healthy controls.

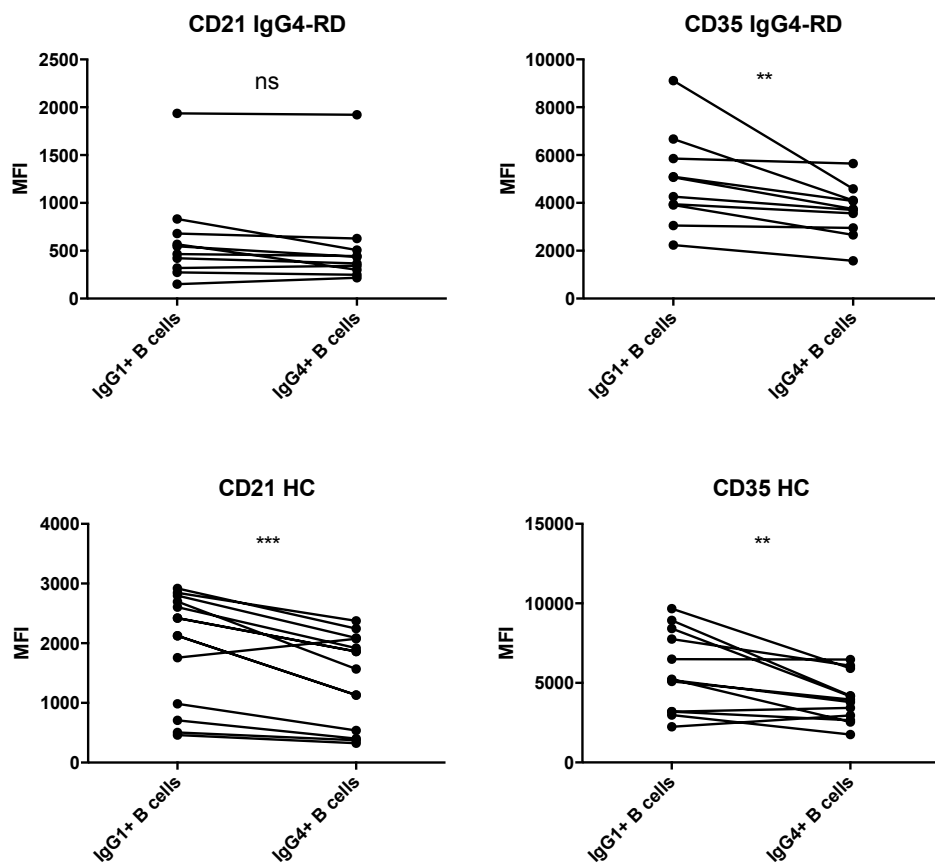


Figure 24: Paired frequency plots showing surface expression of complement receptors CD21 and CD35 in CD20+IgG1+ and CD20+IgG4+ B cells in IgG4-RD patient (IgG4RD) and in healthy controls (HC). Axis and statistics as per **figure 21**.

Given that CD21 and CD35 expression were lower on IgG4+ than IgG1+ cells, when accounting for this using a ratio of IgG1 to IgG4, overall the expression of CR2 (CD21) was lower in IgG4-RD patients than in healthy controls (**Figure 25**).

Figure 25: Complement receptors and ratio of IgG1:IgG4 B cells in IgG4-RD and healthy controls.

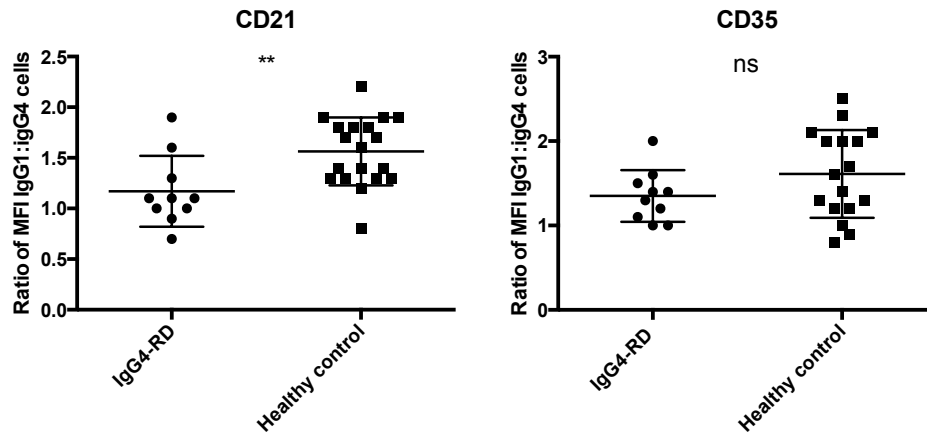


Figure 25: Ratio of complement markers on IgG1:IgG4 B cells in IgG4-RD and healthy controls. Axis and statistics as per figure 22.

Fc receptors in IgG4-RD and healthy controls.

IgG4-RD patients expressed lower inhibitory FcγRIIb (CD32), but there was no difference in FcεRI (CD23) expression, compared with healthy controls (**Figure 26**).

Figure 26: Fc receptor expression on B cells in IgG4-RD and healthy controls.

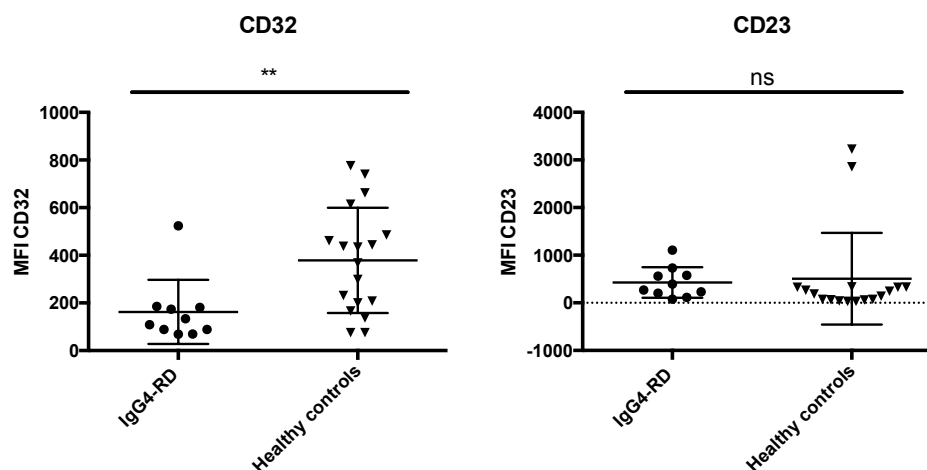


Figure 26: Dot plots showing Fc receptor expression, FcγRIIb (CD32) and FcεRI (CD23) on CD19+CD20+ B cells, in IgG4-RD patients and healthy controls. The axes and statistics as per figure 23.

IgG4 B cells express lower FcγRIIb (CD32) than IgG1 cells in healthy controls, but not IgG4-RD patients. In contrast, IgG4 B cells express higher FcεRI (CD23) in healthy controls, but not IgG4-RD patients (**Figure 27**).

Figure 27: *Fc receptors on IgG1+ and IgG4+ B cells in IgG4-RD and healthy controls.*

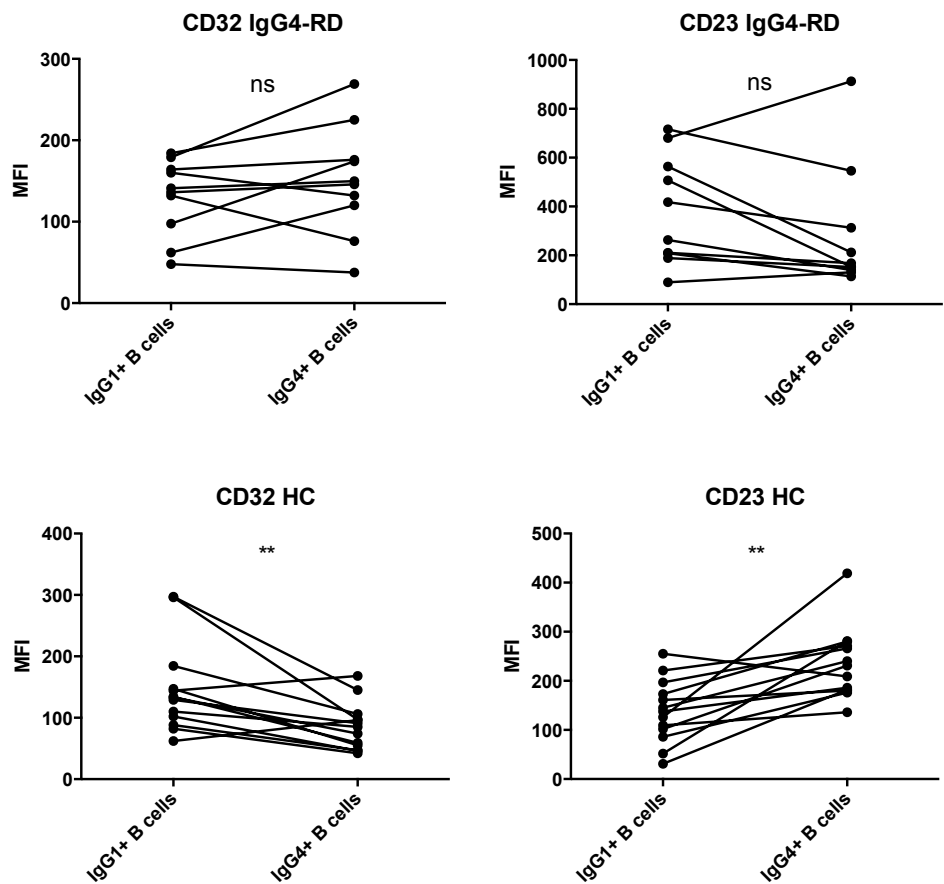


Figure 27: *Paired frequency plots showing surface expression of Fc receptors CD32 and CD23 in CD20+IgG1+ and CD20+IgG4+ B cells in IgG4-RD patients (IgG4RD) and healthy controls (HC). Axes and statistics as per Figure 21.*

When adjusting for CD32 and CD24 on IgG1 and IgG4 cells, CD32 was lower and CD23 was higher in IgG4-RD patients compared with healthy controls (**Figure 28**).

Figure 28: Fc receptors and ratio of IgG1:IgG4 B cells in IgG4-RD and healthy controls.

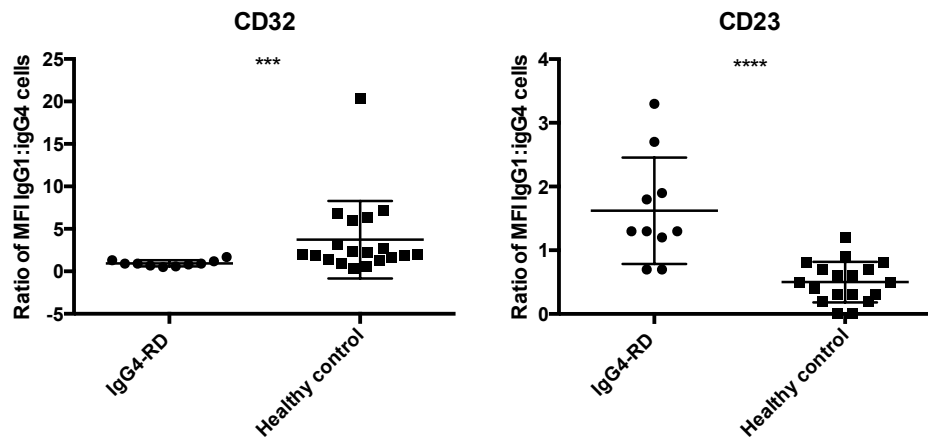


Figure 28: Ratio of Fc receptor markers on IgG1:IgG4 B cells in IgG4-RD and healthy controls. Axes and statistics as per figure 22.

Transitional cells in IgG4-RD and healthy controls.

There was no difference in the expression of transitional cells (CD20+CD24^{hi}CD38^{hi}) in patients with IgG4-RD and healthy controls (Figure 29).

Figure 29: Transitional cell markers in IgG4-RD and healthy controls

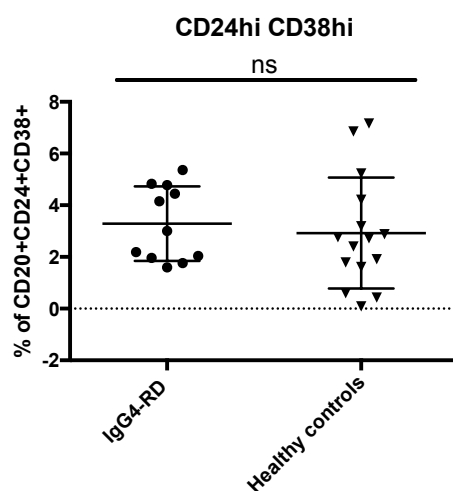


Figure 29: Dot plots showing the percentage of transitional cells in IgG4-RD and healthy controls. The y-axis displays the percentage of CD20+CD24⁺⁺ CD38⁺⁺ cells. Mann-Whitney values, where NS is $p > 0.05$.

IgG4 B cells expressed lower CD38 in both IgG4-RD and healthy controls. IgG4 B cells similarly expressed lower CD24 in healthy controls, but not IgG4-RD patients (Figure 30).

Figure 30: Transitional cell markers on IgG1+ and IgG4+ B cells in IgG4-RD and healthy controls.

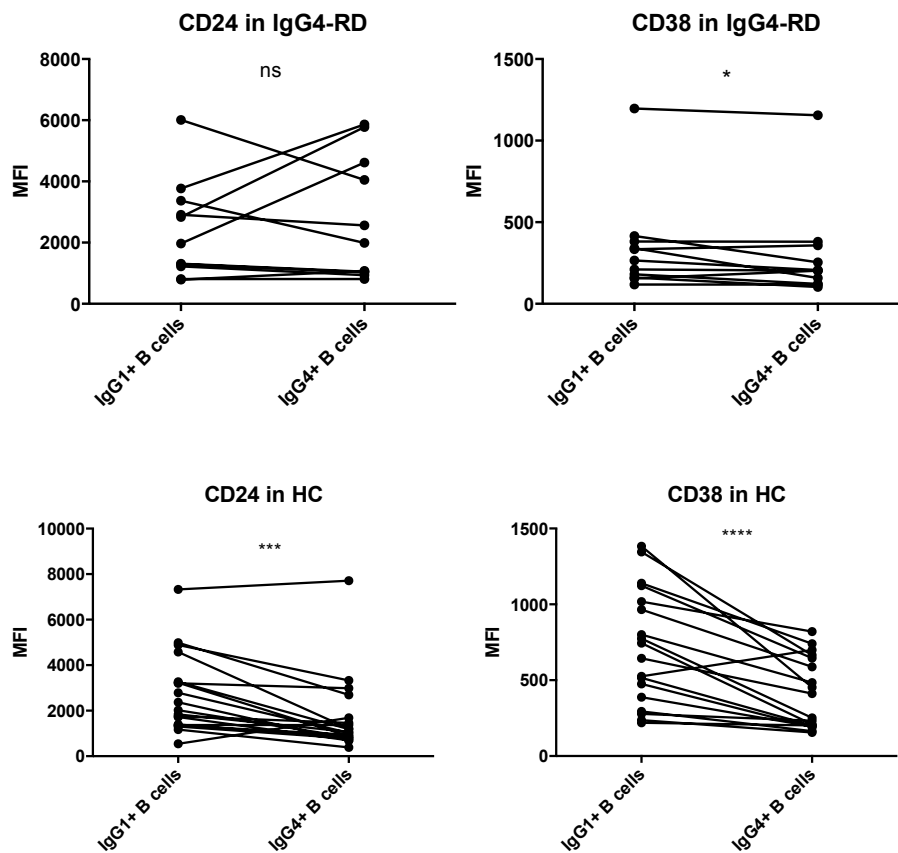


Figure 30: Paired frequency plots showing surface expression of transitional cell markers CD24 and CD38 in CD20+IgG1+ and CD20+IgG4+ B cells in IgG4-RD patients. Axes and statistics as per Figure 21.

As CD38 and CD24 expression were lower on IgG4+ than IgG1+ cells, it was unsurprising that when correcting for this, expression of both CD38 and CD24 were significantly lower in IgG4-RD patients than in healthy controls (Figure 31).

Figure 31: Transitional cells and ratio of IgG1:IgG4 B cells in IgG4-RD and healthy controls.

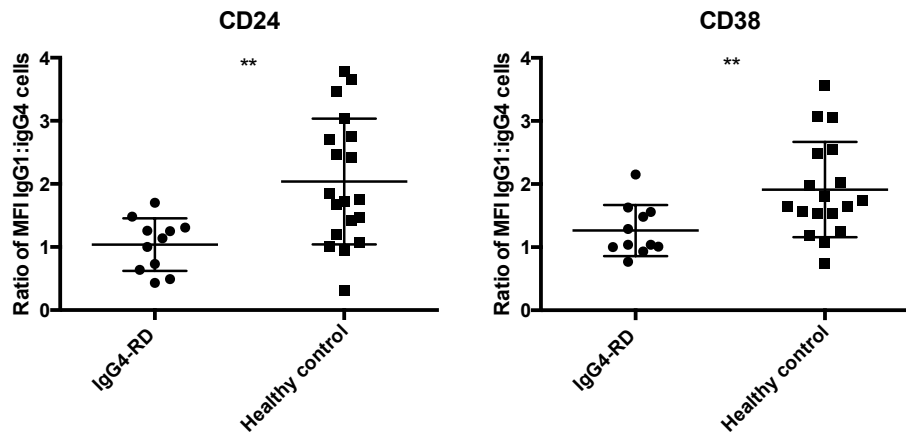


Figure 31: Ratio of transitional cell markers on IgG1:IgG4 B cells in IgG4-RD and healthy controls. Axes and statistics as per figure 22.

9.5 DISCUSSION

After our collaborators identified an IgG4-specific antibody suitable for staining and isolating IgG4 B cells, we were able to use this approach to establish certain similarities but also important differences in the frequency and phenotype of IgG1 and IgG4 B cells in health and disease. IgG1 B cells were more prevalent than IgG4 B cells in health, which correlated with serum IgG subclass levels. Unsurprisingly both IgG1 and IgG4 B cells had a memory phenotype, but there were important differences with regard to their potential to react to complement activation and immune complexes, which may help explain the differential regulation of the IgG4 antibody response. In IgG4-RD patients with active disease, there were more numerous IgG1 and IgG4 memory B cells and IgG4 antibody-producing plasmablasts compared with healthy states. Both the IgG4 memory B cells and plasmablasts correlated with the serum IgG4 level in these patients, suggesting the elevated serum IgG4 level reflects cells of the B cell lineage circulating in the blood.

Complement receptors expressed on B cells act as an interface to transmit signals from the innate to the adaptive immune system (Mauri et al, *Annu Rev Immunol* 2012). In healthy donors, IgG4 B cells expressed significantly less CR2 (CD21) and CR1 (CD35), suggesting a decreased sensitivity to complement components. CD21 is part of the B-cell receptor complex and CD21 co-ligation lowers the threshold for B-cell activation and clonal expansion (Barrington et al, *Proc Natl Acad Sci USA* 2009). Larger amounts of antigen may be needed to induce the production of IgG4 antibodies, which might contribute to the need for prolonged stimulation with antigen. In IgG4-RD patients, CR2 (CD21) expression is similar on both IgG1 and IgG4 B cells, suggesting that either IgG4 B cells have an increased sensitivity for this complement receptor in disease or that the increased number of IgG4 B cells in disease accounts for its increased expression. CR1 (CD35) binds C3b and CD4b, is a negative regulator of the classical and alternative pathways, and is inhibitory to B cell proliferation (Nielsen et al, *Immunol* 2001). In both healthy donors and IgG4-RD patients, IgG4 B cells have lower expression of this receptor, which may allow for B cell proliferation and survival. Interestingly, CD35 expression is also decreased in systemic lupus, sarcoidosis and immune complex disease (Arora et al, *Mol Immunol* 2004; Hamacher et al, *Eur Resp J* 1998).

Fc receptors bind the Fc fractions of antibody both freely circulating and within antibody-containing immune complexes. CD32 (FcγRIIb) is an inhibitory Fc receptor, which modulates B cell activation in response to BCR ligation and counteracts signalling through activating FcRs by suppressing B cell survival and proliferation (Nicholas et al, *Immunol* 2003). All four IgG isotypes have similar low binding affinity to this receptor and therefore the full spectrum of IgG antibody responses can negatively

regulate survival of FcγRIIb⁺ B cells to control an immune response (Karnell et al, J Immunol 2014). In healthy donors, there was a lower expression of inhibitory FcγRIIb on IgG4 compared with IgG1 cells, whereas in IgG4-RD there was no loss of expression on IgG4 B cells. Similarly, CD32 expression is known to be aberrant in autoimmune conditions such as systemic lupus (Means et al, J Clin Invest 2005).

FcεRI (CD23) is the low affinity receptor for IgE and may play a role in the regulation of serum IgE levels (negative regulator), and has been shown to be important in allergy, parasitic infections and chronic lymphocytic leukaemia (Stone et al, JACI 2010). In healthy donors, there was a higher expression of the FcεRI (CD23) on IgG4 compared with IgG1 B cells. Since many allergens are good inducers of IgG4 as well as IgE and CD23 can be up regulated by IL-4 (Acharya et al, Clin Exp Immunol 2010), it is interesting that IgG4 B cells appear to have increased capacity to capture IgE molecules. Signalling via IgE might trigger IgG4 cells and contribute to dampening of allergic IgE responses. Besides IgE antibodies, CD23 binds to CD21 and several integrins (Acharya et al, Clin Exp Immunol 2010). Taken together, IgG4 B cells may stand out in the way they receive signals via the B-cell receptor complex. Our data show that a subset of IgG4-RD patients have an increased prevalence of atopy, and such patients have an elevated serum IgE level during active disease (**Chapter 5**). Interestingly, IgG4-RD patients have a reversal of FcεRI (CD23) expression compared with healthy donors, with a similar expression on IgG4 to IgG1. In IgG4-RD, FcεRI may indeed be downregulated by the high IgE levels or it may account for an inability to negatively regulate IgE levels in active disease states.

The cell surface markers CD24 and CD38 are important in cell adhesion and activation. Circulating B cells expressing CD24^{hi}CD38^{hi} have been termed transitional cells, and in the context of IL10 production, B regulatory cells (Marie-Cardine et al, Clin Immunol 2008; Yanasa et al, J Immunol 2009; Fillatreau, Nat Immunol 2002). Both B regulatory and transitional cells have been described in autoimmune diseases such as systemic lupus and rheumatoid arthritis (Blair et al, Immunity 2010). Previous reports of circulating B regulatory cells in AIP and IgG4-RD have shown conflicting results (Sumimoto et al, Pancreatology 2014; Lin et al, Arthritis Res Ther 2014; Matoo et al, JACI 2014), with different surface markers used to delineate this population. In healthy controls there was lower expression of CD24 and CD38 on IgG4 compared with IgG1 B cells. In IgG4-RD, the IgG4 and IgG1 cells retained a similar expression of these cell adhesion and activated markers, although overall there was no difference in CD20⁺CD24^{hi}CD38^{hi} transitional cells compared with healthy donors.

Importantly, our data suggest that the elevated IgG4 response in IgG4-RD is made up of both IgG4 memory B cells and antibody-producing plasmablasts in the blood, and predominantly IgG4 plasmablasts/plasma cells in the tissues. B cell clones and plasmablast accumulations in the blood have recently been described in IgG4-RD patients (Maillette de Buy Wenniger et al, Hepatology 2013; Matoo et al, JACI 2014) and similarly in a small subset of patients with active systemic lupus and rheumatoid arthritis (Nicholas et al, Clin Immunol 2008; Owczarczyk et al, Sci Transl Med 2008). Given the high levels of surface MHC class II (HLA-DR) on plasmablasts, one could speculate that these cells serve as vital antigen-presenting cells to CD4⁺ T cells in patients with IgG4-RD, but it remains possible that this disease is driven directly by B-lineage cells with little or no role for T cells in pathogenesis. Although we suspect that

activated IgG4 B cells participate in the process of pathogenesis, a demonstration of their direct role in disease pathogenesis is lacking, and one could still argue that IgG4 plasmablasts are secondarily induced bystanders in this disease. The identity of the antigens recognised by these immunoglobulins remains to be determined, and it is not yet known whether the same antigens are recognised during the initial disease process and at the time of relapse. The specific contributions of both B and T cells to this process also remain to be identified. A clearer picture may emerge after the characterization of self-antigens and the clonal identification and characterization of disease associated CD4 and/or B cells both at initial presentation and at the time of relapse.

9.6 SUMMARY

This chapter highlights the differences in phenotype and function between IgG1 and IgG4 B cells in health and in patients with IgG4-RD. It is clear that IgG4⁺ B cells have a distinct phenotype that may allow them to dampen the immune response as a blocking antibody. Importantly, in IgG4-RD the elevated IgG4 response seems to be made up of both IgG4 memory B cells and antibody-producing plasmablasts in the blood, and predominantly IgG4 plasmablasts/plasma cells in the tissues. However, demonstration of a direct role of these activated B cells in disease pathogenesis is still lacking. Further studies investigating the immunological effects of rituximab treatment in active disease, recognised to deplete CD20⁺ B cells with biochemical and radiological disease resolution (Carruthers et al, *Ann Rheum Dis* 2015), may provide further clues to the role of the B cell lineage.

Chapter 10 Gene Expression in IgG4-RD

Extracts from Chapter 10 have been published in abstract form:

EL. Culver, E. Marchi, C. Manganis, W. Smit, M. Makuch, N. Ramamurthy, T. Rispens, P. Klenerman, E. Barnes Gene expression analysis identifies immune signalling and complement pathways in IgG4-related disease. *J Hepatology*. April 2014;60(1):S185-186.

10.1 HYPOTHESES

1. Gene expression analysis in whole blood implicates the innate and adaptive immune system as important in IgG4-RD pathogenesis.
2. Gene Set Enrichment Analysis, used to assess co-expression of gene sets with shared biological function or regulation, highlights the importance of the complement cascade proteins in IgG4-RD.
3. Principle component analysis identifies genes expressed in severe disease phenotypes, as determined by the presence of multiple organ involvement, relapse to medical therapy and development of disease-related complications.

10.2 INTRODUCTION

The challenges in IgG4-RD are in diagnosis, identifying those who will relapse or develop multiple organ involvement, and understanding the pathogenic mechanisms that initiate and drive disease. At present, diagnosis relies on careful interpretation of a

combination of histopathological appearances, radiological features and serological abnormalities, in an appropriate clinical scenario (Stone et al, N Engl J Med 2012). Discrimination between mass-forming lesions and strictures caused by malignancy, inflammatory conditions, and IgG4-RD can be difficult. The disease is corticosteroid-responsive in its inflammatory state, although persistent inflammation can lead to fibrosis, organ dysfunction and failure (Huggett et al, AJG 2014). We have demonstrated that an elevated serum IgG4 >2.8g/l at diagnosis is associated with multiple organ involvement and risk of disease relapse, and that IgG4-SC, the biliary manifestation of the disease, is an independent marker for relapse after corticosteroid therapy (**Chapter 3**). However, we have limited serological or immunological markers of disease, other than serum IgG4 level (**Chapter 4**) and IgG4 plasmablasts and memory B cells (**Chapter 9**).

The assessment of candidate T cell subsets and cytokines suggest that the immune process in IgG4-RD is predominated by CD4⁺ T helper 2 (Th2), T regulatory (Treg) and T follicular helper (TFh) cells. The tissues of affected organs are infiltrated with CD4⁺ T cells and abundant IgG4-positive plasma cells, and display a storiform pattern of fibrosis, obliterative phlebitis and a variable presence of eosinophils (Culver & Bateman, Diagn Histopathol 2013). Th2 and Treg cytokines (IL-4, IL-5, IL-10, TGF- β) and Treg transcription factor (Foxp3) mRNA expression in blood and tissue are substantially higher in IgG4-RD than in other autoimmune conditions (Okazaki et al, Gastroenterol 2000; Miyoshi et al, Pancreas 2008). IL21 production and CXCR5⁺ICOS⁺ TFh cells have also been demonstrated in higher numbers in blood and affected organs in patients with AIP and IgG4-SC, compared to pancreatitis and PSC (Esposito et al, PLoSOne 2008). Importantly, IL4, IL5 and IL13 promote the production

of IgG4 and IgE antibodies, IL10 drives class switching to IgG4, and IL21 induces proliferation of IgG4-switched cells (Kusuda et al, Pancreas 2011; Kuchen et al, J Immunol 2007). TGF- β is an important fibrogenic factor, postulated to contribute to the storiform fibrosis in this disease (Taylor et al, Immunology 2006).

In this chapter, a non-hypothesis driven approach is used to identify the expression of genes and biological pathways associated with IgG4-RD. Gene expression analysis using a whole human genome oligomicroarray was undertaken in patients with active single-organ and systemic IgG4-RD and healthy controls. Gene expression profiles were interpreted using Ingenuity Pathway Analysis and Metacore software, and immunologically relevant genes were confirmed by biomedical literature review. Gene Set Enrichment Analysis was used to assess co-expression of genes in both the original dataset of up/down regulated genes and for cross-comparison with relevant publicly available datasets. Gene expression was subsequently confirmed at the RNA and protein level. A bioinformatics-based deconvolution algorithm was used to suggest the contribution of each peripheral blood cell type to the general expression profile. Lastly, principle component analysis and a dendrogram heat map were used to highlight the differential gene expression in patients with varying degrees of disease severity.

10.3 SPECIFIC METHODS

Methods detailing patient identification, data storage, diagnostic criteria, definitions, serological measurements, blood sampling and processing, whole blood RNA extraction, Aligent platform for gene expression, analysis software packages, enrichment analysis, deconvolution algorithm, principal component analysis, flow cytometry, Taqman RT-PCR, Luminex cytokine array and statistics are described in **Chapter 2**. Dr E Marchi provided bioinformatics assistance for this Chapter.

The pathway for gene expression analysis is shown in **Figure 1**.

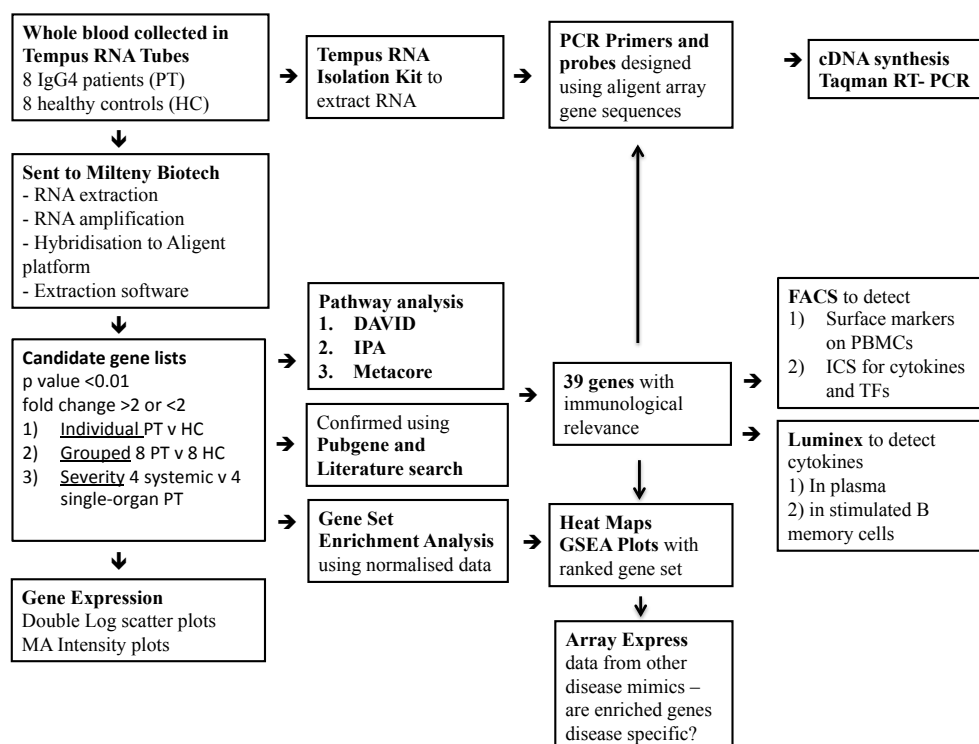
Figure 1: The pathway for gene expression analysis.

Figure 1: The figure shows the pathway used for gene expression analysis. **Abbreviations:** PT: IgG4-RD patient; HC: healthy control; MA: GSEA: gene set enrichment analysis; RNA: ribonucleic acid; PCR: polymerase chain reaction; cDNA: complementary deoxyribonucleic acid; RT-PCR: real time polymerase chain reaction; DAVID; Database for Annotation, Visualisation and Integrated Discovery; IPA: ingenuity pathway analysis; PBMCs: peripheral blood mononuclear cells; ICS: intracellular cytokine staining; TFs: transcription factors.

10.3.1 Patient and control recruitment

A total of 8 treatment-naïve patients with active IgG4-RD (all with AIP; of whom 5 had IgG4-SC and additional systemic manifestations such as salivary gland and parotid disease, orbital pseudo tumour, renal disease, interstitial lung disease and retroperitoneal fibrosis) and 8 age and sex matched healthy controls were included.

10.3.2 Whole blood RNA Extraction

Whole blood was collected in Tempus RNA tubes and RNA was extracted using a Tempus Spin RNA Isolation kit (Applied Biosystems®).

10.3.3 Agilent Platform for Whole Genome Oligo-Microarray

Whole human genome oligo-microarray was performed using a single-colour oligonucleotide array on an Agilent platform (Miltény Biotech). To investigate differential gene expression, the output of individual matched samples and grouped analyses from Miltény Biotech, and an additional LIMMA package analysis was used.

10.3.4 Software to interpret gene expression profiles and pathway analysis

Gene expression profiles were interpreted using three software packages; (1) Database for Annotation, Visualisation and Integrated Discovery (DAVID) web-based program (david.abcc.ncifcrf.gov/), (2) Ingenuity Pathway Analysis (IPA) (Quiagen), and (3) Metacore (Thomson Reuters) software.

10.3.5 Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) of entire gene sets or pathways was then used to; (1) assess co-expression of gene sets sharing common biological functions, chromosomal location or regulation, and (2) cross-compare expression profiles to publicly available data sets. Furthermore, enrichment of our gene expression signatures were compared with selected experiments available in public repository microarray

databases - Array Express www.ebi.ac.uk/arrayexpress/ and Gene Expression Omnibus (GEO) www.ncbi.nlm.nih.gov/geo/.

10.3.6 Deconvolution Algorithm

A deconvolution algorithm was applied to infer the contribution of each cell type in whole blood to the general expression profile (Shen-Orr et al, Nature Methods, 2010; Abbas et al, PLoSOne 2009).

10.3.7 Principle Component Analysis

Principle component analysis (PCA) was used to calculate the co-variance of a set of variables (Ringner, Nature Biotech 2008), producing a PCA map, dendrogram and heat map showing clustering of samples based on expression of activity.

10.3.8 Taqman RT-PCR

Gene expression was investigated at the RNA level by Taqman real-time polymerise chain reaction (RT-PCR).

10.3.9 Luminex Cytokine Array

Quantitative multiplexed immunoassays based on Luminex technology were performed using a Procarta immunoassay kit (polystyrene beads) from Affymetrix.

10.3.10 Flow Cytometry

Gene expression was investigated at the protein level by flow cytometry. Expression of cell surface molecules was determined using a Beckton Dickinson LSR II (BD Biosciences) and analysed with FACS DIVA software (BD Biosciences) and FlowJo software (v7.6.5, Treestar).

10.4 RESULTS

10.4.1 Patient cohort

All 8 IgG4-RD patients were males and had pancreatic disease, and 5 of these had additional systemic manifestations and disease relapse. Nine patients had an elevated serum IgG4 ($>1.4\text{g/l}$), with one patient having an undetectable serum IgG4 and IgE level, but radiological and histological evidence of disease. The controls were age and gender-matched, all with normal serum IgG4 levels (**Table 1**).

Table 1: Characteristics of IgG4-RD patients and healthy controls.

PT	Serum IgG4 (G/L)	Gender	Age (yrs)	Organs	Relapse	CT	Serum IgG4 (G/L)	Gender	Age (yrs)
1	7.86	M	81	AIP, IgG4-SC, Op, P	Yes	1	0.26	M	76
2	17.2	M	68	AIP, SMG, P, ILD, LN	Yes	2	0.84	M	62
3	44.1	M	65	AIP, IgG4-SC TIN	Yes	3	1.2	M	65
4	40.4	M	60	AIP, IgG4-SC	Yes	4	1.12	M	59
5	7.64	M	56	AIP, IgG4-SC	Yes	5	0.35	M	51
6	21.7	M	84	AIP	No	6	0.87	M	87
7	2.14	M	56	AIP	No	7	0.78	M	55
8	<0.01	M	63	AIP	No	8	0.2	M	62

Table 1: The demographics and serum IgG4 levels of IgG4-RD patients and healthy controls, and the organ involvement and relapse status for the IgG4-RD patients. Relapse was defined as disease recurrence after at least 3 months of corticosteroid therapy, confirmed biochemically and/or radiologically. **Abbreviations:** PT: IgG4-RD patient; CT: healthy control; AIP: Autoimmune Pancreatitis; IgG4-SC: IgG4-related sclerosing cholangitis; Op: orbital pseudotumour; P: parotid disease; SMG: IgG4-siloadenitis; ILD: interstitial lung disease; LN: lymphadenopathy; TIN: IgG4-tubulointerstitial nephritis; UC: ulcerative colitis; G/L: grams per litre; M: male; Yrs: years.

10.4.2 Differential gene expression

Gene expression was first compared between individual IgG4-RD patients and healthy controls, and then in a combined group analysis. After normalization of data and adjusting for multiple comparisons, a list of differentially expressed genes between 8 IgG4-RD patients and 8 healthy controls was created.

213 putative candidate genes were identified: 161 up regulated (fold>2, $p<0.01$) and 52 down-regulated (fold<-2, $p<0.01$) (**Appendix 5**). The grouped comparison is illustrated in a double-log scatter plot (**Figure 2**) and MA plot (**Figure 3**).

Figure 2: Double Log scatter plot of differential gene expression in IgG4-RD and healthy controls.

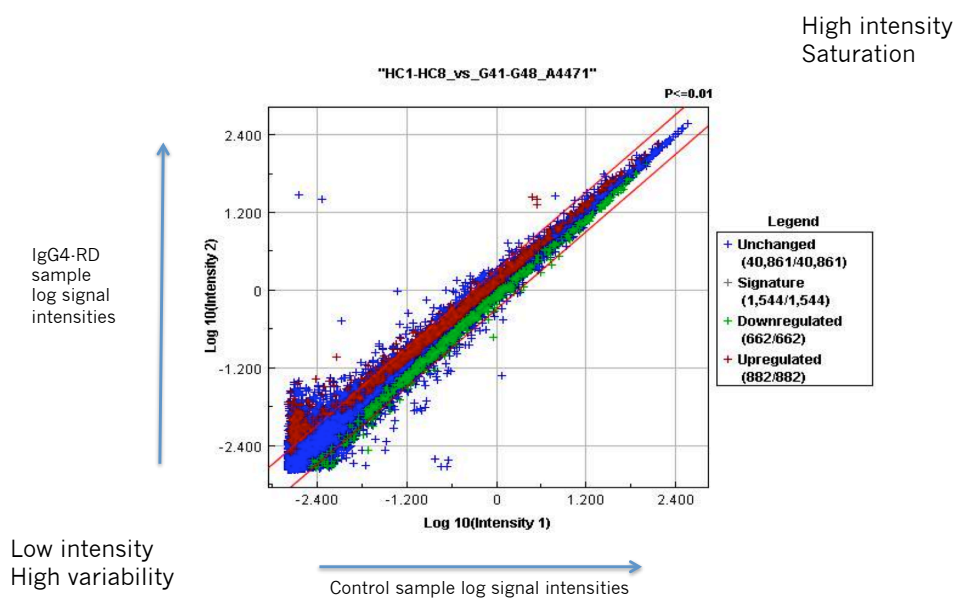


Figure 2: A double Log scatter plot showing differentially expressed candidate genes in IgG4-RD patients compared with 8 healthy controls. The signal intensities of each gene represented by a cross are shown in the double log scale. Red diagonal lines define the area of 2 fold different signal intensities. The red crosses are up regulated genes, green crosses down regulated genes, blue crosses unchanged genes and yellow crosses signature genes. The x-axis represents the healthy control sample log signal intensities and the y-axis the IgG4-RD patient sample log signal intensities.

Figure 3: MA plot of differential gene expression in IgG4-RD and healthy controls.

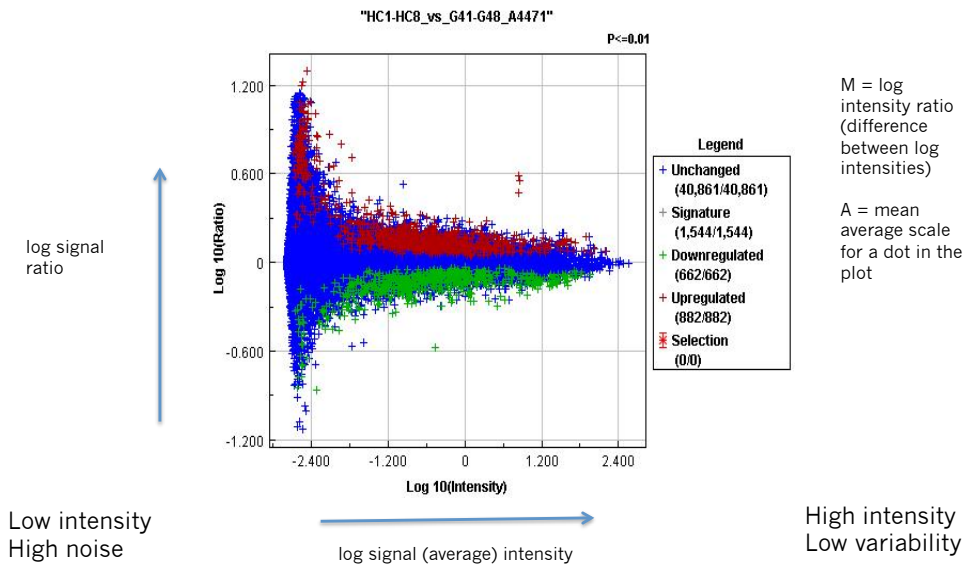


Figure 3: An MA intensity plot (log-ratio of the expression intensities versus the mean log-expression of the intensities) showing differentially expressed candidate genes in IgG4-RD patients compared with 8 healthy matched controls. The x-axis is the Log 10 ratio between IgG4-RD and healthy control samples and the y-axis is the Log 10 average of intensity. The scale, crosses and red diagonal lines are as of figure 2.

10.4.3 Gene expression profiles and pathway analysis

The candidate gene list was interpreted using 3 software packages, each adding depth to the analysis and accounting for variability between tools.

(1) Database for Annotation, Visualisation and Integrated Discovery

From the 213 candidate genes, 145 unique gene symbols were generated in DAVID. The functional annotation tool identified 1 disease category, 3 functional categories, 3 gene ontology categories, 3 pathways and 3 protein domains (**Table 2**). The main disease category was immune-mediated disease incorporating genes involved in complement activation and pathways (CR1, C1Q, C2, SERPING1) (**Figure 4**), the

coagulation cascade (F5, SERPING1), cytokine-cytokine receptor interactions (IL21, IL5), chemokine signalling (CCL23, CCL25, GNG4), and TGF-beta signalling (LTBP1).

There were 47 functional annotation clusters (groups with similar biological meaning sharing similar gene members); 2 of these with an enrichment score of >1.3 (equivalent to $p < 0.05$) based on the individual gene-term enrichment with a modified Fischer exact p value (EASE score). These clusters included complement activation and pathways, humoral immune response, inflammatory response, B cell mediated immunity, immunoglobulin-mediated immune response, protein processing and maturation, disulphide bonds, glycosylation, lectins and carbohydrate binding.

Table 2: DAVID Annotation Summary

Summary	No.	Original resource	No. of Genes	Genes involved	Enriched terms associated with gene list
Disease categories	1	Genetic Association Disease Database	20	9	Immune disease (IL5, IL21, PPARG, IgHCG1, IgHCG3, IgHCG4, IgHCM, CYP2J2, CR1, F5, PTGDR2, FOXJ1)
				2	Non-Hodgkins Lymphoma (IL5, PPARG)
Functional categories	3	SP-PIR-Keyword	133	14	Complement path; Glycoprotein; Lectin; Immunoglobulin Domain; Receptor; Secreted; Disulphide bond; Glycoprotein
		Up-Seq-Feature	133	7	Signal peptide; Disulphide bond; Glycosylation site; Sequence variant; Extracellular, cytoplasmic and fibronectin type III domains
		Cog-Ontology	10	0	
Gene Ontology	3	Goterm-BP-Fat	93	63	Complement activation, classical pathway; lymphocyte-mediated immunity; circulating immunoglobulin and immunoglobulin-mediated immune response; inflammatory response; humoral and innate immune response; somatic recombination of immune receptors built from immunoglobulin domains; cell morphogenesis and maturation.
		Goterm-CC-Fat	96	3	Extracellular region and space
		Goterm-MF-Fat	92	9	Complement binding; Carbohydrate and sugar binding; Prostaglandin receptor activity; Actin and tropomyosin binding; icosanoid and prostanoid binding.
Pathways	3	KEGG path (metabolic)	34		1 Path: Complement cascade (CR1, C1Q, SERPING1)
		BIOCARTA path (signalling)	9		1 Path: Coagulation cascade and Intrinsic prothrombin pathway (F5, SERPING1)
		Biological Biochemical Image Database	4		
Protein domains	3	Interpro	116		
		SP-PIR-Keyword	55		
		SMART	75		
Functional Annotation Clusters	47	Cluster 1 (ES 2.38) Components		15	Signal peptide; Disulphide bond; Glycosylation site; Glycoprotein; Extracellular and cytoplasmic domains.
		Cluster 2 (ES 1.34) Immune response		30	Complement and coagulation cascades; Complement activation, classical pathway; B-cell mediated and lymphocyte mediated immunity; circulating immunoglobulin and immunoglobulin-mediated immune response; acute inflammatory response; humoral, adaptive, innate immune response; protein maturation and processing.
		Cluster 3 (ES 1.12) Lectins		8	C-type lectin; carbohydrate and sugar binding.

Table 2: The functional annotation summary of 213 genes differentially expressed in IgG4-RD patients and healthy controls, run through DAVID web-based program. **Abbreviations:** ES: enrichment score (>1.3 equivalent to a p value of <0.05).

(2) Ingenuity Pathway Analysis (IPA)

From the 213 candidate genes, 150 were mapped to IPA with a fold change from -7.41 to +19.3. IPA identified 5 main diseases and disorders, 45 molecular and cellular functions, 7 physiological system development and functions, 129 canonical pathways and 25 overlapping pathways with common genes, 9 networks and 2 overlapping networks, 131 molecules and 19 upstream regulators (**Table 3**).

The main biological functions were the inflammatory response, immune cell trafficking, hypersensitivity, and cell-to-cell signalling and interactions. The complement system was the key canonical pathway (four-fold higher than other pathways $p=2.85 \times 10^{-5}$), incorporating C2, C1Q, CR1 and SERPING1 (**Figure 4**). Overlapping up-regulated pathways included the cytokine-mediated communication between immune cells and T helper cell differentiation (IL21, IL5), acute phase response (C2, SERPING1), communication between innate and adaptive immune response, Fc Epsilon R1 signalling (IL5), airway inflammation in asthma (IL5), and haematopoiesis of pluripotent stem cells. The main network comprised 35 genes and 11 focus molecules (C1QC, CR1, IL5, IL21, F5, PPARG, PTGDR2, SDC1, TIMP4, GNG4, TRPM3) involved in cell-cell signalling and interaction, overlapping with a second network of 35 genes and 4 focus molecules (CCL23, PPARG, CDC25C, HSD3B7) involved in the inflammatory response and cellular movement. Upstream regulators that may contribute to gene expression changes observed included STAT3 and GATA3 (transcription targeting IL21, IL5), ROR2 (kinase targeting PPARG), IL4 and IL10 (cytokines targeting CCL23, IL5, PPARG, CR1).

Table 3: IPA Functions, Pathways and Networks

	Categories (p<0.05)	Functions	Molecules/Genes	P value	Focus molecules
Diseases and disorders	5	1. Inflammatory response		0.000401	9
		2. Cancer		0.00307	8
		3. Gastrointestinal disease		0.00371	6
		4. Hypersensitivity response		0.000401	4
		5. Haematological disease		0.0128	2
Molecular and Cellular Functions	45	1. Cell-to-cell signalling and interaction		0.000401	12
		2. Cellular Movement		0.000466	6
		3. Cellular Development		0.00043	10
		4. Cell Death and Survival		0.00049	6
Physiological Development and Functions	7	1. Immune cell trafficking		0.000401	8
		2. Cell mediated immune response		0.00472	4
		3. Haematological system function		0.000401	11
		4. Tissue development		0.00351	8
		5. Haematopoiesis		0.00143	7
Canonical Pathways (metabolic and signalling)	129	1. Complement system	C1Q, C2, CR1, SERPING1	0.0000285	4(28)
		2. Role of cytokines mediating communication between immune cells	IL21, IL5	0.0309	2(43)
		3. Inhibition of matrix metalloproteases	SDC1, TIMP4	0.0222	2(36)
		4. Airway inflammation in Asthma	IL5	0.0254	1(4)
		5. T helper cell differentiation	IL21, IL5	0.0669	2
		6. Haematopoiesis from pluripotent stem cells		0.0296	2(42)
		7. Acute Phase Response	C2, SERPING1	0.0582	2(155)
		8. FcEpsilon Receptor Signalling	IL5	0.0302	1(107)
		9. TGFbeta Signalling	ACVR1C	0.039	1(81)
Overlapping Pathways	25	1. Role of cytokine in mediating communication between immune cell		0.0309	
		2. T helper cell differentiation		0.0669	
		3. Communication between innate and adaptive immune cells		0.0892	
		4. Airway inflammation in asthma		0.0254	
		5. Haematopoiesis from pluripotent and multipotent stem cells		0.0296	
Networks	9	1. Cell-To-Cell Signalling and Interaction, Cell death and survival, Haematological System	C1QC, CD200, CD200R1, CEACAM1, CIITA, CR1, CTSC, F5, FABP4, FAM65B, FCER1G, FGF2, GNG4,	ES 16	11

		Development and Function	ICOS, IFNG, IL5, IL13, IL21, IL33, IL18R1, IL18RAP, IL3RA, JAK2, MYB, NFE2, PIM1, PPARG, PTGDR2, QSOX1, SATB1, SDC1, STAT3, TBX21, TIMP4, TRPM3		
		2. Inflammatory Response	CCL2, CCL3, CCL5, CCL23, CDC25C, CFB, CXCL1, CXCL2, CXCL3, CXCL5, EBI3, EGR1, GCLM, HAS1, HBEGF, HSD3B7, IL4, IL6, INHBA, LAMP3, MAFF, MAPK1, MAPK14, NOD2, NR4A2, OASL, PPARG, RNASE1, RNASE2, SLAMF7, THBD, TNF, TNFSF14, TREM1, WNT5A	ES 4	4
		3. Cellular Development	ANG, UCHL1	ES 2	1
		4. Cellular Growth and Proliferation	MYOF, TGFBR2	ES 2	1
Overlapping Networks	2	1. Cell-To-Cell Signalling and Interaction, Cell death and survival, Haematological System Development and Function	C1QC, CD200, CD200R1, CEACAM1, CIITA, CR1, CTSC, F5, FABP4, FAM65B, FCER1G, FGF2, GNG4, ICOS, IFNG, IL5, IL13, IL21, IL33, IL18R1, IL18RAP, IL3RA, JAK2, MYB, NFE2, PIM1, PPARG, PTGDR2, QSOX1, SATB1, SDC1, STAT3, TBX21, TIMP4, TRPM3	ES 16	35
		2. Inflammatory Response	CCL2, CCL3, CCL5, CCL23, CDC25C, CFB, CXCL1, CXCL2, CXCL3, CXCL5, EBI3, EGR1, GCLM, HAS1, HBEGF, HSD3B7, IL4, IL6, INHBA, LAMP3, MAFF, MAPK1, MAPK14, NOD2, NR4A2, OASL, PPARG, RNASE1, RNASE2, SLAMF7, THBD, TNF, TNFSF14, TREM1, WNT5A	ES 4	35
Toxicology List	2	1. Acute Phase Response Proteins		0.0102	2(24)
		2. Cytochrome P450 Panel		0.044	1(7)
Upstream Regulators	19	STAT3 (IL21,IL5,TRPM3)		0.00169	3
		SEMA3A (MAP1B)		0.00624	1
		ICAM3 (PPARG)		0.00624	1
		IL12RB1 (IL21)		0.00624	1
		IL4 (CCL23,IL5,PPARG)		0.0099	3
		LGALS3 (IL5)		0.0124	1
		SATB1 (F5,GNG4,IL5)		0.0146	3
		GATA3 (IL5)		0.0186	1

		ROR2 (PPARG)	0.0186	1
		TBX21 (PTGDR2)	0.0247	1
		CDK9 (PPARG)	0.0308	1
		YAP1 (DIAPH3)	0.0308	1
		RNF40 (PPARG)	0.0369	1
		WAC (PPARG)	0.0369	1
		MYB (IL5)	0.0429	1
		IL10 (CR1,IL5)	0.0447	2
		SPI1 (C1QC)	0.0489	1
		HDAC1 (IL5)	0.0489	1
		FCER1G (IL5)	0.0489	1
Molecules	131	ACVR1C ADORA3 ANG ANGPTL2 ARHGEF26 C2 C1QC CCL2 CCL3 CCL5 CCL23 CCL25 CD200 CD248 CD200R1 CD8A CD8B CDC25C CDK9 CEACAM1 CENPA CFB CIITA CLSPN CPE CR1 CTSC CXCL1 CXCL2 CXCL3 CXCL5 CYP2J2 DIAPH3 DKKL1 DMC1 DNAJC5B EBI3 EGR1 ERG EYA2 F5 FABP4 FAM65B FBN1 FCER1G FGF2 GATA3 GCLM GCM2 GGT5 GNG4 GRIK3 HAS1 HBEGF HDAC1 HS3ST5 HSD3B7 ICAM3 ICOS IFNG IL4 IL5 IL6 IL10 IL13 IL21 IL33 IL12RB1 IL18R1 IL18RAP IL3RA INHBA JAK2 KCNN3 KLRC3 LAMP3 LEFTY1 LEFTY2 LGALS3 LTBP1 MAFF MAP1B MAPK1 MAPK14 MYB MYOF NANOG NEK2 NFE2 NOD2 NODAL NPPC NR4A2 NRN1 OASL OLIG2 PIM1 PLXDC1 PPARG PTCH1 PTGDR2 PYGO1 QSOX1 RNASE1 RNASE2 RNF40 ROR2 SATB1 SDC1 SEMA3A SERPING1 SLAMF7 SLC24A2 SLC29A2 SPI1 STAT3 TBX		
Up-regulated	42	Gene (Fold change)		
		GCM2 (12.097), ANGPTL2 (9.932), SLC24A2 (7.651), GRIK3 (7.576), CCL23 (7.288), NRN1 (7.083), IL5 (6.548), DKKL1 (5.412), UCHL1 (5.028), USP42 (4.797), DMC1 (4.599), UNC5B (4.431), TRIM9 (4.327), KCNN3 (4.323), NPPC (4.182), HS3ST5 (3.958), MAP1B (3.941), EYA2 (3.762), C1QC (3.754), CENPA (3.592), GNG4 (3.296), CPE (3.213), IL21 (3.197), OLIG2 (3.191), PPARG (3.161), ARHGEF26 (2.986), TIMP4 (2.823), DIAPH3 (2.770), NEK2 (2.631), LTBP1 (2.449), HSD3B7 (2.348), C2 (2.301), CLSPN (2.242), MYOF (2.188), CCL25 (2.155), SDC1 (2.132), GGT5 (2.095), PTGDR2 (2.069), SERPING1 (2.068), CDC25C (2.058), CR1 (2.047), F5 (2.023), ADORA3 (2.008).		
Down-regulated	11	PYGO1 (-7.137), DNAJC5B (-2.656), TRPM3 (-2.637), KLRC3 (-2.213), SLC29A2 (-2.165), ACVR1C (-2.152), PLXDC1 (-2.132), CD248 (-2.127), PTCH1 (-2.108), CYP2J2 (-2.057), CD8B (-2.011).		

Table 3: The table shows the functions, pathways, networks and regulators of 213 genes differentially expressed in IgG4-RD patients and healthy controls, run through Ingenuity software.

(3) Metacore Analysis

From the 213 genes, 176 were mapped to Metacore and utilised for enrichment analysis (**Table 4**). The main pathway map group was the immune response, within which the key pathways were the lectin-induced and classical complement pathways (network genes C2b, C2a, C2, CD21, CR1, C1 inhibitor) (**Figure 4**). Other important pathways included signal transduction (CRT2, PPARG, PGD2R), and the immune response T cell subset secreted signals (IL21 and IL5).

The main process networks were those of inflammation – the complement system (6-fold higher than any other network). The main metabolic networks involved insulin and glutamic acid pathways, in keeping with autoimmune pancreatitis and diabetes. The key GO processes were regulation of the inflammatory response, the immune response and the defence response (PPAR-gamma, LRRC32, IL21, C2, CR1L, CR1, C1 inhibitor, FOXJ1, IL5, CCL23, CCL25, CD21, Histone H2, BAFT2); GO molecular functions were complement binding (3-fold higher than other functions) and prostaglandin receptor activity; GO localisations were the plasma membrane, extracellular region, cell periphery and receptor complex.

Based on network analysis, there were 3 key networks (1) Complement, incorporating complement receptors, G-protein coupled receptors, transcription factors RelA (p65 NFkB subunit), receptors CR1 and secreted proteins CCL23, CCL25, GCP2; (2) PPAR-gamma, incorporating PPAR-gamma, arachidonic acid and intracellular fatty acids, transcription factor PPAR-gamma fusion protein, receptor galpha-specific peptide

GPCRs; and (3) p53, incorporating Bcl-XL, transcription factors NFkB, receptors PTCH1 and patched, secreted proteins angiopoietin-like 2.

The 10 genes most highly expressed in network analysis were Angiopoietin-like 2, PTCH1 Patched, UNC5B, PRUNE2, CR1, PPAR-Gamma, Syndecan-1, CCL23 and CCL25. Based on interactome analysis, there was enrichment of proteases, ligands, and receptors.

Table 4: Metacore Enrichment Analysis

Enrichment Analysis		Maps	Total genes	p Value (<0.05)	FDR	Genes in list	Network molecules
Pathway Maps	1	Immune response_ Lectin induced complement pathway	49	3.710E-07	3.410E-05	6	C2b, C2a, C2, CD21, CR1, C1 inhibitor
	2	Immune response_ Classical complement pathway	52	5.328E-07	3.410E-05	6	C2b, C2a, C2, CD21, CR1, C1 inhibitor
	3	Immune response_ Alternative complement pathway	39	2.965E-06	1.265E-04	5	Factor B, Factor Ba, Factor Bb, CD21, CR1
	4	Signal transduction_ Production and main functions of biologically active prostaglandins and Thromboxane A2	59	5.038E-03	1.612E-01	3	CRTH2, PPAR-gamma, PGD2R
	5	Immune response_ T cell subsets: secreted signals	25	9.448E-03	2.152E-01	2	IL-5, IL-21
	6	DNA damage_ ATM / ATR regulation of G2 / M checkpoint	26	1.020E-02	2.152E-01	2	CDC25C, Claspin
	7	Immune response_ Antigen presentation by MHC class I	28	1.177E-02	2.152E-01	2	CD8 beta, CD8
	8	Cell cycle_ The metaphase checkpoint	36	1.904E-02	2.966E-01	2	Nek2A, CENP-A
	9	Development_ Neural stem cell lineage commitment (schema)	38	2.108E-02	2.966E-01	2	PTCH1, MAP-1B
	10	Immune response_ IL-13 signalling via JAK-STAT	44	2.774E-02	2.966E-01	2	CCL23, FOXJ1
GO Processes	1	Regulation of inflammatory response	368	9.141E-09	3.021E-05	16	PPAR-gamma, LRRC32, Adenosine A3 receptor, Gamma-glutamyltranspeptidase, Galpha(q)-specific nucleotide-like GPCRs, Galpha(i)-specific nucleotide-like GPCRs, C2, Factor B, CR1L, Galpha(s)-specific prostanoid GPCRs, Galpha(q)-specific peptide GPCRs, PSMA1, PAX8/PPARgamma fusion protein, CR1, IL-21, C1 inhibitor
	2	Regulation of acute inflammatory response	110	9.492E-08	1.046E-04	9	PPAR-gamma, Galpha(i)-specific nucleotide-like GPCRs, C2, Factor B, CR1L, Galpha(q)-specific peptide GPCRs, PAX8/PPARgamma fusion protein, CR1, C1 inhibitor
	3	Defence response	1798	3.466E-07	2.291E-04	33	IL-5, DEFB108B, TLX, Syndecan-1, CCL23, Histone H2, PPAR-gamma, LRRC32, Adenosine A3 receptor, KLRC3, CRIPTO, Gamma-glutamyl transpeptidase, CD8, Galpha(q)-specific nucleotide-like GPCRs, Galpha(i)-specific nucleotide-like GPCRs, C1qc, C2, Factor B, CR1L, Inotropic glutamate receptor, G-protein gamma, Galpha(s)-specific prostanoid GPCRs, BATF2, Olfactory receptor, Galpha(q)-specific peptide GPCRs, PGD2R, GGT5, Kainate receptor, CD21, PAX8/PPARgamma fusion protein, CR1, CCL25, C1 inhibitor
	4	Regulation of exocytosis	147	1.125E-06	5.112E-04	9	

			Adenosine A3 receptor, TRIM9, Galpha(q)-specific nucleotide-like GPCRs, Rab-3, Galpha(i)-specific nucleotide-like GPCRs, Ionotropic glutamate receptor, Galpha(q)-specific peptide GPCRs, Kainate receptor, Rab-3C				
	5	Negative regulation of humoral immune response	12	1.181E-06	5.112E-04	4	FOXJ1, CR1L, CR1, C1 inhibitor
	6	Regulation of immune effector process	408	1.408E-06	5.112E-04	14	IL-5, FOXJ1, Adenosine A3 receptor, CD8 beta, CD8, Galpha(q)-specific nucleotide-like GPCRs, Galpha(i)-specific nucleotide-like GPCRs, C2, Factor B, CR1L, Galpha(q)-specific peptide GPCRs, CR1, IL-21, C1 inhibitor
	7	Multicellular organismal process	8197	1.529E-06	5.112E-04	87	Nek2A, Carboxypeptidase H, TRPM8, GluR7, CDC25C, TLX, CDC25, PCDHB10, Syndecan-1, TEM7, Evi5, Neuritin1, FOXJ1, Histone H2, PPAR-gamma, UNC5B, SLC24A4, Histone H2A, PTCH1, Fibromodulin, Adenosine A3 receptor, G-protein gamma 4, IRR, SLC29A2, CRIPTO, Angiopoietin-like 2, Gamma-glutamyltranspeptidase, TRIM9, mDIA2(DIAPH3), CD8, Caspase-7, Galpha(q)-specific nucleotide-like GPCRs, SHRM, PYGO1, DMC1, Rab-17, OR13C3, CNP, Rab-3, Galpha(i)-specific nucleotide-like GPCRs, KCNAB2, SLC24A2, USP42, ATP8B3, EYA2, ZBPB, CLN8, CR1L, TBX4, Ionotropic glutamate receptor, G-protein gamma, Galpha(s)-specific prostanoid GPCRs, Calponin-3, COLEC11, BATF2, BRK, BONO1, Troponin T, cardiac, Myoferlin, Olfactory receptor, PGAR, OLIG2, Galpha(q)-specific peptide GPCRs, PGD2R, UCHL1, PRTG, OR13D1, ALK-7, Kainate receptor, FAM20A, CD248, Patched, Coagulation factor V, TIMP4, PCDH8, PAX8/PPARGgamma fusion protein, BM88 antigen, SK3, CR1, KIF3A, SLC26A7, GCM2, Rab-3C, LDB2, MAP-1B, C1 inhibitor, CYP2J2
	8	Adenylate cyclase-inhibiting G-protein coupled receptor signalling pathway	79	1.547E-06	5.112E-04	7	GluR7, CRTH2, Galpha(q)-specific nucleotide-like GPCRs, Galpha(i)-specific nucleotide-like GPCRs, Ionotropic glutamate receptor, Galpha(q)-specific peptide GPCRs, Kainate receptor
Process Networks	1	Inflammation _ Complement system	73	2.538E-08	2.411E-06	9	C2b, C1qc, C2a, C2, Factor B, Factor Bb, CD21, CR1, C1 inhibitor
	2	Transport _ Potassium transport	194	1.076E-02	5.109E-01	6	TRPM8, TRPM3, SLC24A4, KCNAB2, SLC24A2, SK3
	3	Reproduction _ Male sex differentiation	246	3.082E-02	8.008E-01	6	FOXJ1, Histone H2, Histone H2A, IRR, Olfactory receptor, Patched
	4	Transport _ Calcium transport	192	3.741E-02	8.008E-01	5	TRPM8, TRPM3, SLC24A4, SLC24A2, Ionotropic glutamate receptor
	5	Cell cycle _ G2-M	206	4.819E-02	8.008E-01	5	Nek2A, CDC25C, CDC25, PTCH1, Claspin
	6	Blood coagulation	94	6.203E-02	8.008E-01	3	Galpha(q)-specific nucleotide-like GPCRs, Coagulation factor V, C1 inhibitor
	7	Cell cycle _ Meiosis	106	8.239E-02	8.008E-01	3	Nek2A, CDC25C, DMC1
	8	Cell cycle _ Mitosis	179	9.404E-02	8.008E-01	4	Nek2A, CDC25C, CDC25, CENP-A
	9	Development _ Hedgehog signalling	254	9.792E-02	8.008E-01	5	PTCH1, EYA2, OLIG2, Patched, KIF3A
	10	Cytoskeleton _ Cytoplasmic	115	9.932E-02	8.008E-01	3	CDC25C, mDIA2(DIAPH3),

		microtubules					KIF3A
Metabolic Networks	1	CYP2C9-1-Insulin-C/EBP-IRS1	98	1.427E-02	9.374E-02	2	Ionotropic glutamate receptor, Kainate receptor
	2	CYP2C9-2-Insulin-C/EBP-IRS1	99	1.455E-02	9.374E-02	2	Ionotropic glutamate receptor, Kainate receptor
	3	Glutamic acid pathway	103	1.569E-02	9.374E-02	2	Ionotropic glutamate receptor, Kainate receptor
	4	L-ornithine pathways and transport	124	2.227E-02	9.374E-02	2	Ionotropic glutamate receptor, Kainate receptor
	5	L-glutamate pathways and transport	131	2.469E-02	9.374E-02	2	Ionotropic glutamate receptor, Kainate receptor
	6	Lipid metabolism_ HETE, HPETE and Leukotriene4 metabolism	139	2.757E-02	9.374E-02	2	GGT5, CYP2J2
	7	Glutamic acid pathways and transport	145	2.983E-02	9.374E-02	2	Ionotropic glutamate receptor, Kainate receptor
	8	1-alkyl-glycerol_3-phosphoethanolamine pathway	28	5.207E-02	1.432E-01	1	Gamma-glutamyltranspeptidase
	9	1-alkyl-2-lyso-glycerophosphocholine pathway	50	9.122E-02	1.922E-01	1	Gamma-glutamyltranspeptidase
	10	D-glucuronic acid pathway	73	1.306E-01	1.922E-01	1	PPAR-gamma

Table 4: The table shows the enrichment analysis (pathway maps, processes and networks) of 213 genes differentially expressed in IgG4-RD patients and healthy controls, run through Metacore software. **Abbreviations:** FDR: false discovery rate.

Figure 4: Candidate genes identified in the complement pathway in IgG4-RD.

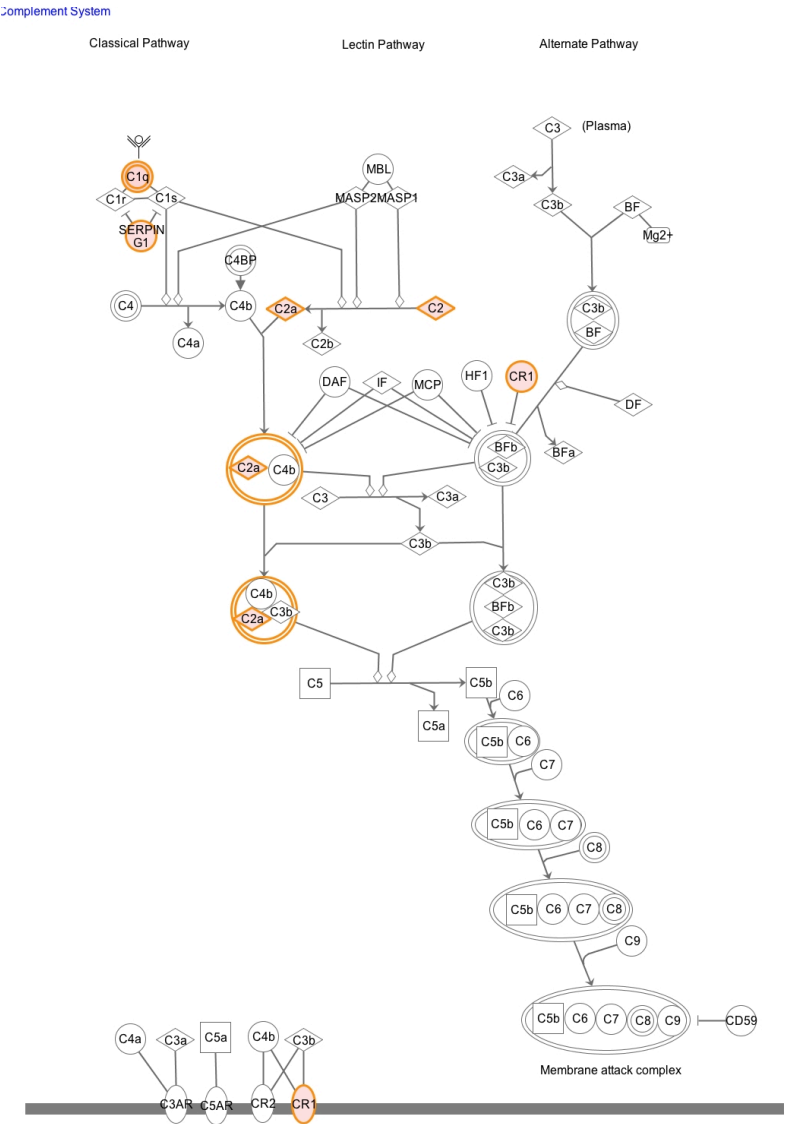


Figure 4: The key components of the classical, alternative and lectin-induced complement pathway identified by DAVID, IPA and Metacore software. Candidate genes involved in IgG4-RD ($P < 0.05$) are C2, CR1, C1QC, and SERPING1.

10.4.4 Biomedical literature search

From the list of 213 genes, 52 ‘unknown’ genes and 24 non-coding RNA, open reading frames, and hypothetical proteins were removed. A literature search of the 137 remaining genes was performed on Pubgene and Pubmed. Those candidate genes related to immunological processes, malignancy, autoimmunity and inflammation were identified (Appendix 6).

10.4.5 Candidate genes with immune function

Using the 3 software packages and biomedical literature search, 39 genes involved in the immune system were targeted for further analysis (Table 5 and Figure 5).

Figure 5: Candidate genes involved in the immune response

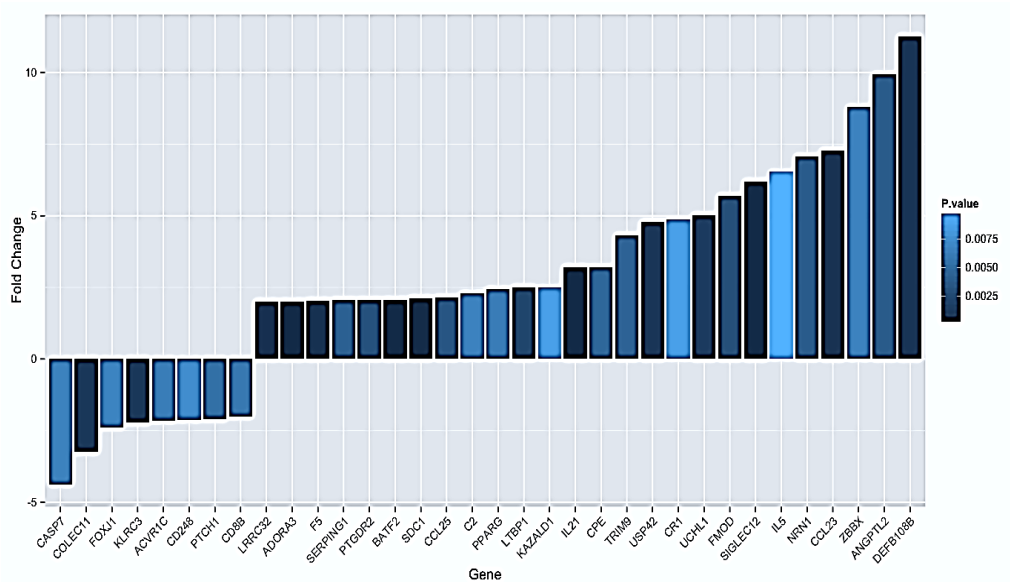


Figure 5: Bar chart of the 39 up regulated and downregulated candidate genes involved in the immune response. The x-axis lists the genes in order of fold change, the y-axis is the fold change between patient and control samples. The p value is illustrated in shades of blue, whereby the darker shade is the higher p value.

Table 5: Candidate genes involved in the immune response

Category	Gene	Full name	Fold change	P-value
Cytokines	IL-5	Interleukin 5	6.54769	0,00979
	IL-21	Interleukin 21	3.19675	0,00002
Chemokines	CCL23	Chemokine (C-C motif) ligand 23		
	CCL25	Chemokine (C-C motif) ligand 25	7.28818	0,00069
Complement cascade proteins	C1QC	Complement component 1, q subcomponent, C chain		
	C2	Complement component 2	3,75443	0,00261
	CR1	Complement component (3b/4b) receptor 1	2,30093	0,00656
	SERPING1	Serpin peptidase inhibitor, clade G (C1 inhibitor)	2,04711	0,00323
C-type lectins	CD248	Endosialin		
	COLEC11	Collectin sub-family member 11	-2,12656	0,00734
	KLRC3	Killer cell lectin-like receptor subfamily C, member 3	-3,25124	0,00112
Plasma cell differentiation	SDC1 (CD138)	Syndecan-1		
TGF- β pathway interaction proteins	LTBP1	Latent transforming growth factor beta binding protein 1		
	FMOD	Fibromodulin	2,49942	0,00211
	LRRC32 (GARP)	Leucine rich repeat containing 32	5,69471	0,00289
	BATF2 (ATF-Like 2)	Basic leucine zipper transcription factor	2,00002	0,00056
	ACVR1C	Activin A receptor, type IC	2,07418	0,00007
Transcription factors	ZBBX	Zinc finger, B-box domain containing 1		
	FOXJ1	Forkhead box protein J1	8,79478	0,00658
Protein cleavage and degradation	UCHL1	Ubiquitin carboxy-terminal hydrolase L1		
	USP42	Ubiquitin carboxyl-terminal hydrolase 42	5,02842	0,00138
	TRIM9	Tripartite motif-containing protein 9	4,79739	0,00098
Apoptosis	CASP7	Caspase 7		
Immune signalling/response molecules	ADORA3	Adenosine A3 receptor		
	CD8B	T cell surface glycoprotein CD8 beta chain	2,00847	0,00004
	DEFB108B	Beta-defensin 108B	-2,01052	0,00583
	KAZALD1	Kazal-type serine protease inhibitor domain-containing protein 1	11,25092	0,00091
	PPARG	Peroxisome proliferator-activated receptor gamma	2,51409	0,0086

	PTGDR2	Prostaglandin D2 receptor 2	2,4307	0,00614
	SIGLEC12	Sialic acid binding Ig-like lectin 12	2,06942	0,00309
Other molecules with possible immune function	ANGPTL2	Angiopoietin-related protein 2		
	NRN1	Neuritin 1	9,9317	0,0038
	CPE	Carboxypeptidase E	7,08321	0,00381
	F5	Coagulation factor V	3,21333	0,00469
	PTCH1	Patched 1	2,02314	0,00063

Table 5: The table lists the category, gene symbol, full name, fold change and *p* value, of the differential candidate genes involved in the immune response in IgG4-RD.

10.4.6 Gene Set Enrichment Analysis

Gene Set Enrichment analysis (GSEA) was performed to determine the rank enrichment score of individual genes in the dataset (32575 probes). This was used as an internal control and as a guide for further functional analysis of individual and groups of genes.

The 39 genes involved in the immune response were mapped on a GSEA Plot; 22 were enriched (Enrichment Score 0.6912; $p=0.0059$) (**Figure 6 and Table 6**). Enriched genes were ranked on a heat map ($p<0.01$) (**Figure 7**).

Figure 6: Gene set enrichment analysis of candidate genes in the immune response.

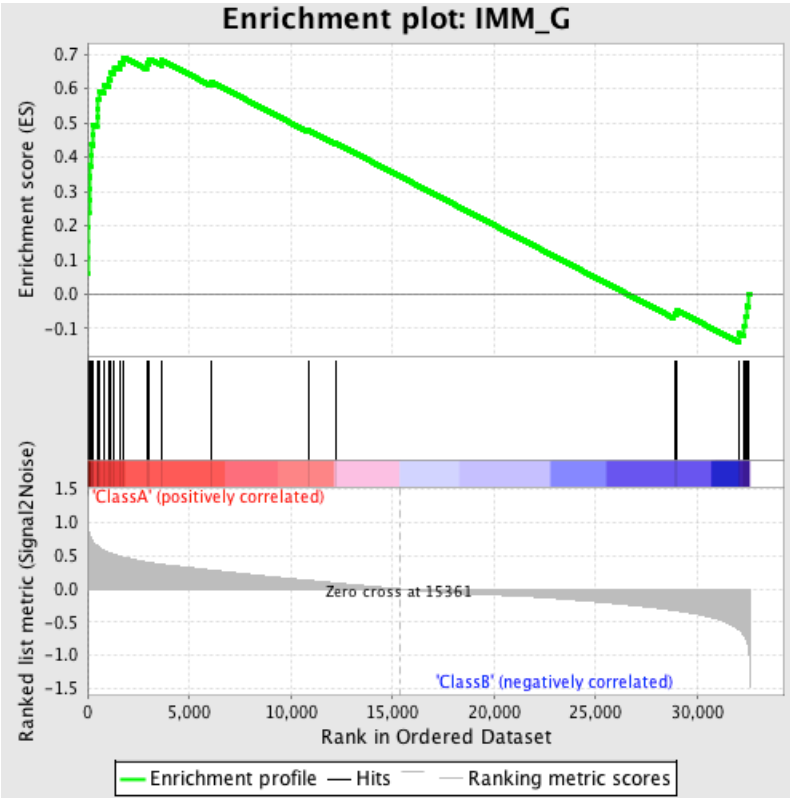


Figure 6. Gene Set Enrichment Plot of the 39 genes of interest ranked across 32,575 probes. The enrichment plot shows the ranking of the genes. The y-axis gives the enrichment score, and the x-axis shows the candidate genes 'hits' and their ranking in the plot. The green line is the enrichment profile. On the x-axis, Class A is IgG4-RD patients and Class B is healthy controls. Red is positively correlated and blue is negatively correlated. On the lower graph, the y-axis shows the ranked list metric score (signal to noise). The enrichment score was 0.69; $P=0.00591$.

Figure 7: Enriched Genes ranked on a heat map

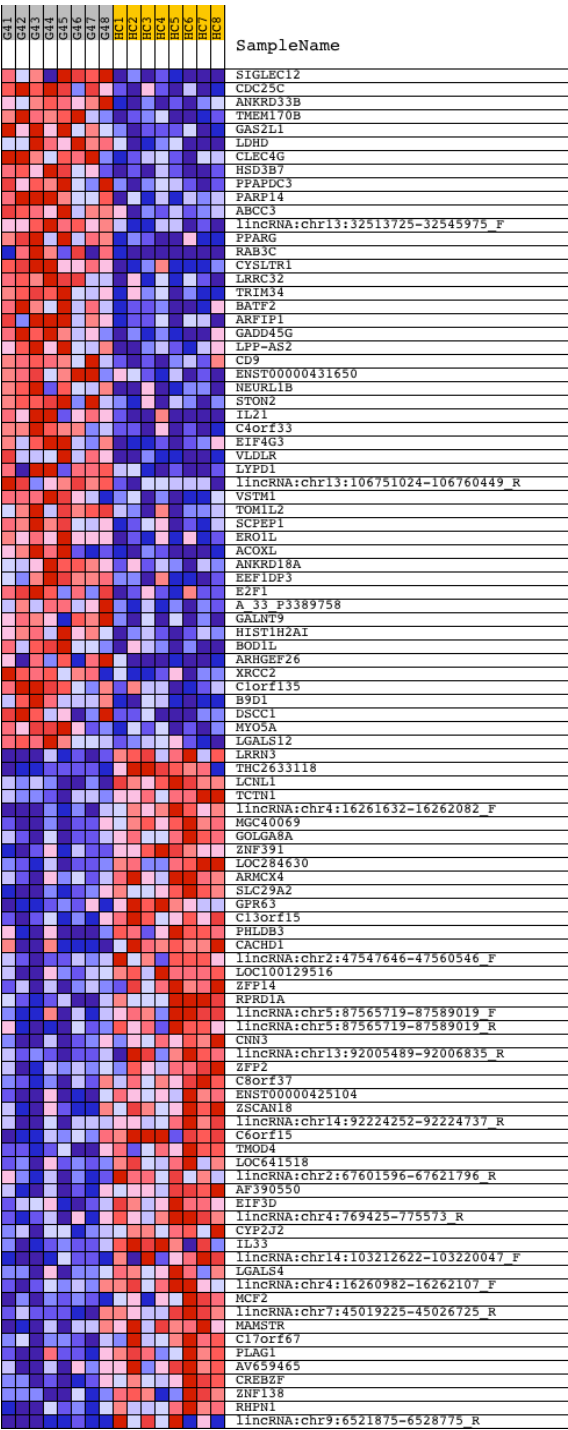


Figure 7. A heat map of the top 50 ranked genes on the GSEA Plot. G1 to 8 are IgG4-RD patients and H1 to 8 are healthy controls. Sample Name is the gene abbreviations

Table 6: Gene Set Enrichment Score in the GSEA Plot

Probe	Rank in gene list	Rank Metric Score	Enrichment Score	Core Enrichment	Full gene name
SIGLEC12	0	1.38892746	0.059140753	Yes	sialic acid binding Ig-like lectin 12
PPARG	12	1.115168571	0.10628675	Yes	peroxisome proliferative activated receptor, gamma
LRRC32	15	1.090517879	0.15265971	Yes	leucine rich repeat containing 32
BATF2	17	1.046002746	0.19716795	Yes	basic leucine zipper transcription factor, ATF-like 2
IL21	25	0.989553213	0.23908815	Yes	interleukin 21
C2	73	0.848787606	0.27378523	Yes	complement component 2
PTGDR2	87	0.828904212	0.3086806	Yes	Null
LTBP1	94	0.815738499	0.3432305	Yes	latent transforming growth factor beta binding protein 1
PRSS33	134	0.780719459	0.37527508	Yes	protease, serine, 33
SDC1	173	0.754830062	0.40624803	Yes	syndecan 1
CCL25	211	0.728804588	0.43614355	Yes	chemokine (C-C motif) ligand 25
F5	260	0.709275186	0.46486944	Yes	coagulation factor V (proaccelerin, labile factor)
USP42	280	0.701265574	0.4941455	Yes	ubiquitin specific peptidase 42
CR1	465	0.647458136	0.51605964	Yes	complement component (3b/4b) receptor 1 (Knops blood group)
SERPING1	497	0.64086926	0.54239523	Yes	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)
UCHL1	515	0.637555301	0.56902	Yes	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)
FMOD	584	0.624128222	0.5935057	Yes	Fibromodulin
NRN1	790	0.584947824	0.6121127	Yes	neuritin 1
ANGPTL2	1059	0.544443429	0.6270589	Yes	angiopoietin-like 2
DEFB108B	1157	0.534167707	0.64682287	Yes	defensin, beta 108B
CCL23	1325	0.518152535	0.66375357	Yes	chemokine (C-C motif) ligand 23
C1QC	1611	0.490258366	0.6758701	Yes	complement component 1, q subcomponent, C chain
CPE	1773	0.47581619	0.69118255	Yes	carboxypeptidase E
ZBBX	2904	0.402196676	0.6735806	No	Null
IL5	3022	0.396224707	0.6868562	No	interleukin 5 (colony-stimulating factor, eosinophil)
ADORA3	3659	0.365815043	0.6828869	No	adenosine A3 receptor
CASP7	6087	0.280965894	0.62026304	No	caspase 7, apoptosis-related cysteine peptidase

TRIM9	10845	0.127572715	0.4795013	No	tripartite motif-containing 9
KAZALD1	12229	0.086035132	0.44066185	No	Kazal-type serine peptidase inhibitor domain 1
PTCH1	28855	-0.322532177	-0.056530066	No	patched homolog 1 (Drosophila)
COLEC11	28946	-0.327158928	-0.045365497	No	collectin sub-family member 11
FOXJ1	32016	-0.604229391	-0.11395491	No	forkhead box J1
CD248	32300	-0.700750291	-0.0928141	No	CD248 molecule, endosialin
CD8B	32318	-0.710189879	-0.06309656	No	CD8b molecule
ACVR1C	32402	-0.755034387	-0.033497863	No	activin A receptor, type IC
KLRC3	32521	-0.910120428	0.001628795	No	Killer cell lectin like receptor C3

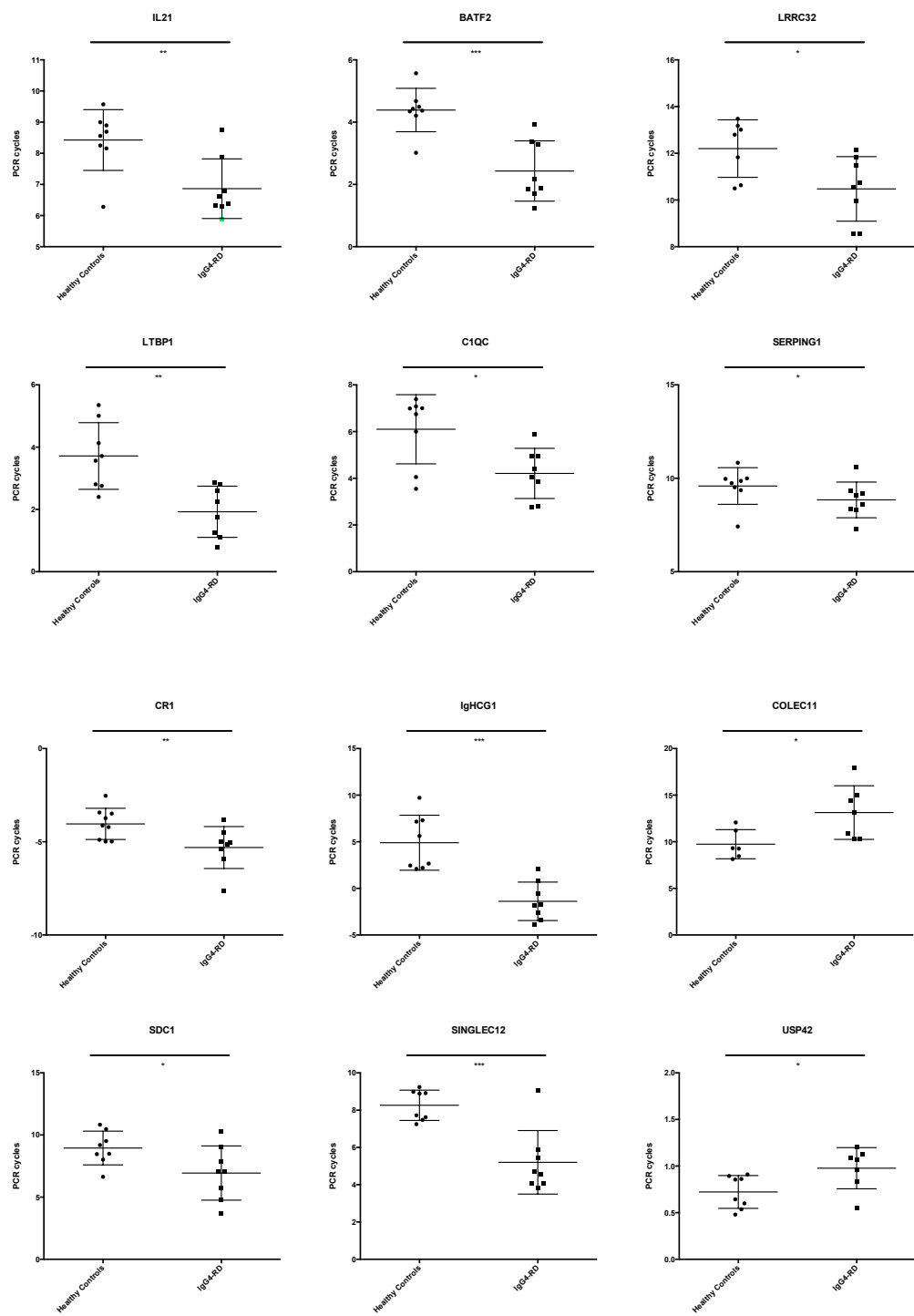
Table 6: Gene Set Enrichment Score in the GSEA Plot. The gene probe, rank in the overall gene list, rank metric score on the enrichment plot, enrichment score, and full gene name are given.

10.4.7 Functional analysis at the RNA and Protein level

(1) Gene expression at the RNA level

Taqman PCR was used to confirm differential gene expression at the RNA level. In total, 19 of 40 genes tested were confirmed to be significantly active in the disease cohort compared to healthy controls (**Table 7**). These included the cytokine (IL21), complement cascade proteins (C1QC, CR1 and SERPING1), lectins (C-type CD248 and KLRC3; Ig-like SIGLEC12), plasma cell differentiation marker (syndecan-1), TGF-beta pathway interaction proteins (LTBP1, BAFT2), coagulation cascade protein (F5), prostaglandin (PTGDR2) and immunoglobulin (IgG heavy chain locus G1) (**Figure 8**).

Figure 8: *Differential gene expression in IgG4-RD patients and healthy controls at the RNA level.*



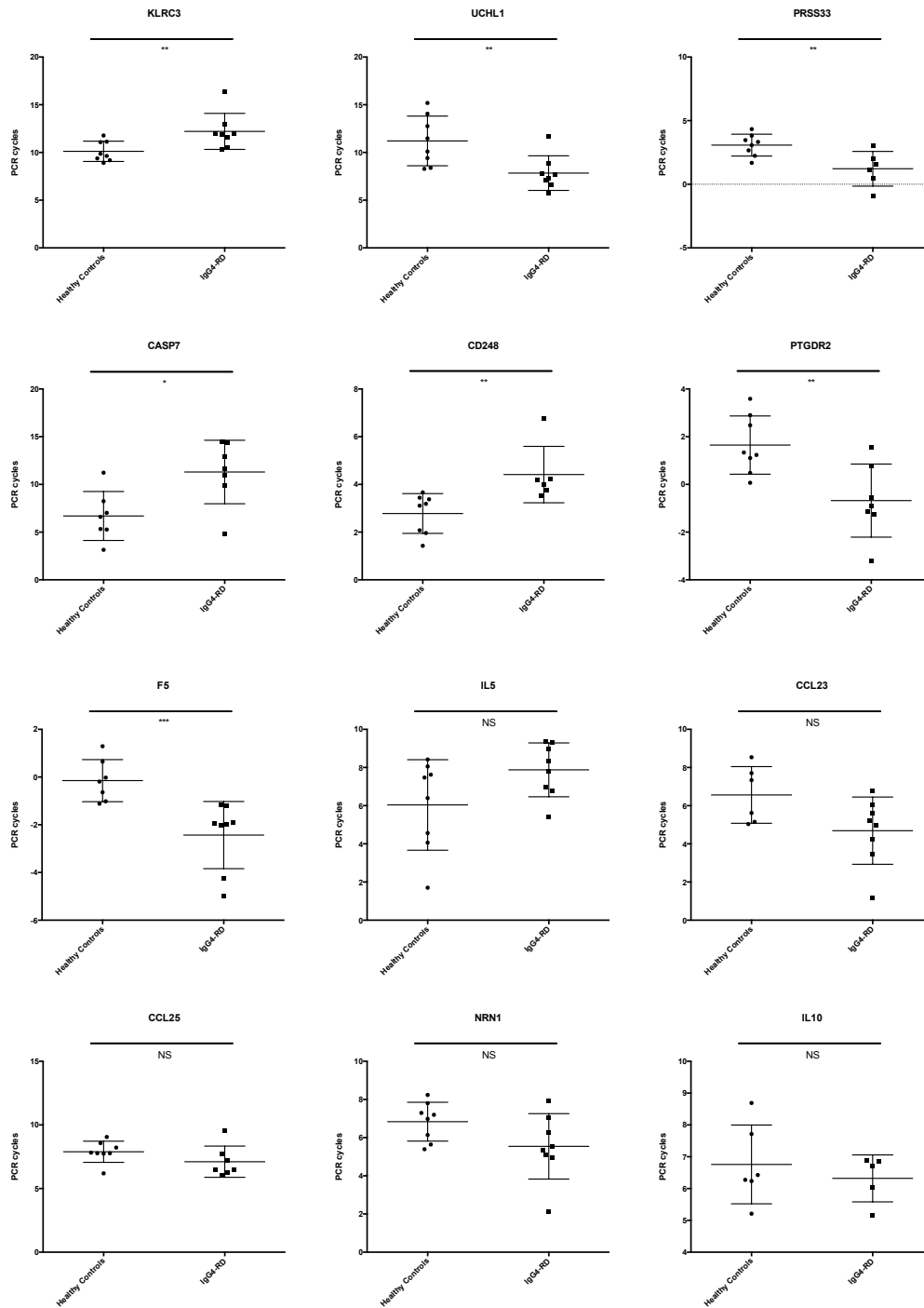


Figure 8. The dot plots show differential gene expression at the RNA level, using Taqman PCR, in 8 IgG4-RD patients and 8 healthy controls. Dot plots of 21 genes (of 40 genes tested) are shown. The x-axis shows IgG4-RD patients and healthy controls. The y-axis shows the number of PCR cycles. Unpaired *t* test *p* values: NS (non significant) $p > 0.05$; * $p \leq 0.05$; ** $p < 0.005$, *** $p < 0.005$, **** $p < 0.0001$.

Table 7: Differential gene expression in IgG4-RD patients and healthy controls at the RNA level.

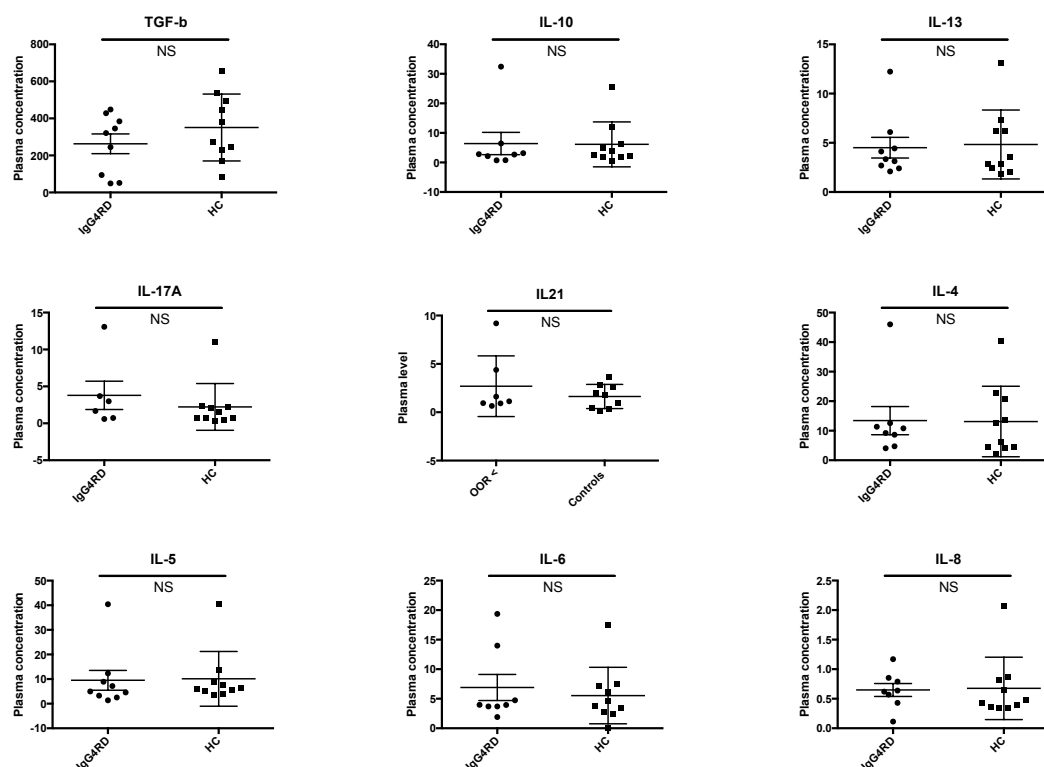
Gene Probe	Unpaired t-test p-value	Significance
IL21	0.0006	**
BATF2	0.0005	***
LRRC32	0.0238	*
LTBP1	0.0024	**
C1QC	0.012	*
FMOD	0.3905	NS
CCL25	0.0932	NS
IL5	0.085	NS
CCL23	0.052	NS
C2	0.7212	NS
SERPING1	0.0499	*
ACVR1C	0.763	NS
CR1	0.0055	**
HLADQB1	0.0929	NS
IgHCG1	0.0003	***
COLEC11	0.0236	*
SDC1	0.0472	*
NRN1	0.0827	NS
TRIM9	0.181	NS
HLADRB1	0.5846	NS
SIGLEC12	0.001	**
ADORA3	0.207	NS
USP42	0.0316	*
KLRC3	0.007	**
UCHL1	0.007	**
PRSS33	0.008	**
PPARG	0.2335	NS
CASP7	0.0141	*
FOXJ1	0.788	NS
CD248	0.0013	**
PTGDR2	0.0077	**
ZBBX	0.7714	NS

F5	0.0003	***
DEFB108B	0.5584	NS
ANGPTL2	0.2152	NS
CPE	0.4456	NS
PTCH1	0.2516	NS
CD8B	0.9349	NS
KAZALD1	0.6461	NS
IL10	0.4870	NS

Table 7: The table shows differential gene expression at the RNA level between 8 IgG4-RD patients and 8 healthy controls, using Taqman PCR. Unpaired *t* test *p* values, as per Figure 9.

(2) Gene expression at the protein level

A Luminex Th1 and Th2 cytokine array was used to assess protein expression. There were no significant differences in plasma cytokine expression between the IgG4-RD patients and healthy controls (**Figure 9**).

Figure 9: Plasma cytokine concentrations in IgG4-RD and healthy controls.**Figure 9.** The dot plots show plasma cytokine concentrations in 8 IgG4-RD patients and 8 healthy controls. The x-axis shows IgG4-RD patients and healthy controls. The y-axis shows the plasma concentration. Mann-Whitney p values: NS (non significant) $p > 0.05$; * $p \leq 0.05$; ** $p < 0.005$, *** $p < 0.005$, **** $P < 0.0001$.

Cytokines were then measured in the supernatants of cultured PBMC's, using non-specific stimuli to enhance B cell memory and plasma cell production. There was significantly higher TGF-beta expression ($p=0.030$) and lower BAFF expression ($p=0.037$) in IgG4-RD patients compared with healthy controls (**Figure 10**).

Figure 10: Supernatant cytokine concentrations in IgG4-RD and healthy controls.

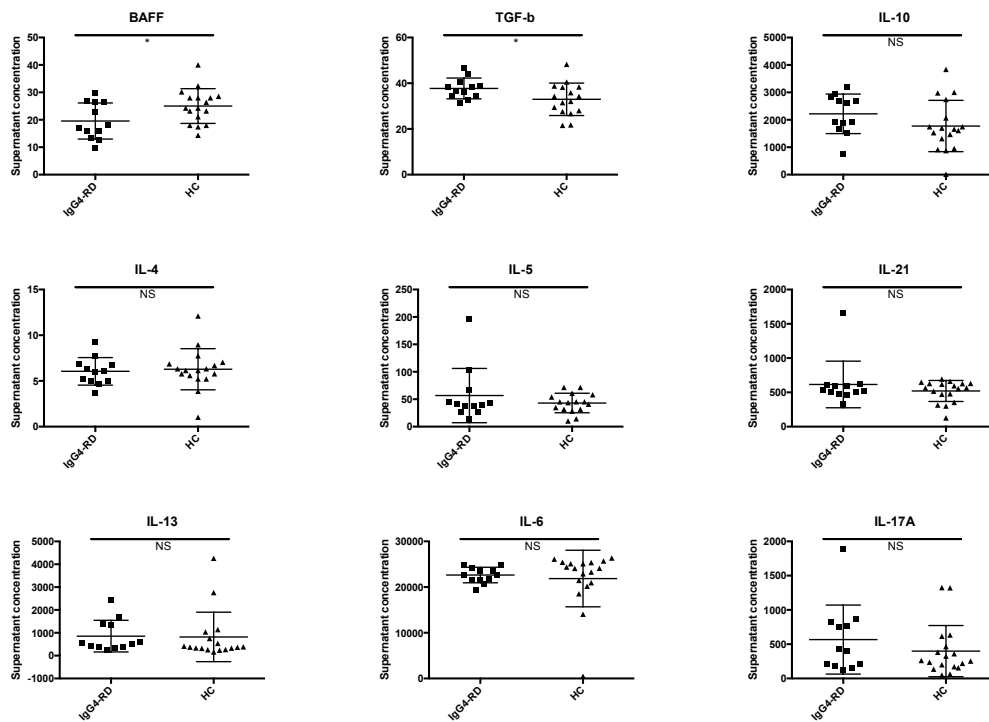
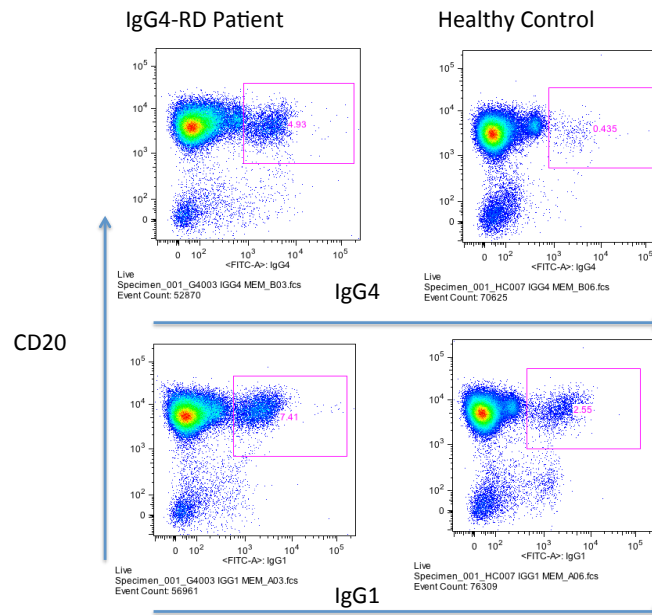


Figure 10. The dot plots show supernatant cytokine concentrations after stimulation with non-specific stimuli (CpG, pokeweed mitogen, SAC) to promote B cell proliferation and plasma cell production, in IgG4-RD patients and healthy controls. The x-axis shows IgG4-RD patients and healthy controls. The y-axis shows the supernatant cytokine concentration. Mann-Whitney p values, as per Figure 10.

Fluorescent activated cell staining was also used to assess surface protein expression. B cells were isolated and stained for immunoglobulin subclasses (IgG1 and IgG4), complement receptors CR1 (CD35) and CR2 (CD21) and Fc receptors Fc Epsilon RII (CD23) and Fc Gamma RIIb (CD32). The IgG4-RD patients had a higher surface expression of IgG1 ($p=0.01$) and IgG4 ($p<0.001$) compared with healthy controls (Figure 11).

Figure 11: Surface expression of IgG4 and IgG1 on B cells.**Figure 11:** The fluorescent-activated cell staining plots show CD19+ isolated B cells, stained for CD20 on the y-axis and for IgG4/IgG1 on the x-axis. The percentage of IgG1 and IgG4-positive surface stained B cells is shown in the pink box, in a representative IgG4-RD patient and healthy control.

IgG4-RD patients expressed lower inhibitory Fc receptor FcγRIIb (CD32) than healthy controls, but similar surface Fc receptor FcεRI (CD23) and complement receptors CR2 (CD21) and CR2 (CD35) (**Figure 12 and 13**). IgG1 and IgG4 B cell surface receptor expression is explored further in **Chapter 9**.

Figure 12: Complement receptors in IgG4-RD and controls.

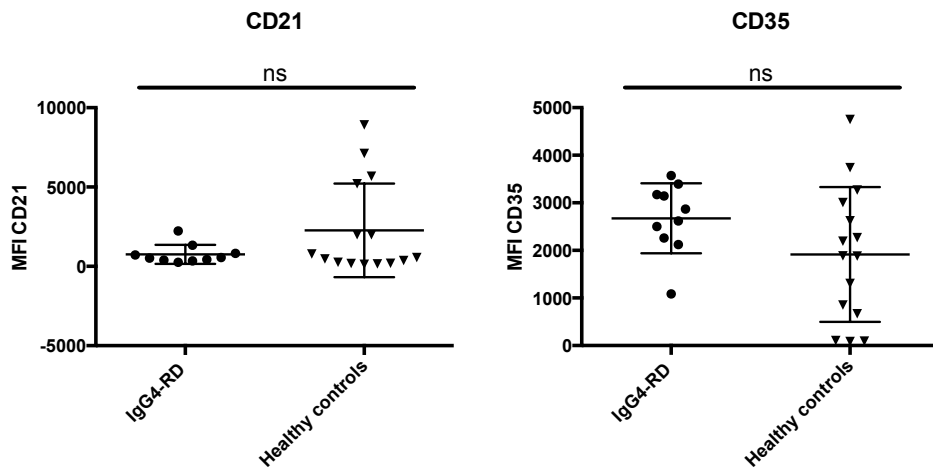


Figure 12: Dot plots showing mean fluorescence intensity (MFI) of CD21 and CD35 in IgG4-RD patients and healthy controls. On the x-axis are the 2 cohorts, and on the y-axis is the MFI. Mann-Whitney values, where NS is $p > 0.05$, $*p < 0.05$, $**p < 0.01$.

Figure 13: Fc receptor expression in IgG4-RD and healthy controls.

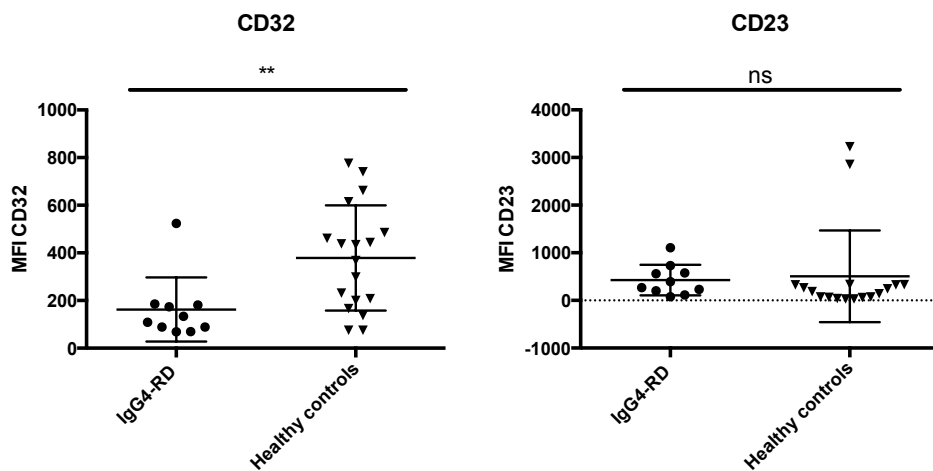


Figure 13: Dot plots showing Fc receptor expression, $Fc\gamma RIIb$ (CD32) and $Fc\epsilon RI$ (CD23), in IgG4-RD patients and healthy controls. The axes and statistics as per figure 12.

10.4.8 Public Repositories: Array Express and Gene Expression Omnibus

We next compared our dataset with those on public repository's, namely Array Express (AE) and Gene Expression Omnibus (GEO) - databases of functional genomic data generated from microarray and high throughput sequencing experiments. A tissue array comparing patients with AIP and alcohol-induced chronic pancreatitis showed peroxiredoxin 2, complement cascade proteins, cytokines, chemokine receptors, immunoglobulins, and immune signalling molecules to be up-regulated and trypsinogens to be down-regulated in AIP patients. (Lohr et al, Am J Gastro 2010). There was overlap in up-regulated genes in this tissue array of AIP patients with our whole blood array, including the classical complement components and receptors (C2 and C1QA and B).

10.4.9 Deconvolution

A deconvolution algorithm was applied to infer the contribution of each cell type in whole blood to the gene expression profile. This was based on the full blood count of the disease and control cohorts at the time of sampling. Using this algorithm, the monocyte and eosinophil lineages were suggested to be important in differential gene expression (**Figure 14**).

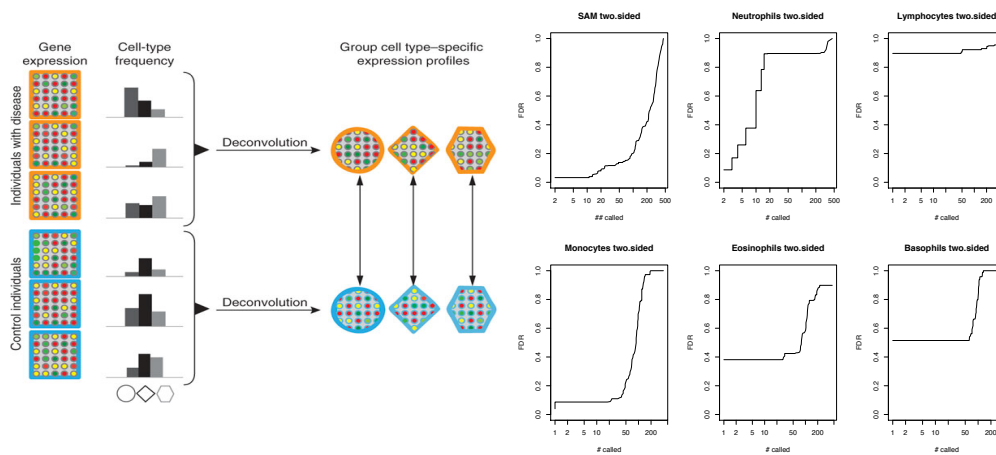
Figure 14: Deconvolution Algorithm

Figure 14. Deconvolution algorithm using an R package for cell-type specific differential expression of a microarray experiment of heterogeneous tissue samples, using SAM (well known statistical test for microarrays). Adapted from *Nature Methods* 7, 287–289 (1 April 2010).

Using genes specific to the monocyte lineage, we then repeated the gene expression analysis in Metacore. Groups of diseases with similar gene profile included lymphoma (mantle cell, B cell, Non-Hodgkin's, lymphoid), lymphoproliferative disease and immunoproliferative diseases.

10.4.10 Principal Component Analysis

The differential gene expression between healthy controls and IgG4-RD patients, and patients with multiple and single organ disease was investigated using principle component analysis (PCA) (**Figure 15**). The principle components in the healthy control and IgG4-RD patients were at distinct ends of the plot. Despite using genes differentially active in all disease samples, severe and less severe conditions were discernible, with less severe samples closer to healthy controls. The patient with an undetectable IgG4 level (G4-5) was isolated on the PCA plot.

Figure 15: Principle component analysis in IgG4-RD and controls.

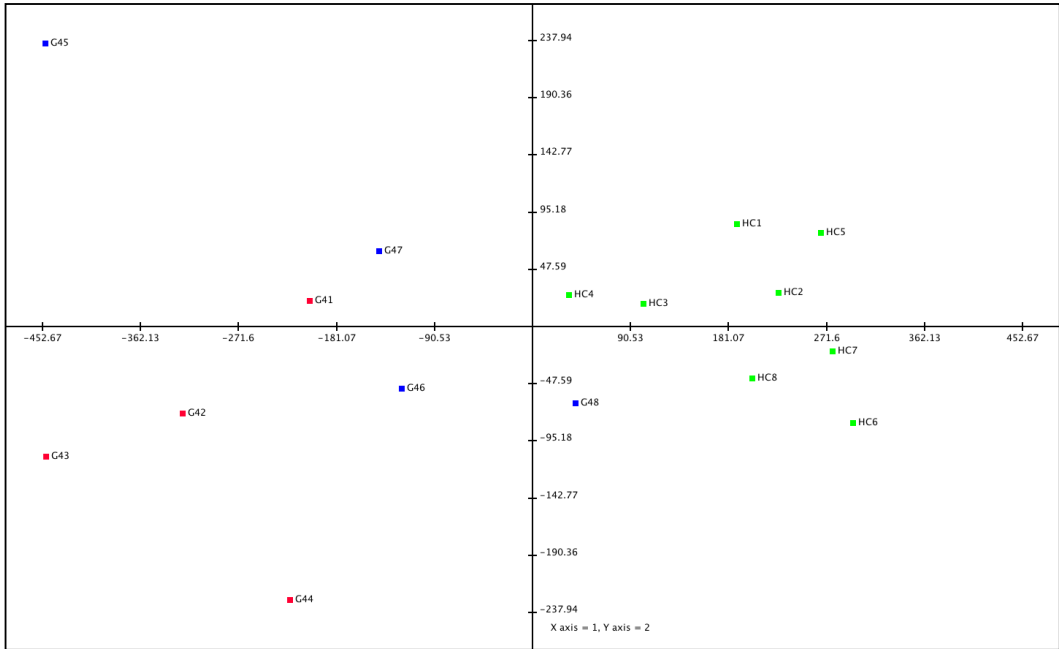


Figure 15: Principle component analysis of differential gene expression in IgG4-RD patients (multi-organ red; single organ blue) and healthy controls (green). IgG4-RD patients labelled G1-G8, and healthy controls labelled HC1-HC8.

This is further illustrated in the dendrogram heat map, where the patients cluster according to ‘severity’ of disease, and the patient with an undetectable IgG4 level is separate (**Figure 16**). Genes in patients with multi-organ involvement and at least one episode of biochemical/radiological disease relapse after initial corticosteroid treatment could be identified, and are illustrated in a Venn diagram (**Figure 17**). Disease relapse was associated with 55 up regulated genes.

Figure 16: Dendrogram heat map of genes in IgG4-RD and healthy controls.

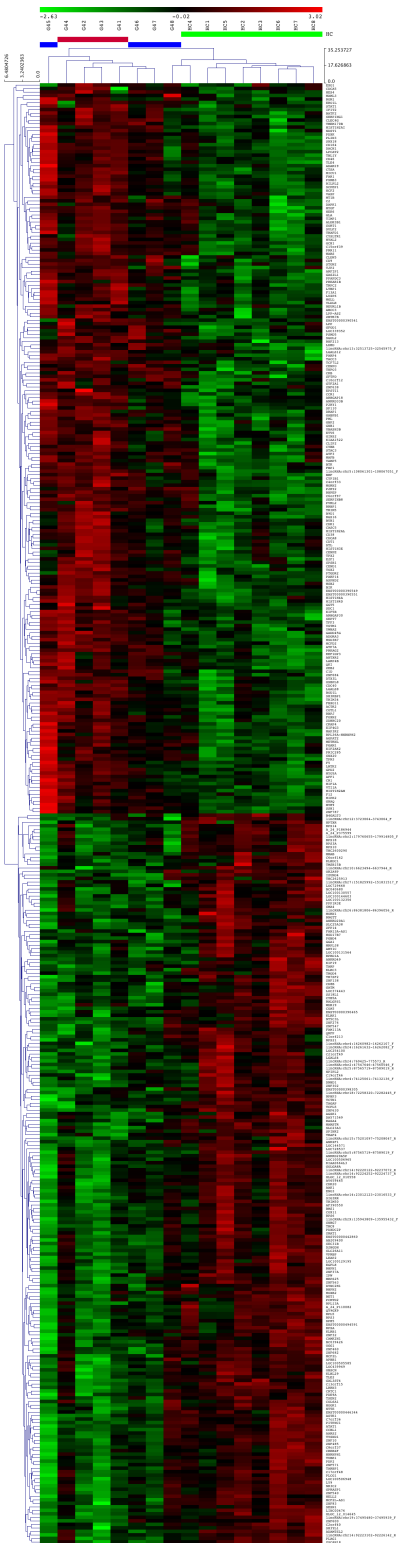


Figure 17: Venn Diagram of genes in IgG4-RD and healthy controls.

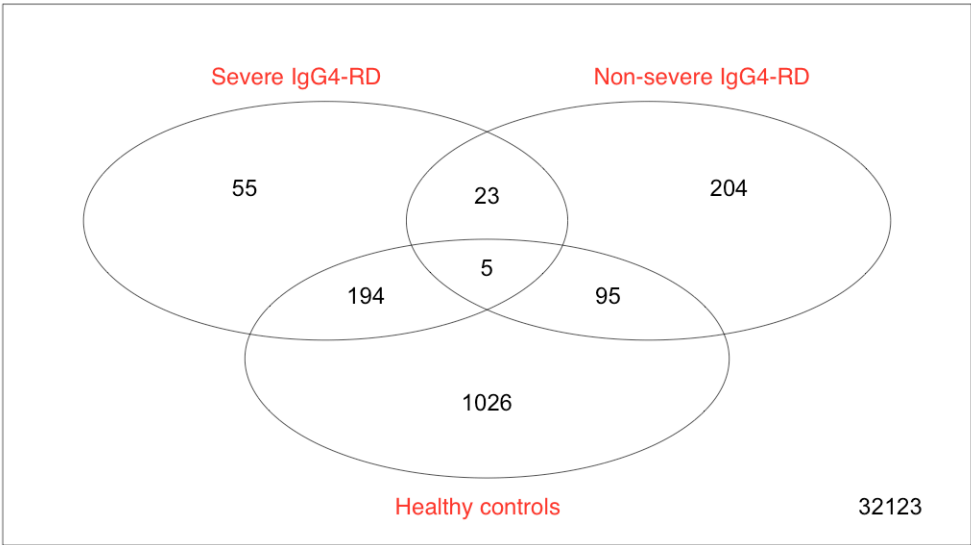


Figure 17: A Venn Diagram showing gene distribution and overlap in IgG4-RD patients and healthy controls. Severe IgG4-RD was defined as those patients who relapsed and developed multiple organ involvement ($n=5$). Non-severe IgG4-RD was defined as those patients who had responded to medical therapy and not relapsed ($n=3$). Healthy controls were age and gender matched to the IgG4-RD patients ($n=8$). There are 32123 gene probes included in this analysis. The number of up regulated genes falling in each category is shown in the Venn diagram.

10.5 DISCUSSION

The aetiology and drivers of IgG4-RD are currently unknown. A non-hypothesis driven approach was used to identify the expression of genes and biological pathways associated with the disease. Using whole-genome microarray analysis of peripheral blood, we were able to characterise altered gene-expression in the inflammatory process of IgG4-RD, and analyse the output using functional annotation programs. 213 differentially expressed candidate genes were identified: 161 up regulated and 52 down regulated in patients versus healthy controls. Pathway analysis and biomedical literature review revealed 39 immunologically functional candidate genes and 2 key pathways,

which may be important in disease pathogenesis. Of these, 19 genes were further confirmed at the RNA and/or protein level. Importantly, these genes encoded cytokines known to drive IgG4 switch and proliferation, complement proteins in the classical and lectin pathways, and members of the TGF beta pathway that may be important in the development of fibrosis.

The genes encoding cytokines IL-21 and IL5 were up regulated in the peripheral blood of patients, and the cytokines IL4 and IL10 and transcription factors STAT3 and GATA3, were identified by Ingenuity as upstream regulators in disease. IL21 is understood to drive proliferation of IgG4-switched B cells (Kuchen et al, J Immunol 2007). Increased IL21 mRNA expression was reported in labial salivary gland biopsies, and IL-21 was detected by immunohistochemistry in and around germinal centres, in patients with IgG4-related sialadenitis (Maehara et al, Ann Rheum Dis 2012). IL5 is involved in eosinophil generation, and both peripheral and tissue eosinophilia is apparent in IgG4-RD (**Chapter 5**). These findings are in line with earlier studies, where increased mRNA ratios of IL-5/IFN in pancreatic and extra-pancreatic lesions and a markedly enhanced *in vitro* IL-5 production by PBMCs in IgG4-RD patients was seen (Zen et al, Hepatology 2007; Kudo-Tanaka et al, Arthr Res 2012). Upstream regulators included STAT3 and GATA3, involved in transcription targeting of IL21 and IL5, and cytokines IL4 and IL10 involved in targeting CCL23, IL5, PPARG and CR1. Both STAT3 and GATA3 transcription factors and IL4 and IL10 cytokines have been shown to be present in the tissue of patients with IgG4-related sialadenitis and AIP (Tsuboi et al, Arthr Res Thep 2012; Eto et al, PLoS One 2011).

The chemokine ligands CCL23 and CCL25 were up regulated in the peripheral blood of patients. This is interesting given that CCL23 is a ligand for the chemokine receptor CCR1 and shows chemotactic activity for monocytes, resting T-lymphocytes, and neutrophils (Berahovich et al, J Immunol 2005). It can be induced by IL-4 and has been shown to be a potential biomarker of disease activity in rheumatoid arthritis (Novac et al, J Immunol 2007; Rioja et al, Arthritis Rheum 2008). CCL25 is important in the gut-liver axis and had been described with its chemokine CCR9 to be important in recruiting gut-homing lymphocytes to the liver in PSC, an immune-mediated condition similar in clinical and radiological characteristics to IgG4-SC (Eksteen et al, J Exp Med 2004). These may be important molecules in trafficking and homing of lymphocytes to end organs in IgG4-RD.

Expression of the C-type lectins CD248, COLEC11 and KLRC3 may indicate involvement of the C-type lectin superfamily. Lectins are carbohydrate-binding proteins highly specific for sugar moieties that play a role in the biological recognition phenomena of cells and proteins. The C-type lectins are known to function as pattern recognition receptors in innate immunity and some C-type lectins can activate complement (Kerrigan et al, Immunobiology 2009). It has been suggested that an endogenous lectin may function as an alternative trigger of the BCR in IgG4-RD, particularly as there are indications of unusual glycosylation of the Fab of (part of) IgG4. The B cell would be activated by the lectin upon cross-linking of the BCR via its Fab glycan. In this way, the lectin would act as an endogenous superantigen, resulting in recruitment of IgG4-switched B cells (Lighaam et al, Int J Rheumaol 2012). Support for this theory comes from work on the activation of B cells in follicular lymphoma (Coelho et al, Proc Natl Acad Sci USA 2010).

Several genes that play a role in the TGF- β pathway (receptor binding and downstream signalling) were dysregulated in the IgG4-RD patients, including LTBP, FMOD, LRRC32, BATF2 (up regulated) and ACVR1C (downregulated). TGF- β is an important pleiotropic cytokine with potent immunoregulatory properties, and is well known for its involvement in fibrosis, one of the key features of the late immune process in IgG4-RD (Leask et al, FASEB 2004). Tissue mRNA expression of TGF- β was substantially higher in inflammatory lesions from patients with AIP, IgG4-SC and tubulointerstitial nephritis, compared with controls (Nakashima et al, Clin Nephrol 2010). TGF- β was also seen in inflamed labial salivary gland tissue of patients with IgG4-sialadenitis (Ohata et al, Acta Otolaryngol 2013). Importantly, TGF- β can contribute to the function and generation of Tregs by inducing FoxP3 (Maynard et al, Nat Immunol 2007). Tregs (more specifically Treg-1 cells) have been shown to induce IgG4 production in B cells *ex vivo* (Boer et al, Clin Exp Immunol 1998). CTLA-4, proposed to be important in susceptibility to AIP in Asian patients (Chang et al, Clin Chemist 2004), contributes to the activation of Tregs (Scalapino et al, Immunol Rev 2008). Furthermore, the gene NRN1 encoding for neuritin 1 (a glycosylphosphatidylinositol-anchored protein named for its induction by neural activity), which was up regulated in peripheral blood in our disease patients, is part of the Treg transcriptional signature and its product promotes expansion and persistence of Tregs *in vitro* and *in vivo*.

Using gene expression software, the main functional pathway identified as important in IgG4-RD was the complement pathway. Several genes involved in both the classical and mannose-binding lectin pathway were up regulated including C1QC, C2, CR1 and

SERPING1. This finding is particularly interesting as IgG4 antibody is believed to be a poor activator of C1q (Van der Zee et al, Clin Exp Immunol 1986). Furthermore, decreased levels of C4, as well as a tendency toward decreased levels of C3, have been reported in serum of AIP patients (Muraki et al, Pancreas 2006) and deposits containing C3, IgG and IgG4 were found in the tubular basement membranes of renal biopsy specimens in IgG4-tubulointerstitial nephritis (Detlefsen et al, Histopath 2010).

Another interesting finding is the high numbers of oncogenic genes enriched in the IgG4-RD patients. This is particularly important due to the association of IgG4-RD with an increased risk of any malignancy (Yamamoto et al, Mod Rheumatol 2012; **Chapter 3**) and the reported co-existence of IgG4-RD lesions with lymphoma (orbital, non-Hodgkin, mantle cell) or carcinoma (Cheuk et al, Am J Surg Pathol 2008; Sato et al, Pathol Int 2008; Takahashi et al, Pancreas 2009; Sato et al, Int J Haematol 2008; Brenner et al, Mod Pathol 2013). This makes diagnosis even more challenging as malignancy is the main differential diagnosis of an IgG4-related mass-like lesion (Law et al, Cleve Clin Med 2009).

Principle component analysis demonstrated a discernible difference between IgG4-RD patients with single and multiple organ involvement; 55 genes enriched in systemic disease were identified. These genes need to be validated in further cohorts of patients and disease controls, but could play an important role as a prognostic marker to identify those who may develop systemic disease and have a higher chance of relapse. Interestingly the single IgG4-RD patient with a normal IgG4 was isolated with differential gene expression to the other patients and controls on the PCA. This finding

needs to be validated for other patients with a normal IgG4 level, but may provide important insights in this group of patients who are challenging to diagnose.

Gene expression microarrays provide a snapshot of all the transcriptional activity in a biological sample, facilitating discovery of totally novel and unexpected functional role of genes, potentially aiding other tools in identification and stratification of samples. Despite this, evidence of gene expression does not confirm a causal relationship, and interpretation of these findings should be made acknowledging this. We performed a whole blood microarray, and further information may be gained using a tissue array or cell-type specific array.

10.6 SUMMARY

Gene expression analysis in IgG4-RD implicates antibody-inducing cytokines, hepatic/biliary chemokines, TGF-beta, the complement pathway as putative immune drivers of disease. This provides novel biological insights into the disease, may guide diagnostic algorithms, as is the case with Ulcerative Colitis (Watanabe et al, Clin Cancer Res 2007), and provides targets for disease stratification and therapeutics. Further validation of these findings will be important.

Chapter 11 HLA Associations in IgG4-RD.

11.1 HYPOTHESES

1. There are distinct HLA haplotypes present in a UK cohort of IgG4-RD patients compared with disease and healthy controls..
2. There are distinct HLA haplotypes present in patients with Primary Sclerosing Cholangitis (PSC) with a normal and elevated serum IgG4 level, which may help to determine severity of disease.

11.2 INTRODUCTION

IgG4-RD is a multi-organ fibro-inflammatory disorder characterised by the accumulation of CD4⁺ T lymphocytes, CD20⁺ B cells and IgG4⁺ plasma cells at disease sites and by the formation of tumour-like masses (Stone et al, N Engl J Med 2012). Genetic factors may contribute to pathogenesis in IgG4-RD, as they do for other autoimmune conditions. Human leukocyte antigen (HLA) molecules are highly polymorphic and have a central role in the T cell response. The genes encoding HLA fall into two major classes, Class I (HLA A, B, and C) and Class II (HLA DP, DQ, and DR), which are clustered within the major histocompatibility complex (MHC) region on the short arm of chromosome 6 (Klein et al, N Engl J Med 2000). HLA-DR and CD4⁺ lymphocytes are expressed on the pancreatic duct and acinar cells in AIP patients (Okazaki et al, Gastroenterol 2000) suggesting a plausible role for the HLA class-II pathway in disease.

In IgG4-RD studies have identified various HLA molecules and other immune-

regulatory genes as determinants of disease susceptibility to AIP, disease relapse after steroid therapy and extra-pancreatic disease (Kawa et al, *Gastroenterol* 2002; Park et al, *Gastroenterol* 2008; Hirano et al, *Gastroenterol* 2009). The first HLA association study reported significantly higher frequencies of the HLA-DRB1*0405-DQB1*0401 haplotype in Japanese AIP patients in comparison to healthy individuals and in patients with chronic calcifying pancreatitis (Kawa et al, *Gastroenterol* 2002). These HLA haplotypes have similarly been reported in other autoimmune diseases, such as rheumatoid arthritis (Al-Swailem et al, *Rheumatol Int* 2006), autoimmune hepatitis (Ota et al, *Immunogen* 1992) and type I diabetes mellitus (Undlien et al, *Diabetes* 1997). Close associations between AIP and other HLA class II antigens such as DQB1*0302, DQB1*0602, DRB1*0701 and DQB1*0202 have also been reported in Japan. A non-aspartic amino acid at DQB1*57 was significantly associated with relapse in patients with AIP in one Korean study (Park et al, *Gastroenterol* 2008), although these findings were not confirmed in a Japanese cohort (Hirano et al, *Gastroenterol* 2009).

In contrast to the small number of candidate genes in AIP and IgG4-SC, genetic associations in PSC have been well defined in large genome wide association studies (GWAS) (Karlson et al, *W J Gastroenterol* 2007). In PSC the key HLA haplotypes implicated in disease are A*01, C*07, B*08, DRB1*0301-DQB1*0201 and HLA-DRB1*1301-DQB1*0603 in different populations of Northern Europe (Farrant et al, *Hepatology* 1992; Mehal et al, *Gastroenterol* 1994). However, the clinical phenotype of PSC is heterogeneous. An elevated serum IgG4 has been reported in 9-15% of PSC patients, and an increased IgG4 is a marker of a severe disease course (Mendes et al, *Am J Gastroenterol* 2006; *Boostr & *Culver et al, *Hepatology* 2014). The relationship of PSC high IgG4 to IgG4-SC is unknown. Recently, a Norwegian study

reported an association between PSC patients with elevated IgG4 levels and specific haplotypes HLA B*07 and HLA DRB1*15, and PSC patients with normal IgG4 levels with HLA B*08 (Bernsten et al, *Gastroenterol* 2015). They validated these findings in a combined Swedish and US cohort. Of note, this study specifically looked at HLA class I B and class II DRB1 alleles only, and no comparisons with IgG4-SC were made.

Non-HLA single nucleotide polymorphisms (SNPs) within genes encoding cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (Scalapino et al, *Immunol Rev* 2008; Chang et al, *Clin Chem* 2007; Umemura et al *AJG* 2008), tumour necrosis factor-alpha (TNF α) and Fc receptor-like 3 (FcR-3) (Umemura et al, *Gut* 2006) have been reported to be associated with AIP disease susceptibility, recurrence and extra-pancreatic manifestations. CTLA-4 49A haplotype, a critical attenuator of T cell activation and an essential component of the regulatory system involved in several autoimmune diseases, was reported at higher frequency in Chinese AIP patients compared with healthy individuals (Chang et al, *Clin Chem* 2007). SNPs of the CTLA-4 +6230G/G genotype in Japan (Umemura et al *AJG* 2008) and CTLA-4 318C/+49A/CT60G haplotype in China (Chang et al, *Clin Chem* 2007) were linked to AIP disease susceptibility, whilst CTLA-4 +6230A in Japan was linked to disease resistance (Umemura et al, *AJG* 2008). The CTLA-4 +49A/A and +6230A/A genotypes were also related to an increased risk of relapse. TNF- α promoter 863A haplotype was suggested to be important in extra-pancreatic disease in Chinese patients (Chang et al, *Clin Chem* 2007). Polymorphisms of the FCRL3 molecule, expressed on B cells and known to augment autoantibody production in individuals with disease-susceptible genotypes, have been shown to contribute to susceptibility to autoimmune diseases (Kochi et al, *Nat Genetics* 2009). FCRL3 -110A/A has been associated with AIP disease susceptibility in Japanese

patients - the number of susceptible alleles correlating with serum IgG4 concentrations (Umemura et al, Gut 2006; Ota et al, Dis Markers 2011).

To date, genetic associations in AIP have not been validated outside of Asia or in patients with systemic disease. Given the recognised genetic variation between ethnic groups, we sought to investigate the HLA associations in our prospective UK cohort of patients with systemic IgG4-RD. We compared HLA haplotypes in a cohort of IgG4-RD patients from Oxford (reference cohort) with healthy controls then validated this in a second cohort of IgG4-RD patients from external sites across the UK (validation cohort). We also compared both the reference and validation IgG4-RD cohorts to patients with PSC, subgrouping the latter into those with a normal serum and elevated serum IgG4 level. Lastly, we compared HLA haplotypes in patients with PSC to our healthy control cohort to confirm previously published associations, and compared the PSC patients with a normal and elevated IgG4 to look for an association with specific haplotypes.

11.3 SPECIFIC METHODS

11.3.1 Patients and Control Cohort for HLA Serotyping

HLA class I and class II allelic genotyping was performed on 954 EDTA samples; 117 patients with IgG4-RD, 122 patients with PSC and 656 healthy subjects from the UK (**Table 1**). In the IgG4-RD cohort, 59 patients were referred to the John Radcliffe Hospital in Oxford (reference cohort) and 58 patients were from other centres across the UK including University College London, Freeman Hospital Newcastle, Leeds Teaching Hospital, Hull Royal Infirmary, Southampton General Hospital and Royal Berkshire in Reading (validation cohort). In the PSC cohort, 92 patients had a normal

serum IgG4 and 30 patients had an elevated serum IgG4, all patients were referred to the John Radcliffe Hospital in Oxford. The healthy subjects were anonymised controls from vaccine studies in Oxford.

Table 1: HLA serotyping cohort

Dataset name	Number of samples	Type of sample
IgG4-RD Reference	59	IgG4-RD patients from Oxford
IgG4-RD Validation	58	IgG4-RD patients from other UK centres
PSC Normal IgG4	92	PSC patients with a normal serum IgG4 from Oxford
PSC High IgG4	30	PSC patients with a high serum IgG4 from Oxford
Healthy controls	656	Healthy subjects from vaccine studies in Oxford

11.3.2 Diagnosis of IgG4-RD and PSC

The diagnosis of IgG4-RD was made in accordance with the HISORT criteria for patients with AIP and IgG4-SC (since 2007) and the Japanese Consensus Criteria for IgG4-RD (since 2011) as described in **Chapter 2**. The diagnosis of PSC was made in accordance with the EASL Guidelines in those with a cholestatic biochemical profile, characteristic cholangiography and/or consistent liver histology. All PSC patients had serum IgG4 level measured and a ratio of IgG1/IgG4 calculated (*Boostra & *Culver et al, Hepatology 2014). Those PSC patients with an elevated serum IgG4 were carefully evaluated with attention to cross sectional imaging for evidence of systemic organ involvement and liver/bile duct biopsy with review of morphology and IgG4 immunostaining where possible, to exclude IgG4-SC.

11.3.3 HLA Serotyping

HLA class I and II allelic serotyping was performed in the Immunotyping Laboratory at the Churchill Hospital in Oxford, as described in **Chapter 2**.

11.3.4 Statistical analysis

The significance of HLA associations was evaluated by determining both the standard and corrected p values after χ^2 analysis and Fishers Exact test. The strength of association was estimated by calculating the odds ratio and confidence intervals. An optimised logistic regression model was created that used all HLA associations with a fisher p-value of <0.2 . A Bonferroni correction was used to account for multiple comparisons (Dr Jacob Hurst, postdoctoral researcher). Further stratification analysis was performed using the Mantel–Haenszel method to determine the association between HLA types between individual groups.

11.4 RESULTS

11.4.1 HLA in IgG4-RD patients compared with healthy controls

Firstly, HLA haplotypes were compared between 59 patients diagnosed with IgG4-RD from Oxford (reference cohort) and a large cohort of 656 healthy controls. Four HLA associations were more prevalent in the IgG4-RD reference cohort than in healthy controls, as shown in **Table 2**.

Two HLA class II haplotypes DRB1*0301 (40.7% v 19.1%; $p=0.0003$) and DQB1*0201 (54.2% v 34.3%; $p=0.002$), and two HLA class I haplotypes A*2501 (8.5% v 3.0%; $p=0.0473$) and A*2402 (5.1% v 3.0%, $p=0.0473$) were more frequent,

with the class I associations weaker than the class II. However, only DRB1*0301 remained significant after Bonferroni correction ($p=0.0478$).

Table 2: HLA typing in IgG4-RD reference cohort and healthy controls.

HLA	IgG4-RD Reference Absolute (%)	Healthy Controls Absolute (%)	Odds Ratio	P value*	Bonferroni Correction
DRB1 0301	24/59 (40.7%)	125/656 (19.1%)	2.9129	0.0003	0.0478
DQB1 0201	32/59 (54.2%)	225/656 (34.3%)	2.2702	0.0002	0.4369
A 2501	5/59 (8.5%)	20/656 (3.0%)	2.9444	0.0473	7.2439
A 2402	3/59 (5.1%)	20/656 (3.0%)	0.3125	0.0473	7.2100

Table 2: The table shows significantly different HLA associations in the IgG4-RD reference cohort in Oxford and the healthy control cohort. A p value and odds ratio was generated using the fisher's exact test and an optimised logistic regression model. A p value* of <0.05 was statistically significant. Blue signifies that the allele was found in both the validation and reference cohort. Red – remains significant after Bonferroni correction.

Secondly, HLA genotypes were compared between 58 patients diagnosed with IgG4-RD from external hospitals across the UK (validation cohort) and the same cohort of 656 healthy controls. Seven HLA associations were more prevalent in the IgG4-RD validation cohort than in healthy controls, shown in **Table 3**.

Four HLA class II haplotypes DRB1*0301 (36.2% v 19.1%; $p=0.0035$), DQB1*0201 (48.3% v 34.3%; $p=0.0441$), DRB1*1502 (6.9% v 1.8%; $p=0.0341$), DQB1*0601 (10.3% v 2.9%; $p=0.0116$), and three HLA class I haplotypes B*5201 (8.6% v 1.7%; $p=0.0065$), B*0801 (34.5% v 18.8%; $p=0.0092$) and C*1202 (6.9% v 2.0%; $p=0.0419$) were more frequent. However, none of these retained significance after Bonferroni correction.

Table 3: HLA typing in IgG4-RD validation cohort and healthy controls

HLA	IgG4-RD Validation Absolute (%)	Healthy Controls Absolute (%)	Odds Ratio	P value	Bonferroni Correction
DRB1 0301	21/58 (36.2%)	125/656 (19.1%)	2.4110	0.0035	0.5328
B 5201	5/58 (8.6%)	11/656 (1.7%)	5.5317	0.0065	0.9863
B 0801	20/58 (34.5%)	123/656 (18.8%)	2.2807	0.0092	1.3992
DQB1 0601	6/58 (10.3%)	19/656 (2.9%)	3.8684	0.0116	1.7644
DRB1 1502	4/58 (6.9%)	12/656 (1.8%)	3.9753	0.0341	5.1840
C 1202	4/58 (6.9%)	13/656 (2.0%)	3.6638	0.0419	6.3757
DQB1 0201	28/58 (48.3%)	225/656 (34.3%)	1.7878	0.0441	6.7016

Table 3: The table shows significantly different HLA associations in the IgG4-RD validation cohort from centres across the UK and the healthy control cohort. A *p* value and odds ratio was generated as in Table 2. Ordered by the *p* value. Blue signifies that the allele was found in both the validation and reference cohort.

Therefore, in both the reference and validation IgG4-RD cohorts, HLA class II associations DRB1*0301 and DQB1*0201 were significantly more prevalent in IgG4-RD patients than in healthy controls. However, only DRB1*0301 retained significance after Bonferroni correction (in the reference cohort).

11.4.2 HLA in IgG4-RD patients compared with PSC with normal serum IgG4

HLA haplotypes in IgG4-RD patients were then compared with PSC patients - an immune-mediated fibro-inflammatory disease with similar clinical and radiological changes seen as in patients with IgG4-SC, the biliary manifestation of IgG4-RD.

HLA haplotypes in 58 patients with IgG4-RD in the reference cohort were compared with 92 patients with PSC and a normal serum IgG4. Six HLA associations were more prevalent in the IgG4-RD reference cohort than in the PSC patients (**Table 4**).

One HLA class II association DQB1*0201 (54.2% v 30.4%, $p=0.004$) and five HLA class I associations A*0201 (44.1% v 23.9%, $p=0.012$), A*2501 (8.5% v 1.1%, $p=0.034$), B*4402 (13.6% v 3.2%, $p=0.024$), C*0702 (28.8% v 13%, $p=0.020$) and C*0501 (13.6% v 3.2%, $p=0.024$) were more frequent. However, none retained significance after Bonferroni correction.

Table 4: HLA typing in IgG4-RD reference cohort and PSC normal IgG4

HLA	Reference IgG4-RD Absolute (%)	PSC normal IgG4 Absolute (%)	Odds Ratio	P value	Bonferroni Correction
DQB1 0201	32/59 (54.2%)	28/92 (30.4%)	2.7090	0.0040	0.4449
A 0201	26/59 (44.1%)	22/92 (23.9%)	2.5068	0.0122	1.3411
C 0702	17/59 (28.8%)	12/92 (13.0%)	2.6984	0.0204	2.2393
C 0501	8/59 (13.6%)	3/92 (3.2%)	4.6536	0.0243	2.6781
B 4402	8/59 (13.6%)	3/92 (3.2%)	4.6536	0.0243	2.6781
A 2501	5/59 (8.5%)	1/92 (1.1%)	8.4259	0.0340	3.7358
DQB1 0301	13/59 (22.0%)	10/92 (10.9%)	2.3173	0.0685	7.5598

Table 4: The table shows significantly different HLA associations in the IgG4-RD reference cohort from Oxford and PSC patients with normal serum IgG4. A p value and odds ratio was generated as in Table 2. Blue signifies that the allele was found in both the validation and reference cohort.

Next, HLA haplotypes were compared between 59 patients with IgG4-RD in the validation cohort and the same 92 patients with PSC and a normal serum IgG4. Thirteen HLA associations were more prevalent in the IgG4-RD validation cohort than in the PSC patients, as shown in **Table 5**.

Five HLA class II associations DQB1*0201 (48.3% v 30.4%, $p=0.037$), DQB1*0301 (24.1% v 10.9%, $p=0.040$), DQB1*0601 (10.3% v 1.1%, $p=0.014$), DRB1*0701 (19.0% v 6.5%, $p=0.032$), DRB1*0401 (19.0% v 7.6%, $p=0.043$), and eight HLA class I associations A*0201 (43.1% v 23.9%, $p=0.019$), A*3101 (12.1% v 3.2%, $p=0.046$), B*4402 (20.7% v 3.3%, $p=0.001$), B*5201 (8.6% v 0%, $p=0.008$), B*5701 (10.3% v 1.1%, $p=0.014$), C*0501 (20.7% v 3.3%, $p=0.001$), C*1202 (6.9% v 0%, $p=0.021$) and C*1502 (12.1% v 2.2%, $p=0.028$) were more frequent. None retained significance after Bonferroni correction.

Table 5: HLA typing in IgG4-RD validation cohort and PSC normal IgG4.

HLA	Validation IgG4-RD Absolute (%)	PSC normal IgG4 Absolute (%)	Odds Ratio	P value	Bonferroni Correction
C 0501	12/58 (20.7%)	3/92 (3.3%)	7.739	0.001	0.130
B 4402	12/58 (20.7%)	3/92 (3.3%)	7.739	0.001	0.130
B 5201	5/58 (8.6%)	0/92 (0%)	0	0.008	0.852
DQB1 0601	6/58 (10.3%)	1/92 (1.1%)	10.5	0.014	1.505
B 5701	6/58 (10.3%)	1/92 (1.1%)	10.5	0.014	1.505
A 0201	25/58 (43.1%)	22/92 (23.9%)	2.410	0.019	2.042
C 1202	4/58 (6.9%)	0/92 (0%)	0	0.021	2.304
C 1502	7/58 (12.1%)	2/92 (2.2%)	6.176	0.028	3.069
DRB1 0701	11/58 (19.0%)	6/92 (6.5%)	3.354	0.032	3.508
DQB1 0201	28/58 (48.3%)	28/92 (30.4%)	2.133	0.037	4.106
DQB1 0301	14/58 (24.1%)	10/92 (10.9%)	2.609	0.040	4.378
DRB1 0401	11/58 (19.0%)	7/92 (7.6%)	2.842	0.043	4.724
A 3101	7/58 (12.1%)	3/92 (3.2%)	4.072	0.046	5.081

Table 5: The table shows significantly different HLA associations in the IgG4-RD validation and PSC with normal serum IgG4. A p value and odds ratio as in Table 2. Blue signifies that the allele was found in both validation and reference cohorts.

In both the reference and validation IgG4-RD cohorts, HLA class II association DQB1*0201 and class I associations B*4402 and C*0501 were significantly more prevalent in IgG4-RD patients than in PSC patients with a normal serum IgG4. However, neither alleles remained significant after Bonferroni correction.

11.4.3 HLA in IgG4-RD patients compared with PSC with high serum IgG4

To investigate if PSC patients with an elevated serum IgG4 had similar HLA haplotypes to PSC patients with a normal IgG4 or those patients with IgG4-SC, HLA typing in patients with PSC and an elevated serum IgG4 were compared with both groups.

Firstly, HLA types in 58 patients with IgG4-RD in the reference cohort were compared with 25 patients with PSC and a high serum IgG4. There were no HLA associations that were more prevalent in the IgG4-RD reference cohort than in the PSC patients. Three HLA associations were less prevalent in the IgG4-RD reference cohort than in the PSC patients with a high IgG4, as shown in **Table 6**.

One HLA class II haplotype DQB1*0301 (22.0% v 48.0%, $p=0.034$) and two class I haplotypes B*0801 (27.1% v 52.0%, $p=0.044$) and C*1203 (0% v 13.6%, $p=0.024$) were less frequent in the IgG4-RD cohort. However, none remained significant after Bonferroni correction.

Table 6: HLA type in IgG4-RD reference cohort and PSC high IgG4

HLA	Reference IgG4-RD Absolute (%)	PSC high IgG4 Absolute (%)	Odds Ratio	P value	Bonferroni Correction
DQB1 0301	13/59 (22.0%)	12/25 (48.0%)	0.306	0.034	3.461
B 0801	16/59 (27.1%)	13/25 (52.0%)	0.343	0.044	4.331
C 1203	0/59 (0%)	3/22 (13.6%)	0	0.024	2.390

Table 6: The table shows significantly different HLA associations in the IgG4-RD reference cohort in Oxford and PSC patients with elevated serum IgG4. A p value and odds ratio was generated as in Table 2. Blue signifies that the allele was found in both the validation and reference cohort.

Secondly, HLA haplotypes in 59 patients with IgG4-RD in the validation cohort were compared with the same 25 patients with PSC and a high IgG4. Similarly, there were no prevalent HLA haplotypes in the IgG4-RD reference cohort compared with the PSC patients (**Table 7**). One class II HLA haplotype DQB1*0301 (24.1% v 48.0%, $p=0.041$) was less prevalent in the IgG4-RD validation cohort than in the PSC high IgG4 patients, however this was not significant after Bonferroni correction.

Table 7: HLA type in IgG4-RD validation cohort and PSC high IgG4

HLA	Validation IgG4-RD Absolute (%)	PSC high IgG4 Absolute (%)	Odds Ratio	P value	Bonferroni Correction
DQB1 0301	14/58 (24.1%)	12/25 (48.0%)	0.350	0.041	4.368
C 0701	20/58 (34.5%)	15/25 (60.0%)	0.351	0.051	5.506
A 0101	21/58 (36.2%)	15/25 (60.0%)	0.378	0.056	5.961

Table 7: The table shows significantly different HLA associations in the IgG4-RD validation cohort from centres across the UK and PSC patients with elevated serum IgG4. A p value and odds ratio was generated as in Table 2. Blue signifies that the allele was found in both the validation and reference cohort.

Therefore, in both the reference and validation IgG4-RD cohorts, HLA class II association DQB1*0301 was significantly less frequent in IgG4-RD patients than in PSC patients with a high serum IgG4, but did not remain significant after Bonferroni correction.

11.4.4 HLA in PSC patients with high and normal serum IgG4

Next, HLA haplotypes were compared between 98 patients with PSC and a normal serum IgG4 and 25 patients with PSC and a high serum IgG4. There were eight HLA associations more prevalent in the PSC high IgG4 than PSC normal IgG4 cohort, as shown in **Table 8**.

Four HLA II associations, DQB1*0301 (48% v 10.9%, $p=0.0001$), DRB1*0401 (24% v 7.6%, $p=0.0316$), DRB1*1101 (16% v 2.2%, $p=0.0186$) and *1102 (8% v 0%, $p=0.0442$) and four HLA I associations A*0101 (60% v 23.9%, $p=0.0012$), A*0201 (64% v 23.9%, $p=0.0005$), B*1501 (20% v 5.4%, $p=0.0356$), B*1302 (8% v 0%, $p=0.0442$) were more prevalent in the PSC high IgG4 cohort. Although DQB1*0201 (52% v 30.4%) and B*0801 (52% v 29.3%) were more prevalent in the PSC high IgG4 group, values were non-significant.

Overall, in the PSC high IgG4 versus PSC normal IgG4 cohort, HLA DQB1*0301 ($p=0.012$) and A*0201 ($p=0.048$) retained significance after Bonferroni correction.

Table 8: HLA typing in PSC high IgG4 and PSC normal IgG4.

HLA	PSC high IgG4 Absolute (%)	PSC normal IgG4 Absolute (%)	Odds Ratio	P value	Bonferroni Correction
DQB1 0301	12/25 (48%)	10/92 (10.9%)	7.569	0.0001	0.012
A 0201	16/25 (64%)	22/92 (23.9%)	5.657	0.0005	0.048
A 0101	15/25 (60%)	22/92 (23.9%)	4.773	0.0012	0.120
DRB1 1101	4/25 (16%)	2/92 (2.2%)	8.571	0.0186	1.818
DRB1 0401	6/25 (24%)	7/92 (7.6%)	3.835	0.0316	3.092
B 1501	5/25 (20%)	5/92 (5.4%)	4.350	0.0356	3.486
DRB1 1102	2/25 (8%)	0/92 (0%)	0	0.0442	4.332
B 1302	2/25 (8%)	0/92 (0%)	0	0.0442	4.332
B 0801	13/25 (52%)	27/92 (29.3%)	2.608	0.0554	5.433
DQB1 0201	13/25 (52%)	28/92 (30.4%)	2.476	0.0590	5.784

Table 8: The table shows significantly different HLA associations in the PSC patients with a high and normal serum IgG4. A p value and odds ratio was generated as in Table 2. Red – remains significant after Bonferroni correction.

11.4.5 HLA in PSC patients compared with healthy controls

Susceptibility to PSC is linked to known HLA haplotypes from genome-wide association studies. To look for evidence of these in our Oxford-based PSC cohort, we compared HLA haplotypes between patients with PSC with a normal and elevated IgG4 and healthy controls.

Three HLA haplotypes were more prevalent in the PSC patients with a normal serum IgG4 than in healthy controls, as shown in **Table 9**. One HLA class II association DRB1*0301 (38.0% v 25.6%, $p=0.05$) fell just short of significance, and two HLA class I associations B*0801 (29.3% v 19.1%, $p=0.025$) and C*0701 (38% v 25.6%, $p=0.017$) were more frequent.

Thirteen HLA types were less prevalent in the PSC patients with a normal serum IgG4 than in healthy controls, as shown in **Table 9**. Seven HLA type II associations DRB1*0401 (7.6% v 18.3%, $p=0.010$) and *0404 (2.2% v 9.0%, $p=0.024$), DRB1*0701 (6.5% v 25.5%, $p=0.0003$), DRB1*1101 (2.2% v 9.6%, $p=0.016$), DQB1*0301 (10.9% v 31.4%, $p=0.000002$) and *0302 (6.5% v 17.1%, $p=0.009$), DQB1*0501 (16.3% v 28.2%, $p=0.017$), and six HLA class I associations A*0201 (23.9% v 48.3%, $p=0.000001$), A*2901 (2.2% v 8.8%, $p=0.023$), B*4402 (3.3% v 14.9%, $p=0.0009$), B*5701 (1.1% v 6.7%, $p=0.033$), C*0501 (3.3% v 14.7%, $p=0.0009$), C*0602 (6.5% v 15.5%, $p=0.018$) were less frequent.

Overall, in PSC patients with a normal IgG4 versus healthy donors, only DQB1*0301 (more prevalent; $p=0.003$) and A*0201 (less prevalent; $p=0.001$) retained significance after Bonferroni correction.

Table 9: HLA typing in PSC normal IgG4 patients and healthy controls

HLA	PSC normal IgG4 Absolute (%)	Healthy Controls Absolute (%)	Odds Ratio	P value	Bonferroni Correction
C 0701	35/92 (38.0%)	168/656 (25.6%)	1.784	0.0169	2.531
B 0801	27/92 (29.3%)	123/656 (18.8%)	1.800	0.0252	3.786
DRB1 0301	26/92 (28.3%)	125/656 (19.1%)	1.673	0.0512	7.684
A 0201	22/92 (23.9%)	317/656 (48.3%)	0.336	0.000001	0.001
DQB1 0301	10/92 (10.9%)	206/656 (31.4%)	0.266	0.000002	0.003
DRB1 0701	6/92 (6.5%)	141/656 (21.5%)	0.255	0.0003	0.056
B 4402	3/92 (3.3%)	98/656 (14.9%)	0.192	0.0009	0.141
C 0501	3/92 (3.3%)	97/656 (14.7%)	0.194	0.0009	0.141
DQB1 0302	6/92 (6.5%)	112/656 (17.1%)	0.339	0.0087	1.307
DRB1 0401	7/92 (7.6%)	120/656 (18.3%)	0.368	0.0109	1.641
DRB1 1101	2/92 (2.2%)	63/656 (9.6%)	0.209	0.0160	2.401
DQB1 0501	15/92 (16.3%)	185/656 (28.2%)	0.496	0.0165	2.474
C 0602	6/92 (6.5%)	102/656 (15.5%)	0.379	0.0177	2.648
A 2901	2/92 (2.2%)	58/656 (8.8%)	0.229	0.0234	3.508
DRB1 0404	2/92 (2.2%)	59/656 (9.0%)	0.225	0.0235	3.528
B 5701	1/92 (1.1%)	44/656 (6.7%)	0.153	0.0326	4.893

Table 9: The table shows significantly different HLA associations in the PSC patients with normal serum IgG4 and healthy controls. A p value and odds ratio was generated as in Table 2.

Five HLA haplotypes were more prevalent in the PSC high serum IgG4 patients than healthy controls, as shown in **Table 10**. One HLA class II association DRB1*0301 (44.0% v 19.1%, p=0.0081) and four class I associations A*0101 (60.0% v 31.9%, p=0.0078), B*0801 (52.0% v 18.8%, p=0.0003) and B*0705 (8.0% v 0.6%, p=0.0177), and C*0701 (60.0% v 25.6%, p=0.0004) were more frequent.

Overall, in PSC patients with a high IgG4 versus healthy donors, only B*0801 (more prevalent; $p=0.041$) retained significance after Bonferroni correction.

Table 10: HLA typing in PSC high IgG4 patients and healthy controls

HLA	PSC high IgG4 Absolute (%)	Healthy Controls Absolute (%)	Odds Ratio	P value	Bonferroni Correction
B 0801	13/25 (52.0%)	123/656 (18.8%)	4.694	0.0003	0.041
C0701	15/25 (60.0%)	168/656 (25.6%)	4.357	0.0004	0.059
A 0101	15/25 (60.0%)	209/656 (31.9%)	3.208	0.0078	1.152
DRB1 0301	11/25 (44.0%)	125/656 (19.1%)	3.338	0.0081	1.213
B 0705	2/25 (8.0%)	4/656 (0.6%)	14.174	0.0177	2.661

Table 10: The table shows significantly different HLA associations in the PSC patients with elevated serum IgG4 and healthy controls. A p value and odds ratio was generated as in Table 2. Red – remains significant after Bonferroni correction.

Therefore, in both PSC cohorts regardless of the serum IgG4 level, HLA class II DRB1*0301 and class I B*0801 and C*0701 haplotypes were more frequent than in the healthy controls, which are the haplotypes that have been shown in PSC genome-wide association studies in Europe. However, only DRB1*0301 was more prevalent in PSC normal IgG4, and B*0801 more prevalent in PSC high IgG4, than in healthy controls after Bonferroni correction. Furthermore, only A*0201 was less prevalent in PSC normal IgG4 patients than healthy controls after Bonferroni correction.

An overall summary of the HLA associations in IgG4-RD, PSC patients with an elevated and normal serum IgG4 and healthy controls is shown in **Table 11**.

Table 11: Summary of HLA associations in IgG4-RD, PSC and healthy controls.

Cohort	HLA allele	Association
IgG4-RD patients v Healthy controls	DRB1*0301	+
	DQB1*0201	+
IgG4-RD patients v PSC norm serum IgG4	DQB1*0201	+
	B*4402	+
	C*0501	+
PSC high serum IgG4 v IgG4-RD patients	DRB1*0301	+
PSC norm serum IgG4 v Healthy controls	DRB1*0301	+
	A*0201	-
PSC high serum IgG4 v Healthy controls	B*0801	+
PSC high serum IgG4 v PSC norm serum IgG4	DRB1*0301	+
	A*0201	+

Table 11: The table shows significantly different HLA associations between each of the main cohorts. For IgG4-RD, significantly different HLA associations are shown when present in both the reference and validation cohorts. Red – signifies remains positive after Bonferroni correction. Key: + positive association (more prevalent) and – negative association (less prevalent).

11.5 DISCUSSION

In autoimmune diseases, associations have been demonstrated for several genetic variants within the HLA complex, usually linked with risk and/or severity of the disease, for example in multiple sclerosis, type 1 diabetes mellitus and systemic lupus erythematosus (Handunnetth et al, Genes & Immunity 2010). In some autoimmune diseases, such as coeliac disease, the HLA genes are regarded as virtually essential for the disease (Kaukinen et al, AJG 2002). However, in other diseases such as sarcoidosis, HLA provides modifying effects on the risk and/or severity of the disease (Sato et al, Am J Resp Cell Molec Biol 2002). In AIP, the pancreatic manifestation of IgG4-RD, HLA class II alleles have been linked to susceptibility and risk of relapse in single-

centre retrospective Asian studies (Kawa et al, *Gastroenterol* 2002; Park et al, *Gastroenterol* 2008; Hirano et al, *Gastroenterol* 2009).

In our prospective UK cohort of systemic IgG4-RD patients, HLA DRB1*0301 and HLA-DQB1*0201 were more frequent in IgG4-RD patients (reference and validation cohorts) than in healthy controls. HLA-DQB1*0201 allele is in tight linkage disequilibrium with the HLA-DRB1*0301 allele, making it difficult to determine which of these two alleles is responsible for the association with IgG4-RD. However, after Bonferroni correction only DRB1*0301 retained significance.

Since the haplotype HLA-DRB1*0301-DQB1*0201 includes specific alleles at HLA-DRB3, genes encoding tumour necrosis factor- α , lymphotoxin- α , I- κ -B-like protein, HLA-B-associated transcript 1 loci, and to a lesser extent HLA-B*0801 and C*0701, additional fine mapping across the MHC region is required for a precise localisation. HLA-DRB1*0301-DQB1*0201 has been associated with other autoimmune diseases, such as Lambert-Eaton myasthenic syndrome (with and without IgG4 muscle-specific receptor tyrosine kinase (MuSK) antibodies) (Parsons et al, *Human Immunol* 2000), milder forms of sarcoidosis (Sato et al, *Am J Resp Cell Molec Biol* 2002) and diabetes type I (Lambert et al, *J Clin Endocrinol Metab* 2004). This is interesting given the potential pathogenic similarities in terms of IgG4 autoantibodies in IgG4-RD and MuSK-positive myasthenic syndromes, and the clinical overlap with steroid-responsive hilar lymphadenopathy found in sarcoidosis.

The HLA susceptibility haplotypes in our UK cohort are different to those described in AIP patients in Japan, namely the HLA DRB1*0405 (58% vs. 21%, $p=0.00003$) allele and DQB1*0401 (58% vs. 21%, $p=0.00002$) allele (Kawa et al, *Gastroenterol* 2002).

This is likely due to ethnic differences in HLA associations that are very common in other diseases, such as multiple sclerosis. In the Japanese cohort, almost all patients with DRB1*0405 also had the DQB1*0401 allele, resulting in the formation of a 2-loci haplotype DRB1*0405- DQB1*0401, with linkage disequilibrium of 100% and high prevalence (>21%) in the Japanese population (Saito et al, Tissue Antigens 2000). DRB1*0405-DQB1*0401 is associated with other autoimmune disorders (autoimmune hepatitis, type 1 diabetes, rheumatoid arthritis) and DR4 itself is increased in patients with chronic pancreatitis (Katahira et al, Hormone Research 2008). Antigen studies supporting the role for gastric *H. pylori* infection triggering AIP in genetically predisposed individuals were based on sequence homology between human carbonic anhydrase-II and α -carbonic anhydrase of *H. pylori*, the homologous segments containing the binding motif of DRB1*0405 (Guarneri et al, J Cell Mol Med 2005).

In our study, HLA alleles DRB1*0301, B*0801 and C*0701 were more frequent in PSC patients (with either a normal or elevated serum IgG4) compared with healthy controls. These findings are supported by published genome wide studies in PSC patients in Europe (Karlson et al, W J Gastroenterol 2007; Farrant et al, Hepatology 1992; Mehal et al, Gastroenterol 1994; Spurkland et al, Tissue Antigens 1999), although in these studies the level of serum IgG4 was not accounted for. After Bonferroni correction, however, in our cohort only DRB1*0301 was more prevalent in PSC normal IgG4, and B*0801 more prevalent in PSC high IgG4, than in healthy controls.

HLA DR*03 and B*08 are in linkage disequilibrium, and this haplotype is also associated with several organ-specific autoimmune diseases including lupoid chronic active hepatitis, Graves disease thyrotoxicosis, type I diabetes mellitus and myasthenia

gravis (de Boer et al, *Gastroenterology* 2014; Maciel et al, *Thyroid* 2001). An increased frequency of the DRB1*0301 allele has also been reported in UC patients with extensive colitis, a characteristic feature of UC in patients with PSC and AIP (Park et al, *J Clin Gastroenterol* 2013). Studies have shown no difference in HLA haplotypes between PSC patients with and without autoimmune diseases outside the liver and colon, suggesting the association of PSC with autoimmune disease is not secondary to HLA but rather a primary phenomenon (Saarinen et al, *Am J Gastro* 2000). Furthermore, HLA-DR, DQ and DP are aberrantly expressed on target cells in PSC, whereas normal biliary cells express HLA class I and not class II (Chapman et al, *W J Gastro* 2008).

Importantly, HLA DQB1*0301 was more prevalent in PSC patients with an elevated serum IgG4 than in IgG4-RD patients, PSC patients with a normal serum IgG4 and healthy controls. This haplotype has been found with increased frequency in Southern European PSC patients (Neri et al, *Dig Dis* 2003). In our study, other alleles associated with PSC, namely HLA A*0101, B*0801 and C*0701 were also more frequent in PSC with an elevated serum IgG4 than in IgG4-RD patients. Furthermore, B*0801 was more frequent in PSC high IgG4 than healthy controls, retaining significance upon Bonferroni correction. This suggests that PSC high IgG4 more closely resembles PSC normal IgG4 than IgG4-SC.

The increased frequency of HLA DQB1*0301 and A*0201 in the PSC high IgG4 compared with PSC normal IgG4 cohorts may signify disease severity alleles. This is important, as PSC patients with an elevated serum IgG4 have a worse clinical outcome than PSC patients with a normal serum IgG4 or IgG4-SC patients (Mendes et al, *Am J Gastro* 2006). Interestingly, DQB1*0301 haplotype confers more severe disease in

multiple sclerosis on MRI imaging (Zivadinov et al, *Int Rev Neurobiol* 2007) and variants of bullous pemphigoid (Delgado et al, *Proc Natl Acad* 1996). However, a recent study with similar numbers of Norwegian PSC patients suggested higher frequencies of B*07 and DRB1*15 in PSC high IgG4 compared with PSC normal IgG4 (with a higher cut off serum IgG4 of >2.01g/l – associations were non significant at a cut off of 1.35g/l) and lower frequencies of B*0801 (cut off of serum IgG4 of 1.35g/l) (Bernsten et al, *Gastroenterol* 2015). This may be explained by different definitions of an elevated serum IgG4, overlap in their PSC group with IgG4-SC patients, and loco-regional variations.

Two models are used to explain the association between HLA antigens and a predisposition to a disease. One is that specific HLA antigens play a functional role in presenting a specific peptide to T cells, resulting in the induction of an autoimmune response. The other model is that HLA is only a genetic marker for the non-HLA gene-associated disease susceptibility, which is located within the HLA gene region. Owing to the strong linkage disequilibrium of genes on common HLA haplotypes, it has been difficult to dissect the relative contribution of individual HLA genes to the overall risk attributed to the HLA region in this disease using population-based immunogenetic analysis alone. In this instance, transgenic rodents have been used to conduct in vivo studies of the function of single HLA genes. This strategy has led to the development of several humanized animal models of autoimmune diseases concentrating on MHC class II genes, such as in HLA-DR*0301 and autoimmune thyroiditis (Kong et al, *J Exp Med* 1996). This could be applied to AIP and systemic IgG4-RD allowing careful interrogation of HLA-DR and DQ regions (Freitag et al, *Gastroenterol* 2010).

This study provides evidence for the genetic basis of IgG4-RD. It may help to tease out the complexity of the initiation of IgG4-RD. The characteristics of the peptide-binding groove of the HLA molecules are at least in part determined by the amino acid sequences of its chains, which are encoded by genes. Specific peptides may be presented in the initial phase of the pathogenesis of IgG4-RD, and certain peptides might give rise to particular disease phenotypes, such as single organ disease or relapsing disease. Whether the class II HLA DQ or DR allele is directly involved in the binding of a particular IgG4-RD-triggering peptide in a more favourable way, or merely indirectly, by operating as a marker for other immune-regulating molecules, needs further elucidation. Knowledge of the involvement of particular allelic variants in IgG4-RD might help in finding a particular peptide motif, which could be the key to identifying the antigenic trigger(s) of this disease. These findings are clinically relevant in that they may inform the future development of genetic screening to identify likely disease severity and prognosis when patients first present. These tests might therefore ultimately help physicians in making disease management decisions on a more individualised basis.

Our study and others illustrate the difficulty of ascertaining associations between HLA class I and class II molecules with a specific disease because of the need for multiple comparisons and the complex genetics operant in studying HLA, alleles/disease associations. Although this is the largest HLA study of IgG4-RD patients to date, the numbers are small, which can limit the ability to recognize associations. The identification of spurious associations may be found simply by chance and rare variants may be missed unless the odds ratio for the variant is very high. To account for this we used a Bonferroni correction, which sets the p value proportionately lower depending on

the number of comparisons being made. Indeed, in the Korean study DQB1*0201 was present on a univariate basis but lost after Bonferroni correction. Lastly there were some observed differences between the reference and validation IgG4-RD cohorts. These may be explained as the criteria for inclusion in the reference cohort was strict with availability of a histopathologist and clinicians with a special interest and multidisciplinary team diagnosis and management in a condition which can be challenging to diagnose.

11.6 SUMMARY

This Chapter suggests novel HLA associations in a UK cohort of IgG4-RD patients, and in cohorts of PSC patients with an elevated and normal serum IgG4 (**Table 11**). This suggests that IgG4 concentrations may be determined genetically and that phenotypic heterogeneity may contribute to the complexity of the HLA associations observed in both IgG4-RD and PSC.

Chapter 12 Conclusions

12.1 NATURAL HISTORY OF IGG4-RD

12.1.1 Progress in diagnosis

Over recent years a developing consensus on the clinical characteristics, histopathology and diagnostic features of this disease has led to important advances, including recognition that AIP is only one component of a multi-system IgG4-RD (Kamisawa et al, *Am J Gastroenterol* 2003; Kamisawa et al, *Clin Gastroenterol Hepatol* 2008), and it has a world-wide distribution (Chari et al, *Pancreas* 2009; Kamisawa et al, *Pancreas* 2011). Although serum IgG4 was originally reported to be highly sensitive and specific for AIP in Japan (Hamano et al, *N Engl J Med* 2001), our data suggest this is not the case for IgG4-RD in the UK. In evaluating the utility of serum IgG4 levels in diagnosis, IgG4-RD diagnostic criteria were met in only 5.1% of patients whom had IgG4 measured and in 22.4% of patients found to have an elevated IgG4 level (**Chapter 4**). An IgG4 level of 1.4g/l had a sensitivity of 82.8% and specificity of 82.1% for differentiating IgG4-RD from other disease mimics, whereas a level of 2.8g/l had a lower sensitivity of 56.9% but higher specificity of 95.6%. Furthermore, increasing this to a level of 5.6g/l (4 times ULN) gave almost 100% specificity to differentiate IgG4-SC from PSC (*Boostra & *Culver et al, *Hepatology* 2014) and cholangiocarcinoma (Oseini et al, *Hepatology* 2011), which is important given their similar clinical and radiological appearances (Webster et al, *J Hepatol* 2009). Recently, we reported that a serum IgG4:IgG1 ratio cut-off of 0.24 yielded a specificity of 74% and sensitivity of 80% to discriminate IgG4-SC and PSC high IgG4 in those patients with a serum IgG4 between 1.4g/l and 2.8g/l, based on data from 3 independent cohorts from the

UK, Amsterdam and Rotterdam (*Boostra & *Culver et al, Hepatology 2014) (**Appendix 3**).

Recently, a subset of PSC patients with an elevated serum and/or tissue IgG4 have been described, who have more severe liver disease and more rapid progression to transplantation (Mendes et al, Am J Gastroenterol 2006; Culver et al; Hepatology (abstract) 2013) (**Appendix 3**). The main distinguishing features between IgG4-SC and PSC with an elevated IgG4 are the presence of extra-biliary organ involvement, radiological response to corticosteroid therapy (in the inflammatory stage), and the presence of storiform fibrosis and phlebitis on histopathological analysis in IgG4-SC (Culver et al, Aliment Pharm Therap 2011) (**Appendix 3**). However, discrimination is not always possible - PSC can be associated with other autoimmune diseases, those with autoimmune hepatitis overlap or autoimmune cholangitis in children may respond to corticosteroids (Culver et al, Aliment Pharm Therap 2011; Culver et al, J Hepatology (abstract) 2013) (**Appendix 3**), and the morphological features on biopsy specimens are variable whereby onion skin (PSC) and storiform fibrosis (IgG4-SC) can be indistinguishable and not all of the 'IgG4-RD Boston criteria' are seen (Zen et al, Semin Diagn Pathol 2012; Deshpande et al, Mod Pathol 2012). The extent of pathogenic overlap between the 2 conditions also remains uncertain (Zen et al, Hepatology 2014). Our data highlighted that HLA DQB1*0301 was more frequent in the PSC patients with elevated serum IgG4 than in IgG4-RD and PSC with normal serum IgG4 (**Chapter 11**). Additional alleles were also more frequent in PSC high IgG4 than in IgG4-RD patients (A*0101, B*0801 and C*0701), and in PSC high IgG4 than PSC normal IgG4 (DRB1*0401, *1101 and *1102), which may provide additional means of discrimination.

Distinguishing IgG4-RD from malignant disease remains challenging. In our prospective cohort, one-fifth of AIP and IgG4-SC patients had surgical operations for suspected pancreaticobiliary cancer (**Chapter 3**). Although recognition of the disease has increased, the lack of a sensitive and specific diagnostic marker has hampered progress in reliable discrimination. Almost 30% of IgG4-RD patients will have a normal serum IgG4 at diagnosis, so it is important that serum IgG4 is not used alone (**Chapter 3 and 4**). Diagnostic criteria have evolved to include extra-pancreatic organs and strict histopathology characteristics (Deshpande et al, Mod Pathol 2012). An abundance of IgG4 positive plasma cells infiltrating the involved organs of IgG4-RD patients is well described, but not specific for the disease (Culver et al, Diagn Histopath, 2013; Culver & Bateman, J Clin Path 2012) (**Appendix 3**). It is therefore recommended that all biopsy and resection specimens should be reviewed for the key triad of morphological features and immunostaining for IgG4 and IgG performed, with a mean count per high power field and IgG4 to IgG ratio calculated (Deshpande et al, Modern Pathol 2012). Furthermore, radiological evidence of multiple organ involvement and steroid-response should be sought.

The advent of the hepatopancreatobiliary multidisciplinary team (MDT) meeting in many centres and more recently of the IgG4-RD MDT in Oxford has allowed for all patients to be discussed with medical, surgical, radiology and histopathology input in an attempt to reduce the number of misdiagnosed patients. These meeting have allowed for more appropriate histological sampling and supervised therapeutic trials of corticosteroid in cases with uncertainty. However, abundant IgG4 counts can occur in the context of malignancy and other inflammatory disease, and steroid-related

improvement in symptoms, a fall in serum IgG4, improvements in liver function tests and reduction of a mass on imaging are not disease-specific. Indeed, even in the MDT setting, cases where the diagnosis has been missed or over-called leading to surgery and inappropriate therapy are still reported, so there is pressing need for a better diagnostic tool.

The original study of serum IgG4 levels suggested no role for IgE in the diagnosis of AIP (Hammano et al, *N Engl J Med* 2001). Subsequent studies using IgE to differentiate AIP from pancreatic cancer in small series received limited attention (Umemura et al, *Hepatology*, 2007; Kamisawa et al, *Euro J Gastro Hep*, 2009). Our data revealed 56.8% of IgG4-RD patients have an elevated serum IgE level at diagnosis, and a level of 480KU/L (4 times the upper limit of normal) has a specificity of 85.7% and sensitivity of 35.9% in differentiating IgG4-RD from disease mimics. Furthermore, the IgE level was higher in those with a history of allergy/atopy, and there was a positive correlation of serum IgE with serum IgG4 and eosinophil count (**Chapter 5**). However, although an elevated IgE level and a history of atopy are useful in identifying IgG4-RD patients with an elevated IgG4 level, the majority of patients with a normal IgG4 also had a normal IgE, so it is of limited value in this group. Interestingly, we have also shown tissue infiltration of IgE, mast cells and eosinophils in involved organs, which may be included in future diagnostic criteria (**Chapter 5**).

Recently, we identified IgG4⁺ memory B cells and antibody-producing plasmablasts in the blood of patients with active IgG4-RD, both of which correlated with serum IgG4. However, in contrast to recent data published by a Boston group (Mattoo et al, *JACI* 2014), we found no expansion of plasmablasts in active IgG4-RD patients with normal

serum IgG4 levels (**Chapter 9**). Finally, our collaborators at AMC Amsterdam have employed a method using PCR in clonally expanded cells that reportedly distinguishes active IgG4-SC patients from PSC elevated IgG4 and normal IgG4 patients, which has been validated in samples from our Oxford IgG4-RD cohort (L Hubers, J Hepatol (abstract) 2015) (**Appendix 3**). A head-to-head comparison with serum IgG4 levels and serum IgG4:IgG1 ratios in IgG4-SC and PSC high IgG4 is still awaited.

12.1.2 Risk factors and Associations

Observational data from the Oxford and Amsterdam cohorts led to the theory that occupational and environmental risk factors may be important in disease pathogenesis. We observed that the majority of IgG4-RD patients (61% in Oxford and 88% in the AMC) had had a career in blue-collar occupations and/or had prolonged exposures to potentially hazardous occupational antigens (**Chapter 6**). The older male distribution seen in this disease may also be explained by the occupational history (**Chapter 3**). Interestingly, environmental risk factors have also been described in pemphigus foliaceus (chronic exposure to black fly antigens - from simuliidae bites), an epidermal organ-specific autoimmune disease mediated by IgG4 autoantibodies (Lombardi et al, J Invest Dermatol 1992; Warren et al, N Engl J Med 2000). However, this hypothesis requires further substantiation and an international registry of IgG4-RD are being created with input from specialists in occupational medicine to define this further.

The relationship between allergy and IgG4-RD has received recent interest. Our data suggests that 62.5% of IgG4-RD patients report a clinical history of allergy and/or atopy, compared with 17% of healthy controls and 20% national population statistics. Furthermore, IgE-specific allergen testing confirmed response in 40% of IgG4-RD

patients. The concentration of total serum IgE was higher in atopic individuals than those deemed non-atopic (**Chapter 5**). This data is reinforced by a recent study showing elevated IgE levels and peripheral blood eosinophilia predominantly seen in atopic IgG4-RD patients (Della Torre et al, Allergy, 2014) with such atopic patients expressing up regulated Th2 cytokines in the peripheral blood, in contrast to those who were non-atopic (Mattoo et al, Allergy, 2014). In those patients with a high IgE and IgG4 without atopic manifestations, IgG4 may block IgE-mediated immune function as an important anti-inflammatory response (Aalberse et al, J Immunol, 1983; Aalberse et al, Clin Exp Allergy, 2009).

12.1.3 Response to Treatment

Corticosteroids are currently the first line medication for IgG4-RD. In our study, both serum IgG4 and IgE concentrations decreased whilst on corticosteroid therapy in the first 12 weeks, with a parallel fall in disease activity in the patients. However, normalisation of serum IgG4 levels with treatment did not predict risk of relapse and the fall was not disease-specific (**Chapter 3 and 4**). Rituximab, an anti-CD20 B cell depletion therapy used in refractory IgG4-RD, similarly results in a decrease in serum IgG4 and IgE levels with regression of active disease; in fact the concentration of IgE showed a steeper decline than serum IgG4 levels (Khosroshahi et al, Med Baltimore, 2012). However, the fall in immunoglobulins is not specific to IgG4-RD, and we have shown that this is similarly seen in rheumatoid arthritis patients treated with rituximab (Lighaam et al, Keystone (abstract) 2014) (**Appendix 3**). Other second line immunosuppressive agents such as azathioprine and mycophenolate are often used in those patients who relapse, without any real evidence base and no published data on

serum IgG4 and IgE levels. Whether these levels are important in determining response to therapy remains to be clarified.

12.1.4 Disease Relapse and Multi-Organ Involvement

Early studies of AIP focused on the gratifying clinical and radiological response to an initial course of steroids, and for this reason systemic IgG4-RD is commonly perceived to run a benign disease course. However in the largest prospective cohort of IgG4-RD patients reported from Europe, we have provided evidence of disease relapse, organ dysfunction and failure, malignancy and mortality (**Chapter 3**). Disease relapse following an initial course of steroids occurs in 50% of patients (*Huggett & *Culver et al, Am J Gastro 2014) and relapse frequently arises in organs other than the pancreas (**Appendix 3**). Extra-pancreatic disease was present in the majority (73%) of patients, most frequently proximal biliary disease, and was a predictor for disease relapse and a more complicated course. Furthermore, a serum IgG4 of >2 times the upper limit in patients with IgG4-RD at diagnosis was associated with multi-organ involvement and disease relapse (**Chapter 4**). An elevated IgE concentration >4 times the upper limit was similarly a marker of relapse, with a non-significant trend to identify those with multiple organ involvement, although this was lost on multivariate analysis (**Chapter 5**). Thus, stratification of risk of complications on the basis of IgG4 level may be possible. An elevated serum IgG4 >2.8g/l at diagnosis may guide further imaging to search for subclinical organ involvement and early consideration of prolonged corticosteroid or second line treatments to prevent relapse. Conversely, normal serum IgG4 often suggests single organ involvement in whom relapse is low, and these patients may require less stringent follow-up with annual outpatient appointments.

Regardless, serum IgG4 cannot be used in isolation and the identification and validation of additional diagnostic markers requires careful attention.

12.1.5 Organ Dysfunction and Failure

In our prospective cohort we identified a number of patients with organ dysfunction and failure with associated morbidity and mortality, despite close clinical follow up and treatment of clinically active IgG4-RD (**Chapter 3**). Particular sites of organ dysfunction included the pancreas, liver, and kidneys. Pancreatic exocrine insufficiency occurred in > 50% of cases, and this included patients who had an excellent clinical and radiological response to steroid therapy, and without disease relapse. This reinforces the extent to which inflammation and subsequent fibrosclerosis can be a rapid process in IgG4-RD. Although steroid therapy undoubtedly has a dramatic and widely reported effect in resolving the pancreatic mass in AIP (Hirano et al, Gut 2007; Uchida et al, J Gastroenterol 2009; Maruyama et al, J Gastroenterol 2012), it remains uncertain the extent to which steroids impact on preserving pancreatic structure and function. Pancreatic exocrine failure can be managed with enzyme supplementation, however we found a number of cases of other organ dysfunction and failure that were irreversible such as cerebral involvement, pulmonary fibrosis, stage 4 chronic kidney disease requiring renal dialysis, and liver cirrhosis resulting in liver transplantation or death.

12.1.6 Malignancy

There is increasing realisation that IgG4-RD itself may be associated with an increased risk of malignancy and an overall reduced life expectancy (*Huggett & *Culver et al, Am J Hepatol 2014) (**Appendix 3**). Follow-up of our prospective IgG4-RD cohort showed an increased incidence ratio of 2.25 (P=0.01) for all cancers compared to the

general population (**Chapter 3**). There was a risk of ascertainment bias in our cohort, as the rate of malignancy may have been elevated by earlier discovery of cancer in patients having scans for evaluation of IgG4-RD and comparison with national statistics are not adjusted for other risk factors such as smoking, alcohol intake and obesity. However, the data is supported by large retrospective analyses in AIP patients (Uchida et al, *J Gastroenterol* 2009; Hart et al, *Gut* 2013; Shiokawa et al, *Am J Gastroenterol* 2013; Yamamoto et al, *Mod Rheumatol* 2012). Further support from our data comes from the high numbers of oncogenic genes enriched in IgG4-RD patients using microarray gene expression analysis (**Chapter 10**). IgG4-RD lesions have also been reported to co-exist with orbital, non-Hodgkin's and mantle cell lymphoma or carcinoma (Cheuk et al, *Am J Surg Pathol* 2008; Sato et al, *Pathol Int* 2008; Takahashi et al, *Pancreas* 2009; Sato et al, *Int J Haematol* 2008; Brenner et al, *Mod Pathol* 2013). This makes diagnosis even more challenging as malignancy is the main differential diagnosis of an IgG4-related mass-like lesion (Law et al, *Cleve Clin Med* 2009).

The underlying cause and pathogenesis of cancer in IgG4-RD remains unanswered. The concept of inflammation leading to malignancy is well recognised and has been described in a variety of disease states such as hepatocellular cancer and viral hepatitis, colorectal cancer in inflammatory bowel disease, and cholangiocarcinoma on a background of PSC. Therefore, in IgG4-RD it is plausible that malignancy develops in a setting of chronic inflammation. Other authors have suggested that IgG4-RD itself is a paraneoplastic phenomenon, whereby an intense IgG4+ve infiltrate can be identified in the cancer stroma at diagnosis, and resolves without relapse after steroid therapy. More recently, a potential cellular mechanism has been proposed whereby IgG4 antibodies are specifically generated in response to malignant disease (Karagiannis et al, *J Clin*

Invest 2013). In human melanoma patients, tumour-associated B cells are polarised to produce IgG4, stimulated by a Th2 (IL-10 secreting) tumour microenvironment. Strikingly, antigen-specific and non-specific IgG4 inhibited IgG1-mediated tumoricidal functions, mediated through reduction of Fc γ RI activation, both in vitro and in a human melanoma xenograft mouse model. Higher serum IgG4 levels were associated with decreased patient survival. This study provides evidence for an alternative hypothesis linking malignancy to IgG4-RD whereby the generation of IgG4 antibodies, in the context of malignancy, may restrict effector cell function against tumours and represents a mechanism of tumour-induced immune escape. This theory requires further evaluation.

12.1.7 Surveillance and Follow-up

Currently there are no guidelines to direct the surveillance and follow-up of IgG4-RD patients with regards to development of complications or malignancy. This is in contrast to other disease groups, such as patients with inflammatory bowel disease (IBD) who undergo interval colonoscopy for colorectal carcinoma surveillance, or PSC patients who undergo annual ultrasounds and colonoscopy for gallbladder cancer and colorectal carcinoma screening, respectively. Given the evidence that IgG4-RD is associated with organ dysfunction, failure and malignancy, we would recommend a policy of close observation early after initiating steroid therapy to confirm symptomatic improvement and steroid tolerance, and then assessment at weeks 6-8 with imaging and biochemical assessment where appropriate to confirm steroid responsiveness. Patients may then be assessed at 3 and 6 months to wean and discontinue steroids assuming a primary response to steroid therapy. In the absence of firm guidelines, all patients should be discussed in a joint multi-disciplinary team meeting to decide on further therapy on an

individual basis. One approach may be to repeat imaging (CT or MR) at 3 months and 1 year after steroid discontinuation to assess the maintenance of the response and evidence of subclinical radiological relapse, based on our data that 50% of patients will relapse at a median of 4.6 months (range 0-64) after steroid discontinuation. Further imaging should be dictated by clinical or biochemical evidence of relapse. In theory the use of low-dose CT scanning could be used to survey for malignancy within the first year of diagnosis, as has been suggested by a large randomised trial for lung malignancy in long-term smokers (Gould et al, NEJM 2014), but this has not been validated.

The high relapse rate at 50% after corticosteroid therapy and the need for re-treatment or prolonged therapy, especially with IgG4-SC, raises two important issues. Firstly, corticosteroid-related side effects should be anticipated and preventative measures taken. This process has been implemented into our routine clinical management of patients over the last four years. Secondly, there is an urgent need for international prospective studies to evaluate rational treatment strategies, including the early use of second line treatment regimens and B cell depletion therapy to prevent relapse and long term complications of disease. Ultimately, prospective studies of natural history and novel therapeutic agents, in very carefully defined patient cohorts, will be required to establish the true clinical course of the disease.

12.2 PATHOGENESIS

12.2.1 Genetics: HLA associations

HLA associations in autoimmune diseases are usually linked with risk and/or severity of disease (Handunnetth et al, Genes & Immunity 2010). HLA class II alleles DRB1*0405 and DQB1*0401 have been linked to susceptibility, risk of relapse and extra-pancreatic

involvement in single-centre Asian studies in AIP (Kawa et al, *Gastroenterol* 2002; Park et al, *Gastroenterol* 2008; Hirano et al, *Gastroenterol* 2009). In the UK, HLA DRB1*0301 and DQB1*0201 were linked with susceptibility in both a reference and validation IgG4-RD cohort (**Chapter 11**). The different associations in Asia and the UK are likely due to ethnic variation, which is seen in diseases such as multiple sclerosis. DQB1*0201-DRB1*0301 is in tight linkage disequilibrium, making it difficult to determine which of these alleles are primarily associated with disease. They have also been associated with other autoimmune disease, including Lambert-Eaton myasthenic syndrome, sarcoidosis and type I diabetes (Parsons et al, *Human Immunol* 2000; Sato et al, *Am J Resp Cell Molec Biol* 2002; Lambert et al, *J Clin Endocrinol Metab* 2004).

Two models are used to explain the association between HLA antigens and a predisposition to a disease. One is that specific HLA antigens play a functional role in presenting a specific peptide to T cells, resulting in the induction of an autoimmune response. The other model is that HLA is only a genetic marker for the non-HLA gene-associated disease susceptibility, which is located within the HLA gene region. Using population-based immunogenetic analysis it is difficult to dissect the relative contribution of individual HLA genes to the overall risk attributed to the HLA region in this disease, specifically owing to the strong linkage disequilibrium of genes on common HLA haplotypes. For other autoimmune diseases, transgenic rodents have been used to conduct in vivo studies of the function of single HLA genes allowing careful interrogation of HLA-DR and DQ regions (Freitag et al, *Gastroenterol* 2010). This could be applied to IgG4-RD, perhaps utilising a recently described canine model (English cocker spaniels) of IgG4-RD with IgG4+ cell infiltration and multiple organ involvement (Penny Watson, Cambridge, personal communication). Whether the class

II HLA DQ or DR allele is directly involved in the binding of a particular IgG4-RD-triggering peptide in a more favourable way, or merely indirectly, by operating as a marker for other immune-regulating molecules, needs further elucidation. Knowledge of the involvement of particular allelic variants in IgG4-RD might help in finding a peptide motif, which could be the key to identifying the antigenic trigger(s) of this disease.

12.2.2 Antigen Targets: Initiation and Maintenance

Since its discovery, there has been a sustained quest for an antigen initiating and/or driving IgG4-RD. Antibodies against a range of auto-antigens have been proposed including anti-nuclear antigens, lactoferrin, carbonic anhydrase II and IV, pancreatic secretory inhibitor, trypsinogens, and an unknown 13.kDa protein (Lohr et al, Am J Gastroenterol 2010; Asada et al, Pancreas 2006). However, none has been consistently found in the disease, and all are of IgG1 and not IgG4 subclass. Next generation sequencing of whole blood in IgG4-SC patients demonstrated clonal expansions of IgG4-switched cells, with the authors concluding that specific B cell responses are pivotal to disease pathogenesis, yet no specific antigen was identified (Maillette de Buy Wenniger et al, Hepatology 2013). A role for *Helicobacter pylori* plasminogen binding peptide, through a process of molecular mimicry in genetically predisposed individuals, was proposed in AIP (Frulloni et al, N Engl J Med 2009). However, using antigen-specific IFN- γ and IL4-secreting T cell responses and B cell (IgG and IgG4) immunological memory to overlapping peptides derived from *H.pylori* PBP we showed no positive response (**Chapter 8**). Furthermore, the prevalence of peptic ulceration (7.2%) and *H.pylori* infection (23.6%) in IgG4-RD patients was similar to those with other autoimmune and inflammatory conditions, and in gastric tissue of AIP patients with *H.pylori*-associated chronic active gastritis there were <10/HPF

of IgG4-positive plasma cells, which were not seen to co-localise with *H.pylori* bacteria. Given the relevance placed on sequence homology of the binding motif of the susceptibility HLA DRB1*0405 haplotype in Asia, it is important to note we demonstrated different HLA haplotypes in the UK (**Chapter 11**).

More recently oligoclonal expansion of plasmablasts in the blood of IgG4-RD patients (Mattoo et al, J Allergy Clin Immunol 2014) and oligoclonal expansions of IgG4+ cells in whole blood of IgG4-RD patients in our Oxford cohort and IgG4-SC/AIP patients in a Dutch cohort (Lowiek et al, J Hepatol (abstract) 2015) have been demonstrated (**Appendix 3**). Using a radioimmunoassay supported by electrophoresis and immunofixation, we demonstrated a polyclonal IgG4 response to multiple non-infectious environmental antigens in IgG4-RD (**Chapter 7**). Antigen-specific responses correlated with the serum IgG4 level, suggesting that elevated IgG4 could be the result of a polyclonal expansion of many IgG4 B cells irrespective of their specificity, and thus that pre-existing subsets of IgG4 B cells expand and differentiate in the course of developing IgG4-RD. Furthermore, observational data highlighted a clinical history of multiple occupational and environmental exposures in IgG4-RD, supported by the production of IgG4 antibodies in the context of chronic antigenic stimulation (**Chapter 6**). Factors other than antigen include IL21 driving proliferation and expansion of IgG4-switched cells, IL4, IL13 and IL10 that enhance class switch to IgG4, and/or the up-regulation of activation-induced cytidine deaminase (AICDA), B lymphocyte induced maturation protein 1 (BLIMP1) and X-box protein 1 (XBP1), all of which have been shown in IgG4-RD patients (Kuchen et al, J Immunol 2007; Tsuboi et al, Arthritis Res Ther 2012; Jeannin et al, J Immunol 1998; Miyoshi et al, Pancreas 2008). Further evidence for a non-clonal population of B cells is provided by PCR using multiplex

primers in the involved tissue of patients with IgG4-RD (**Chapter 7**). Whilst the poly/oligo clonality of the IgG4 response makes it unlikely that these B cells initiate IgG4-RD, it does not preclude it to be an important contributing factor to maintenance of disease progression once established, and it does not exclude the possibility that a single antigen (self or exogenous) served as the initial trigger.

12.2.3 Role of B cells

Immunological work in AIP has focussed predominantly on T cells lineages including helper cell and regulatory phenotypes, with resultant cytokine production thought to drive IgG4 switch and production. However, there has been limited attention paid to the phenotype and function of the IgG4+ve B cells themselves. After identifying an IgG4-specific antibody suited for staining B cells and isolating IgG4 B cells (Lighaam et al, JACI 2014), we were able to establish certain similarities but also important differences in the frequency and phenotype of IgG1 and IgG4 B cells in health and disease (**Chapter 9 and Appendix 3**). In particular, differences in their potential to react to complement activation and immune complexes may help explain the differential regulation of the IgG4 antibody response.

Complement and Fc receptors are important in the humoral immune response. IgG4 B cells expressed less complement receptors CR2 (CD21) and CR1 (CD35) than IgG1 cells in health, but CR2 expression on IgG4 cells was not decreased in IgG4-RD. As CD21 co-ligation lowers the threshold for B-cell activation and clonal expansion (Barrington et al, Proc Natl Acad Sci USA 2009), this may explain why the IgG4 B cell population preferentially expands in IgG4-RD (**Chapter 7**). IgG4 B cells expressed less inhibitory FcγRIIb (CD32) and more IgE receptor FcεRI (CD23) than IgG1 cells, but

the reverse was true in IgG4-RD. This lower expression of FcεRI on IgG4 B cells in IgG4-RD may account for an inability to negatively regulate IgE levels in active disease (**Chapter 5**). IgG4 B cells also expressed less cell adhesion and activation markers CD24 and CD38 than IgG1 B cells, and did not have a traditional B regulatory phenotype lacking IL10 production. Whilst in IgG4-RD, IgG4 and IgG1 cells had similar CD24 and CD38 expression there was no increased CD20⁺CD24^{hi}CD38^{hi} transitional cell population.

The frequency of IgG4 memory B cells and antibody-producing plasmablasts in the blood correlated with serum IgG4 levels in both health and disease, providing support that these cell types are responsible for the elevated serum IgG4 level (**Chapter 7**). Expansions of plasmablasts in the blood have recently been reported by the Boston group in active disease (Mattoo et al, JACI 2014). Given the high levels of surface MHC class II (HLA-DR) on plasmablasts, one could speculate that these cells serve as vital antigen-presenting cells to CD4⁺ T cells in patients with IgG4-RD, but it remains possible that this disease is driven directly by B-lineage cells with little or no role for T cells in pathogenesis and that IgG4 plasmablasts are secondarily induced bystanders in this disease.

Corticosteroids interfere with the production of cytokines critical for T cell proliferation, and the binding of interleukins to B cells suppressing proliferation and antibody production. Using a radioimmunoassay, antigen-specific responses were higher in treatment-naïve IgG4-RD patients compared to those on corticosteroids (**Chapter 7**). Serum IgG4 also falls with corticosteroid therapy (**Chapter 4**), and correlates with IgG4⁺ B cells and plasmablasts in the blood (**Chapter 9**). This may be

explained by suppressed proliferation of expanded IgG4-switched B cells or suppressed differentiation into IgG4 plasmablasts. The steroid effect highlights the rapid turn over of the IgG4-producing plasma cells, as opposed to production by long-lived plasma cells in the bone marrow. This is also reported by observational data in those patients treated with Rituximab (Khosroshahi et al, *Medicine (Baltimore)* 2012).

12.2.4 Role of IgE and Mast cells

An elevated serum IgE and the presence of IgE-positive mast cells in involved tissues of patients with IgG4-RD points to the role of an IgE-mediated response. The strong links between IgG4 and IgE are well established; both are induced by IL4 and IL13, whilst IL5 and IL6 enhance IL4-dependent IgE synthesis, and IL13 induces and IL7 potentiates IL4-independent IgG4 and IgE synthesis (Jeaninin et al, *Blood*, 1998; Pene et al, *Euro J Immunol*, 1988). Factors that differentiate IgG4 and IgE include regulatory IL10, which promotes the production of IgG4-switched cells and down regulates IgE (Jeanin et al, *J Immunol*, 1998). Studies have shown that Th2-dominant IL4, IL5, IL13 and T regulatory immune responses IL10 and TGF-beta are more activated in IgG4-RD in both the peripheral blood and affected tissues of patients (Zen et al, *Hepatology*, 2007; Tanaka, *Arthritis Rheum*, 2012; Nakashima et al, *Clin Nephrol*, 2010). Importantly, IL4, IL13 and IL10 are secreted not only by T helper and regulatory cells but also by mast cells, of which IgE is a key stimulator. IL4 and IL5 also control the accumulation of eosinophils in blood and tissue.

Non-specific polyclonal IgE alone can induce cytokine secretion, independent of any antigen (Kashiwakura et al, *Allergol Int*, 2009). Chronic elevation of antigen-independent non-specific IgE up regulates the high-affinity IgE receptor FcεRI on mast

cells, which inhibits mast cell apoptosis and promotes cytokine production (Bax et al, Front Immunol, 2012). The number of FcεRI receptors on mast cells increases in the presence of elevated serum IgE levels. It is therefore interesting that IgG4 B cells also appear to have increased capacity to capture IgE molecules, by up-regulation of the FcεRI on their surface (**Chapter 9**). Signalling via IgE might trigger IgG4 cells and contribute to dampening of allergic IgE responses. This antigen-independent non-specific IgE may bind to FcεRI on mast cells or B cells to promote the production of T helper 2 IL4 and regulatory T-cell IL10 and TGF-beta1 cytokines, which may induce the distinctive histological features of IgG4-RD - CD4+CD138+ infiltration and abundant IgG4-switched cells, and interstitial fibrosis. Mast-derived IL10, an anti-inflammatory cytokine that can suppress acquired or innate immune responses, may also reduce allergic inflammation in the affected organs (Grimbaldeston et al, Nat Immunol 2007). The inflammatory milieu itself may indeed sustain the IgE elevation observed, whereby IL4 and IL13 induce IgE production, which further stimulates mast cells. Therefore, in a subset of individuals with IgG4-RD with a history of allergy/atopy and elevated IgE levels, it is plausible that elevated serum IgG4, resulting from polyclonal expansion of many IgG4 B cells irrespective of antigen specificity (**Chapter 9**), may be linked to IgE B cell expansion, and that corticosteroids and rituximab may exert their effects through inhibition of both IgE and hence IgG4 producing B cells and plasmablasts.

A possible therapeutic target is the prostaglandin D2 receptor, CRTH2 (chemoattractant receptor homologous molecule expressed on Th2 cells), important in allergic inflammation. It is expressed on Th2 cells and innate cells such as eosinophils and responds to mast cell derived factors (Nagata et al, J Immunol 1999). The frequency of

CRT_h2 cells in CD4⁺ T cells was reportedly increased in patients with IgG4-RD, specifically IgG4-siloadenitis, with a non-significant correlation of these cells with the serum IgE and peripheral blood eosinophils (Saito et al, *Int Arch Allergy Immunol*, 2012). We also report the up regulation of PGD2 and CRT_h2 in gene expression analysis of IgG4-RD patients (**Chapter 10**). Furthermore, blockade of CRT_h2 can reduce allergic inflammation in rodent models of antigen-induced airway inflammation, allergic rhinitis, atopic dermatitis, and hyper-responsiveness in asthma. These agents may be important in IgG4-RD patients with a history of allergy, elevated IgE and eosinophilia.

12.2.5 Gene Expression: Immunological drivers of disease

Using a whole-genome microarray approach we performed gene expression analysis in the peripheral blood of IgG4-RD patients, aiming to identify biological pathways associated with the disease. This implicated antibody-inducing cytokines important in driving IgG4 switch and proliferation, chemokines important in trafficking and homing of lymphocytes to end organs, complement proteins in the classical and lectin pathways, and members of the TGF beta pathway as putative immune drivers of disease (**Chapter 10**). Out of 213 differentially expressed candidate genes, 39 immunologically functional genes and 2 pathways were identified by network analysis and biomedical literature review. Of these, 19 genes were further confirmed at the RNA and/or protein level.

Of particular importance were genes encoding cytokines IL4, IL5, IL10, IL21, and transcription factors STAT3 and GATA3, which are induced by Th2 and mast cells and have been shown to be important in IgG4 switch and proliferation, eosinophil generation and targeting of chemokines and complement receptors (Zen et al,

Hepatology 2007; Kudo-Tanaka et al, Arthr Res 2012; Tsuboi et al, Arthr Res Thep 2012; Eto et al, PLoS One 2011). Furthermore, chemokine ligands CCL23 and CCL25 were up regulated, which are important in homing to the gut-liver axis (Eksteen et al, J Exp Med 2004) and as biomarkers for other autoimmune diseases (Rioja et al, Arthritis Rheum 2008). Genes that play a role in the TGF- β pathway, known for its involvement in fibrosis and the generation of regulatory T cells, were similarly up regulated (Leask et al, FASEB 2004; Maynard et al, Nat Immunol 2007). Several genes involved in both the classical and mannose-binding lectin pathway were up regulated including C1QC, C2, CR1 and SERPING1. This finding is particularly interesting as IgG4 antibody is believed to be a poor activator of C1q (Van der Zee et al, Clin Exp Immunol 1986). Furthermore, decreased levels of C4, as well as a tendency toward decreased levels of C3, have been reported in serum of AIP patients (Muraki et al, Pancreas 2006) and deposits containing C3, IgG and IgG4 were found in the tubular basement membranes of renal biopsy specimens in IgG4-tubulointerstitial nephritis (Detlefsen et al, Histopath 2010).

Gene expression microarrays provide a snapshot of all the transcriptional activity in a biological sample, facilitating discovery of novel and unexpected functional roles of genes, and providing targets for disease stratification and therapeutics. Despite this, evidence of gene expression does not confirm a causal relationship, and interpretation of these findings should be made acknowledging this. We performed a whole blood microarray, and further information may be gained using a tissue array or cell-type specific array. Further validation of these findings in extra-mural cohorts will be important.

12.3 FUTURE CONSIDERATIONS

Over a decade after the first reports of elevated serum IgG4 concentrations in AIP, our understanding of this disease and its triggers is gradually progressing. Reliable diagnosis remains a significant challenge, as there is currently no validated non-invasive test with good diagnostic accuracy. The recently described PCR-based assay based on clonal analysis requires careful validation and scrutiny. Whilst we now understand that this is not a benign disease, the ability to reliably target those patients who require longer duration and early escalation of therapy is in its early stages. The pathophysiology underlying the recently described association with malignancy also requires further attention. Treatment options are not supported by prospective randomized controlled trials, and none are in progress, and this will require international collaboration. The role and action of rituximab and other disease-modifying biological agents needs to be understood.

The initial trigger that sets off aberrant IgG4 production is not known, although evidence points to proliferation of polyclonal non-specific IgG4⁺ B cells and plasmablasts in the disease. The supportive role of IgE and mast cells driving this disease in subsets of patients with allergy and/or atopy requires further clarification. The role of T cell subsets in initiation and maintenance of the inflammatory state has received recent attention, and our on-going work looks specifically at the role of T follicular helper cells in this capacity (unpublished data). Ultimately, finding the trigger for inflammation and the driver for fibrosis-induced tissue destruction, as well as developing novel therapeutic steroid-sparing approaches to prevent long-term sequelae must be the immediate aim.

Appendix 1 Criteria for enrolment

The inclusion and exclusion criteria for the study were as follows:

INCLUSION CRITERIA

1. Male or female subjects
2. Aged between 18 and 110 years
3. Elevated serum IgG4 levels or
4. (A) A previous diagnosis of systemic IgG4 disease or
(B) A condition that results in high IgG4 levels or
(C) A disease that mimics IgG4 systemic disease (for example PSC)

Healthy volunteers must have no medical conditions

EXCLUSION CRITERIA

Clinical judgement that the patient is unlikely to participate in a long-term study

Appendix 2 Ethical documents

The following documents were approved by Oxfordshire Research Ethics Committee and given to recruits for the IgG4 study;

- (1) Participant invitation letter
- (2) Participant information sheet (patients)
- (3) Informed consent sheet (patients)



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PARTICIPANT INVITATION LETTER

Dear

I am writing to you to invite you to participate in a study entitled “Natural history and pathogenesis of systemic IgG4 disease”. We do not really know what causes this disease, or how common it is. It is very important to recognise it so that we can offer the right treatment.

The fact that we have written to you does not mean that you have this disease as we are inviting people with other conditions, as well as people with the disease for comparison.

The study involves having your medical details recorded on a database and also a giving a blood sample-usually just once a year. The research doctors would try to coordinate the research visit with a visit that you would normally make to the hospital.

If you are willing to consider taking part in the study please read the enclosed information sheet. You may then contact the study doctors using the details on the patient information sheet.

Thank you for considering this study.

Kind Regards

Dr Roger Chapman, Consultant Hepatologist

Dr Emma Culver, Specialist Registrar to Dr Chapman



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PARTICIPANT INFORMATION SHEET (PATIENTS)

6th August 2010 v2

IgG4 systemic disease

You are being invited to take part in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it if you wish with family and friends. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether you wish to take part.

What is the purpose of the study?

Recently, a disease known as “IgG4 systemic disease” has been described. IgG4 is a specific type of antibody that is found in all people, and is part of the immune system. In this disease IgG4 infiltrates different organs in the body such as the pancreas, liver and kidneys, and is associated with organ scarring and malfunction. In some people, systemic IgG4 disease is also associated with high levels of IgG4 in the blood. Diagnosing and understanding this disease is important so that the correct treatment may be given. Often people improve with a course of steroid treatment but we do not yet know what happens to people when steroids are stopped. A recent report has suggested that IgG4 systemic disease may be associated with a bacteria infection in the stomach called helicobacter pylori.

Not everybody with high levels of IgG4 in the blood has IgG4 systemic disease. Elevated levels of IgG4 can be caused by many other things such as bee stings, allergy treatments, and blistering skin diseases. If you have elevated levels of IgG4 this does not mean that you have, or that you will develop a disease.

Currently we do not understand what happens to people with IgG4 systemic disease over time, or why IgG4 levels are high. Other people may have high levels of blood IgG4 that are not associated with IgG4 systemic disease.

In this study we wish to see what happens to people that have IgG4 systemic disease over time and to assess the immune response to further understand why IgG4 levels are high, and what role these play in the disease process. We wish to study people with IgG4 systemic disease, and compare these to people with high blood levels of IgG4

without IgG4 systemic disease, patients with diseases that may mimic IgG4 disease, and healthy individuals.

The aims of the study are to establish a database of people with IgG4 systemic disease and assess the nature of the disease and response to treatment over time. We also hope to understand more about the reason why cells are making IgG4 and why these cause disease in some people but not in others.

Why have I been chosen?

We are asking you to participate as someone who has been found to have high levels of IgG4 in blood or tissue, or because you have another condition that is known to be associated with, or to mimic IgG4 systemic disease. The fact that you have been chosen does not necessarily mean that you have or that you will develop IgG4 systemic disease.

In total we are hoping to recruit 200 people with IgG4 disease, 50 people with high levels of IgG4 in the blood, 200 people with conditions that may mimic IgG4 disease and 35 volunteers with no disease and normal IgG4 levels.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason.

What will happen to me if I take part?

You will be given or sent this patient information sheet in advance. If you wish to participate we will ask you to contact us and we will arrange to meet you at the John Radcliffe hospital or at University College London hospital. At this visit we will answer any questions. If you decide to take part we will ask you to sign a consent form. We will then follow and record your progress and your treatment over time and include you in a database of IgG4 research subjects. To do this we will request access to your medical notes.

We will also ask you for a blood sample (60 mls-12 teaspoons) so that we can assess your immune system further. We may also take some blood (6 mls) to see if you have been infected with a bacteria in the stomach called helicobacter pylori, as this has been associated with IgG4 systemic disease. If we find that you have high levels of IgG4 in your blood we will ask a doctor to contact you. If we find that you do have helicobacter infection we may suggest that you have treatment, which would consist of a short course of antibiotics.

Depending on the results we may ask you to give further blood samples, up to a maximum of four times a year. If you are receiving treatment then we will ask for a blood sample before, during and after treatment. We will ask to see you annually thereafter where we will take a history and a blood sample. If you have had previous tests and have stored tissue we will ask if we may look at this tissue to assess IgG4 levels.

How long is the study?

We would like to see you at least once a year for your lifetime. However, you can leave the study at any time, for any reason, without any effects on your future medical care and without being obliged to give a reason for your decision to withdraw from the study.

What will happen to my samples?

Your blood will be used to assess your immune response further to try to understand further why levels of IgG4 are high in some people. Some blood or blood cells will be frozen down to use in this project in the future. Genetic tests may be performed only to answer questions about IgG4 disease. All your samples will be labelled with a number rather than your name, so that research staff and investigators in the laboratory will not be able to identify you.

If you have had previous tissue samples taken, for example, liver, colon and kidney biopsies, we would like to use these to look to see if they have IgG4 in them, or perform other tests that are relevant to the diagnosis of IgG4 systemic disease. These will be returned to storage after use.

What are the possible disadvantages of taking part?

The only procedure will be blood tests. These may be associated with bruising. It may be that we make a diagnosis of IgG4 systemic disease. If this happens we will inform your doctor who may refer you for appropriate treatment.

What are the benefits of taking part?

The information we obtain may not be of direct benefit to you but it will help us to understand this disease. You will be tested for helicobacter pylori-the doctor who usually looks after you will be informed and you may be offered treatment if this is present.

Will my GP be informed about this study?

We will, with your permission, inform your GP that you are taking part in this study. Your physician (GP or hospital doctor) would be informed about any results if they have implications for your health or health care.

What will happen if I don't want to carry on with the study?

You can leave the study at any time, for any reason, without any effects on your future medical care and without being obliged to give a reason for your decision to withdraw from the study. Should this happen, we will destroy the research samples or continue to use them and data about you, depending on your preference. In the unlikely event that you lose the ability to give consent during the study, data or tissue/blood already collected with consent would be retained and used in the study.

What if there is a problem?

The University of Oxford has appropriate insurance-related arrangements in place in respect of the University's role as Research Sponsor of this study. If you wish to complain about any aspect of the way in which you have been approached or treated during the course of this study, you can either contact Dr. Ellie Barnes (01865 271199) directly or contact the University of Oxford Clinical Trials and Research Governance office on 01865 857939.

Will my taking part in the study be kept confidential?

Your data and blood samples will be collected by clinical researchers and anonymised which means that it will be coded and only the study investigators will have access to the code. All the investigators have a duty of confidentiality towards you. Your research records may be inspected by University staff for audit purposes to ensure that researchers are following good research practice guidelines. All will have a duty of confidentiality to you as a research participant. All study data will be stored for twenty years and then disposed of securely unless we obtain further ethical committee approval to use it in a subsequent study. Data will be kept on a secure, password protected computer with restricted access in a locked room.

What will happen to the results of the research study?

We hope to publish the results of the study in the scientific literature. You will not be identified in the publication. We will be happy to send you a summary of the results of the study if you wish.

Who is funding the research?

The Medical research Council and Wellcome trust are funding the study investigators and the research is being sponsored and organised by the University of Oxford. No payment is made to either the doctors conducting the research or the hospitals where this research is carried out if you were to decide to help with the study.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee to protect your safety, rights, well-being and dignity. This study has been reviewed and given a favourable opinion by the Oxfordshire Research Ethics Committee.

If you have any questions please get in touch with

Dr Ellie Barnes (tel: 01865-271199 or ellie.barnes@ndm.ox.ac.uk) or

Dr Emma Culver (emma.culver@ndm.ox.ac.uk).

INFORMED CONSENT SHEET (PATIENTS)**Study Title: IgG4 systemic disease****Ethics Ref: 10/H0604/51****Date and Version No: 6th August 2010 v2****Please tick box**

1. I confirm that I have read and understand the information sheet dated _____ for the above project and have had the opportunity to ask questions. ☐
2. I agree to take part in the above research project. ☐
3. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, with no effect to my rights. ☐
4. I understand why blood samples are being taken, how the samples will be collected, that giving samples for this research is voluntary and that I am free to withdraw my approval for use of the sample at any time without giving a reason and without my medical treatment or legal rights being affected. ☐
5. I understand that any data collected from the analyses of the samples will be retained for use in the results of the research study. ☐
6. I understand that my GP will be informed that I am participating in this study ☐
7. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the University of Oxford, regulatory authorities and the NHS trust, where it is relevant to my taking part in this research. I permit these individuals access to my research records. ☐
8. I understand that this research is being conducted by clinical researchers who may not be otherwise responsible for my healthcare. ☐
9. I agree that these individuals may have access to my medical records to carry out this research. ☐
10. I agree to give blood samples taken for the purpose of the research study to the University of Oxford. If a commercial product were developed as a result of this study, I will not profit financially from such a product. ☐
11. I agree to allow the researchers access to previously collected samples of tissue and blood for the purpose of this research study. ☐

Name of Participant_____
Date_____
Signature_____
Name of person taking consent_____
Date_____
Signature

Appendix 3 Published Abstracts

The following list of abstracts is relevant to this thesis have been published as full text original articles, case discussions, reviews, book chapters, and/or presented at international conferences.

ABSTRACT 1

EL Culver, E Vermeulen, M Makuch, A van Leeuwen, R Sadler, T Cargill, P Klenerman, R Aalberse, SM van Ham, E Barnes, T Rispens. Increased IgG4 responses to multiple food and animal antigens indicate a polyclonal expansion and differentiation of pre existing B cells in IgG4-related disease. *Annals Rheum Dis* 2015;74(5):944-947.

Background: IgG4-related disease (IgG4-RD) is a systemic fibroinflammatory condition, characterised by an elevated serum IgG4 concentration and abundant IgG4-positive plasma cells in the involved organs. An important question is whether the elevated IgG4 response is causal or a reflection of immune-regulatory mechanisms of the disease. *Objectives:* To investigate if the IgG4 response in IgG4-RD represents a generalised polyclonal amplification by examining the response to common environmental antigens. *Methods:* Serum from 24 patients with IgG4-RD (14 treatment-naive, 10 treatment-experienced), 9 patients with primary sclerosing cholangitis and an elevated serum IgG4 (PSC-high IgG4), and 18 healthy controls were tested against egg white and yolk, milk, banana, cat, peanut, rice and wheat antigens by radioimmunoassay. *Results:* We demonstrated an elevated polyclonal IgG4 response to multiple antigens in patients with IgG4-RD and in PSC-high IgG4, compared with

healthy controls. There was a strong correlation between serum IgG4 and antigen-specific responses. Responses to antigens were higher in treatment-naïve compared with treatment-experienced patients with IgG4-RD. Serum electrophoresis and immunofixation demonstrated polyclonality. *Conclusions:* This is the first study to show enhanced levels of polyclonal IgG4 to multiple antigens in IgG4-RD. This supports that elevated IgG4 levels reflect an aberrant immunological regulation of the overall IgG4 response, but does not exclude that causality of disease could be antigen-driven.

ABSTRACT 2

JP Corcoran, **EL Culver**, I Psallidas, RJ Hallifax, SJ Davies, AC Bateman, E Barnes, NM Rahman. A 63yr old man with a right recurrent pleural effusion: IgG4-pleural disease. *Thorax*. Epub Jan 2015;1-4.

A case based discussion of a 63 year-old patient presenting with exertional dyspnoea and a dry cough. Imaging confirmed a right sided pleural effusion and pleural thickening. Serum IgG4 was elevated, and thorascopy with pleural biopsy showed abundant IgG4 plasma cells and morphology consistent with IgG4-related pleural disease. Symptomatic and radiological response to high dose corticosteroids was seen.

ABSTRACT 3

M Huggett*, **EL Culver***, M Kumar, J Hurst, M Chapman, S Pereira, RW Chapman, G Webster, E Barnes. IgG4-related autoimmune pancreatitis and cholangitis is associated with extra-pancreatic organ failure, malignancy and mortality in a prospective UK cohort. *Am J Gastro* 2014;109:1675-1683.

Introduction: Type I autoimmune pancreatitis (AIP) and IgG4-related sclerosing cholangitis (IgG4-related SC) are now recognized as components of a multisystem IgG4-related disease (IgG4-RD). *Objectives:* We aimed to define the clinical course and long-term outcomes in patients with AIP / IgG4-SC recruited from two large UK tertiary referral centers. *Methods:* Data were collected from 115 patients identified between 2004 and 2013, and all were followed up prospectively from diagnosis for a median of 33 months, and evaluated for response to therapy, the development of multi-organ involvement, and malignancy. Comparisons were made with national UK statistics. *Results:* Although there was an initial response to steroids in 97%, relapse occurred in 50% of patients. IgG4-SC was an important predictor of relapse ($p<0.01$). Malignancy occurred in 11% shortly before or after the diagnosis of IgG4-RD, including three hepatopancreaticobiliary cancers. The risk of any cancer at diagnosis or during follow-up when compared with matched national statistics was increased (OR 2.25, CI 1.12–3.94, $p=0.02$). Organ dysfunction occurred within the pancreas, liver, kidney, lung, and brain. Mortality occurred in 10% of patients during follow-up. The risk of death was increased compared with matched national statistics (OR 2.07, CI 1.07–3.55, $p=0.02$). *Conclusions:* Our findings suggest that AIP and IgG4-SC are associated with significant morbidity and mortality owing to extra-pancreatic organ failure and malignancy. Detailed clinical evaluation for evidence of organ dysfunction

and associated malignancy is required both at first presentation and during long-term follow-up.

ABSTRACT 4

K Boostra*, **EL Culver***, L Maillette de Buy Wenniger*, MJ Heerde, KJ Erpecum, A Poen, KM Nieuwkerk, M Spanier, BJ Witteman, H Tuynman, N Geloven, H van Buuren, RW Chapman, E Barnes, U Beuers, C Ponsioen. Serum IgG4 and IgG1 for distinguishing IgG4-Associated Cholangitis from Primary Sclerosing Cholangitis. *Hepatology* 2014;59(5):1954-1963.

Introduction: The recent addition of immunoglobulin (Ig)G4-associated cholangitis (IAC), also called IgG4-related sclerosing cholangitis (IgG4-SC), to the spectrum of chronic cholangiopathies has created the clinical need for reliable methods to discriminate between IAC and the more common cholestatic entities, primary (PSC) and secondary sclerosing cholangitis. The current American Association for the Study of Liver Diseases practice guidelines for PSC advise on the measurement of specific Ig (sIg)G4 in PSC patients, but interpretation of elevated sIgG4 levels remains unclear.

Objectives: We aimed to provide an algorithm to distinguish IAC from PSC using sIgG analyses. *Methods:* We measured total IgG and IgG subclasses in serum samples of IAC (n=73) and PSC (n=310) patients, as well as in serum samples of disease controls, primary biliary cirrhosis (n=22). Serum IgG4 levels were elevated above the upper limit of normal (ULN>1.4 g/L) in 45 PSC patients (15%; 95% confidence interval [CI]: 11-19). The highest specificity and positive predictive value (PPV; 100%) for IAC were reached when applying the 4xULN (sIgG4 > 5.6 g/L) cutoff with a sensitivity of 42% (95% CI: 31-55). However, in patients with a sIgG4 between 1x and 2x ULN (n=38/45),

the PPV of sIgG4 for IAC was only 28%. In this subgroup, the sIgG4/sIgG1 ratio cutoff of 0.24 yielded a sensitivity of 80% (95% CI: 51-95), a specificity of 74% (95% CI: 57-86), a PPV of 55%(95%CI: 33-75), and a NPV of 90% (95% CI: 73-97). *Conclusion:* Elevated sIgG4 (>1.4 g/L) occurred in 15% of patients with PSC. In patients with a sIgG4 >1.4 and <2.8 g/L, incorporating the IgG4/IgG1 ratio with a cutoff at 0.24 in the diagnostic algorithm significantly improved PPV and specificity. We propose a new diagnostic algorithm based on IgG4/IgG1 ratio that may be used in clinical practice to distinguish PSC from IAC.

ABSTRACT 5

LJ Maillette de Buy Wenniger*, **EL Culver***, U Beuers. Exposure to occupational antigens might predispose to IgG4-RD. *Hepatology* 2014;60(4):1453-1454.

Evidence is mounting that the immune system of patients with IgG4-related disease (IgG4-RD) shows indications of chronic antigenic stimulation. Hypothesizing a possible role for occupational antigenic exposure, we observed in two independent cohorts of patients with IgG4-RD that the majority had had a career in blue-collar occupations with prolonged exposures to potentially hazardous occupational antigens. Our finding of clonal expansions of IgG4-switched B cells in patients with IgG4-RD is compatible with the presence of an antigen-driven immune process in these individuals. Our observed high rate of chronic occupational exposure suggests that chronic exposure to occupational antigens may play a role in the initiation and/or maintenance of IgG4-RD in susceptible individuals. We postulate that the chronic antigenic load associated with ‘dirty’ jobs could be one of the factors contributing to the development of IgG4-RD.

ABSTRACT 6

LC Lighaam, E Vermeulen, T Bleker, KJ Meijlink, R Aalberse, E Barnes, **EL Culver**, Marieke van Ham, T Rispens. Phenotypic differences between IgG4+ and IgG1+ B cells point to distinct regulation of the IgG4 response. *J Allerg Clin Immunol* 2014;133(1):267-270.

Background: IgG4 antibodies are a marker for immune tolerance upon chronic antigenic stimulation. Although the properties of soluble IgG4 have been studied in some detail, the characteristics of the B cells that produce these antibodies are unknown. *Aim:* To identify and characterise IgG4-switched memory B cells. *Methods:* We carried out FACS experiments using fluorescently labelled antibodies specific to IgG4 and IgG1 to investigate phenotypic differences in IgG4 and IgG1 B cells. *Results:* Here we show for the first time that IgG4+ B cells can be surface stained and sorted. We show phenotypical differences between IgG4+ B cells and IgG1+ B cells. Strikingly, IgG4+ B cells expressed more of the low affinity FcεR and less of the inhibitory FcγRIIb compared to IgG1 cells. Interestingly, expression of complement receptors is reduced on IgG4+ B cells, possibly making them less sensitive to regulation via complement. Furthermore, we show that IgG4+ B cells are not classical B-regulatory cells, since they are not CD24^{hi}CD38^{hi} and do not produce IL-10 or express IL10 receptor. *Conclusion:* Taken together, these findings may provide insight into the tolerogenic role of IgG4+ B cells in the immune response, as it is likely that IgG4+ cells are differentially regulated to IgG1+ cells.

ABSTRACT 7

P Hart, T Kamisawa, W Brugge, **EL Culver**, L Czakó, L Frulloni, V Go, T Gress, M Kim, S Kawa, K Lee, M Lerch, W Liao, M Löhr, K Okazaki, J Ryu, N Schleinitz, K Shimizu, T Shimosegawa, R Soetikno, G Webster, D Yadav, Y Zen, S Chari. Long-term outcomes of autoimmune pancreatitis: a multicentre, international analysis. *GUT* 2013;62:1771-1776.

Objective: Autoimmune pancreatitis (AIP) is a treatable form of chronic pancreatitis that has been increasingly recognised over the last decade. We set out to better understand the current burden of AIP at several academic institutions diagnosed using the International Consensus Diagnostic Criteria, and to describe long-term outcomes, including organs involved, treatments, relapse frequency and long-term sequelae. *Design:* 23 institutions from 10 different countries participated in this multinational analysis. A total of 1064 patients meeting the International Consensus Diagnostic Criteria for type 1 (n=978) or type 2 (n=86) AIP were included. Data regarding treatments, relapses and sequelae were obtained. *Results:* The majority of patients with type 1 (99%) and type 2 (92%) AIP who were treated with steroids went into clinical remission. Most patients with jaundice required biliary stent placement (71% of type 1 and 77% of type 2 AIP). Relapses were more common in patients with type 1 (31%) versus type 2 AIP (9%, $p<0.001$), especially those with IgG4-related sclerosing cholangitis (56% vs 26%, $p<0.001$). Relapses typically occurred in the pancreas or biliary tree. Retreatment with steroids remained effective at inducing remission with or without alternative treatment, such as azathioprine. Pancreatic duct stones and cancer were uncommon sequelae in type 1 AIP and did not occur in type 2 AIP during the study period. *Conclusions:* AIP is a global disease, which uniformly displays a high

response to steroid treatment and tendency to relapse in the pancreas and biliary tree. Potential long-term sequelae include pancreatic duct stones and malignancy, however they were uncommon during the study period and require additional follow-up. Additional studies investigating prevention and treatment of disease relapses are needed.

ABSTRACT 8

AC Bateman, **EL Culver**, R Chetty. Intraduct papillary mucinous neoplasm of the pancreas: a tumour linked with IgG4-related disease. *J Clin Pathol* 2013;66(8):671-675.

Intraduct papillary mucinous neoplasm (IPMN) is a pancreatic tumour that is often associated with chronic pancreatitis (CP) in the surrounding pancreas. Type 1 autoimmune pancreatitis (AIP) is a fibro-inflammatory condition with characteristic histological features and prominent IgG4+ plasma cells. We identified two consecutive cases of pancreatic IPMN in which the adjacent pancreas showed morphological features suggestive of AIP. The diagnosis of type I AIP was reached in one case after immunohistochemistry and detailed assessment of the clinical and radiological features. A subsequent clinical review of 12 additional cases of IPMN in which the tumour or the surrounding pancreas showed significant lymphoplasmacytic inflammation and/or fibrosis revealed no further cases showing co-existent AIP. We conclude that while pancreatic IPMN and AIP may co-exist, most CP associated with IPMN does not represent AIP.

ABSTRACT 9

EL Culver, E Barnes, AJ Ellis, B George, RW Chapman. A rare cause of ileocaecal mass and lymphadenopathy: IgG4-mesenteric fibrosclerosis. *GUT*, March 2012; 61;6:819-820.

A 56 year-old gentleman presented with a six-week history of fatigue, drenching night sweats and weight loss. Clinical examination revealed a palpable right iliac fossa mass. CT demonstrated a mass at the ileocaecal junction involving the ascending colon with local and mesenteric lymphadenopathy. Ileocolonoscopy revealed extrinsic compression and normal mucosal biopsies. Laparoscopic-assisted resection revealed a mesenteric mass extending posteriorly to the serosal surface of the caecum and ascending colon forming a mesenteric cake around the bowel. He underwent a right hemicolectomy removing the mass and lymph nodes. Histology was consistent with IgG4-related mesenteric fibrosclerosis. Retrospective serum IgG was raised at 18.5g/l and IgG4 was 2.06g/l. Treatment with oral prednisolone was commenced and tapered over 3 months. Serum IgG4 normalised and repeat imaging at 10 weeks showed resolution of lymphadenopathy, no recurrence or other organ involvement.

ABSTRACT 10

EL Culver, A Hunt, E Crewe, K Shah, P Martinez-Davos. IgG4-related chronic sclerosing sialadenitis. *J Laryng Otolology*. 2015;129:226-231.

Background: ENT surgeons may be the first specialists to encounter and diagnose patients with salivary gland disease. A new entity involving the salivary glands has recently been described of which ENT surgeons need to be aware: IgG4-related chronic sclerosing sialadenitis. *Method:* A literature search of Medline, Embase and Cochrane Library databases was performed, using the search terms ‘IgG4’, ‘hyperIgG4 syndrome’ and ‘IgG4 related chronic sclerosing sialadenitis’. *Results:* Knowledge concerning immunoglobulin G4 related chronic sclerosing sialadenitis is rapidly increasing. This new entity is part of a fibro-inflammatory corticosteroid-responsive systemic disease (immunoglobulin G4 related disease) and has been described in almost every organ. Biopsy of the submandibular gland can be diagnostic. However, the diagnosis can easily be overlooked if: clinical suspicion is not high, one is unaware of the classical morphology and/or immunoglobulin G4 staining is not performed. This paper presents a summary of the current understanding of the disease and its management. *Conclusion:* ENT surgeons should be aware of this new disease entity. Patients with systemic disease should be managed under a multidisciplinary team, with input from clinicians who have an interest in such diseases (such as gastroenterologists and rheumatologists), and input from histopathologists and radiologists.

ABSTRACT 11

EL Culver, AC Bateman. General principles of IgG4-related disease. *Diagn Histopathol* 2013;19:111-118.

The concept of IgG4-related disease is rapidly evolving. The disease has been described in virtually every organ system and can mimic a number of other important malignant and inflammatory conditions. Consensus on nomenclature was proposed at an international IgG4-related disease symposium in Boston in 2011. Diagnostic criteria have been established to include characteristic histopathology, clinical and radiological manifestations, serology and response to treatment. Consensus histopathology guidelines have been agreed by an international panel of experts. Treatment options continue to expand and include corticosteroids, immunosuppressive therapy and B-cell depletion strategies. The role of IgG4 in pathogenesis remains uncertain but elucidating this will be crucial in the further understanding of this disease. This review summarises the principles of IgG4-related disease from a clinical perspective.

ABSTRACT 12

K Orlitzka, E Barnes, **EL Culver**. Prevention of Infection caused by Immunosuppressive drugs in Gastroenterology. *Therap Adv Chronic Disease*, July 2013;4(4):1-8.

Immunosuppressive therapy is frequently used to treat gastrointestinal diseases such as inflammatory bowel disease, autoimmune hepatitis, IgG4-related disease (autoimmune pancreatitis and sclerosing cholangitis) and in the post-transplantation setting. These drugs interfere with the immune system. The main safety concern with their use is the risk of infections. Certain infections can be prevented or their impact minimised. Physicians must adopt preventative strategies and should have a high degree of suspicion to recognize infections early and treat appropriately. This article reviews the risk factors for infections, the mechanism of action of immunosuppressive therapy and proposes preventive strategies.

ABSTRACT 13

EL Culver & AC Bateman. IgG4-related disease: can non-classical histopathological features or examination of clinically involved tissues be helpful in diagnosis. *J Clin Pathol.* Oct 2012;65:963-969.

IgG4-related disease (IgG4-RD) is an increasingly recognised inflammatory and fibrosing condition that commonly shows multi-system involvement. The disease may mimic malignancy and other inflammatory or immune-mediated disorders, but usually has a good response to corticosteroid therapy, underlining the requirement for recognition of the condition. Accurate diagnosis requires careful interpretation of varying combinations of serum IgG4 levels, radiological features and characteristic histopathological appearances within an appropriate clinical setting. The presence of ‘classical’ histopathological features together with an elevated tissue IgG4+ plasma cell count and IgG4 to IgG ratio is often diagnostic and at the very least can strongly support a clinicopathological diagnosis of IgG4-RD. We describe the most recent diagnostic criteria for IgG4-RD, especially the histopathological features. We then discuss the merits of examining tissues that may be more easily obtainable than those that commonly show the ‘classical’ histopathological features, but within which not all of these ‘diagnostic’ features may be present. We conclude that while a combination of ‘classical’ histopathological features and an elevated tissue IgG4+ plasma cell count is the gold standard for diagnosis, examination of tissues that show some but not all of these features can, in the appropriate context, provide useful supporting evidence for a clinicopathological diagnosis of IgG4-RD.

ABSTRACT 14

EL Culver & RW Chapman. Systematic Review: Management of primary sclerosing cholangitis and its variants: IgG4-associated cholangitis and overlap with autoimmune hepatitis. *Aliment Pharmacol Therap.* 2011 Jun;33(12):1273-91.

Background: Primary sclerosing cholangitis (PSC) remains a challenging disease to manage. The main goals are prevention of disease progression and reduction of the increased cancer risk. *Aims:* To review the management strategies for PSC and its variant forms based on published studies. *Methods:* Publications were identified using Pubmed, Medline and Ovid search engines. *Results:* Distinguishing PSC from variants such as IgG4-associated cholangitis and overlap with autoimmune hepatitis is essential to guide treatment decisions. There is no proven efficacious medical treatment for PSC. Ursodeoxycholic acid has been disappointing in low and moderate doses, and potentially dangerous in higher doses, although its role and optimal dose in chemoprevention requires investigation. The novel bile acid 24-norursodeoxycholic acid has shown promise in mouse models; human trials are in progress. Dominant strictures are optimally managed by dilatation and stenting to relieve obstructive complications, although exclusion of biliary malignancy is essential. Liver transplantation is the only proven therapy for those with advanced disease. Cholangiocarcinoma remains the most unpredictable and feared complication. In highly selected groups, neo-adjuvant chemoradiation with liver transplantation seems promising but requires further validation. Screening for inflammatory bowel disease and surveillance for colorectal carcinoma should not be overlooked. *Conclusion:* The effective management of PSC and its variants is hindered by uncertainties regarding pathogenesis of disease and factors responsible for its progression. Genome studies may

help to identify further targets for drug therapy and factors leading to malignant transformation.

ABSTRACT 15

S Almamari, **EL Culver**, RW Chapman. Chapter 5: IgG4 associated cholangitis. E Carey & K Lindor, Editors: Carey & Lindor's: Cholestatic Liver Disease, 2nd Edition. SpringerLink. 2014:87-110.

IgG4 associated cholangitis (IAC) is a distinct form of chronic cholangitis that is responsive to corticosteroids and has characteristic histological manifestations including a dense infiltration of the affected bile duct walls with IgG4 positive plasma cells. It is the biliary manifestation of the novel multisystem IgG4 related disease (IgG4-RD). Although isolated cases of IAC are reported, it occurs most frequently in association with autoimmune pancreatitis (AIP). The clinical significance of IAC originates from the similar clinical, biochemical, radiological and even histological features that it shares with primary sclerosing cholangitis (PSC) and cholangiocarcinoma (CCA). In contrast to these disorders, IAC patients respond dramatically to corticosteroid therapy when treated in its inflammatory phase. Distinguishing IAC from these disease mimics is important in order to institute appropriate therapy early enough to prevent fibrotic complications and unnecessary major surgical interventions. Nevertheless, this distinction is not straightforward and can be challenging. This is related to the lack of pathognomonic features of IAC even on histology. This chapter will focus on the current available knowledge on pathogenesis, prevalence, clinical features, radiology and histology findings, diagnosis, treatment and natural history of IAC.

ABSTRACT 16

EL Culver & AC Bateman. Chapter 8 IgG4-related disease. C Probert, Editor. Recent Advances in Gastroenterology. JP Medical Ltd. Feb 2014:93-112.

IgG4-related disease (IgG4-RD) is a recently recognised systemic fibroinflammatory condition characterised by characteristic histopathological findings and an abundant IgG4-positive plasma cell infiltrate in involved organs. The disease often manifests with tumour-like lesions that can mimic malignancy and other inflammatory diseases. IgG4-RD is believed to be immune-mediated, suggested by serological abnormalities (presence of autoantibodies and elevated levels of IgG4) and a dramatic response to corticosteroid therapy. However, the pathogenetic mechanisms and the precise nature of the underlying immune response have not been well clarified. This chapter covers the importance of IgG4 biology and IgG4 autoantibodies, and highlights the putative role of the humoral and cellular immune response in disease pathogenesis. The epidemiology and demographics, clinical features including organ involvement, radiological manifestations and histological features are described. The role of recently adapted diagnostic criteria and current treatment strategies and response to these are discussed.

ABSTRACT 17

FS van de Bovenkamp, **EL Culver**, LC Lighaam, E Vermeulen, E Barnes, RC Aalberse, T Rispens. The role of IgG4 Fab glycosylation in IgG4-related disease. Arch Allerg Symposium Proceedings. May 2015.

Introduction: Preliminary data show that IgG4 Fab glycosylation is increased compared to that of the other subclasses. Additionally, there are indications that there are IgG4 antibodies with high mannose structures. We postulate that B cells with BCRs containing high mannose structures at their Fab are a target for an endogenous lectin and that IgG4⁺ B cells with high mannose structures at the Fab of their BCRs are preferentially expanding and differentiating in IgG4-RD, possibly caused by over-expression of an endogenous mannose-binding lectin. *Methods:* We analysed sera of 14 healthy controls (HCs), 6 primary sclerosing cholangitis (PSC) patients and 9 IgG4-RD (IRD) patients. Binding of IgG4 to BanLec in the presence or absence of mannoside, was determined by a radioimmunoassay. *Results:* Binding of IgG4 to BanLec is partially mannoside-resistant, indicating that the interaction between IgG4 and BanLec is partially through binding of this lectin to the high mannose structures on IgG4, but that other factors are also involved in this interaction. The mannoside-susceptible fraction (i.e. IgG4 with high mannose structures) was not significantly higher in IRD (median = 67%) compared to HCs (median = 47%) and PSC patients (median = 37%). A large fraction of IgG4 antibody binding to BanLec is mannoside-resistant, even in IRD patients. It is possible that these are IgG4 antibodies produced by B cells of which the BCR is stimulated by the antigen (BanLec) instead of by the interaction between this lectin and high mannose structures on the BCR. *Conclusion:* In this preliminary study the mannoside-susceptible fraction is not significantly higher in IRD patients compared

to HCs and PSC patients. However, our results do not rule out that Fab glycosylation of IgG4 on the surface of B cells makes these cells more susceptible to stimulation by endogenous lectins.

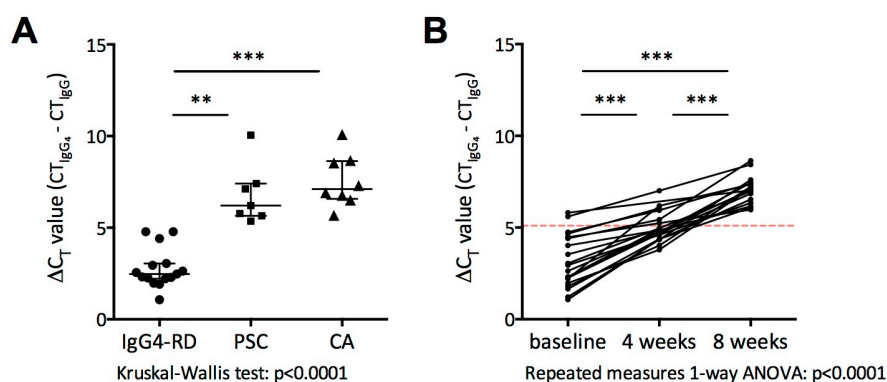
ABSTRACT 18

L.M. Hubers*, M.E. Doorenspleet*, **EL Culver**, L.J. Maillette de Buy Wenniger, P.L. Klarenbeek, R. Chapman, S.F. van de Graaf, J. Verheij, T. van Gulik, F. Baas, E. Barnes, N. de Vries, U. Beuers. The IgG/IgG4 mRNA Ratio by quantitative PCR accurately diagnoses IgG4-RD and predicts treatment response. *Hepatology* (abstract) 2015.

Introduction: IgG4-associated cholangitis (IAC) and autoimmune pancreatitis (AIP) are major manifestations of IgG4-related disease (IgG4-RD). Misdiagnosis and inadequate treatment are common since IAC and AIP mimic other inflammatory and malignant pancreatobiliary diseases, and accurate diagnostic biomarkers are lacking. Moreover, since relapse after tapering of immunosuppressive therapy occurs in 50% of patients, there is a need for biomarkers monitoring disease activity. Recently, using Next-Generation Sequencing, we observed that dominant IgG4+ B-cell receptor clones in peripheral blood distinguish patients with active IAC/AIP from primary sclerosing cholangitis (PSC) and pancreatobiliary malignancies (CA) (*Hepatology* 2013;57:2340). Here, we report on a simple quantitative PCR (qPCR) protocol for diagnosing IAC/AIP and monitoring disease activity. *Methods:* 15 patients with IAC and/or AIP according to HISORT criteria, 7 patients with PSC and 8 with CA formed the test cohort. Intra- and extramural replication cohorts consisted of 16 IAC/AIP, 5 PSC and 13 CA patients (Dutch cohort), and 8 IAC/AIP and 8 PSC patients (British cohort). In 20 Dutch

IAC/AIP patients, follow-up samples after 4 and 8 weeks of corticosteroid therapy were available. RNA was isolated and the constant region of the B-cell receptor was amplified using a generic forward IgG primer together with either a generic IgG or a IgG4-specific reverse primer. The ratio total IgG/IgG4 mRNA was calculated and expressed as ΔC_T . *Results:* ΔC_T as measure of IgG/IgG4 mRNA expression in peripheral blood of the test cohort was 2.8 ± 1.1 (mean $\pm$ SD) in IAC/AIP patients, compared to 6.8 ± 1.6 in PSC and 7.6 ± 1.4 in CA (**Figure 1A**, $p < 0.0001$). ROC analysis revealed a ΔC_T cut-off value of 5.1 in the test cohort distinguishing all cases from controls. In the replication cohort sensitivity was 95% and specificity 100% ($p = 8.6 \times 10^{-21}$). ΔC_T increased after 4 weeks (5.1 ± 0.8) and 8 weeks (7.1 ± 0.7) of corticosteroid treatment, compared to pre-treatment (mean 3.1 ± 1.4 , **Figure 1B**, $p < 0.0001$). *Conclusion:* IgG4-related disease of the biliary tree and pancreas can be accurately distinguished from PSC or pancreatobiliary malignancies by ΔC_T based on an affordable qPCR test. ΔC_T can also be used as a marker for treatment response and disease activity in IgG4-associated cholangitis and autoimmune pancreatitis.

Figure 1: The ratio of total IgG/IgG4 mRNA in IgG4-RD compared to PSC patients



ABSTRACT 19

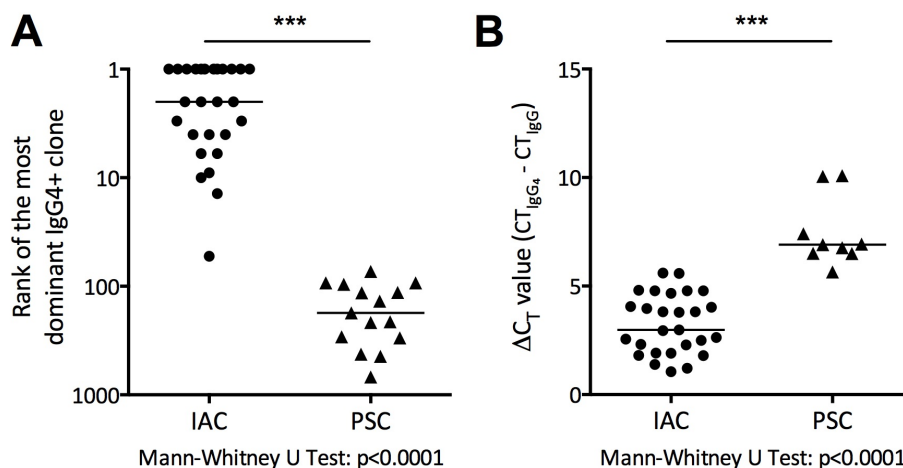
LM Hubers*, ME Doorenspleet*, **EL Culver**, LJ Maillette de Buy Wenniger, PL Klarenbeek, SF van de Graaf, J Verheij, T van Gulik, F Baas, E Barnes, N de Vries, U Beuers. IgG4+ B cell receptor clones in peripheral blood distinguishes IgG4-associated cholangitis/autoimmune pancreatitis from primary sclerosing cholangitis.

J Hepatology (abstract) May 2015.

Introduction: Immunoglobulin G4-related disease (IgG4-RD) predominantly affects the biliary tract (IgG4-associated cholangitis, IAC) and pancreas (autoimmune pancreatitis, AIP). Its clinical presentation often mimics primary sclerosing cholangitis (PSC) and/or pancreatobiliary malignancies, and an accurate diagnostic marker is lacking. Recently, we identified dominant IgG4+ B-cell receptor (BCR) clones in blood of 6 patients with active IAC, but not in controls, using next-generation sequencing (NGS; Hepatology 2013;57:2340). Here, we aimed to validate our findings in larger patient cohorts and to test a novel qPCR protocol in comparison to NGS. *Methods:* 27 patients with IAC/AIP fulfilling HISORT criteria were included in one Dutch (n=17) and one English (n=10) academic hospital. Also, 15 PSC controls were included. The total BCR heavy-chain repertoire was analysed using primers for all V(ariable)-genes and total RNA from peripheral blood. All amplified products encoded CDR3, the region defining a unique clone. The number of identical sequences reflects the size of the BCR clone. In addition, qPCR was performed on 27 IAC/AIP patients and 9 disease controls using forward primers for all IgG subtypes and IgG4 specifically; one generic reverse primer was used within the constant region of the receptor. Differences between the specific IgG4 and total IgG message in peripheral blood were calculated and expressed as differences in

CT value. *Results:* IgG4+ BCR clones were detected in all individuals included. 25/27 of all IAC/AIP patients had IgG4+ BCR clones that were among the 10 most dominant IgG+ BCR clones in blood (median 2nd, range 1-53; **Figure 1A**), whereas the most dominant IgG4+ BCR clone in PSC patients ranked 176th (median, range 73-686, $p < 0.0001$). All IgG4+ clones together covered 16.6% of all IgG+ clones, and 19.9% of the IgG+ repertoire (compared to 1.6% and 1.0% resp. in PSC controls). This difference was confirmed using qPCR: in IAC/AIP patients the median ΔCT value was 3.0 (1.0-5.6), compared to 6.9 in PSC patients (range 5.6-10.1, $p < 0.0001$; **Figure 1B**). *Conclusion:* Identification of highly dominant IgG4+ BCR clones by NGS in patients with active IAC/AIP clearly distinguishes IAC/AIP from PSC. Our novel qPCR protocol, in comparison to NGS, could be a simple and inexpensive alternative diagnostic tool. Expansion of patient cohorts (IAC/AIP, PSC, pancreatobiliary malignancies) for test validation is underway.

Figure 1: (A) Dominant IgG4+ B cell clones in IgG4-RD and PSC patients. (B) The ratio of total IgG/IgG4 mRNA using qPCR in IgG4-RD compared to PSC patients.



ABSTRACT 20

EL Culver, M Makuch, E Vermeulen, T Cargill, R Sadler, R Aalberse, T Rispens.
Increased IgG4 responses to multiple food and animal antigens in IgG4-related disease.
J Hepatology (abstract), May 2015.

Background: Autoimmune pancreatitis and IgG4-related sclerosing cholangitis are the pancreatic and biliary manifestations of a systemic fibro-inflammatory condition (IgG4-related disease; IgG4-RD), characterized by an elevated serum IgG4 concentration and abundant IgG4-positive plasma cells in the involved organs. An important question is whether the elevated IgG4 response is causal, or a mere reflection of immune-regulatory mechanisms of the disease. *Aims:* We sought to investigate if the IgG4 response represents a generalised polyclonal amplification by examining the response to common environmental antigens. *Methods:* Serum from 24 patients with IgG4-RD (14 treatment-naïve, 10 treatment-experienced), 9 patients with primary sclerosing cholangitis and an elevated serum IgG4 (PSC-high IgG4), and 18 healthy controls were tested against egg white and yolk, milk, banana, cat dander and serum, peanut, rice and wheat antigens by radioimmunoassay. Serum levels of IgG subclasses and IgE were measured and serum electrophoresis and immunofixation performed. *Results:* The concentration of serum total IgG, IgG4, IgE, and to a lesser extent IgG1, was higher in IgG4-RD patients versus healthy controls, as well as in the PSC-high IgG4 patients versus healthy controls. We demonstrated an elevated polyclonal IgG4 response to multiple antigens in patients with IgG4-RD (egg ($p=0.004$); milk ($p=0.04$); peanut ($p=0.0003$); cat dander and serum ($p=0.012$); rice and wheat (0.006)) and in PSC-high IgG4 (egg ($p=0.03$); cat dander and serum ($p=0.04$); rice and wheat (0.01)), compared to healthy controls. There was a strong positive correlation between serum IgG4 and multiple antigen-specific responses.

Responses to antigens (banana ($p=0.001$), egg ($p=0.039$), peanut ($p=0.003$), and cat ($p=0.006$)) were higher in treatment-naïve compared to treatment-experienced IgG4-RD patients, as were serum total IgG ($p=0.017$) and IgG4 ($p=0.001$). Serum electrophoresis and immunofixation demonstrated polyclonality in all patients. *Conclusions:* This is the first study to show enhanced levels of polyclonal IgG4 to multiple antigens in patients with IgG4-RD. This supports that elevated IgG4 levels reflect an aberrant immunological regulation of the overall IgG4 response, but does not exclude that causality of disease could be antigen-driven.

ABSTRACT 21

EL. Culver, E. Marchi, C. Manganis, W. Smit, M. Makuch, N. Ramamurthy, T. Rispen, P. Klenerman, E. Barnes Gene expression analysis identifies immune signalling and complement pathways in IgG4-related disease. *J Hepatology* (abstract). April 2014;60(1):S185-186.

Introduction: IgG4-related disease is a corticosteroid-responsive systemic fibro-inflammatory condition of unknown aetiology. Diagnosis relies on careful interpretation of a combination of histopathological appearances, radiological features and serological abnormalities, in an appropriate clinical scenario. To identify the potential mediators, effector molecules and biological pathways implicated in disease pathogenesis we used a systems biology approach. *Methods:* Whole blood genome oligo-microarray was performed in 8 treatment-naïve patients with active IgG4-RD (IgG4 related cholangitis and autoimmune pancreatitis; of whom 4 had additional systemic manifestations) and 8 healthy age and sex-matched controls. Gene expression profiles were interpreted using Ingenuity Pathway Analysis (IPA) software and the Database for Annotation,

Visualisation and Integrated Discovery (DAVID) web-based program. Gene Set Enrichment Analysis was used to assess co-expression of gene sets with shared biological function or regulation. Gene Set Enrichment plots and Heat Maps were created. Gene expression was confirmed at the RNA level by Taqman RT-PCR and at the protein level by ELISA, cytokine array, and flow cytometry. A deconvolution algorithm was applied to infer the contribution of each cell type to the general expression profile, using an R package for cell-type specific differential expression of microarray experiments in heterogeneous samples. *Results:* 213 putative candidate genes were identified: 161 up regulated (fold>2, p<0.01) and 52 down-regulated (fold<-2, p<0.01). Pathway analysis revealed 16 immunologically functional genes and 2 pathways implicated in disease pathogenesis. Genes encoding cytokines (IL5 and IL21), the chemokine ligands (CCL23 and CCL25), and the classical complement and TGF beta pathways were shown to be active in the disease cohort compared to healthy controls, which was confirmed by RT-PCR. Gene set enrichment analysis confirmed enriched gene sets with shared biological function in IgG4-RD patients compared with controls. Genetic markers were identified which were specifically enriched in the group with systemic manifestations, compared to single organ disease. Deconvolution indicated the monocytic lineage was important in differential gene expression. *Conclusion:* Gene expression analysis in IgG4-RD implicates antibody inducing cytokines, hepatic/biliary chemokines and the complement pathway as putative immune drivers of disease, providing novel targets for disease stratification and therapeutics.

ABSTRACT 22

L Lighaam, S Kruithof, M Makuch, E Vermeulen, **EL Culver**, E Barnes, J Neefjes, SM van Ham, T Rispen. Difference in co-localisation of IgG4 cells and kinetics of IgG4 plasma cell biology. Poster at Biology of B cell responses, Keystone Symposia, Colorado, Feb 2014.

Background: Although the properties of soluble IgG4 have been studied in some detail, the characteristics of the B cells that produce these antibodies are unknown. *Aim:* This study aims to identify, isolate, and characterize IgG4⁺ B cells in peripheral blood and determine their abundance in tissue. *Methods:* We developed a protocol to sort IgG4⁺ B cells using an antibody that recognizes the membrane-bound form of IgG4. We carried out cell sorting, RNAseq, and flow cytometry experiments to investigate phenotypic characteristics of IgG4⁺ B cells. *Results:* Here we show for the first time that IgG4⁺ B cells can be specifically identified in blood isolates and sorted to high purity and that there are phenotypic differences between IgG4⁺ B cells and IgG1⁺ B cells. The number of IgG4⁺ memory B cells correlated with the serum IgG4 levels in both healthy controls and patients with IgG4-related disease. Expression of CD21 is reduced on IgG4⁺ B cells, possibly rendering them less sensitive to regulation via complement. Furthermore, we show that IgG4⁺ B cells are not classical Bregs, since they are not CD24^{hi}CD38^{hi} and more importantly, do not produce IL-10. Strikingly, IgG4⁺ B cells express more CD23 (the low affinity FcεR) compared to IgG1⁺ B cells, potentially increasing their sensitivity to IgE. Using RNAseq, we show that expression of chemokine receptors CCR1 and CCR5 is increased on IgG4⁺ B cells. Analysis of lymph node and spleen samples showed that, while IgG1⁺ B cells are enriched in these tissues compared to peripheral blood, IgG4⁺ memory cells are not. *Conclusion:* These findings show that IgG4⁺ B cells have a distinctive expression pattern of receptors that are involved in B cell regulation by immunoglobulins and complement. Furthermore, these data indicate

that IgG4+ cells have an atypical localization. Understanding where IgG4+ cells reside will help to elucidate the local events that control differentiation of IgG4+ B cells into IgG4 plasma cells, and may contribute to clarifying the mechanisms by which IgG4 related disease emerges.

ABSTRACT 23

EL Culver*, K Orlicza* A Bateman, R Sadler, E Barnes, RW Chapman, SPL Travis. IgG4-positive plasma cells in the colon of patients with ulcerative colitis are markers of endoscopic and histological disease activity. J Crohns Colitis (abstract) 2013.

Background: Elevated serum IgG4 and abundant tissue IgG4+ plasma cells are features of IgG4-RD. The relevance of prominent colonic mucosal tissue IgG4 in IgG4-RD and (non-IgG4-RD-related) ulcerative colitis (UC) is uncertain. *Aims:* This study aimed to (1) establish the relationship between serum and tissue IgG4 and disease severity in UC; and (2) determine whether colonic mucosal tissue IgG4 supports a diagnosis of IgG4-RD. *Methods:* Patients with IgG4-RD and UC were prospectively recruited. Patients with extensive UC were identified from a prospective biobank. Controls were patients with diverticular disease undergoing colonoscopy and age-matched healthy individuals. All patients included had serum and tissue stored before treatment with corticosteroids or immunosuppression. Endoscopy reports were scored for disease severity. Serum IgG4 was measured by nephelometry; elevated defined as >1.4g/l. Biopsies were reviewed for the morphological characteristics of IgG4-RD and colitis severity. Tissue IgG4 was assessed using immunohistochemistry (cells/mean of 3 high power fields - HPF) and the IgG4+/IgG+ ratio (%) calculated. *Results:* In 54 UC patients, serum IgG4 was elevated in 6% (3/54) (median 0.43g/L, 0-3.78g/L) and associated with male gender

(13% v 0%, $p=0.046$) and presence of primary sclerosing cholangitis (33% v 4%, $p=0.031$). Tissue IgG4 was $>10/\text{HPF}$ in 22% (12/54) (median 2/HPF, 0-65/HPF) and was associated with endoscopic ($p=0.012$) and histological ($p=0.001$) disease severity. Tissue IgG4 was $>20/\text{HPF}$ in 9% (5/54) and $>50/\text{HPF}$ in 2% (1/54); associated with histological severity ($p<0.0001$). Tissue IgG4 $>10/\text{HPF}$ and $>20/\text{HPF}$ was associated with the requirement for corticosteroids (50% versus 16%, $p=0.022$) and other immunosuppressants (38% v 11%, $p=0.03$). In all 12 cases with tissue IgG4 $>10/\text{HPF}$, the IgG4+/IgG+ ratio was $<40\%$ (median 15%, 10%-35%). In 51 controls, serum IgG4 was elevated in 2% (1/50) (median 0.34g/L, 0.04-2.4g/L) and tissue IgG4 $>10/\text{HPF}$ in none (0/20) (median 1/HPF, 0-5/HPF). Five of 62 IgG4-RD patients had colitis (8.1%); four with UC and 1 with Crohn's disease. Serum IgG4 was elevated in all 4 patients (median 6.98g/l, 2.14-67.4g/l). Tissue IgG4 was $>10/\text{HPF}$ in 100%, $>20/\text{HPF}$ in 75% and $>50/\text{HPF}$ in 50%. However, the morphological features of IgG4-RD were not present. *Conclusion:* In UC, elevated serum IgG4 and prominent tissue IgG4 were found more commonly in patients (with or without IgG4-RD) than controls and were associated with disease severity. In IgG4-RD with colitis, elevated serum IgG4 and prominent IgG4 was universally present, but without the morphological features on biopsy.

ABSTRACT 24

R Aalberse, **EL Culver**, L Lighaam, E Vermeulen, T Rispens. The fate of IL-4-switched B cells: dynamics of IgE and IgG4 in the hyper-IgG4 syndrome. *Inter Arch Allergy Immunol*; Sept 2013. *Collegium Internationale Allergologicum (CIA)*, Sept 2013.

The behaviour of the post-switch B cell is markedly isotype-dependent. IgE and IgG4 are the human isotypes that are most strongly dependent on TH2 conditions. We postulate that the local environment at the site of switching influences the post-switch fate of the B cell. Since IgE switched B cells are rare, we focused on IgG4-switched B cells. Their fate is still uncertain, largely because there is no close mouse homologue, but also because a reliable detection of membrane-anchored IgG4 was a technical challenge. With the availability of an antibody suitable for FACS procedures, ex-vivo and in-vitro analyses of IgG4-switched B cells are now feasible. As a significant clinical problem, but also as a model system for TH2-driven immune responses, we are investigating IgG4-related disease (IRD), also known as the hyper-IgG4 syndrome. This is characterized by marked expression of IgG4-positive polyclonal plasma cells in non-lymphoid organs (e.g. pancreas), with severe organ dysfunction due to the tumour-like mass of plasma cells, without evidence of antibody-related damage. Treatment with corticosteroids, methotrexate and/or rituximab rapidly normalizes not only the number of circulating IgG4 B cells, but also plasma IgG4 levels, indicating a high turnover of IgG4 plasma cells. These results suggest a deranged migration, expansion, differentiation and turnover of IgG4-switched B cells in the affected organ(s). However, no plausible antigenic stimulus has been identified to date. Based on the marked reactivity of IgG4 to a mannose-specific lectin from banana, our working hypothesis for

this polyclonal B cell activation is that Fab-glycosylation of the B cell receptor makes these B cells susceptible to a tissue-expressed lectin. This was investigated in IRD patients during therapy by analysing the dynamics of lectin-positive and lectin-negative IgG4 in plasma. The results indicate that lectin-positive and lectin-negative IgG4 change in parallel during treatment, which does not support the hypothesis that the aberrant IgG4 production is exclusively derived from B cells with lectin-positive IgG4. A highly significant correlation was found between total IgG4 and total IgE. This suggests a significant contribution of the indirect switching pathway of IgE production (from IgM via IgG4 to IgE) in this condition.

ABSTRACT 25

EL Culver, K Williamson, J Halliday, AC Bateman, R Sadler, E Barnes, RW Chapman. Elevated serum and tissue IgG4 in PSC are associated with a worse clinical outcome. Hepatology (abstract). Nov 2012.

Introduction: IgG4-related cholangitis (IC) is the biliary manifestation of IgG4-related disease; an increasingly recognised phenomenon with an elevated serum and tissue IgG4 incorporated in the current diagnostic criteria. Less is known about patients with primary sclerosing cholangitis (PSC) with elevated serum and tissue IgG4 who do not meet criteria for IC. *Aims:* This study aimed to describe the characteristics and outcome of patients with PSC with an elevated serum and/or tissue IgG4 compared to those with a normal level. *Methods:* Serum IgG and IgG4 were prospectively measured in 194 PSC patients at the John Radcliffe Hospital from 2008 to 2011. Liver and colonic tissue from these patients, where available, was stained immune-histochemically for monoclonal IgG and IgG4. Demographic, clinical, laboratory, imaging, endoscopic, treatment and

outcomes were recorded. The groups with an elevated serum IgG4 ($>1.4\text{g/l}$) and elevated tissue IgG4 ($>20/\text{HPF}$) were compared.

Results: Eight of the 194 patients originally diagnosed with PSC (4.1%) were reclassified as IC on the basis of serum IgG4, tissue IgG4, histology, imaging and presence of systemic disease. 186 patients (53.8% male) with a median age of 55.5yrs (range 16-92yrs) and follow-up of 7.5 years (range 0.5-30.5yrs) were included in the analysis. 118 (63.4%) of these had inflammatory bowel disease (IBD). 26 (14%) had an elevated serum IgG4 (median 2.45g/l). Of 76 patients for whom biopsy specimens were available, 8 (10.5%) had an elevated liver and/or colon tissue IgG4 level. Elevated serum IgG4 was significantly associated with elevated liver ($p=0.02$) and/or colon ($p=0.001$) tissue IgG4 counts. Patients with an elevated serum IgG4 were more likely than those with normal serum levels to; i) have abnormal extra-hepatic (11.5% v 1.4% $p=0.002$), and normal intra-hepatic ducts (84.6% v 58.1%; $p=0.002$) on cholangiogram ii) decompensate (34.6% v 8.1%; $p<0.0001$), and iii) reach the combined end-point of death or liver transplantation (11.5% v 2.5%; $p=0.025$). Patients with an elevated tissue IgG4 were more likely than those with normal tissue levels to reach the combined end-point of death or liver transplantation (25% v 2.9%; $p=0.008$). There were no significant differences between those with normal and elevated serum IgG4 levels in clinical presentation, liver function, immunological markers, the presence of autoimmune diseases or IBD (including type and extent), a family history of PSC, hepatobiliary, or colorectal cancer 7 of 26 (26.9%) patients with elevated serum IgG4 received a trial of steroid therapy. Five (71.4%) of these had a favourable clinical, biochemical and immunological response, of which 3 are now maintained on azathioprine. *Conclusion:* A significant proportion of patients with PSC have elevated serum and tissue IgG4 levels, both of which are associated with a worse clinical outcome. This supports previous studies investigating serum IgG4 in PSC and liver tissue IgG4 in explants, in a

non-transplant tertiary referral centre. Corticosteroid treatment in selected patients with elevated IgG4 may be beneficial and should be the subject of prospective studies.

ABSTRACT 26

EL Culver, A Rodriguez, A Bateman, J Collier, RW Chapman, P Klenerman, E Barnes. IgG4-plasma cell infiltrate in the liver and colon of children with PSC. J Hepatology (abstract). April 2012.

Background: Children diagnosed with primary sclerosing cholangitis (PSC) more frequently have features of overlap with steroid-responsive autoimmune hepatitis (AIH) than adults with PSC. Since IgG4-associated cholangitis (IAC) is difficult to distinguish from PSC, we sought to determine whether children diagnosed with PSC may in fact have steroid-responsive IgG4-related systemic disease (IgG4-RSD). *Methods:* Retrospective controlled study of 14 children with PSC in Oxford Radcliffe Hospital from 2001 to 2011. Controls included 5 children with AIH, 10 children with colitis, 12 adults with PSC and 10 adults with colitis and normal bile ducts. Serum IgG and IgG4 were measured by nephelometry. Tissue from the liver, colon, stomach and duodenum from diagnosis were immunostained with monoclonal IgG4 and CD138. A blinded pathologist calculated the mean number of IgG4-plasma cells in three high power fields (HPF). Clinical records were reviewed. Fishers Exact test was used. *Results:* Fourteen children, median age 13.5years (9-16yr) and 79% male, had sclerosing cholangitis on liver histology. Six (43%) had large duct involvement and 1 (7%) had an abnormal pancreatogram. Four (29%) had autoimmune overlap and 11 of 13 (85%) had colitis. Liver IgG4-cells were >10/HPF in 6 of 14 (43%); 50% of these had >20/HPF in a peri-ductal distribution. IgG4 count was independent of fibrosis or

inflammation on biopsy. Colonic IgG4-cells were >10/HPF in 8 of 13 (62%); 50% of these had >20/HPF. This was significantly associated with an elevated IgG4-cell count in the liver ($p=0.016$; OR4.0; 95%CI1.2-13.3) but independent of the presence or severity of colitis or inflammatory markers. There was no difference in demographics, clinical presentation or treatment outcome in the groups with an elevated or normal serum/tissue IgG4 after mean follow-up of 38 months (4.6-183m).

One child had an elevated serum and tissue IgG4, autoimmune overlap, lymphadenopathy, biliary strictures and dilated pancreatic duct, and was steroid-responsive. This may represent a childhood variant of IgG4-RSD. *Conclusion:* An IgG4-cell infiltrate in the liver and colon is a common feature in children previously diagnosed with PSC. Long-term follow-up of this cohort is warranted to determine the natural evolution of this disease into adulthood and long-term prognosis.

ABSTRACT 27

EL Culver, R Sadler, B Ferry, E Barnes. Elevated serum IgG4 in multiple hepatobiliary pathologies. J Hepatology (abstract). April 2012.

Background: Elevated serum IgG4 levels have been reported to be highly specific for the diagnosis of autoimmune pancreatitis (AIP) and IgG4-associated cholangitis (IAC) as manifestations of IgG4-related systemic disease (IgG4-RSD). Other conditions have since been associated with an elevated IgG4. We sought to characterize the different pathologies associated with an elevated serum IgG4 and define factors that favour a diagnosis of IgG4-RSD. *Methods:* Systematic review of all serum IgG4 requests to the Oxford Radcliffe Hospital from Jan 2008 to Oct 2011. Total IgG, IgG1 and IgG4 subclasses were recorded. Reason for referral and final diagnosis for all patients with an elevated serum IgG4 (greater than 1.4g/l) were reviewed. The HISORt criteria were used to diagnose AIP and IAC and Japanese Research Committee criteria were used to diagnose IgG4-RSD. Unpaired t test and one-way ANOVA were used to compare means. *Results:* One thousand and twenty IgG4 subclass levels were requested on 969 patients over 3 years 10 months. Of these, 146 patients (15%) had an elevated serum IgG4 level (range 1.4-21.0g/l, median IgG4 2.4g/l, IgG 15.0g/l). The majority (84%) of requests were made with the aim of distinguishing between IgG4-RSD and pancreatobiliary carcinoma, primary sclerosing cholangitis (PSC), benign biliary strictures, unexplained pancreatitis or pancreatic insufficiency. The final diagnosis in patients with an elevated serum IgG4 was variable. Criteria for IgG4-RSD were met in 28.7%, pancreatic carcinoma or cholangiocarcinoma were histologically confirmed in 9.8%, PSC with no evidence of IAC in 14% and chronic pancreatitis in 11.1% of cases. Other causes included cryptogenic cirrhosis (7.7%), ulcerative colitis (1.4%),

autoimmune hepatitis (1.4%), Addison's (3.3%), rheumatoid arthritis (3.3%) and infection (1.4%). There was no definite cause in 7.1% of patients. The mean levels of serum IgG4 were significantly higher in IgG4-RSD (5.38g/l; $p=0.0152$; 95%CI 3.79-6.9) compared to all other causes (2.27g/l). The IgG/IgG4 ratio was lower in IgG4-RSD (4.26g/l; $p=0.0163$; 95%CI 3.31-5.22) compared to other causes (6.32g/l). *Conclusion:* Elevated serum IgG4 is not specific to IgG4-RSD. However, the mean serum IgG4 level is significantly higher and mean IgG/IgG4 ratio significantly lower in IgG4-RSD than other pathologies associated with an elevated serum IgG4 level.

ABSTRACT 28

EL Culver, R Sadler, D Simpson, F Highfield, S Yates, RW Chapman, E Barnes, B Ferry. Elevated serum IgG4 and IgE in IgG4-RD and other disease mimics. IgG4-RD Symposium, Hawaii.

Background: Elevated serum IgG4 levels have been associated with IgG4-RD for over a decade, yet the lack of specificity at the currently accepted upper limit of 1.4g/l undermines its diagnostic potential. IgG4 and IgE have been associated with a range of type I hypersensitivity conditions due to their similar requirements for class-switch in the Th2 immune response, but the involvement of IgE in this disease remains uncertain.

Aims: We sought to characterize the relationship of IgG4 and IgE in patients with IgG4-RD and other disease mimics. *Methods:* We performed a systematic review of all serum IgG subclass measurements in the Oxford Immunology Laboratory over 5 years. Total IgG, IgG1 and IgG4 and IgE were recorded. Medical records, histopathology and radiology were reviewed to confirm the final diagnosis for each patient and the temporal relationship of serum measurements to corticosteroids and immunosuppression. The

HISORT and Japanese Consensus Criteria were used to diagnose IgG4-RD prospectively, and the International Consensus Diagnostic Criteria were retrospectively applied. A two-tailed Mann-Whitney test and a one-way Kruskal Wallis test with post hoc Dunns test were used to compare individual and multiple groups, respectively.

Results: 1854 samples from 1347 unique patients had serum IgG subclass levels measured. Of these, 550 samples from 224 unique patients had an elevated serum IgG4 (>1.4 g/l). The majority of requests were made with the aim of distinguishing between IgG4-RD and disease mimics. The final diagnosis met IgG4-RD criteria in only 53 of 224 (23.7%) patients. In IgG4-RD the serum IgG4 was elevated in 84% of patients. Serum IgG4 (median 3.83g/l, range 1.41-54.1g/l) and IgE (median 425.5KU/L, range 1711-6.08KU/L) were significantly higher compared to other conditions (IgG4 median 1.98g/l, range 1.4-6.3g/l and IgE median 54.3KU/L, range 5.1 – 4457KUL). The significant difference in IgE was independent of IgG4 serum levels in the two groups (median IgE/IgG4 59.8 versus 17.5KU/g/L). Using a serum IgG4 level of 2.8g/l (2xULN) the specificity for IgG4-RD was 97.9%, sensitivity 54%, PPV 47.2% and NPV 98.4%. At 5.6g/l (4xULN) the specificity was 99.8%, sensitivity 31.7%, PPV 87% and NPV 97.7%, AUC 0.79. *Conclusions:* Elevated serum IgG4 with an upper limit of 1.4g/l is not specific for IgG4-RD, although an elevated serum IgG4 >5.6g/l increases specificity to over 99% in those where there is diagnostic uncertainty. Elevated serum IgE is similarly non-specific, but may help to support a diagnosis and suggests a Th2 mediated immune response.

Appendix 4 Helicobacter Pylori PBP sequence

The amino acid sequences derived from *H.pylori* Plasminogen Binding Peptide.

*peptide sequence of interest (AKEERRY) is highlighted in red - number 74 and 75.

Peptide Number	Sequence
1	MLRLLIGLLLSFIS
2	LIGLLLSFISLQSA
3	LLMSFISLQSASWQE
4	FISLQSASWQEPLRV
5	QSASWQEPLRVSIEF
6	WQEPLRVSIEFVDLP
7	LRVSIEFVDLPKKII
8	IEFVDLPKKIIRFPA
9	DLPKKIIRFPAHDLQ
10	KIIRFPAHDLQVGEF
11	FPAHDLQVGEFGFVV
12	DLQVGEFGFVVTKLS
13	GEFGFVVTKLSDYEI
14	FVVTKLSDYEIVNSE
15	KLSDYEIVNSEVVII
16	YEIVNSEVVIIAVEN
17	NSEVVIIAVENG VAT
18	VIIAVENG VATAKFR
19	VENG VATAKFRAFES
20	VATAKFRAFESMKQR
21	KFRAFESMKQRHLPT
22	FESMKQRHLPTPRMV
23	KQRHLPTPRMVARKG
24	LPTPRMVARKGDLVY
25	RMVARKGDLVYFRQF
26	RKGDLVYFRQFNNQA
27	LVYFRQFNNQAFLIA
28	RQFNNQAFLIAPNDE
29	NQAFLIAPNDELYEQ
30	LIAPNDELYEQIRAT
31	NDELYEQIRATNTDI

32	YEQIRATNTDINFIS
33	RATNTDINFISDLL
34	TDINFISDLLVTFL
35	FISDLLVTFLNGFD
36	DLLVTFLNGFDPKIA
37	TFLNGFDPKIANLRK
38	GFDPKIANLRKACNV
39	KIANLRKACNVYSVG
40	LRKACNVYSVGVIYI
41	CNVYSVGVIYIVTTN
42	SVGVIYIVTTNTLNI
43	IYIVTTNTLNLSCE
44	TTNTLNLSCESEI
45	LNLSCESEILEKR
46	SECESEILEKRELD
47	FEILEKRELDTSVGT
48	EKRELDTSVGTST
49	LDSVGTSTPFFS
50	GVTKSTPFFSRVEG
51	TSTPFFSRVEGIDAG
52	FFSRVEGIDAGTLGK
53	VEGIDAGTLGKLFSG
54	DAGTLGKLFSGSQSK
55	LGKLFSGSQSKNYFA
56	FSGSQSKNYFAYYDA
57	QSKNYFAYYDALVKK
58	YFAYYDALVKKEKRK
59	YDALVKKEKRKEVRI
60	VKKEKRKEVRIKKRE
61	KRKEVRIKKREEKID
62	VRIKKREEKIDSREI
63	KREEKIDSREIKREI
64	KIDSREIKREIKQEA
65	REIKREIKQEAKEP
66	REIKQEAKEPKKAN
67	QEAKEPKKANQGTQ
68	KEPKKANQGTQNAPT
69	KANQGTQNAPTLEEK
70	GTQNAPTLEEKNYQK
71	APTLEEKNYQKAERK
72	EEKNYQKAERKLDK
73	YQKAERKLDKEERR

74	ERKLD AKEERRY LRD
75	DAKEERRY LRDERKK
76	ERRYLRDERKKAKAT
77	LRDERKKAKATKKAM
78	RKKAKATKKAMEFEE
79	KATKKAMEFEEREKE
80	KAMEFEEREKEHDER
81	FEEREKEHDERDEQE
82	EKEHDERDEQETEGR
83	DERDEQETEGRRKAL
84	EQETEGRRKALEMDK
85	EGRRKALEMDKGDKK
86	KALEMDKGDKKEERV
87	MDKGDKKEERVKPKKE
88	DKKEERVKPKENERE
89	ERVKPKENEREIKQE
90	PKENEREIKQEAIKE
91	EREIKQEAIKEPSDG
92	KQEAIKEPSDGNNAT
93	IKEPSDGNNATQQGE
94	SDGNNATQQGEKQNA
95	NATQQGEKQNAQKEN
96	QGEKQNAQKENNAQK
97	QNAQKENNAQKEENK
98	KENNAQKEENKPNKSK
99	AQKEENKPNKSKKEER
100	ENKPNKSKKEERRLKE
101	NSKEERRLKEEKKK
102	EKRRLKEEKKKAKAE
103	LKEEKKKAKAEQRRAR
104	KKKAKAEQRAREFEQ
105	KAEQRAREFEQRARE
106	RAREFEQRAREHQER
107	FEQRAREHQERDEKE
108	AREHQERDEKELEER
109	QERDEKELEERRKAL
110	EKELEERRKALEAGK
111	KELEERRKALEAGKK

Appendix 5 Candidate gene list

The original candidate list of differentially expressed genes in 8 IgG4-RD patients and 8 healthy donors.

Primary Sequence Name	Sequence Description	Log(Ratio)	Fold Change	P-value
T cell receptor delta variable 3	T cell receptor delta variable 3	-0.87039	-7.41978	0.0062
PYGO1	Pygopus homolog 1	-0.85353	-7.13728	0.00086
LOC730159	Uncharacterized protein LOC730159	-0.78217	-6.05576	0.00174
LINC00483	Long intergenic non-protein coding RNA 483	-0.78216	-6.05567	0.00348
PCDH8	Protocadherin 8 transcript variant 1	-0.69667	-4.97357	0.00044
GOLT1A	Golgi transport 1A	-0.6572	-4.54152	0.00256
CASP7	Caspase 7, apoptosis-related cysteine peptidase	-0.64103	-4.37548	0.00654
LRRN3	Leucine rich repeat neuronal 3 transcript variant 3	-0.58696	-3.86331	0.00027
TRPM8	Transient receptor potential cation channel, subfamily M, member 8	-0.56653	-3.68576	0.00879
COLEC11	Collectin sub-family member 11 transcript variant 2	-0.51205	-3.25124	0.00112
LOC256880	Uncharacterized LOC256880 non-coding RNA	-0.48351	-3.04445	0.00956
OR13C3	Olfactory receptor, family 13, subfamily C, member 3	-0.47471	-2.98339	0.00174
MGC40069	Hypothetical protein MGC40069	-0.46164	-2.89495	0.0027
CNN3	Calponin 3, acidic	-0.44073	-2.75884	0.0023
ZCCHC18	Zinc finger, CCHC domain containing 18 transcript variant 1	-0.42782	-2.67804	0.00632
XLOC_010390	BROAD lincRNAs version v2	-0.42461	-2.65832	0.00285
DNAJC5B	Homo sapiens DnaJ (Hsp40) homolog, subfamily C, member 5 beta	-0.42429	-2.65635	0.00475
TRPM3	Transient receptor potential cation channel, subfamily M, member 3 transcript variant 7	-0.4211	-2.63696	0.00619
SUMF1	sulfatase modifying factor 1 [Source:HGNC Symbol;Acc:20376] [ENST00000534863]	-0.41226	-2.5838	0.00413

ZNF816-ZNF321P	Homo sapiens ZNF816-ZNF321P readthrough (ZNF816-ZNF321P), mRNA [NM_001202473]	-0.40232	-2.52534	0.00972
SCAPER	S-phase cyclin A-associated protein in the ER [Source:HGNC Symbol;Acc:13081] [ENST00000303521]	-0.39147	-2.46301	0.00349
LOC284630	Homo sapiens cDNA FLJ39065 fis, clone NT2RP7014721. [AK096384]	-0.39129	-2.46201	0.00018
FOXJ1	Homo sapiens forkhead box J1 (FOXJ1), mRNA [NM_001454]	-0.38043	-2.40121	0.00669
KRTAP9-1	Homo sapiens keratin associated protein 9-1 (KRTAP9-1), mRNA [NM_001190460]	-0.37716	-2.3832	0.00859
ZNF248	Homo sapiens zinc finger protein 248 (ZNF248), mRNA [NM_021045]	-0.36842	-2.33573	0.00038
GOLGA8A	Homo sapiens golgin A8 family, member A (GOLGA8A), transcript variant 1, mRNA [NM_181077]	-0.35132	-2.24556	0.00003
SEC13	SEC13 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:10697] [ENST00000479868]	-0.34759	-2.22635	0.00807
KLRC3	Homo sapiens killer cell lectin-like receptor subfamily C, member 3 (KLRC3), transcript variant 2, mRNA [NM_007333]	-0.34499	-2.21306	0.00108
ZFP2	Homo sapiens zinc finger protein 2 homolog (mouse) (ZFP2), mRNA [NM_030613]	-0.33615	-2.16844	0.00042
SLC29A2	Homo sapiens solute carrier family 29 (nucleoside transporters), member 2 (SLC29A2), mRNA [NM_001532]	-0.3355	-2.16522	0.00021
KIF3A	kinesin family member 3A [Source:HGNC Symbol;Acc:6319] [ENST00000541316]	-0.33537	-2.16458	0.00294
ACVR1C	Homo sapiens activin A receptor, type IC (ACVR1C), transcript variant 1, mRNA [NM_145259]	-0.33294	-2.1525	0.00619
PLXDC1	Homo sapiens plexin domain containing 1 (PLXDC1), mRNA [NM_020405]	-0.32871	-2.13161	0.00195
CD248	Homo sapiens CD248 molecule, endosialin (CD248), mRNA [NM_020404]	-0.32768	-2.12656	0.00734
TMEM132C	Homo sapiens transmembrane protein 132C (TMEM132C),	-0.32653	-2.12093	0.00782

	mRNA [NM_001136103]			
PLEKHA6	Homo sapiens pleckstrin homology domain containing, family A member 6 (PLEKHA6), mRNA [NM_014935]	-0.32532	-2.11505	0.00857
PTCH1	patched 1 [Source:HGNC Symbol;Acc:9585] [ENST00000375290]	-0.32397	-2.10846	0.00531
LOC641518	Homo sapiens uncharacterized LOC641518 (LOC641518), transcript variant 1, non-coding RNA [NR_029373]	-0.32315	-2.1045	0.00089
CYP2J2	Homo sapiens cytochrome P450, family 2, subfamily J, polypeptide 2 (CYP2J2), mRNA [NM_000775]	-0.31322	-2.05693	0.00098
ZNF391	Homo sapiens zinc finger protein 391 (ZNF391), mRNA [NM_001076781]	-0.31265	-2.05423	0.00136
LOC284408	Homo sapiens cDNA FLJ37005 fis, clone BRACE2009122. [AK094324]	-0.30356	-2.01169	0.00938
CD8B	Homo sapiens CD8b molecule (CD8B), transcript variant 4, mRNA [NM_172102]	-0.30331	-2.01052	0.00583
C17orf67	Homo sapiens chromosome 17 open reading frame 67 (C17orf67), mRNA [NM_001085430]	-0.30276	-2.00796	0.00182
LRRC32	Homo sapiens leucine rich repeat containing 32 (LRRC32), transcript variant 1, mRNA [NM_005512]	0.30104	2.00002	0.00056
ADORA3	Homo sapiens adenosine A3 receptor (ADORA3), transcript variant 2, mRNA [NM_000677]	0.30287	2.00847	0.00004
ANKRD18A	Homo sapiens ankyrin repeat domain 18A (ANKRD18A), mRNA [NM_147195]	0.30595	2.02279	0.00621
F5	Homo sapiens coagulation factor V (proaccelerin, labile factor) (F5), mRNA [NM_000130]	0.30603	2.02314	0.00063
CR1	Homo sapiens complement component (3b/4b) receptor 1 (Knops blood group) (CR1), transcript variant S, mRNA [NM_000651]	0.31114	2.04711	0.00323
CDC25C	Homo sapiens cell division cycle 25 homolog C (S. pombe) (CDC25C), transcript variant 1, mRNA [NM_001790]	0.31354	2.05846	0.00499
SERPING1	Homo sapiens serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 (SERPING1), transcript	0.3155	2.06774	0.00422

	variant 1, mRNA [NM_000062]			
PTGDR2	Homo sapiens G protein-coupled receptor 44 (GPR44), mRNA [NM_004778]	0.31585	2.06942	0.00309
BATF2	Homo sapiens basic leucine zipper transcription factor, ATF-like 2 (BATF2), mRNA [NM_138456]	0.31685	2.07418	0.00007
INSRR	Homo sapiens insulin receptor-related receptor (INSRR), mRNA [NM_014215]	0.31706	2.07522	0.00379
LDHD	Homo sapiens lactate dehydrogenase D (LDHD), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA [NM_153486]	0.31911	2.08502	0.00002
GGT5	Homo sapiens gamma-glutamyltransferase 5 (GGT5), transcript variant 1, mRNA [NM_001099781]	0.32124	2.09529	0.00169
HIST1H2AL	Homo sapiens histone cluster 1, H2a1 (HIST1H2AL), mRNA [NM_003511]	0.32679	2.12219	0.00907
SDC1	Homo sapiens syndecan 1 (SDC1), transcript variant 1, mRNA [NM_001006946]	0.32888	2.13247	0.00021
CCL25	Homo sapiens chemokine (C-C motif) ligand 25 (CCL25), transcript variant 1, mRNA [NM_005624]	0.33354	2.15548	0.00338
NYNRIN	Homo sapiens NYN domain and retroviral integrase containing (NYNRIN), mRNA [NM_025081]	0.3379	2.1772	0.00517
MYOF	Homo sapiens myoferlin (MYOF), transcript variant 2, mRNA [NM_133337]	0.34001	2.1878	0.00036
CEND1	Homo sapiens cell cycle exit and neuronal differentiation 1 (CEND1), mRNA [NM_016564]	0.34573	2.21683	0.00803
TMEM170B	Homo sapiens transmembrane protein 170B (TMEM170B), mRNA [NM_001100829]	0.34982	2.23779	0.00003
CLSPN	Homo sapiens claspin (CLSPN), transcript variant 1, mRNA [NM_022111]	0.35072	2.24245	0.00889
C2	Complement component 2, transcript variant 3	0.3619	2.30093	0.00656
TMED8	Transmembrane emp24 protein transport domain containing 8	0.36801	2.33353	0.00264
LYPD1	LY6/PLAUR domain containing 1 transcript variant 1	0.36909	2.3393	0.00225
HSD3B7	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and	0.37078	2.34844	0.00002

	steroid delta-isomerase 7 transcript variant 2			
PNPLA1	Patatin-like phospholipase domain containing 1 transcript variant 3	0.38446	2.4236	0.00127
PPARG	Peroxisome proliferator- activated receptor gamma (PPARG), transcript variant 3	0.38573	2.4307	0.00614
GALNT9	UDP-N-acetyl-alpha-D- galactosamine:polypeptide N- acetylgalactosaminyltransferas e 9, transcript variant B	0.38771	2.44178	0.00625
LTBP1	Latent transforming growth factor beta binding protein 1 transcript variant 1	0.39784	2.49942	0.00211
KAZALD1	Kazal-type serine peptidase inhibitor domain 1	0.40038	2.51409	0.0086
PTK6	PTK6 protein tyrosine kinase 6	0.41113	2.57709	0.0041
FAM20A	Family with sequence similarity 20, member A, transcript variant 1	0.41325	2.58971	0.00042
NEK2	NIMA (never in mitosis gene a)-related kinase 2 transcript variant 1	0.42017	2.6313	0.00717
ANKRD33B	Ankyrin repeat domain 33B	0.42717	2.67407	0.00063
MYBPHL	Myosin binding protein H-like	0.43906	2.74827	0.00838
DIAPH3	Diaphanous homolog 3 (Drosophila) (DIAPH3), transcript variant 1	0.44253	2.7703	0.00367
TIMP4	TIMP metalloproteinase inhibitor 4	0.45073	2.82314	0.00662
NPSA	Prostate-specific antigen	0.45449	2.84768	0.00542
EVI5	Ecotropic viral integration site 5	0.46201	2.89742	0.00199
IgHCG1	immunoglobulin heavy constant gamma 1 (G1m marker)	0.46234	2.89961	0.00034
PRSS33	Protease, serine, 33	0.46636	2.92655	0.00558
ARHGEF26	Rho guanine nucleotide exchange factor (GEF) 26 transcript variant 1	0.47515	2.98638	0.00011
PPARG	Peroxisome proliferator- activated receptor gamma transcript variant 3	0.49987	3.16134	0.00114
OLIG2	Oligodendrocyte lineage transcription factor 2	0.50397	3.1913	0.00651
SHROOM3	Shroom family member 3	0.50433	3.19398	0.00274
IL21	Interleukin 21 transcript variant 1	0.50471	3.19675	0.00002
CPE	Carboxypeptidase E	0.50695	3.21333	0.00469
GNG4	Guanine nucleotide binding protein (G protein), gamma 4 transcript variant 1	0.51804	3.29643	0.00639
PRUNE2	Prune homolog 2 (Drosophila)	0.54233	3.48602	0.00419
IgHCG1	immunoglobulin heavy constant gamma 1 (G1m marker)	0.5443	3.50189	0.00028

CENPA	Centromere protein A transcript variant 1	0.55538	3.59233	0.00397
C1QC	Complement component 1, q subcomponent, C chain transcript variant 2	0.57454	3.75443	0.00261
EYA2	Eyes absent homolog 2 (Drosophila) transcript variant 1	0.57539	3.76177	0.00517
IgHCG3	immunoglobulin heavy constant gamma 3 (G3m marker)	0.57725	3.7779	0.00063
CKAP2	Homo sapiens cDNA FLJ38908 fis, clone NT2NE2005358. [AK096227]	0.5797	3.79931	0.00504
MAP1B	Microtubule-associated protein 1B	0.59563	3.94123	0.00268
HS3ST5	Heparan sulfate (glucosamine) 3-O-sulfotransferase 5	0.5975	3.95822	0.00392
OR13D1	Olfactory receptor, family 13, subfamily D, member 1	0.60148	3.99467	0.00647
NPPC	Natriuretic peptide C	0.62135	4.18166	0.00585
SLC26A7	Homo sapiens solute carrier family 26, member 7 transcript variant 2	0.62769	4.24321	0.00996
LDB2	LIM domain binding 2 transcript variant 1	0.63186	4.28412	0.00338
KCNN3	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 transcript variant 1	0.63574	4.32259	0.00426
TRIM9	Tripartite motif containing 9 transcript variant 2	0.63615	4.32662	0.00436
CLN8	Ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	0.63962	4.36134	0.00552
LOC152274	Homo sapiens cDNA FLJ31836 fis, clone NT2RP7000041. [AK056398]	0.64433	4.40888	0.00177
C6orf154	Homo sapiens chromosome 6 open reading frame 154 (C6orf154), mRNA [NM_001012974]	0.64635	4.42948	0.00929
UNC5B	Unc-5 homolog B (C. elegans) transcript variant 1	0.64651	4.43112	0.00575
PCDHB18	Protocadherin beta 18 pseudogene non-coding RNA [NR_001281]	0.64868	4.4533	0.00725
RAB17	RAB17, member RAS oncogene family transcript variant 1	0.65258	4.49342	0.00342
DMC1	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)	0.65891	4.55947	0.00248
ATP8B3	ATPase, aminophospholipid transporter, class I, type 8B, member 3, transcript variant 1	0.67356	4.71585	0.00134
PRTG	Protogenin	0.67564	4.73853	0.00459

LOC100131129	Homo sapiens cDNA FLJ45249 fis, clone BRHIP2006921. [AK127184]	0.6797	4.78295	0.00434
USP42	Ubiquitin specific peptidase 42	0.681	4.79739	0.00098
LOC400940	Homo sapiens uncharacterized LOC400940 (LOC400940), non-coding RNA [NR_026833]	0.68103	4.79771	0.00737
VCX2	Variable charge, X-linked 2	0.68351	4.82513	0.00002
NR2E1	Nuclear receptor subfamily 2, group E, member 1	0.68782	4.87327	0.00859
UCHL1	Ubiquitin carboxyl-terminal esterase L1	0.70143	5.02842	0.00138
PLCXD3	Phosphatidylinositol-specific phospholipase C, X domain containing 3	0.70666	5.08927	0.00584
HEATR4	HEAT repeat containing 4 transcript variant 2	0.70931	5.12044	0.00121
ACOXL	Acyl-CoA oxidase-like	0.71348	5.16988	0.00012
POF1B	Premature ovarian failure, 1B	0.72096	5.25969	0.00072
BPIFC	BPI fold containing family C	0.72846	5.35132	0.00464
MORN5	MORN repeat containing 5	0.73255	5.40195	0.00249
DKKL1	Dickkopf-like 1 (DKKL1), transcript variant 1	0.73338	5.41231	0.00658
FMOD	Fibromodulin	0.75547	5.69471	0.00289
HCAR1	Hydroxycarboxylic acid receptor 1	0.76793	5.86044	0.00783
TBX4	T-box 4 (TBX4)	0.77102	5.90231	0.00944
SIGLEC12	Sialic acid binding Ig-like lectin 12 (gene/pseudogene) transcript variant 1	0.79107	6.18114	1.02E-06
LOC100130597	Homo sapiens cDNA FLJ45767 fis, clone NETRP2000439. [AK127669]	0.80038	6.31507	0.00464
LOC100506127	Hypothetical LOC387790, mRNA (cDNA clone IMAGE:4811654). [BC040665]	0.80102	6.32439	0.00896
IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	0.81609	6.54769	0.00979
LOC100130256	cDNA FLJ45491 fis, clone BRTHA2006720. [AK127400]	0.81803	6.57705	0.0052
TNNT2	Troponin T type 2 (cardiac) transcript variant 1	0.83116	6.77895	0.0097
ARMC4	Armadillo repeat containing 4	0.8383	6.89126	0.00417
LOC100131232	cDNA FLJ33442 fis, clone BRACE2021936	0.84199	6.95001	0.00682
ZNF280D	Zinc finger protein 280D transcript variant 3	0.84293	6.9651	0.00102
C2orf83	Chromosome 2 open reading frame 83 (C2orf83), transcript variant 1	0.84969	7.07441	0.0029
NRN1	Neuritin 1	0.85023	7.08321	0.00381
WFDC10B	WAP four-disulfide core domain 10B transcript variant	0.85757	7.20394	0.00225

	1			
CCL23	Chemokine (C-C motif) ligand 23 transcript variant CKbeta8-1	0.86262	7.28818	0.00069
GRIK3	Glutamate receptor, ionotropic, kainate 3	0.87942	7.57566	0.00531
SLC24A2	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 2 transcript variant 1	0.88374	7.65146	0.00546
ZBPB	Zona pellucida binding protein	0.88388	7.65387	0.00104
GSG1L	GSG1-like transcript variant 1	0.89369	7.82866	2.28E-06
IGFL2	IGF-like family member 2 transcript variant 1	0.91519	8.22599	2.96E-06
FANK1	Fibronectin type III and ankyrin repeat domains 1	0.93799	8.66946	0.00482
ZBBX	Xinc finger, B-box domain containing transcript variant 2	0.94423	8.79478	0.00658
C7orf69	Chromosome 7 open reading frame 69	0.95879	9.09466	0.0029
TTLL10	Tubulin tyrosine ligase-like family, member 10 transcript variant 2	0.96741	9.27711	0.0057
ANGPTL2	Angiopoietin-like 2	0.99702	9.9317	0.0038
RAB3C	RAB3C, member RAS oncogene family	1.0022	10.05072	1.42E-10
LOC100144597	Uncharacterized LOC100144597 (LOC100144597), non-coding RNA [NR_033369]	1.01384	10.32371	0.00137
FAM43B	Family with sequence similarity 43, member B	1.05073	11.23916	0.0026
DEFB108B	Defensin, beta 108B	1.05119	11.25092	0.00091
XLOC_003457	BROAD lincRNAs version v2	1.06837	11.70488	2.05E-06
GCM2	Glial cells missing homolog 2	1.08267	12.09678	0.00012
KRT77	Keratin 77	1.212	16.29286	0.00028
LOC339529	Uncharacterized LOC339529 non-coding RNA	1.28562	19.30287	0.00295

Appendix 5 table: List of differential candidate genes, between 8 patients with IgG4-RD and 8 healthy matched controls, after normalization and adjustment for multiple comparisons. Gene abbreviations, full name, fold change for expression (> 2 fold or < 2 fold) and p values (< 0.01 were considered significant) are shown.

Appendix 6 Immunologically active gene list

A list of immunological genes in relevant biological categories.

Primary Sequence Name	Sequence Description	Log(Ratio)	Fold Change	P-value
T cell receptor delta variable 3	T cell receptor delta variable 3	-0.87039	-7.41978	0.0062
PYGO1	Pygopus homolog 1	-0.85353	-7.13728	0.00086
PCDH8	Protocadherin 8 transcript variant 1	-0.69667	-4.97357	0.00044
GOLT1A	Golgi transport 1A	-0.6572	-4.54152	0.00256
CASP7	Caspase 7, apoptosis-related cysteine peptidase	-0.64103	-4.37548	0.00654
LRRN3	Leucine rich repeat neuronal 3 transcript variant 3	-0.58696	-3.86331	0.00027
TRPM8	Transient receptor potential cation channel, subfamily M, member 8	-0.56653	-3.68576	0.00879
COLEC11	Collectin sub-family member 11 transcript variant 2	-0.51205	-3.25124	0.00112
OR13C3	Olfactory receptor, family 13, subfamily C, member 3	-0.47471	-2.98339	0.00174
CNN3	Calponin 3, acidic	-0.44073	-2.75884	0.0023
ZCCHC18	Zinc finger, CCHC domain containing 18 transcript variant 1	-0.42782	-2.67804	0.00632
DNAJC5B	DnaJ (Hsp40) homolog, subfamily C, member 5 beta	-0.42429	-2.65635	0.00475
TRPM3	Transient receptor potential cation channel, subfamily M, member 3 transcript variant 7	-0.4211	-2.63696	0.00619
SUMF1	Sulfatase modifying factor 1	-0.41226	-2.5838	0.00413
SCAPER	S-phase cyclin A-associated protein in the ER	-0.39147	-2.46301	0.00349
FOXJ1	Forkhead box J1	-0.38043	-2.40121	0.00669
KRTAP9-1	Keratin associated protein 9-1	-0.37716	-2.3832	0.00859
ZNF248	Zinc finger protein 248	-0.36842	-2.33573	0.00038
GOLGA8A	Golgin A8 family, member A transcript variant 1	-0.35132	-2.24556	0.00003
SEC13	SEC13 homolog (S. cerevisiae)	-0.34759	-2.22635	0.00807
KLRC3	Killer cell lectin-like receptor subfamily C, member 3 transcript variant 2	-0.34499	-2.21306	0.00108
ZFP2	Zinc finger protein 2 homolog (mouse)	-0.33615	-2.16844	0.00042
SLC29A2	Solute carrier family 29 (nucleoside transporters), member 2	-0.3355	-2.16522	0.00021
KIF3A	Kinesin family member 3A	-0.33537	-2.16458	0.00294
ACVR1C	Activin A receptor, type IC	-0.33294	-2.1525	0.00619

	transcript variant 1			
PLXDC1	Plexin domain containing 1	-0.32871	-2.13161	0.00195
CD248	CD248 molecule, endosialin	-0.32768	-2.12656	0.00734
TMEM132C	Transmembrane protein 132C	-0.32653	-2.12093	0.00782
PLEKHA6	Pleckstrin homology domain containing, family A member 6	-0.32532	-2.11505	0.00857
PTCH1	Patched 1	-0.32397	-2.10846	0.00531
CYP2J2	Cytochrome P450, family 2, subfamily J, polypeptide 2	-0.31322	-2.05693	0.00098
ZNF391	Zinc finger protein 391	-0.31265	-2.05423	0.00136
CD8B	CD8b molecule , transcript variant 4	-0.30331	-2.01052	0.00583
LRRC32	Leucine rich repeat containing 32 transcript variant 1	0.30104	2.00002	0.00056
ADORA3	Adenosine A3 receptor transcript variant 2	0.30287	2.00847	0.00004
ANKRD18A	Ankyrin repeat domain 18A	0.30595	2.02279	0.00621
F5	Coagulation factor V (proaccelerin, labile factor)	0.30603	2.02314	0.00063
CR1	Complement component (3b/4b) receptor 1 (Knops blood group) transcript variant S	0.31114	2.04711	0.00323
CDC25C	Cell division cycle 25 homolog C (S. pombe) transcript variant 1	0.31354	2.05846	0.00499
SERPING1	Serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 transcript variant 1	0.3155	2.06774	0.00422
PTGDR2	G protein-coupled receptor 44	0.31585	2.06942	0.00309
BATF2	Basic leucine zipper transcription factor, ATF-like 2	0.31685	2.07418	0.00007
INSRR	Insulin receptor-related receptor	0.31706	2.07522	0.00379
LDHD	Lactate dehydrogenase D (LDHD), nuclear gene encoding mitochondrial protein, transcript variant 1	0.31911	2.08502	0.00002
GGT5	Gamma-glutamyltransferase 5 transcript variant 1	0.32124	2.09529	0.00169
HIST1H2AL	Histone cluster 1, H2al	0.32679	2.12219	0.00907
SDC1	Syndecan 1 transcript variant 1	0.32888	2.13247	0.00021
CCL25	Chemokine (C-C motif) ligand 25 transcript variant 1	0.33354	2.15548	0.00338
NYNRIN	NYN domain and retroviral integrase containing	0.3379	2.1772	0.00517
MYOF	Myoferlin transcript variant 2	0.34001	2.1878	0.00036
CEND1	Cell cycle exit and neuronal differentiation 1	0.34573	2.21683	0.00803
TMEM170B	Transmembrane protein 170B	0.34982	2.23779	0.00003
CLSPN	Claspin transcript variant 1	0.35072	2.24245	0.00889
C2	Complement component 2, transcript variant 3	0.3619	2.30093	0.00656
TMED8	Transmembrane emp24 protein transport domain containing 8	0.36801	2.33353	0.00264

LYPD1	LY6/PLAUR domain containing 1 transcript variant 1	0.36909	2.3393	0.00225
HSD3B7	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7 transcript variant 2	0.37078	2.34844	0.00002
PNPLA1	Patatin-like phospholipase domain containing 1 transcript variant 3	0.38446	2.4236	0.00127
PPARG	Peroxisome proliferator-activated receptor gamma (PPARG), transcript variant 3	0.38573	2.4307	0.00614
GALNT9	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 9, transcript variant B	0.38771	2.44178	0.00625
LTBP1	Latent transforming growth factor beta binding protein 1 transcript variant 1	0.39784	2.49942	0.00211
KAZALD1	Kazal-type serine peptidase inhibitor domain 1	0.40038	2.51409	0.0086
PTK6	PTK6 protein tyrosine kinase 6	0.41113	2.57709	0.0041
FAM20A	Family with sequence similarity 20, member A, transcript variant 1	0.41325	2.58971	0.00042
NEK2	NIMA (never in mitosis gene a)-related kinase 2 transcript variant 1	0.42017	2.6313	0.00717
ANKRD33 B	Ankyrin repeat domain 33B	0.42717	2.67407	0.00063
MYBPHL	Myosin binding protein H-like	0.43906	2.74827	0.00838
DIAPH3	Diaphanous homolog 3 (Drosophila) (DIAPH3), transcript variant 1	0.44253	2.7703	0.00367
TIMP4	TIMP metalloproteinase inhibitor 4	0.45073	2.82314	0.00662
NPSA	Prostate-specific antigen	0.45449	2.84768	0.00542
EVI5	Ecotropic viral integration site 5	0.46201	2.89742	0.00199
IgHCG1	immunoglobulin heavy constant gamma 1 (G1m marker)	0.46234	2.89961	0.00034
PRSS33	Protease, serine, 33	0.46636	2.92655	0.00558
ARHGEF26	Rho guanine nucleotide exchange factor (GEF) 26 transcript variant 1	0.47515	2.98638	0.00011
PPARG	Peroxisome proliferator-activated receptor gamma transcript variant 3	0.49987	3.16134	0.00114
OLIG2	Oligodendrocyte lineage transcription factor 2	0.50397	3.1913	0.00651
SHROOM3	Shroom family member 3	0.50433	3.19398	0.00274
IL21	Interleukin 21 transcript variant 1	0.50471	3.19675	0.00002
CPE	Carboxypeptidase E	0.50695	3.21333	0.00469
GNG4	Guanine nucleotide binding protein (G protein), gamma 4 transcript variant 1	0.51804	3.29643	0.00639
PRUNE2	Prune homolog 2 (Drosophila)	0.54233	3.48602	0.00419
IgHCG1	immunoglobulin heavy constant gamma 1 (G1m marker)	0.5443	3.50189	0.00028
CENPA	Centromere protein A transcript variant 1	0.55538	3.59233	0.00397
C1QC	Complement component 1, q subcomponent, C chain transcript	0.57454	3.75443	0.00261

	variant 2			
EYA2	Eyes absent homolog 2 (Drosophila) transcript variant 1	0.57539	3.76177	0.00517
IgHCG3	immunoglobulin heavy constant gamma 3 (G3m marker)	0.57725	3.7779	0.00063
MAP1B	Microtubule-associated protein 1B	0.59563	3.94123	0.00268
HS3ST5	Heparan sulfate (glucosamine) 3-O-sulfotransferase 5	0.5975	3.95822	0.00392
OR13D1	Olfactory receptor, family 13, subfamily D, member 1	0.60148	3.99467	0.00647
NPPC	Natriuretic peptide C	0.62135	4.18166	0.00585
SLC26A7	Homo sapiens solute carrier family 26, member 7 transcript variant 2	0.62769	4.24321	0.00996
LDB2	LIM domain binding 2 transcript variant 1	0.63186	4.28412	0.00338
KCNN3	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3 transcript variant 1	0.63574	4.32259	0.00426
TRIM9	Tripartite motif containing 9 transcript variant 2	0.63615	4.32662	0.00436
CLN8	Ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	0.63962	4.36134	0.00552
UNC5B	Unc-5 homolog B (C. elegans) transcript variant 1	0.64651	4.43112	0.00575
RAB17	RAB17, member RAS oncogene family transcript variant 1	0.65258	4.49342	0.00342
DMC1	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)	0.65891	4.55947	0.00248
ATP8B3	ATPase, aminophospholipid transporter, class I, type 8B, member 3, transcript variant 1	0.67356	4.71585	0.00134
PRTG	Protogenin	0.67564	4.73853	0.00459
USP42	Ubiquitin specific peptidase 42	0.681	4.79739	0.00098
VCX2	Variable charge, X-linked 2	0.68351	4.82513	0.00002
NR2E1	Nuclear receptor subfamily 2, group E, member 1	0.68782	4.87327	0.00859
UCHL1	Ubiquitin carboxyl-terminal esterase L1	0.70143	5.02842	0.00138
PLCXD3	Phosphatidylinositol-specific phospholipase C, X domain containing 3	0.70666	5.08927	0.00584
HEATR4	HEAT repeat containing 4 transcript variant 2	0.70931	5.12044	0.00121
ACOXL	Acyl-CoA oxidase-like	0.71348	5.16988	0.00012
POF1B	Premature ovarian failure, 1B	0.72096	5.25969	0.00072
BPIFC	BPI fold containing family C	0.72846	5.35132	0.00464
MORN5	MORN repeat containing 5	0.73255	5.40195	0.00249
DKKL1	Dickkopf-like 1 (DKKL1), transcript variant 1	0.73338	5.41231	0.00658
FMOD	Fibromodulin	0.75547	5.69471	0.00289
HCAR1	Hydroxycarboxylic acid receptor 1	0.76793	5.86044	0.00783
TBX4	T-box 4 (TBX4)	0.77102	5.90231	0.00944

SIGLEC12	Sialic acid binding Ig-like lectin 12 (gene/pseudogene) transcript variant 1	0.79107	6.18114	1.02E-06
IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	0.81609	6.54769	0.00979
TNNT2	Troponin T type 2 (cardiac) transcript variant 1	0.83116	6.77895	0.0097
ARMC4	Armadillo repeat containing 4	0.8383	6.89126	0.00417
ZNF280D	Zinc finger protein 280D transcript variant 3	0.84293	6.9651	0.00102
NRN1	Neuritin 1	0.85023	7.08321	0.00381
WFDC10B	WAP four-disulfide core domain 10B transcript variant 1	0.85757	7.20394	0.00225
CCL23	Chemokine (C-C motif) ligand 23 transcript variant CKbeta8-1	0.86262	7.28818	0.00069
GRIK3	Glutamate receptor, ionotropic, kainate 3	0.87942	7.57566	0.00531
SLC24A2	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 2 transcript variant 1	0.88374	7.65146	0.00546
ZBPB	Zona pellucida binding protein	0.88388	7.65387	0.00104
GSG1L	GSG1-like transcript variant 1	0.89369	7.82866	2.28E-06
IGFL2	IGF-like family member 2 transcript variant 1	0.91519	8.22599	2.96E-06
FANK1	Fibronectin type III and ankyrin repeat domains 1	0.93799	8.66946	0.00482
ZBBX	Zinc finger, B-box domain containing transcript variant 2	0.94423	8.79478	0.00658
TTLL10	Tubulin tyrosine ligase-like family, member 10 transcript variant 2	0.96741	9.27711	0.0057
ANGPTL2	Angiopoietin-like 2	0.99702	9.9317	0.0038
RAB3C	RAB3C, member RAS oncogene family	1.0022	10.05072	1.42E-10
FAM43B	Family with sequence similarity 43, member B	1.05073	11.23916	0.0026
DEFB108B	Defensin, beta 108B	1.05119	11.25092	0.00091
XLOC_003457	BROAD lincRNAs version v2	1.06837	11.70488	2.05E-06
GCM2	Glial cells missing homolog 2	1.08267	12.09678	0.00012
KRT77	Keratin 77	1.212	16.29286	0.00028

Appendix 6 table: List of immunological genes in relevant biological categories.

Gene abbreviations, full name, fold change for expression and p values shown.

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