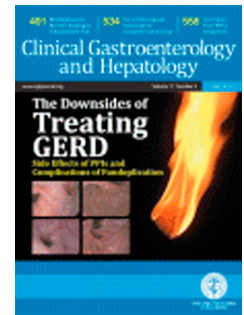


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Increases in IgE, Eosinophils, and Mast Cells can be Used in Diagnosis and to Predict Relapse of IgG4-related Disease

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TITLE PAGE

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Title: Increases in IgE, Eosinophils, and Mast Cells can be Used in Diagnosis and to Predict Relapse of IgG4-related Disease

Short Title: IgE, eosinophils, and mast cells in IgG4-RD.

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Abbreviations: AIP: autoimmune pancreatitis; AUC: area under the curve; DC: disease controls; FcεRI: Fc Epsilon Receptor 1. HC: healthy controls; HPF: high

power field; IgE: immunoglobulin E; IgG4: immunoglobulin G subclass 4; IgG4-RD: IgG4-related disease; IgG4-SC: IgG4-related sclerosing cholangitis; LR: likelihood ratio; NPV: negative predictive value; PPV: positive predictive value; ROC: Receiver Operator Curve.

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Author Contributions: EC, EB, TR and RA were involved in the study concept and design. EC obtained funding, collected, analysed and interpreted data, performed statistical analysis, and drafted the original manuscript. RS processed samples for serological measurement, and RS and MM were involved in analysis and interpretation of this data. ACB was responsible for histological interpretation and immunostaining of specimens. TC collected patient samples and validated clinical details. BF is the head of the clinical immunology department and responsible serological processing and interpretation. EB is the principal investigator on the IgG4-RD NIHR Portfolio study and EB and TR co-supervised the project. All authors critically reviewed and approved the final manuscript for intellectual content.

Keywords: inflammation, pancreas, immune response, detection

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Abstract:

Background & Aims: Immunoglobulin G4-related disease (IgG4-RD) is characterized by increased serum levels of IgG4 and infiltration of biliary, and pancreatic, and other tissues by IgG4-positive plasma cells. We assessed the prevalence of allergy and/or atopy, serum and tissue IgE antibodies, and blood and tissue eosinophils in patients with IgG4-RD. We investigated the association between serum IgE and diagnosis and relapse of this disease.

Methods: We performed a prospective study of 48 patients with IgG4-RD, 42 patients with an increased serum level of IgG4 with other inflammatory and autoimmune conditions (disease controls), and 51 healthy individuals (healthy controls) recruited from Oxford, United Kingdom from March 2010 through March 2014, and followed for a median of 41 months (range 3–73 months). Serum levels of Ig were measured at diagnosis, during steroid treatment, and at disease relapse for patients with IgG4-RD; levels at diagnosis were compared with baseline levels of controls. Allergen-specific IgEs were measured using the IgE ImmunoCAP. Levels and distribution of IgG4 and IgE antibodies in lymphoid, biliary, and pancreatic tissues from patients with IgG4-RD and disease controls were measured by immunohistochemistry. We analyzed data using the Spearman rank correlation and receiver operator characteristic curves.

Results: Serum levels of IgG4 increased to 1.4 g/l or more, and IgE increased to 125 kIU/L or more, in 81% and 54% of patients with IgG4-RD, respectively, compared with 6% and 16% of healthy controls ($P<.0001$). Peripheral blood eosinophilia was detected in 38% of patients with IgG4-RD vs 9% of healthy controls ($P=.004$). Of

patients with IgG4-RD, 63% had a history of allergy and 40% had a history of atopy with an IgE-specific response—these values were 60% and 53% in patients with increased serum levels of IgE ($P<.05$). Level of IgE at diagnosis above 480 kIU/L distinguished patients with IgG4-RD from disease controls with 86% specificity, 36% sensitivity, and a likelihood ratio of 3.2. Level of IgE at diagnosis above 380 kIU/L identified patients with disease relapse with 88% specificity, 64% sensitivity, and a likelihood ratio of 5.4. IgE-positive mast cells and eosinophilia were observed in lymphoid, biliary, and pancreatic tissue samples from 50% and 86% of patients with IgG4-RD, respectively.

Conclusion: In a prospective study, we associated IgG4-RD with allergy, atopy, eosinophilia, increased serum levels of IgE, and IgE-positive mast cells in lymphoid, biliary, and pancreatic tissue. An IgE-mediated allergic response therefore appears to develop in most patients with IgG4-RD; levels of IgE might be used in diagnosis and predicting relapse.

KEY WORDS: inflammation, pancreas, immune response, detection

MAIN TEXT

INTRODUCTION

IgG4-related disease (IgG4-RD) is a systemic fibro-inflammatory condition, of which IgG4-related sclerosing cholangitis (IgG4-SC) and autoimmune pancreatitis (AIP) are the biliary and pancreatic manifestations. An elevated serum IgG4 and abundance of IgG4-positive plasma cells infiltrating the involved organs is well described¹.

Elevated serum IgE concentrations were first reported in a series of Japanese patients with AIP; retrospective studies suggesting a prevalence of between 34% and 86%^{2,3}. Peripheral and tissue eosinophilia are traditionally seen in the context of atopic and allergic conditions, but have also been described in AIP⁴. Furthermore, a clinical history of allergy can be ascertained in 40% to 80% of AIP patients^{2,5}.

Strong links between IgG4 and IgE are well established. IgG4 responses are often associated with IgE-mediated allergy, but these responses are distinct. Both IgG4 and IgE are induced by Th2 cytokines IL-4 and/or IL-13⁶, however production of IgE antibodies often occurs well before IgG4 antibodies appear⁷. It is also common to find IgG4 antibodies in the absence of IgE antibodies, a process called the 'modified Th2 response'⁸. An important regulatory component in the modified Th2 response is IL-10, which promotes the switch to IgG4 and inhibits IgE production⁹. 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 (10-100 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 organs¹⁰⁻¹².

In this paper, we set out to establish the prevalence of allergy and atopy, blood and tissue eosinophilia, serum total and allergen-specific IgE responses, and tissue IgE antibodies, in a prospective UK cohort of IgG4-RD patients. We sought to determine the utility of serum IgE in differentiating patients with IgG4-SC/AIP from disease

mimics with an elevated serum IgG4, such as primary sclerosing cholangitis, and its relationship with corticosteroid use and disease relapse.

METHODS

Patient and Control Recruitment

For this study, 48 IgG4-RD patients, 42 disease controls with an elevated serum IgG4 (DC) and 51 healthy controls (HC) were recruited from the John Radcliffe Hospital, Oxford, UK, between March 2010 and March 2014, and followed-up for a median of 41 months (range 3-73 months). Diagnostic criteria for each group are defined in the **Supplementary Methods**.

Serological testing for IgG, IgG4 and IgE

Serum immunoglobulins were measured at diagnosis, during steroid treatment and at disease relapse within the IgG4-RD cohort. Comparisons between IgG4-RD patients, DC and HC were made at diagnosis before steroid initiation. Total serum IgG and IgG4 were measured by nephelometry (BNII analyser, Siemens, UK). Total IgE was measured by the automated Immucap method (Phadia 250, UK). Elevated serum IgG ($\geq 16\text{g/l}$), IgG4 ($\geq 1.4\text{g/l}$) and IgE ($\geq 125\text{kIU/L}$) were defined by institution range.

IgE-specific ImmunoCAP

An IgE ImmunoCAP method was used to determine and quantify allergen-specific IgE antibodies. IgE-specific allergen testing was performed using a set of allergen mixes: grass pollen, mould, tree pollen and nut (Phadia, Thermo Scientific), as specified in the **Supplementary Methods**.

Peripheral and Tissue Eosinophilia

Peripheral blood eosinophil count was measured at diagnosis. An elevated eosinophil count was defined as >500 cells/ul (count >0.5). Tissue eosinophil number was graded as 'none' (0), 'occasional' (<10), 'moderate' (10-25) or 'marked' (>25) on H&E specimens by the reporting pathologist.

Immunostaining for IgG, IgG4 and IgE antibodies

Tissues were assessed for morphology and immunostained with IgG and IgG4 monoclonal antibody and IgE polyclonal antibody, as described in **Supplementary Methods**. Double immunostaining for IgE and CD138 (plasma cells) or mast cell tryptase (mast cells) was performed.

Statistical analysis

Comparisons between groups were analyzed with a two-tailed Mann Whitney test; correlations quantified as Spearman's rank correlation coefficient. Statistics and Receiver Operator Curve (ROC) analysis were performed using Graphpad Prism v6.0. A P-value of <0.05 was considered significant.

RESULTS

Prevalence of serum IgG4 and IgE elevation.

The serum IgG, IgG1, IgG4 and IgE levels at diagnosis were higher in IgG4-RD patients than in HC ($p<0.0001$), as shown in **Table 1(Figure S1A-B)**. There was no

difference in the IgE/IgG4 ratio between the two groups (**Figure S1C**). Elevated IgG4 concentrations were demonstrated in 81% (39/48), and elevated IgE in 54% (26/48) of the cohort, both more prevalent compared with HC ($p < 0.0001$)(**Table 1**). There was a positive correlation between serum IgE and IgG4 (Sp.rank 0.46, CI0.18-0.66; $p = 0.001$)(**Figure 1A**), and serum IgE and IgG (Sp.rank 0.32, CI0.028-0.56; $p = 0.028$)(**Figure S2A**). However, there was no difference between serum IgE levels in IgG4-RD patients with a normal and high IgG level ($p = 0.178$)(**Figure S2B**). Serum IgE levels were increased in the high IgG4 compared to normal IgG4 group ($p = 0.017$)(**Figure 1B**). In the 9 IgG4-RD patients with a normal serum IgG4 at diagnosis, only 1 had an elevated IgE.

Prevalence of peripheral eosinophilia.

Peripheral blood eosinophilia was present in 38% of IgG4-RD (median 0.44, range 0.0-5.05) versus 8.6% of HC (median 0.16, range 0.05-0.78)($p = 0.004$)(**Table 1**). There was a positive correlation between eosinophil count and serum IgE (Sp.rank 0.45, CI0.18-0.65; $p = 0.001$)(**Figure 1C-D**) and serum IgG4 (Sp.rank 0.34, CI0.049-0.57; $p = 0.019$)(**Figure S2C**). However, there was no difference between serum IgG4 levels in IgG4-RD patients with a normal and high eosinophil count ($p = 0.298$)(**Figure S2D**).

Prevalence of allergy and atopy.

A history of allergy was identified in 63% of patients with IgG4-RD compared with 17% of HC ($p < 0.001$)(**Table 1; Figure S3A**), and national UK statistics of 20% (<http://www.allergyuk.org/allergy-statistics/allergy-statistics>). There was a higher

serum IgE in IgG4-RD patients with an allergic history than those without ($p=0.031$)(**Figure S3B**). An elevated serum IgE level was present in 60% of IgG4-RD patients with allergy. No patient had a history or evidence of parasitic infection.

Atopy was defined by evidence of an IgE antibody response in addition to clinical symptoms. An allergen-specific IgE response was identified in 52% (25/48) of IgG4-RD patients and 40% (19/48) of IgG4-RD patients were defined as 'atopic'. An elevated serum IgE level was present in 53% of IgG4-RD patients with atopy. In total, 62% (16/26) of IgG4-RD with an elevated total serum IgE had a positive allergen-specific IgE response and 41% (9/22) with a normal IgE had a positive IgE-allergen specific response ($p=0.25$).

Allergen-specific IgE responses.

In order to establish if there was a particular allergen prevalent in IgG4-RD and to investigate whether an IgE-specific response was more prevalent in those with an elevated total IgE, we tested the serum of 16 patients with elevated IgE and 17 patients with normal IgE levels to four broad allergen panels (grass, mould, tree and nut mixes). Three-quarters (75%) of those with an elevated IgE (12/16) were positive to at least one of these panels, whereas 24% of those with a normal IgE (4/17) were positive to a single grass allergen panel only ($p=0.005$)(**Table S1**).

Serum IgE to differentiate IgG4-RD from DC with an elevated serum IgG4.

Serum IgE levels were measured in IgG4-RD patients with an elevated IgG4, and DC who had other autoimmune, inflammatory and infective diseases and an elevated IgG4, to investigate if IgE levels may help to discriminate the two groups. The groups

were well matched in age ($p=0.21$) and gender ($p=1.0$). In those IgG4-RD patients with an elevated IgG4 ($n=39$), serum IgE was higher (median 323kIU/L, range 3.31-2024kIU/L) than in the non-IgG4-RD DC's ($n=42$) (median 55.7kIU/L, range 5.11-4457kIU/L)($p=0.003$)(**Figure 2A**). The ability of serum IgE to distinguish IgG4-RD from DC is shown on the ROC curve; area under the curve (AUC) was 0.69 ($p=0.004$, 95% CI 0.57-0.81)(**Figure 2B**).

At a serum IgE cut-off of 125kIU/L, sensitivity was 67%, specificity was 64%, NPV was 68%, and PPV was 63%, with a likelihood ratio of 1.8 to distinguish IgG4-RD from non-IgG4-RD DC's with an elevated IgG4. At a serum IgE of 480kIU/L (4xULN), the sensitivity fell to 36%, specificity increased to 86%, PPV was 70% and NPV was 59%, with a likelihood ratio of 3.2.

Serum IgE and corticosteroid treatment.

Of 48 IgG4-RD patients, 79% (38/48) received corticosteroid therapy, 15% (10/48) underwent surgical resection, and 6.2% (3/48) had conservative management due to severe diabetes(1), end-stage cirrhosis(1), and patient preference(1). In those who received corticosteroid therapy with available serum samples ($n=15$), IgE levels were lower compared to pre-treatment levels by 12 weeks ($p<0.001$)(**Figure S4A**). Over time, serum IgE levels plateaued or increased, corresponding to corticosteroid discontinuation and clinical remission (**Figure S4B**).

Serum IgE and disease relapse.

Of 48 IgG4-RD patients, 43 had sufficient follow-up (>6 months) to determine evidence of relapse. At least one episode of biochemical and/or radiological relapse was recorded in 54% (23/43). Serum IgE at diagnosis was higher in those IgG4-RD patients who relapsed than those who did not ($p=0.001$)(**Figure 3A**). ROC analysis yielded an AUC of 0.76 ($p=0.005$, 95%CI 0.62-0.91). Using an IgE cut-off of 380 kIU/L, the sensitivity was 64%, specificity was 88%, with a likelihood ratio of 5.4, to predict relapse (**Figure 3B**). An IgE of >125kIU/L at diagnosis did not predict relapse.

Prevalence of tissue eosinophilia.

Histological specimens were available from 77% (37/48) of IgG4-RD patients for morphological review and immunostaining; 15 resection and 32 biopsies. Tissue eosinophils were present in 86% (32/37) of specimens. Eosinophil infiltration was graded as 'moderate' (10-25 eosinophils/HPF) or 'marked' (>25 eosinophils/HPF) in 73% (27/37) of specimens.

Prevalence and distribution of tissue IgG4 and IgE-positive cells.

Tissue IgG4 immunohistochemistry was performed in all 37 IgG4-RD specimens; the IgG4-positive plasma cell count >10/HPF (range 5-130/HPF) in 95% (35/37). The two patients with biopsy IgG4 counts <10/HPF had resection specimens with morphological characteristics and ratios 'highly-suggestive' of IgG4-RD.

Tissue IgE immunohistochemistry was performed in 8 IgG4-RD and 6 DC specimens (**Table S2**). The IgE-positive cell count in IgG4-RD (median 10 cells/HPF, range 1-30 cells/HPF) was higher than DC (median 4 cells/HPF, range 0-10 cells/HPF)($p=0.15$) tissues. The IgE-positive cell count was >10 /HPF in 50% of samples stained (4/8). In IgG4-RD tissues, IgE-positive cells were localised to the mantle zone of the germinal centre, within lymphoid aggregates and also scattered throughout the inflammatory infiltrate (**Figure 4A**). The IgE-positive cell staining was both nuclear and surface. Mast cells were also scattered throughout the inflammatory infiltrate (**Figure 4B**). IgE-positive cells and mast cells had a similar distribution, and co-staining demonstrated evidence of IgE-positive mast cells (mast cell cytoplasmic staining and IgE peripheral staining) in IgG4-RD tissue (**Figure 4C**). IgE-positive cells stained separately to CD20-positive B cells (**Figure 4D**) and CD138-positive plasma cells (**Figure 4E**). This suggests IgE-positive cells represent IgE attachment to mast cells, although we cannot exclude the possibility that a proportion could be IgE-producing B cells.

In three IgG4-RD patients with the highest mean count of IgE-positive mast cells, a retrospective serum mast cell tryptase was performed (pre steroid samples), which were within the normal range.

Red flags for the diagnosis of IgG4-RD

Red flags, incorporating these findings, are shown in **Figure 5**.

DISCUSSION

In the original landmark study of elevated serum IgG4 in AIP patients in Japan, there was no difference reported in IgE levels between AIP and HC¹³. Subsequent retrospective studies reported an elevated IgE in 34-86% of AIP patients^{2,3}. In our prospective study of IgG4-RD, an elevated IgE was found in 57% at diagnosis, and a positive correlation was shown between IgE and both IgG4 and eosinophils count. We report a frequent clinical history of allergy (63%) and atopy (40%) in IgG4-RD, supported by retrospective data in Japanese AIP patients², but not by other groups^{3,14,15}. Peripheral eosinophilia and elevated IgE have also been described in subsets of non-atopic IgG4-RD patients¹⁵. The main discrepancy lies in the definition of atopy; symptoms plus an IgE-specific protein response, which has not been characterised in other studies. We also show a higher IgE level in those allergy/atopic individuals, supported by studies showing atopic IgG4-RD patients express up-regulated Th2 cytokines in peripheral blood^{15,16}. We have also reported polyclonal elevations of IgG4 to multiple food allergens in IgG4-RD patients¹⁷.

Our results support the utility of serum IgE in diagnosis of IgG4-RD, with a level of >480KU/L differentiating IgG4-RD from DC with an elevated serum IgG4 (specificity 86%, sensitivity 36%). This two-dimensional diagnostic approach (i.e. IgE combined with IgG4) was supported by the observation that patients with a normal serum IgG4 did not have an elevated IgE. There was no DC's with malignancy in this dataset, however serum IgE levels may be used to distinguish organ-specific IgG4-RD from malignant lesions, potentially minimising unnecessary surgical intervention. This approach will require further validation. An elevated IgE at diagnosis >380KU/L was also a marker of disease relapse (specificity 88%,

sensitivity 64%), defining a subgroup requiring careful follow-up. This is supported by findings in a recent retrospective cohort study of predictors of disease relapse in IgG4-RD after rituximab therapy; in 21 (37%) patients experiencing disease relapse, baseline serum IgG4, IgE and circulating eosinophils predicted relapse¹⁸.

Interestingly, IgE levels decreased during the first 12 weeks of corticosteroid therapy, with a parallel fall in disease activity (as well as IgG4 levels¹⁹). This finding is in line with observations in a retrospective cohort of AIP⁵. Furthermore, the anti-CD20 B cell depletion therapy rituximab, used in refractory disease, results in a decrease in serum IgG4 and IgE with regression of active disease²⁰. However, IgE levels do not fall with steroid treatment in atopic dermatitis²¹ and do not reflect disease activity in asthma or allergic rhinitis²². This complex interplay between allergic disease and IgE levels indicates that a correlation between IgE and response to treatment does not necessarily point to a pathogenic role for IgE in IgG4-RD.

The presence of tissue IgE-positive mast cells in affected organs represents a novel finding, and suggests a possible role of an IgE-mediated response. Mast cells are involved in a variety of immune responses including chronic inflammation and autoimmune disease²³. IgE is a key stimulator of mast cells via binding to the high-affinity IgE receptor (FcεRI). Mast cells secrete mediators including Th2 and regulatory cytokines in response to allergens binding to specific cell-bound IgE. Non-specific polyclonal IgE can also induce cytokine secretion, independent of antigen²⁴. Furthermore, chronic elevation of IgE induces upregulation of FcεRI on mast cells, resulting in inhibition of mast cell apoptosis and promotion of cytokine production²⁵. Indeed, this may explain the predominantly surface IgE-positive mast cell staining

seen in our tissue specimens, due to high levels of membrane-associated IgE. Mast cells might also contribute to fibrosis²⁶, supported by our observation of IgE-positive mast cell infiltration in an IgG4-related fibrosclerotic mesenteric mass (**Table S2**).

The aetiology of the elevated IgG4 and IgE in the blood and tissue of IgG4-RD patients remains unexplained. Both IgG4 and IgE require Th2 cytokines, IL4 and IL13, for induction. By contrast, IL10 promotes IgG4-switched cells, but downregulates IgE⁹. Whilst we have shown that tissue IgE-positive cells represent IgE attachment to mast cells, a proportion could also be IgE-producing B cells. IgE-producing memory B cells and plasma cells may arise directly through a germinal centre IgE-intermediate cell^{27,28} but also indirectly from class switching of IgG4 or IgG1 B cells²⁹. Both situations could lead to elevated IgE and IgG4 in IgG4-RD, the former by sharing of Th2 conditions and the latter in a higher frequency switch from IgG4 cells. Of note, IgG4 B cells appear to have increased capacity to capture IgE molecules, by up-regulation of the FcεRII on their surface³⁰; suggesting a functional link between IgE and IgG4 B cell responses. Importantly, dysfunction of immune cells, such as Th2 and T regulatory cells, and cytokines IL4, IL13 and IL10, may pave the way for increasing serum IgE levels and IgE-positive cells in the tissues of patients with IgG4-RD.

The discovery of IgE, eosinophilia and mast cell infiltration in IgG4-RD highlights novel therapeutic options. The prostaglandin D2 receptor(CRTh2), important in allergic inflammation, is expressed on Th2 cells, innate cells such as eosinophils, and responds to mast cell derived factors^{31,32}. The frequency of CRTh2 cells was increased in IgG4-siloadenitis, with a non-significant correlation with IgE and

eosinophils³³. We also report up-regulation of PGD2 and CRTh2 in gene expression analysis of IgG4-SC/AIP patients³⁴. Blockade of CRTh2 can reduce allergic inflammation in rodent models of antigen-induced airway inflammation, allergic rhinitis, and atopic dermatitis, and may be effective in a subgroup of atopic IgG4-RD patients.

Table Legend

Table 1: Clinical characteristics and laboratory measurements of IgG4-RD patients 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; kIU/L: equivalent to 1 international unit per millilitre.

Figure Legends

Figure 1: The relationship of serum IgE with serum IgG4 and peripheral blood eosinophil counts in IgG4-RD (A) Correlation plots showing serum IgE (kIU/L) plotted against serum IgG4 (G/L) in IgG4-RD. **(B)** Dot plots of serum IgE in IgG4-RD patients with a high ($\geq 1.4\text{g/l}$) or normal serum IgG4. Dashed line is the serum IgE upper limit of normal ($\geq 125\text{kIU/L}$). **(C)** Correlation plots showing blood eosinophil count (cells/ul) plotted against serum IgE (kIU/L) in IgG4-RD. **(D)** Dot plots of serum IgE in IgG4-RD patients with a high ($\geq 500\text{cells/ul}$) or normal eosinophil count. Dashed line is the serum IgE upper limit of normal ($\geq 125\text{kIU/L}$). Spearman's Rank correlation and p values are expressed as NS ≥ 0.05 , * $P < 0.05$, ** $P < 0.01$. Mann-Whitney p values, where NS $p \geq 0.05$, * $p < 0.05$.

Figure 2: Serum IgE levels and ROC Curve to differentiate IgG4-RD patients and non-IgG4-RD disease controls with an elevated serum IgG4. (A) Dot plot showing serum IgE in IgG4-RD patients and non-IgG4-RD disease controls with an elevated serum IgG4. The y-axis shows the serum IgE concentration (kIU/L). Dashed

line is the serum IgE upper limit of normal (≥ 125 kIU/L). Mann-Whitney p values * $p < 0.05$. **(B)** Receiver Operating Characteristic (ROC) curve shows the sensitivity and specificity of IgE in distinguishing IgG4-RD from non-IgG4-RD conditions, with an elevated serum IgG4.

Figure 3: Serum IgE levels and ROC Curve for disease relapse in IgG4-RD

(A) Dot plot showing serum IgE in IgG4-RD patients with and without evidence of biochemical and radiological disease relapse. The y-axis shows the serum IgE concentration (kIU/L). Dashed line is the serum IgE upper limit of normal (≥ 125 kIU/L). Mann-Whitney p values NS $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$. **(B)** ROC curve shows the sensitivity and specificity of IgE at diagnosis in determining disease relapse in IgG4-RD patients.

Figure 4. Immunohistochemical staining for inflammatory cell subsets and IgE

in IgG4-RD (A). Type 1 autoimmune pancreatitis, showing IgE-positive cells (brown) within the inflammatory cell infiltrate. IgE immunohistochemistry. Magnification x200. **(B).** IgG4-related sialadenitis, showing mast cells (red; marked with arrows) within the inflammatory cell infiltrate. Mast cell tryptase immunohistochemistry. Magnification x400. **(C).** Type 1 autoimmune pancreatitis, showing a mast cell (red cytoplasm) expressing surface IgE (pale blue – in contrast to dark blue haematoxylin counter-stain; marked with arrow) within the inflammatory infiltrate. Mast cell tryptase (red) and IgE (pale blue) double immunohistochemistry. Magnification x400. **(D).** Type 1 autoimmune pancreatitis, showing CD20-positive B-cells (red; marked with short arrows) not expressing IgE; and a separate inflammatory cell (not CD20-positive) expressing IgE (pale blue – in contrast to dark blue

haematoxylin counter-stain; marked with long arrow) within the inflammatory cell infiltrate. CD20 (red) and IgE (pale blue) double immunohistochemistry. Magnification x400. **(E).** Nasal polyp, showing CD138-positive plasma cells (bright red; marked with long arrows) and a separate population of CD138-negative and IgE-positive cells (brown; marked with short arrows) within the inflammatory cell infiltrate. CD138 (bright red) and IgE (brown) double immunohistochemistry. Magnification x400.

Figure 5: Red flags in the diagnosis of IgG4-RD. A set of red flags to raise suspicion of a diagnosis of IgG4-RD.

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Table 1: 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) kIU/L	142.0 (0.0-2024)	25.1 (1.99-491.0)	<0.0001 ****
IgE/IgG4 ratio median (range) kIU/G	69.19 (0-705.1)	57.93 (0-2728)	0.6491 NS
Eosinophils median (range) count	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 >1.4 G/L (%)	39/48 (81.3)	3/51 (5.9)	<0.0001 ****
High serum IgE >125kIU/L (%)	26/48 (54.2)	8/51 (15.7)	0.0001 ***
Serum Eosinophilia >0.5 count (%)	18/48 (37.5)	3/35 (8.6)	0.0042 **

Figure 1: The relationship of serum IgE with serum IgG4 and peripheral blood eosinophil counts in IgG4-RD.

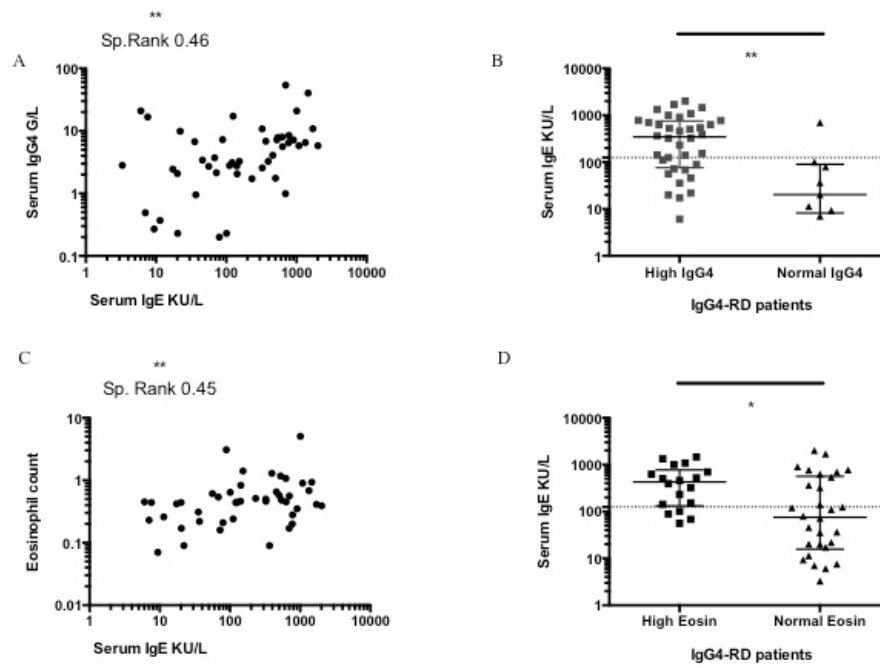


Figure 2: Serum IgE levels and ROC Curve to differentiate IgG4-RD patients and non-IgG4-RD disease controls with an elevated serum IgG4.

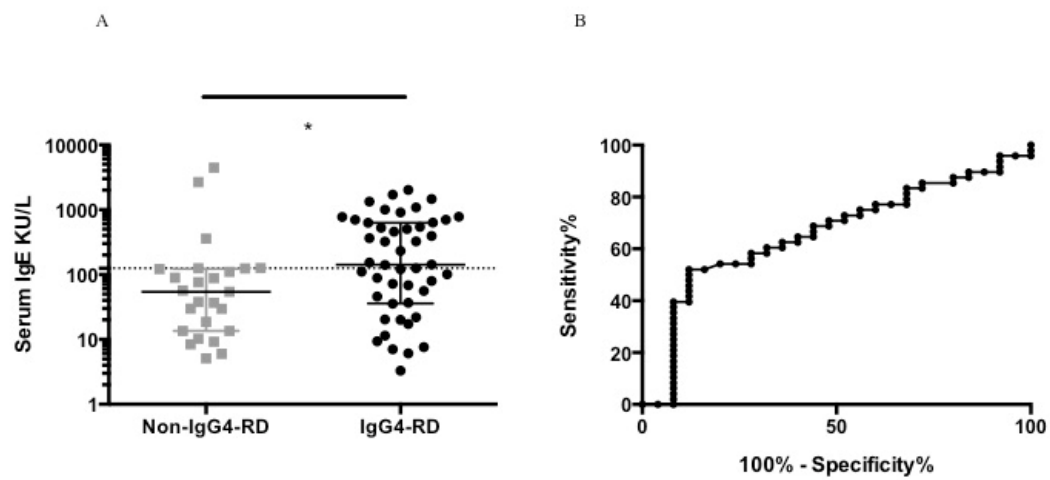
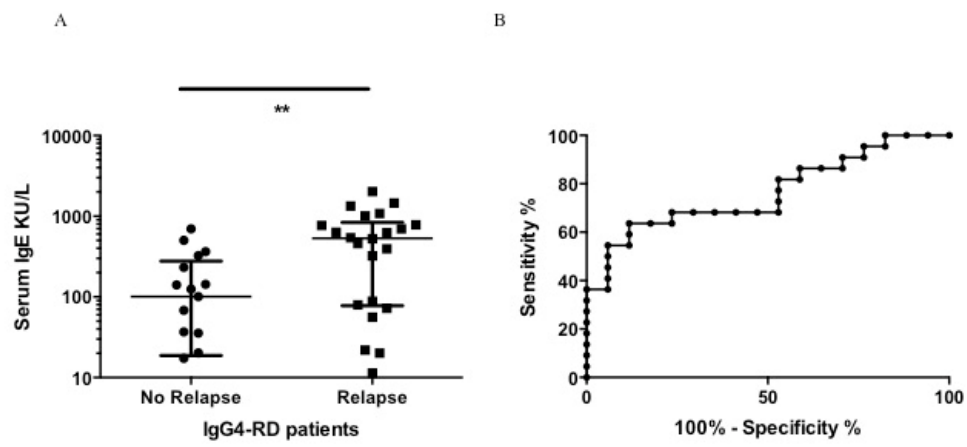
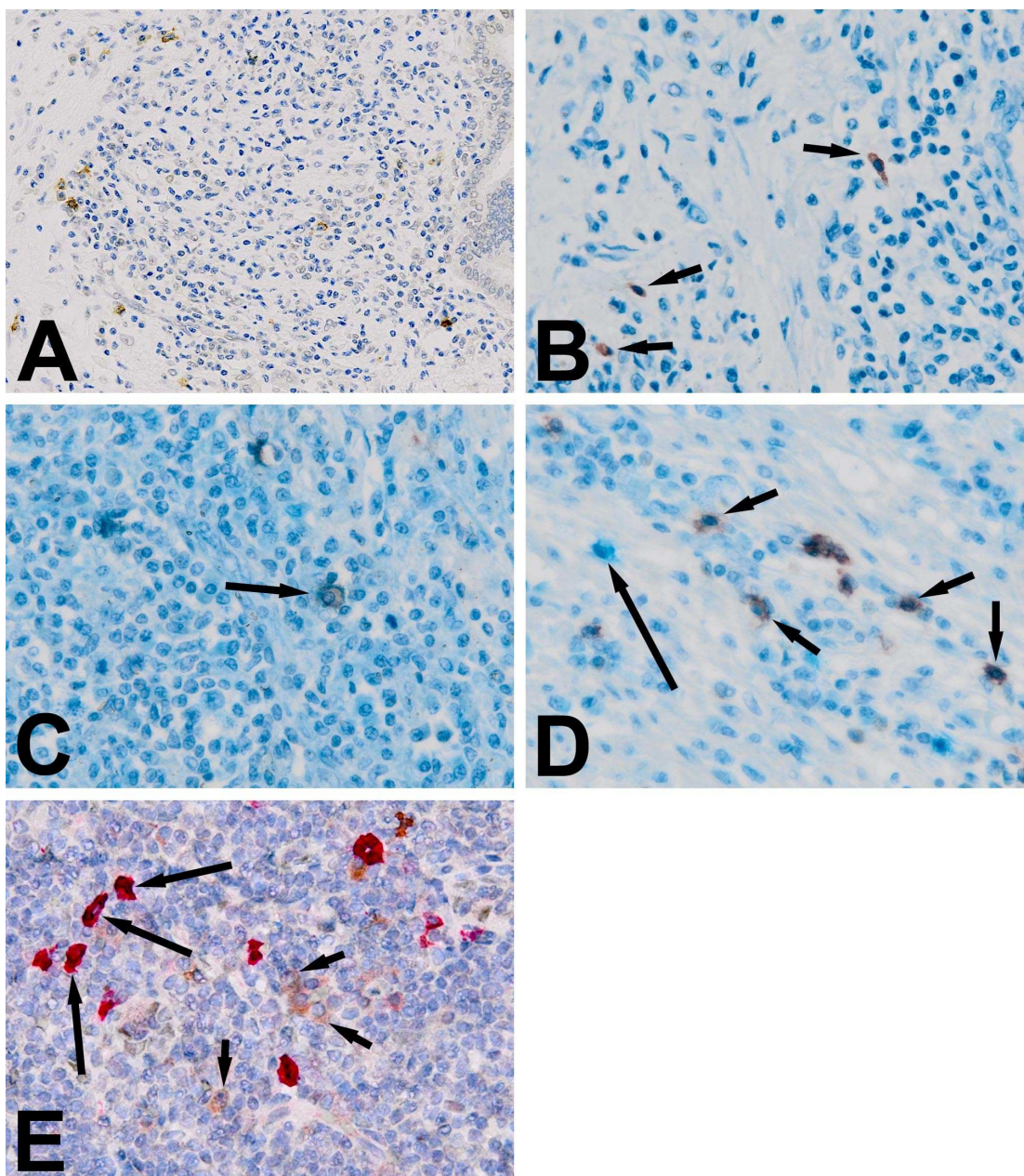
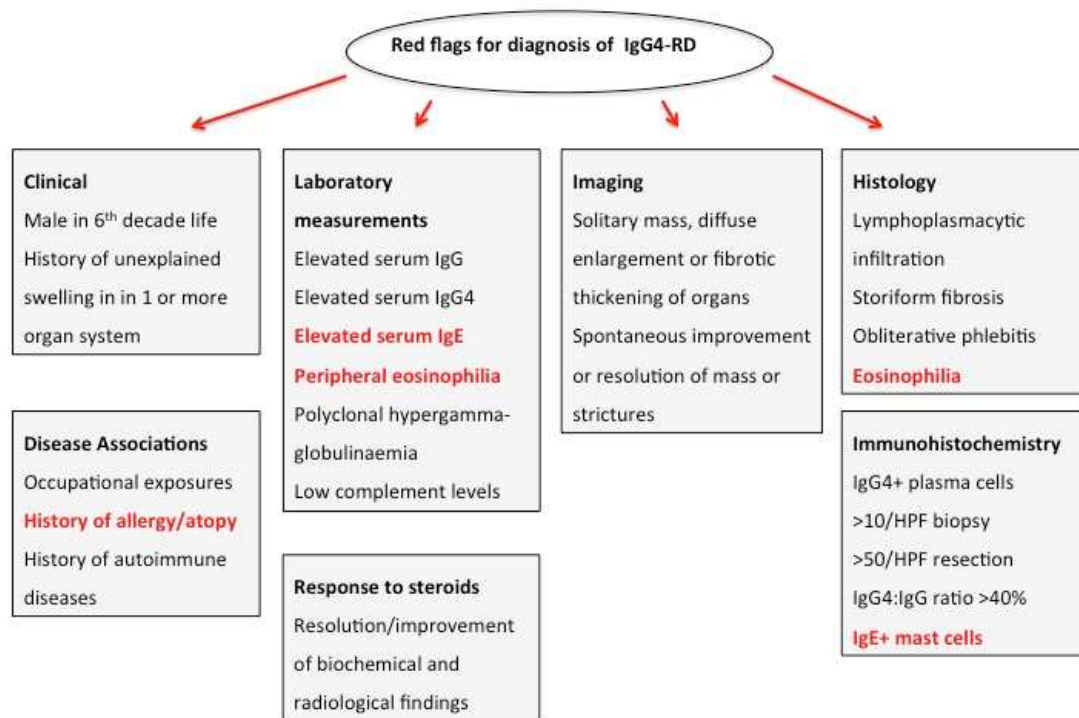


Figure 3: Serum IgE levels and ROC Curve for disease relapse in IgG4-RD patients.







Supplementary Methods

Diagnostic Criteria

The diagnosis of AIP and IgG4-SC was made in accordance with the Mayo HISORT criteria¹ and the International Consensus Diagnostic Criteria (ICDC)². Patients with type II AIP were excluded³. Patients with extra-pancreatic disease were diagnosed using the Japanese Comprehensive Diagnostic Criteria (JCDC) for systemic IgG4-RD⁴. The Boston Consensus Histopathological Criteria for IgG4-RD were applied to all patients with biopsy and resection specimens available⁵. The majority of patients in the cohort had AIP and/or IgG4-SC (85%) with extra-pancreatic manifestations in 72% of patients.

Disease controls had an elevated level of serum IgG4 but had no other evidence to support a diagnosis of IgG4-RD. These included 25 primary sclerosing cholangitis (PSC) patients⁶, 4 hepatitis (1 autoimmune, 2 viral, 1 alcoholic) patients, 3 chronic cholecystitis patients, 4 with cirrhosis (3 alcoholic, 1 cryptogenic), 1 with sarcoidosis, 1 with hypereosinophilic syndrome, 1 with coeliac disease, 1 with chronic pancreatitis, 1 with recurrent pneumonia and 1 with a pleural effusion. Healthy donors had no known immune or inflammatory disease, and were gender-matched to IgG4-RD patients and DC. All comparisons between DC and IgG4-RD patients were made at the time of diagnosis of IgG4-RD (pre steroids or immunosuppressive therapy). Only DC that had not had steroid or immunosuppressive therapy at recruitment were included.

Definitions of Allergy and Atopy

Allergy and atopy were defined in accordance with the European Academy of Allergy and Clinical Immunology classification. Allergy is a hypersensitivity reaction mediated by immunological mechanisms (antibody- or cell-mediated). Atopy is a personal or family tendency to produce IgE antibodies in response to low-dose allergen, and as a consequence develop typical symptoms.

Serological testing for IgG, IgG4 and IgE

For patients with multiple immunoglobulin measurements, the first IgG4 level recorded was used. The prozone effect (falsely low IgG4 values) was accounted for using serial dilutions where necessary⁷. For IgE, units are expressed as kIU/L, equivalent to 1 IU/mL, and equivalent to 2.4ng/mL.

IgE-specific ImmunoCAP allergen mixes

The allergen mixes contained the following; Grass Mix (GX1): Timothy Grass, Rye Grass, Meadow Fescue, Meadow Grass (Kentucky Blue); Tree mix (TX1) Birch, Box Elder, Oak, Cedar Elm, Walnut; Nut Mix (FX1): Peanut, Coconut, Hazelnut, Brazil Nut, Almond; Mould Mix (MX1): *Penicillium chrysogenum*, *Cladosporium herbarum*, *Aspergillus fumigatus*, *Alternaria alternata*.

If a particular allergen was highlighted from the clinical history additional allergen testing was performed to confirm this, including house dust mite, cat dander, wheat, milk and latex.

Tissue Eosinophilia

Tissue eosinophil number was graded as 'none' (0), 'occasional' (<10), 'moderate' (10-25) or 'marked' (>25) on H&E specimens by the reporting pathologist (ACB). The number of eosinophils considered 'physiologically normal' in a tissue sample depends upon 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 situation⁸.

Tissue morphology

All biopsy and resection specimens were assessed for classical morphological features of IgG4-RD, specifically a lymphoplasmacytic infiltrate, storiform fibrosis and obliterative phlebitis in accordance with the Boston histological criteria⁵. The background inflammatory infiltrate was determined using CD4 and CD8 (T cells), CD20 (B cells), CD68 (macrophages) and CD138 (plasma cells) antibodies.

Tissue IgG4 immunostaining

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 elevated IgG4 count, an IgG4 to total IgG ratio was calculated; an elevated IgG4:IgG ratio was defined as >40% in accordance with the Boston histological criteria⁵.

Tissue IgE immunostaining

Eight IgG4-RD specimens (7 resection, 1 biopsy) and 6 disease control specimens (5 resection, 1 biopsy) were immunostained for IgE (**Table S1**). An elevated IgE count was defined arbitrarily as >10/HPF by the reporting pathologist (ACB), using the diagnostic cut off for IgG4-positive cells in biopsy specimens in IgG4-RD. Further characterisation and double immunostaining for IgE and plasma cells (CD138) or IgE and mast cells (mast cell tryptase) was performed at the Histopathology Department at Southampton General Hospital, in order to further define IgE-positive cells.

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), CD68 (macrophages) and CD138 (plasma cells) to determine the background inflammatory infiltrate. In brief, 4 micro-meter (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 Prepstin 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 by two histopathologists, using a multi-headed microscope, to reduce inter-observer variation.

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Supplementary Tables

Table S1: Serum IgE and allergen-specific IgE responses to four allergen panels.

Serum IgE	Number (%) of patients				
	Grass mix	Mould mix	Tree mix	Nut mix	Total
Elevated IgE n=16	8/16 (50%)	2/16 (12.5%)	3/16 (18.8%)	7/16 (43.8%)	12/16 (75%)
Normal IgE n=17	4/17 (23.5%)	0/17 (0%)	0/17 (0%)	0/17 (0%)	4/17 (23.5%)

Table S2: IgE immunostaining in IgG4-RD patients 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.

Supplementary Figure and Table Legends

Figure S1: Serum IgG4 and IgE levels and IgE/IgG4 ratio in IgG4-RD and healthy controls. Dot plots showing (A) serum IgG4 concentrations, (B) serum IgE concentrations, (C) IgE/IgG4 ratio (kIU/G), in IgG4-RD patients and healthy controls (HC). The y-axis shows the serum IgE concentration (kIU/L), serum IgG4 (G/L), or IgE/IgG4 ratio. Dashed line is the serum IgE upper limit of normal (≥ 125 kIU/L) and serum IgG4 upper limit of normal (≥ 1.4 G/L). Mann-Whitney p values; NS $p < 0.05$, **** $p < 0.0001$.

Figure S2: The relationship of serum IgE with serum IgG, and serum IgG4 with peripheral blood eosinophil count, in IgG4-RD (A) Correlation plots showing serum IgE (kIU/L) concentration plotted against serum IgG (G/L) concentrations in IgG4-RD. (B) Dot plots of serum IgE concentrations in IgG4-RD patients with a high (≥ 16.0 g/l) or normal serum IgG. Dashed line is the serum IgE upper limit of normal (≥ 125 kIU/L). (C) Correlation plots showing blood eosinophil count (cells/ul) plotted against serum IgG4 (G/L) concentrations in IgG4-RD. (D) Dot plots of serum IgG4 concentrations in IgG4-RD patients with a high (≥ 500 cells/ul) or normal eosinophil count. Dashed line is the serum IgE upper limit of normal (≥ 125 kIU/L). Spearman's Rank correlation co-efficient and p values are expressed as NS ≥ 0.05 , * $P < 0.05$, ** $P < 0.01$. Mann-Whitney p values, where NS $p \geq 0.05$, * $p < 0.05$.

Figure S3: Allergy and/or Atopy and serum IgE levels in IgG4-RD patients and healthy controls. (A) Bar chart showing the number of IgG4-RD patients and healthy controls with (black) or without (grey) a history of allergy and/or atopy. (B) Dot plot

showing serum IgE concentration (kIU/L) in IgG4-RD patients with and without a history of allergy and/or atopy. Dashed line is the serum IgE upper limit of normal (≥ 125 kIU/L). Mann-Whitney p values, where * $p < 0.05$, $p < 0.0001$ ****.

Figure S4: Serum IgE and relationship to corticosteroid therapy in IgG4-RD. (A)

Serum IgE concentrations in IgG4-RD patients at diagnosis before corticosteroids (0 weeks) and after 12 weeks of corticosteroid therapy (n=15). On the y-axis is serum IgE concentrations kIU/L. Dashed line is the upper limit of IgE (≥ 125 kIU/L). Two-tailed paired t-test p values, where ** $p < 0.01$ (B) Serum IgE concentrations in IgG4-RD patients during follow-up over 12 months of corticosteroid therapy. The dashed line is the same as in (A).

Table S1: Serum IgE and allergen-specific IgE responses to four allergen panels.

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 (> 125 kIU/L) concentration.

Table S2: IgE Immunostaining in IgG4-RD and disease control tissue specimens.

The specimen, mean IgE count and cell distribution in tissue sections from IgG4-RD patients (n=8) and disease controls (n=6). 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.

Figure S1 (A-C) Serum IgG4 and IgE levels and IgE/IgG4 ratio in IgG4-RD and healthy controls.

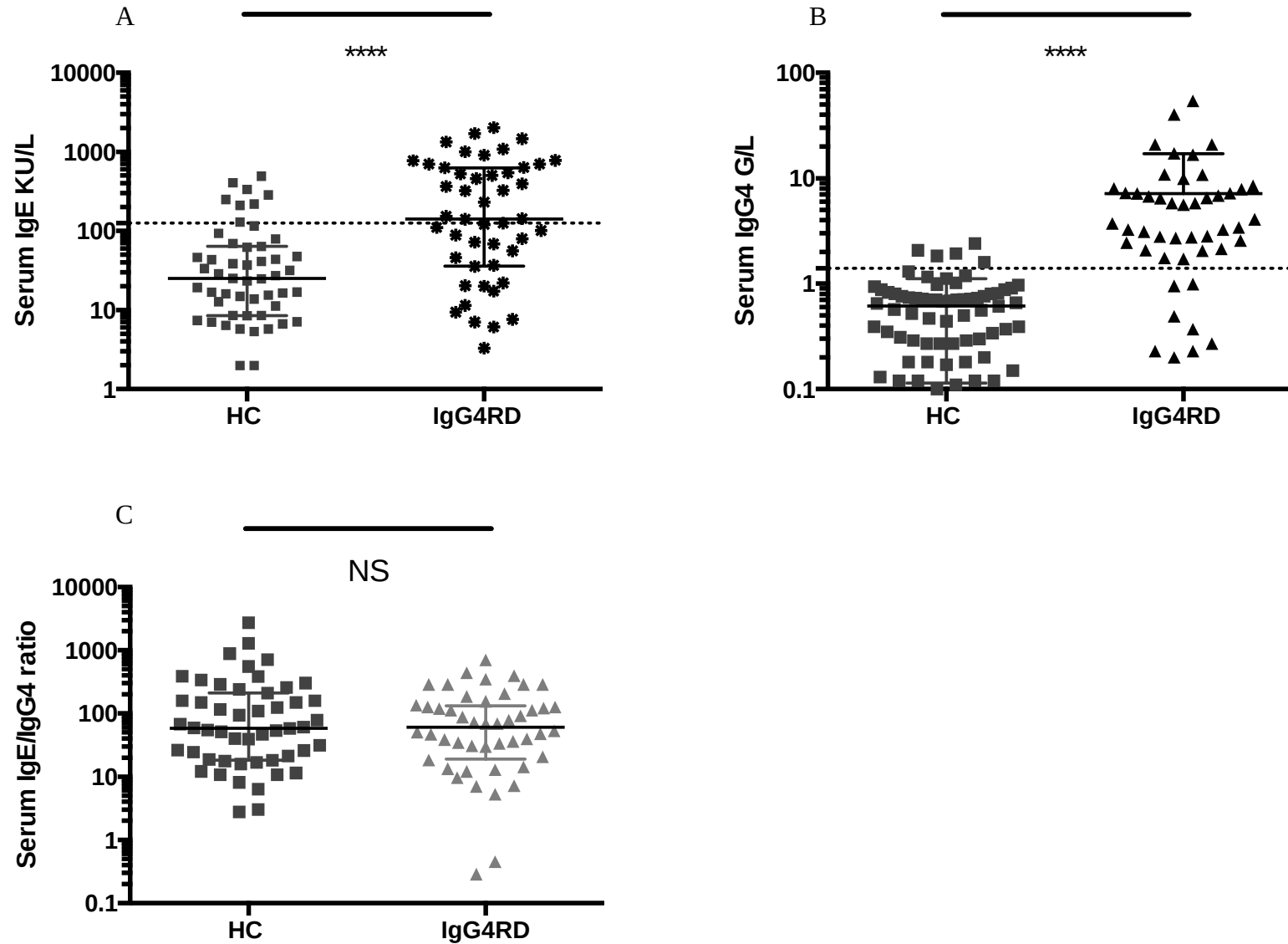


Figure S2: The relationship of serum IgE with serum IgG, and serum IgG4 with peripheral blood eosinophil count, in IgG4-RD.

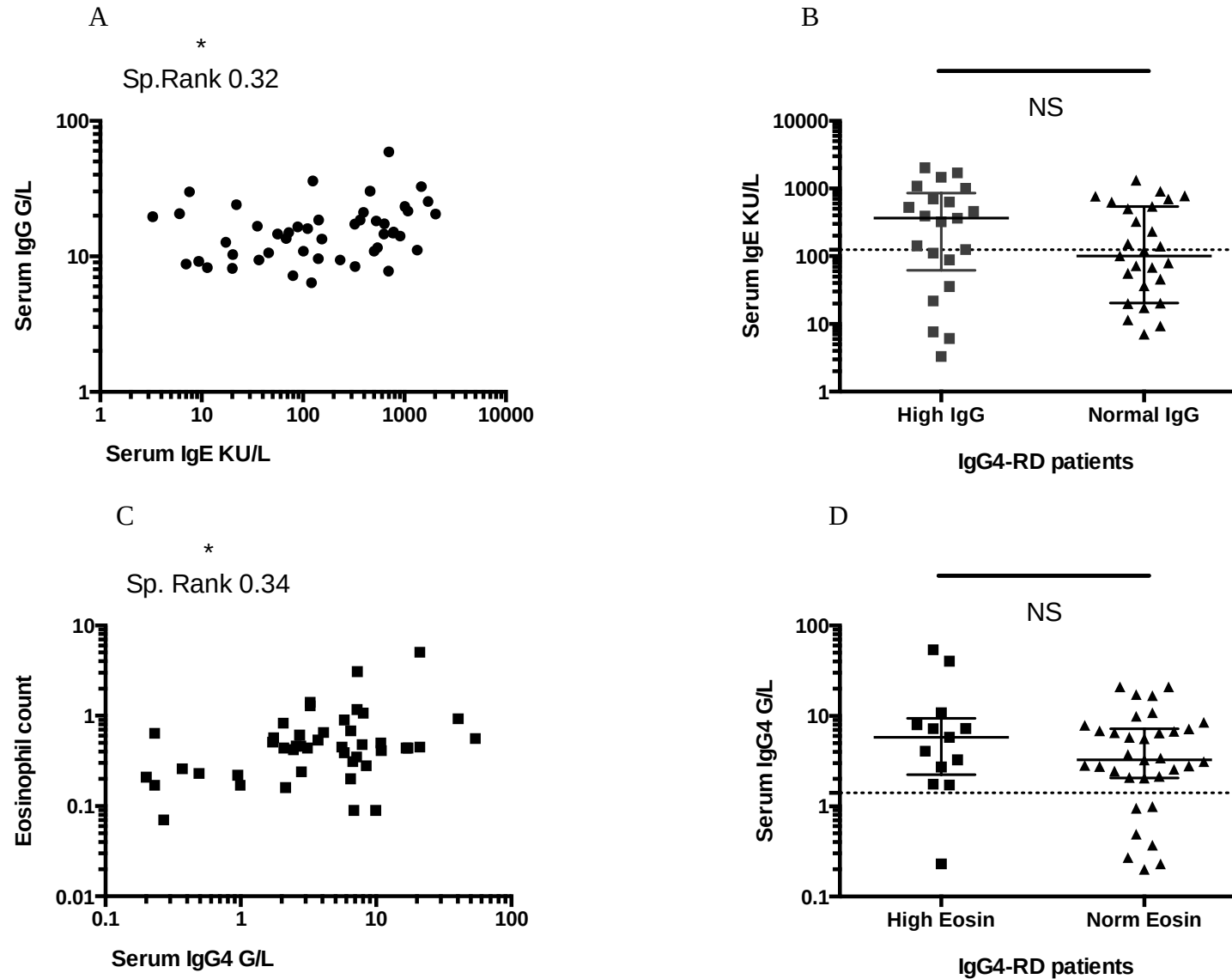


Figure S3: Allergy and/or Atopy and serum IgE levels in IgG4-RD patients and healthy controls.

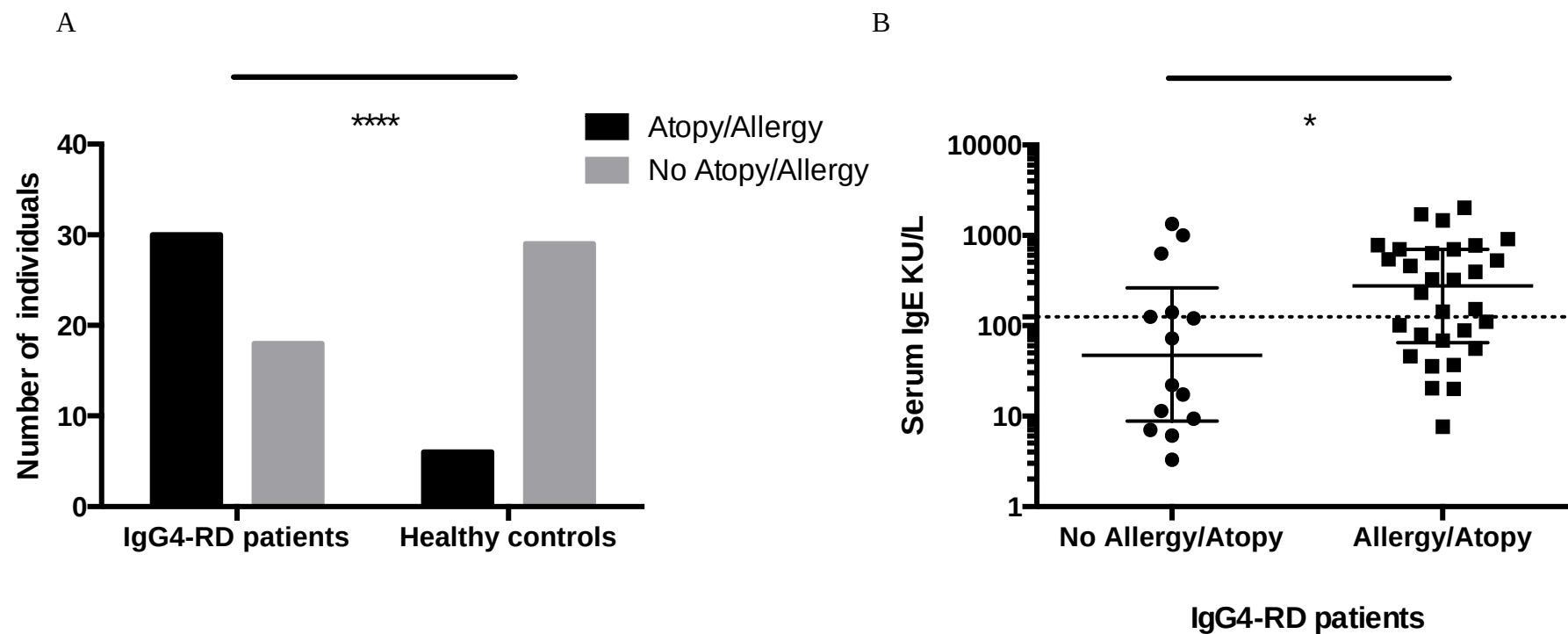


Figure S4: Serum IgE and relationship to corticosteroid therapy in IgG4-RD.

