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Immunohistochemical expression of DNAJB9 in IgG4-related disease

Emily Stuchfield-Denby

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Emily Stuchfield-Denby. Immunohistochemical expression of DNAJB9 in IgG4-related disease. Human health and pathology. 2022. dumas-03945194

HAL Id: dumas-03945194

<https://dumas.ccsd.cnrs.fr/dumas-03945194v1>

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UNIVERSITÉ CLERMONT AUVERGNE
UFR DE MÉDECINE ET DES PROFESSIONS PARAMÉDICALES

THÈSE D'EXERCICE
pour le
DIPLOME D'ÉTAT DE DOCTEUR EN MÉDECINE
par

Emily STUCHFIELD-DENBY

Présentée et soutenue publiquement le 6 octobre 2022

Immunohistochemical expression of DNAJB9 in IgG4-related disease

Directeur de thèse :

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REMERCIEMENTS

Au Professeur André, pour votre accompagnement depuis le début, le partage de vos connaissances, vos conseils et avertissements. Merci de présider ce jury.

Au Professeur Ruivard, pour m'avoir fait confiance et m'avoir accompagnée avec une aide sans faille, enthousiasme, écoute et décision, merci pour votre encadrement précieux.

Au Professeur Schleinitz, de me faire l'honneur de juger ce travail de votre œil expert.

Au Professeur Godfraind, merci d'avoir accepté de juger ce travail et de faire partie de ce jury.

Au Docteur Grobost, pour tes encouragements réconfortants.

A Bruno Pereira, pour votre aide indispensable.

A l'équipe d'anatomopathologie : Marie, Marie-Pierre, Walter, pour tout votre travail ; Victoria, pour ton aide plus que nécessaire.

Au Professeur Aumaître, pour votre présence à nos côtés et votre soutien.

A Perrine, pour ta confiance, pédagogie, vivacité d'esprit et ton humanité ; Hervé, pour ton attention chaleureuse et tes conseils ; Louis, pour ta gentillesse et bienveillance ; Julien, pour ta gentillesse et modestie ; Mélissa, pour ton soutien ; Ludovic, pour ton aide (et l'IFN α). A Magali, Natacha, Paule, Claire, Olivier, pour mon très agréable semestre passé avec vous.

A Damien, pour le chemin parcouru, les délires innombrables, la hideur et l'esclaffe ; Alycia, pour les stétho bien mis ; Clôt, pour les goûters-confidences ; Agathe, pour ta sagesse ; Billy, pour la débrouille et le fun ; Jordan, pour le thé et la folie ; Domitile, 'just no way'. A Florence et à Marie d'être là. A mes co-internes, pour ce trail parcouru, les horaires tardifs et certains goûters biquotidiens. A Paul, Méryl, Sara, Louis, Hugo, pour les débuts. Merci pour vos amitiés.

A Lise et Alexis, pour ce trio précieux ; Céline, Nicolas, Alexandre pour les poulperies éternelles ; Adrien, là depuis toujours ; Céline et Victoria, pour les activités partagées et à venir. A Julie, Emeline, Parag qui, même loin, sont proches. Merci d'être mes amis.

A celui qui est là (presque) tous les jours pour voir comme c'est dur parfois d'être moi, entendre les idées incongrues et inopinées et les blagues apparemment pas drôles. Allez, courage.

A Isabelle, pour ta présence, comme si depuis toujours.

A mes parents d'être là, toujours, de m'avoir accompagnée et d'avoir bien essayé de comprendre ce que c'était que la MAG4...

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LIST OF ABBREVIATIONS

ACR/EULAR: American College of Rheumatology and the European League Against Rheumatism

ADICAP: Association pour le Développement de l'Informatique en Cytologie et en Anatomie Pathologiques

AIP: autoimmune pancreatitis

ANA: antinuclear antibodies

ANCA: anti-neutrophil cytoplasmic antibodies

CDC: Comprehensive Diagnostic Criteria

CRP: C-reactive protein

CS: cytoplasmic staining

DNAJB9: DnaJ Heat Shock Protein Family B Member 9

ENA: Extractable Nuclear Antigen

ER: endoplasmic reticulum

ERdj4: endoplasmic reticulum DNAJ domain-containing protein 4

FGN: fibrillary glomerulonephritis

ICDC: International Consensus Diagnostic Criteria

IgG4-RD: immunoglobulin G4-related disease

IQR: interquartile range

Mdg-1: microvascular endothelial differentiation gene 1 protein

MGN: membranous glomerulonephritis

NAI: non-auto-immune

RCD: Revised Comprehensive Diagnostic

SCS: systemic corticosteroids

SS: Sjögren's syndrome

TIN: tubular interstitial nephropathy

INTRODUCTION

Immunoglobulin G4-related disease (IgG4-RD) is a rare, multisystem, fibro-inflammatory, immune-mediated condition(1) presenting with mass-forming lesions and strictures that can affect almost any organ(2) (**Appendix I**). Autoimmune pancreatitis (AIP) was the first disease manifestation to be described but others have since been reported, including sclerosing cholangitis, lymphadenopathy, dacryoadenitis, sialadenitis, tubulointerstitial nephritis, retroperitoneal fibrosis and pseudotumors(3,4). The diagnosis is made by the association of clinical, biological and histological features(5).

IgG4-RD is characterised by elevated serum IgG4 levels, a dense polyclonal IgG4⁺ plasma cell infiltration(2) and abnormalities in the differentiation of the B-cell lineage(6). A specific subset of circulating B cells was shown to be increased in IgG4-RD patients(7). These CD19⁺CD24⁻CD38^{hi} plasmablasts/plasma cells correlated positively with serum IgG4 levels and the number of organs involved, and decreased significantly after steroid treatment. Differential gene expression analysis in CD19⁺CD24⁻CD38^{hi} plasmablasts/plasma cells showed upregulation of several genes, including the DnaJ Heat Shock Protein Family B Member 9 (*DNAJB9*) gene, coding for co-chaperone protein DNAJB9(6).

DNAJB9 – also named endoplasmic reticulum DNAJ domain-containing protein 4 (ERdj4) or microvascular endothelial differentiation gene 1 protein (Mdg-1) – is a co-chaperone protein located in the nuclei(8) and endoplasmic reticulum (ER)(9). It is expressed at low levels in all human tissues, with the highest levels of expression in tissues with cells with well-developed endoplasmic reticulum (ER) such as the liver, placenta, and kidneys. DNAJB9 protects against cell death and governs the integrity and functions of different cells(10) (**Appendix II**)

Recently, DNAJB9 was shown to be abundant in the glomeruli of patients with fibrillary glomerulonephritis (FGN), a rare proliferative form of glomerulonephritis, characterised by IgG, C3 and light chain deposits in the mesangium and/or along the glomerular basement membranes(11). It was shown to co-localise in large proportions with IgG4 deposits(12). Histological similarities can be found between FGN and IgG4-related kidney disease, such as granular IgG and C3 deposits along the tubular basement membrane in 80% of IgG4-related tubular interstitial nephropathy cases, and IgG4 sub-epithelial or mesangial deposits in IgG4-related membranous glomerulonephritis(13). FGN is associated with inflammatory and autoimmune diseases, such as Sjögren's syndrome (SS), in 13 to 30% of cases(10).

These findings suggest a pathophysiological link between FGN and autoimmune or inflammatory disorders, DNAJB9 being a putative autoantigen(10), which may also trigger IgG4 production in IgG4-RD.

As there is no specific marker for the diagnosis of IgG4-RD(3), evidence of DNAJB9 tissue upregulation in IgG4-RD patients could be a turning point for the diagnosis and treatment-response monitoring of IgG4-RD. Tissue expression of DNAJB9 in IgG4-RD has, to our knowledge, never been explored.

In this study we question DNABJ9 immunohistochemical expression in tissues from patients with IgG4-RD to determine if it may play a role in IgG4-RD pathophysiology.

METHODS

1. Patients

1.1. IgG4-RD patients

IgG4-RD cases were selected by examining all high serum IgG4 measurements (high range 0.86g/L, immunoturbidimetric method, Optilite®, The Binding Site) assessed between 2016 and 2020 in the Immunology department of *Gabriel Montpied* Teaching Hospital, Clermont-Ferrand. Only serum IgG4 levels greater than 1.35g/L were considered, and each corresponding patient's medical record was examined to determine whether a diagnosis of IgG4-RD had been made. Additional IgG4-RD cases were included by asking clinicians for details of known IgG4-RD patients who had been followed in the Internal Medicine departments of *Gabriel Montpied* and *Estaing* Teaching Hospitals, Clermont-Ferrand.

Only definite IgG4-RD cases were selected by meeting either the 2020 Revised Comprehensive Diagnostic (RCD) criteria for IgG4-RD (**Appendix III**), the American College of Rheumatology and the European League Against Rheumatism (ACR/EULAR) international classification criteria (**Appendix IV**) or the International Consensus Diagnostic Criteria (ICDC) for AIP (**Appendix V**).

Each selected IgG4-RD patient's medical record was examined and information was collected regarding sex, age at tissue biopsy, significant medical history, organs involved in IgG4-RD, lab tests results at sampling (C-Reactive Protein [CRP], eosinophils, serum IgG4), pathology reports regarding the tissue sample showing evidence for IgG4-RD, the organ sampled, RCD, ACR/EULAR or ICDC scoring, number and nature of treatments undertaken to achieve remission.

1.2. Non-auto-immune controls

To select non-auto-immune (NAI) tissue controls corresponding to each IgG4-RD affected organ, we generated for each organ a list of samples examined from 2016 to 2021 in the Pathology laboratories of *Gabriel Montpied* and *Estaing* Teaching Hospitals, Clermont-Ferrand, using the ‘Association pour le Développement de l’Informatique en Cytologie et en Anatomie Pathologiques’ (ADICAP) coding system. Where several controls were available, the nearest in age was selected. Cases and controls were not matched on sex.

For each tissue sample selected, the patient’s medical record was examined, and information was collected regarding sex, age at biopsy, significant medical history, lab tests results at sampling (CRP and eosinophil levels), pathology reports regarding the tissue sample, and the organ sampled.

1.3. Sjögren’s syndrome controls

Each IgG4-RD salivary gland tissue sample was paired with a SS control. We also used the ADICAP coding system to go through salivary gland biopsies and select patients compatible with SS, according to the ACR/EULAR classification criteria(14).

For each tissue sample selected, the patient’s medical record was examined and information was collected regarding sex, age at biopsy, significant medical history, organs involved in SS, lab tests results at sampling (CRP and eosinophil levels), pathology reports regarding the sample, the organ biopsied, the number of organs involved in the disease, ACR/EULAR scoring, and the number and nature of treatments undertaken to achieve remission.

2. Immunohistochemistry of DNAJB9

We collected the existing hematoxylin-eosin-stained slides for each patient, and the existing IgG4-stained slides for IgG4-RD patients. Extra IgG4-stained slides were made for SS controls to rule out IgG4-RD. For each IgG4-RD case, non-auto-immune and SS control, a new slide was made from the corresponding tissue block. Anti-DNAJB9 immunohistochemistry (IHC) was then performed on 4µm-thick, 4% formaldehyde-fixed and paraffin-embedded tissue sections, mounted on positively charged slides. Slides were stained with an anti-DNAJB9 polyclonal antibody (catalogue no. HPA040967; 1/75 titre; Sigma-Aldrich, St Louis, MO) on a Ventana BenchMark XT machine. The staining protocol included deparaffinisation, epitope retrieval with Cell Conditioner for 32 minutes, incubation with 100µL of the primary antibody for 32 minutes at 37°C, then OptiView HQ Universal Linker for 8 minutes and OptiView HRP Multimer for 8 minutes. OptiView DAB chromogen and hydrogen peroxide were then added for signal generation. Finally, counterstaining was performed with Hematoxylin II for 8 minutes and then bluing reagent for 4 minutes. The slides were then analysed using a Carl Zeiss microscope. All were examined blinded, by 2 readers, including a junior pathologist, and a third opinion was sought if they disagreed. To recognise normal staining, we referred to The Human Protein Atlas website and *Nasr et al.* descriptions of IHC of DNAJB9 in normal tissues (**Appendix VI**). As DNAJB9 is known to be a specific marker for FGN, there were no extra-renal positive controls available. Our positive control consisted of a kidney tissue sample from a patient with FGN. Information regarding tissue structure, staining pattern, intensity and distribution was sought. We designed an immunohistochemical intensity scoring system with the following grading: 0 (negative), 1 (very weak), 2 (weak), 3 (moderate) and 4 (strong).

3. Statistics

Patient characteristics were described by headcounts and percentages for categorial variables, and by mean and standard deviation or median and interquartile range for quantitative variables, according to their statistical distribution. Assumption of normality was studied using a Shapiro-Wilk test. For quantitative variables, comparisons between independent groups (IgG4-RD vs. NAI) were made using a Student t test, or a Mann-Whitney test if the conditions to use a t test were not met. Homoscedasticity (equality of variables) was studied with a Fisher-Snedecor test. Comparisons between groups regarding categorial parameters were made using a Chi2 test, or a Fisher exact test where applicable. Given the headcounts, we preferred non-parametric tests. All statistical analyses were conducted with Stata software (version 15, StataCorp, College Station, USA), taking into consideration a bilateral Type I error risk of 5%.

4. Ethics

This study was retrospective and all investigations were led using data and samples collected at the time of patients' original disease investigation and diagnosis, and did not necessitate any extra data collection or sampling. Therefore, this study did not require validation from the ethics committee. However, each patient was sent a no-objection letter by post. None refused to participate.

RESULTS

1. Patient characteristics

We collected 11 IgG4-RD cases, 21 non-auto-immune and 6 SS controls. Nine slides were rejected because the staining procedure failed (1 prostatic sample and 1 salivary gland sample from the IgG4-RD group; 1 salivary gland sample, 1 pancreatic and 1 lung samples from the NAI control group; and 3 salivary gland samples from the SS control group) or because sample quality was poor (1 salivary gland sample from the NAI control group). Two extra slides were rejected because they were controls for the IgG4-related prostatitis sample.

After exclusion of the 11 faulty slides, 9 IgG4-RD cases (5 salivary gland biopsies, 1 pleural biopsy, 1 lung biopsy, 1 pancreatic biopsy and 1 retroperitoneal fibrosis sample), 15 NAI controls (10 salivary gland biopsies, 1 pleural biopsy, 1 lung biopsy, 1 pancreatic biopsy and 2 samples of fibrotic nodules), and 3 SS controls (salivary gland samples) were available. **Table I** and **Table II** show characteristics for IgG4-RD patients and NAI controls, respectively. Because too few SS cases were available, they were not included in further results (characteristics of SS patients are shown in **Table III**).

Males represented 66.7% in each group (IgG4-RD patients and NAI controls). The mean age at biopsy was similar in each group (65 ± 12 years in the IgG4-RD group and 67 ± 14 years in the NAI control group, $p = 0.86$). Regarding IgG4-RD organ involvement, 44.4% had 1 organ involved, 22.2% had 2 organs involved and 33.3% had 3 organs involved. Mean serum IgG4 concentrations were 2.8 ± 1.1 g/L in the IgG4-RD group, and 33.3% of IgG4-RD patients had an eosinophil count above 0.4G/L (8.3% in the NAI control group; data was unavailable for 3 patients). The median ACR/EULAR score was 24 (interquartile range [IQR] = 23 – 28). There was no significant difference regarding the inflammatory status between our groups: CRP levels

were greater than 5mg/L in 55.6% of IgG4-RD patients and 30% of NAI controls (CRP levels were unavailable for 5 NAI patients) ($p = 0.63$). For 2 IgG4-RD patients with elevated CRP levels at the time of biopsy (16 and 105mg/L), the elevation was possibly due to concomitant synovitis in the first case and infectious prostatitis in the second. One IgG4-RD patient with well-defined pancreatic IgG4-RD according to the ICDC for AIP had rheumatoid arthritis with positive antinuclear antibodies (ANA) (1:2560), anti-SSA ($> 8U$; normal range $< 1U$) and anti-CCP antibodies. He did not have sufficient ACR/EULAR scoring for SS. Among our NAI controls, 1 patient had positive anti-MPO antibodies (2.9U, normal range $< 1U$, multiplex flow immunoassay, BioPlex 2200®, Bio-Rad), negative anti-neutrophil cytoplasmic antibodies (ANCA) on fluorescence, without symptoms for ANCA-associated-vasculitis. Two patients (1 IgG4-RD patient and 1 NAI patient) were under immune-suppressive therapy (systemic corticosteroids, methotrexate) at the time of biopsy. The median number of treatments undertaken to achieve remission of IgG4-RD was 1 (IQR = 0 – 3).

| Patient | Sex | Age at biopsy | Significant history | IgG4-RD manifestations | Organ biopsied | Serum IgG4 at diagnosis (g/L) | ACR/EULAR score (IgG4-RD)* | Treatments |
|---------|-----|---------------|---|---|--------------------------|-------------------------------|----------------------------|-------------------|
| 1 | M | 77 | Dilated cardiomyopathy | AIP, sialadenitis, sclerosing cholangitis | Minor salivary gland | 2.9 | 20 | SCS |
| 2 | M | 74 | Rheumatoid arthritis, lymphocytic colitis | AIP | Pancreas | 2.8 | 23 | RTX |
| 3 | F | 72 | Hypertension, hypothyroidism, breast and uterine cancer | AIP, sialadenitis, lymphadenopathy | Minor salivary gland | 5.5 | 63 | SCS |
| 4 | M | 64 | Hypertension, type 2 diabetes, ischemic heart disease, colon adenocarcinoma | Pleural, rétro-péritonéal fibrosis | Pleura | 2.0 | 23 | SCS, RTX, AZA, MA |
| 5 | F | 62 | Asthma | Sialadenitis, lymphadenopathy | Submandibular gland | 3.1 | 24 | None |
| 6 | M | 47 | Hypertension, pericarditis | Pulmonary, posterior uveitis | Lung | 2.2 | 24 | SCS, RTX, MA |
| 7 | F | 74 | Unsignificant | Sialadenitis | Submandibular gland | 2.4 | 28 | None |
| 8 | M | 44 | Unsignificant | Retroperitoneal fibrosis | Retroperitoneal fibrosis | 1.7 | 28 | SCS, MTX, surgery |
| 9 | M | 75 | Unsignificant | Sialadenitis | Submandibular gland | 2.6 | 26 | None |

Table I: Characteristics of IgG4-related disease patients. M: male, F: female, AIP: autoimmune pancreatitis, SCS: systemic corticosteroids, RTX: rituximab, AZA: azathioprine, MA: mycophenolic acid, MTX: methotrexate, *threshold (specificity 99.2%, sensitivity 85.5%) ≥ 20

| Patient | Sex | Age at biopsy | Significant history | Organ biopsied |
|----------------|------------|----------------------|--|--------------------------|
| 10 | F | 81 | Type 2 diabetes, hypertension, gout, colon high-grade dysplasia polyp | Minor salivary gland |
| 11 | M | 68 | Type 2 diabetes, hypertension, intraductal papillary mucinous neoplasm, psoriasis | Pancreas |
| 12 | M | 69 | Hypertension, idiopathic sensitive neuropathy | Minor salivary gland |
| 13 | M | 70 | Mixed liver cirrhosis, cardiac and renal insufficiency | Minor salivary gland |
| 14 | M | 83 | Infectious endocarditis, renal insufficiency | Pleura |
| 15 | M | 68 | Central retinal artery occlusion, uveitis, metastatic cutaneous fibrosarcoma, idiopathic chronic pachymeningitis | Minor salivary gland |
| 16 | M | 63 | Type 2 diabetes, hypertension | Minor salivary gland |
| 17 | F | 45 | Unsignificant | Minor salivary gland |
| 18 | F | 59 | Unsignificant | Lung |
| 19 | F | 72 | Type 2 diabetes, hypertension | Minor salivary gland |
| 20 | F | 72 | Breast adenocarcinoma, unicentric Castleman's disease *, paraneoplastic pemphigus* | Minor salivary gland |
| 21 | M | 69 | Colon carcinoma, lung adenocarcinoma | Retroperitoneal fibrosis |
| 22 | M | 32 | Unsignificant | Retroperitoneal fibrosis |
| 23 | M | 78 | Type 2 diabetes, hypertension, IgA type multiple myeloma, prostatic neoplasm**, acute ischemic anterior optic neuropathy | Minor salivary gland |
| 24 | M | 77 | Hypertension, idiopathic membranoproliferative glomerulonephritis | Minor salivary gland |

Table II: Characteristics of non-auto-immune controls. M: male, F: female, *9 years prior to biopsy and of full recovery, **cured

| Patient | Sex | Age at biopsy | Significant history | SS manifestations | Organ biopsied | ACR/EULAR score (SS)* | Treatments |
|---------|-----|---------------|---|----------------------------------|----------------------|-----------------------|------------|
| 25 | F | 72 | Chronic heart failure | Sicca symptoms, pulmonary, renal | Minor salivary gland | 6 | SCS, RTX |
| 26 | F | 58 | Unsignificant | Sicca symptoms, pulmonary | Minor salivary gland | 5 | HC |
| 27 | M | 66 | Hyperferritinemia, ischemic heart disease | Sicca symptoms | Minor salivary gland | 6 | SCS |

Table III: Characteristics of Sjögren's syndrome controls. *M: male, F: female, SS: Sjögren's syndrome, SCS: systemic corticosteroids, RTX: rituximab, HC: hydroxychloroquine, *threshold (specificity 95%, specificity 96%) ≥ 4*

2. Immunohistochemical expression of DNAJB9

Figure 1 is an example of each staining intensity score category. **Figure 2** shows the immunohistochemical aspect of our positive control, with glomerular homogenous, smudgy, extracellular deposits.

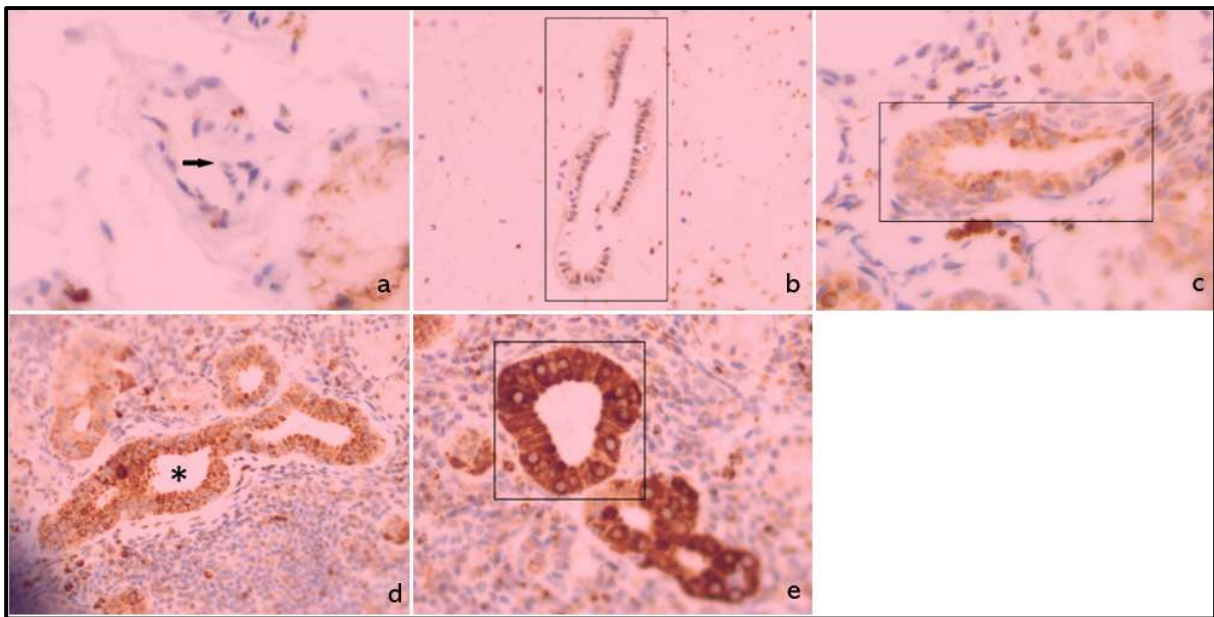


Figure 1: Representation of the immunohistochemical scoring system for DNAJB9. Immunohistochemistry of DNAJB9 showing (a) negative endothelial cells (arrow) in a blood vessel of a minor salivary gland biopsy from a non-auto-immune control patient, (b) very weak staining of ductal epithelial cells in a pancreatic duct (rectangle) from a patient with IgG4-related autoimmune pancreatitis, (c) weak, (d) moderate and (e) strong staining of ductal epithelial cells in salivary glands from patients with IgG4-related sialadenitis. (a-e, magnification x400.)

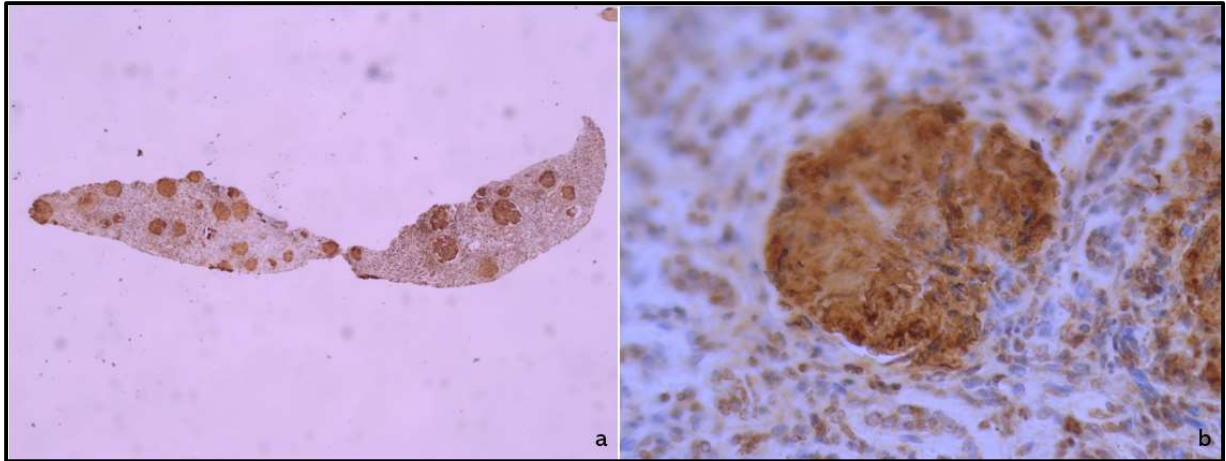


Figure 2: Immunohistochemistry of DNAJB9 revealing fibrillary glomerulonephritis. Immunohistochemistry of DNAJB9 in a renal sample (a,b) showing strong glomerular, smudgy, extracellular staining suggestive of fibrillary glomerulonephritis. (a, magnification x20, b, magnification x400.)

Among all patients included, the median intensity score for endothelial cells was 0 (IQR = 0 – 0) in the IgG4-RD group and 2 (IQR = 1 – 2) in NAI controls ($p < 0.01$). For ductal epithelial cells, the median intensity score was 3 (IQR = 2 – 3) in each group ($p = 0.60$). For glandular epithelial cells it was 2 (IQR = 2 – 2) in each group ($p = 0.26$). For inflammatory cells (including lymphocytes, plasma cells and undifferentiated inflammatory cells) it was 3 (IQR = 2 – 3) in each group ($p = 0.74$). These results are represented in **Figure 3**.

We did the same analysis considering salivary gland alone (5 IgG4-RD patients and 10 NAI controls) to put aside the potential influence of tissue type on IHC intensity. The median intensity score for endothelial cells was 0 (IQR = 0 – 0) in the IgG4-RD group and 1 (IQR = 1 – 2) in NAI controls ($p = 0.01$); for inflammatory cells, the median intensity score was 2 (IQR = 2 – 2) in the IgG4-RD group and 3 (IQR = 2 – 3) in NAI controls ($p = 0.11$); for ductal epithelial cells it was 3 (IQR = 3 – 3) in each group ($p = 0.76$); and for glandular epithelial cells, the median intensity score was 2 (IQR = 2 – 2) in each group ($p = 0.11$) (data not shown). We could not draw conclusions regarding smooth muscle cells, myoepithelial cells, mesothelial

cells, pneumocytes, respiratory epithelial cells and fibroblasts due to the small number of patients available (data not shown).

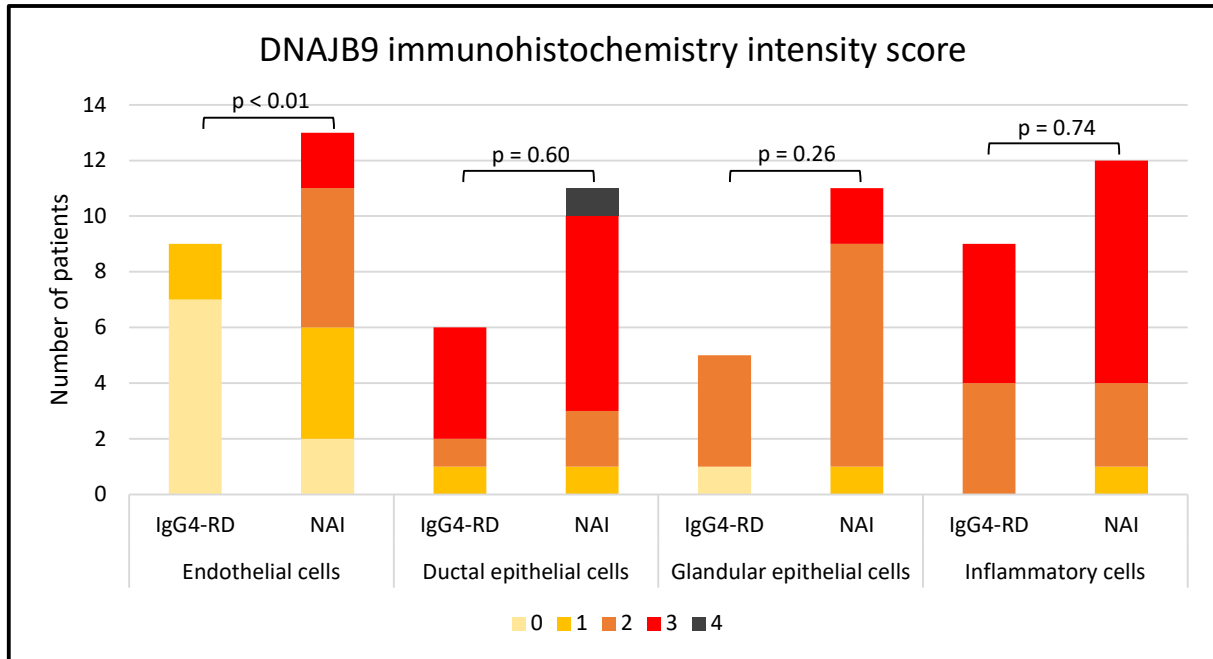


Figure 3: Representation of DNJAB9 immunohistochemistry results. Illustration of DNAJB9 immunohistochemical staining intensity in endothelial cells, ductal and glandular epithelial cells, and inflammatory cells in patients with IgG4-related disease and non-auto-immune controls. Medians of staining intensity were compared using a Mann-Whitney test.

Furthermore, in IgG4-RD patients, endothelial staining intensity was negatively correlated with serum IgG4 concentrations ($r = -0.72$, $p = 0.03$) and the number of treatments required to achieve disease remission ($r = -0.70$, $p = 0.04$).

All our patients displayed a cytoplasmic and granular IHC staining pattern, regardless of their study group. **Table IV** describes the staining pattern in cells of interest in normal tissue from NAI patients.

Secondarily, we identified 3 patients with positive plasma cells, 2 of which belonged to the IgG4-RD group and 1 to the NAI control group (**Appendix VII**).

| Organ system | Location and characteristics of DNAJB9 staining |
|---------------------------|---|
| Digestive system | |
| Pancreas | Acinar cells (very weak, granular, CS), ductal epithelial cells (very weak, granular, CS) |
| Salivary gland | Acinar cells (weak to moderate, granular, CS), ductal epithelial cells (weak to strong, granular, CS) |
| Respiratory system | |
| Lung | Epithelial respiratory cells (moderate, granular, CS), pneumocytes (moderate, granular, CS) |
| Pleura | Fibroblasts (negative to moderate, granular, CS), mesothelial cells (negative) |
| Immune system | |
| Immune cells | Lymphocytes (negative to moderate, granular, CS), plasma cells (moderate, granular, CS) |
| Vascular system | |
| Vessels | Endothelial cells (negative to moderate, granular, CS) |

Table IV: Immunochemical expression of DNAJB9 in normal tissue. CS: cytoplasmic staining

DISCUSSION

This study was to our knowledge the first to explore tissular expression of DNAJB9 in IgG4-RD.

In this study, all patients displayed a cytoplasmic and granular staining pattern, showing there was no difference in DNAJB9 expression patterns between IgG4-RD patients and NAI controls. This aspect coincided with the description of immunochemical expression of DNAJB9 in normal tissue by *Nasr et al.*(11) (**Appendix VI**).

We also evidenced that there was no significant difference in terms of staining intensity for DNAJB9 between IgG4-RD patients and NAI controls regarding ductal and glandular epithelial cells and inflammatory cells. The same conclusion was drawn as to salivary glands alone.

However, endothelial cells showed significantly weaker staining in the IgG4-RD group compared to NAI controls, suggesting possible loss of DNAJB9 expression in those cells. Staining intensity seemed to be negatively correlated with baseline concentrations of serum IgG4 and the number of treatments needed to achieve disease remission.

Our findings regarding endothelial cells seemed paradoxical as IgG4-RD is known to be an inflammatory disease, and inflammatory vascular lesions have been evidenced, with adventitial fibrous thickening and infiltration of inflammatory cells(15), and we expected to show higher expressions of DNAJB9 in IgG4-RD tissues compared to NAI controls.

Although the mechanisms underlying DNAJB9 loss of expression in endothelial cells remain unclear, the endostatin pathway could be partially explanatory. Inflammation leads to tissue hypoxemia which ultimately activates angiogenesis(16). Under such stressful conditions, endothelial cells increase their secretory activity(17), leading to higher levels of ER-resident chaperones and co-chaperones, such as DNJAB9, minimising the amount of misfolded proteins, and contributing to perpetual inflammation via the secretion of pro-inflammatory cytokines(17,18). DNAJB9 was shown to be strongly induced in endothelial cells submitted to stress(18). In IgG4-RD, endostatin – a protein with profibrotic and antiangiogenic activities – was shown to be significantly upregulated compared to healthy controls(19). The implications of endostatin could explain lowered levels of DNJAB9 through its antiangiogenic action, reducing the need for DNAJB9 activation.

In IgG4-RD, pro-inflammatory pathways are upregulated but the implication of regulatory T lymphocytes has also been evidenced(20). Investigating the immune and inflammatory regulations in IgG4-RD at an endothelial level may help to explain our findings and understand their pathological implications in IgG4-RD pathophysiology and management.

High baseline concentrations of serum IgG4 have been associated with a greater risk of disease relapse(21). A negative correlation between endothelial staining intensity and serum

IgG4 levels may suggest lower expression of DNAJB9 in endothelial cells in patients with a higher risk of disease relapse.

Our study did have limitations. First, we studied a small number of patients, no auto-immune controls, and mixed organ types. Two patients were under immune suppressive therapy (SCS, MTX) at the time of biopsy. Secondly, we submitted a semi-quantitative intensity scoring system that might be examined further by considering more patients and a single organ type – or even patients with IgG4-related arterial disease – and auto-immune controls, to see if endothelial loss of expression for DNAJB9 is specific to IgG4-RD. Increasing the number of patients may evidence differences in DNJAB9 tissue expression in other cell types.

CONCLUSIONS

Immunohistochemical expression of DNAJB9 was studied in patients with IgG4-related disease and in non-auto-immune controls, regarding its staining pattern and intensity. No difference was shown in terms of staining pattern between the two groups, and there was no difference in terms of staining intensity in the different tissue cell types, except in endothelial cells in which it was significantly weaker in the IgG4-related disease group. These results were unexpected as IgG4-related disease is an inflammatory disorder with the potential for vascular involvement and we anticipated overexpression of DNAJB9 in IgG4-related disease. The pathophysiological mechanisms underlying this potential loss of expression of DNAJB9 in endothelial cells in IgG4-related disease remain unclear. Our scoring system could be applied to additional patients to confirm its usefulness and determine the specificity of our findings. The endostatin and regulatory T cell pathways could be ones to explore further.

CONCLUSION

Nous avons étudié l'expression immunohistochimique du DNAJB9 en termes d'aspect et d'intensité de marquage chez des patients atteints de la maladie associée aux IgG4 et chez des témoins non auto-immuns. Il n'y avait aucune différence d'aspect de marquage entre les deux groupes, ni d'intensité de marquage des différents types cellulaires, hormis pour les cellules endothéliales pour lesquelles l'intensité était significativement plus faible dans le groupe de patients avec une maladie associée aux IgG4 par rapport aux témoins non auto-immuns. Ces résultats étaient inattendus étant donné le caractère inflammatoire de cette pathologie et son possible tropisme vasculaire et nous nous attendions à une surexpression du DNAJB9. Les mécanismes physiopathologiques sous-tendant cette potentielle perte d'expression du DNAJB9 dans les cellules endothéliales dans la maladie associée aux IgG4 restent incertains. Notre système de gradation d'intensité de marquage pourrait être appliqué à davantage de patients pour confirmer son utilité et vérifier la spécificité de nos résultats. Les voies de l'endostatine et des lymphocytes T régulateurs pourraient être explorées davantage.

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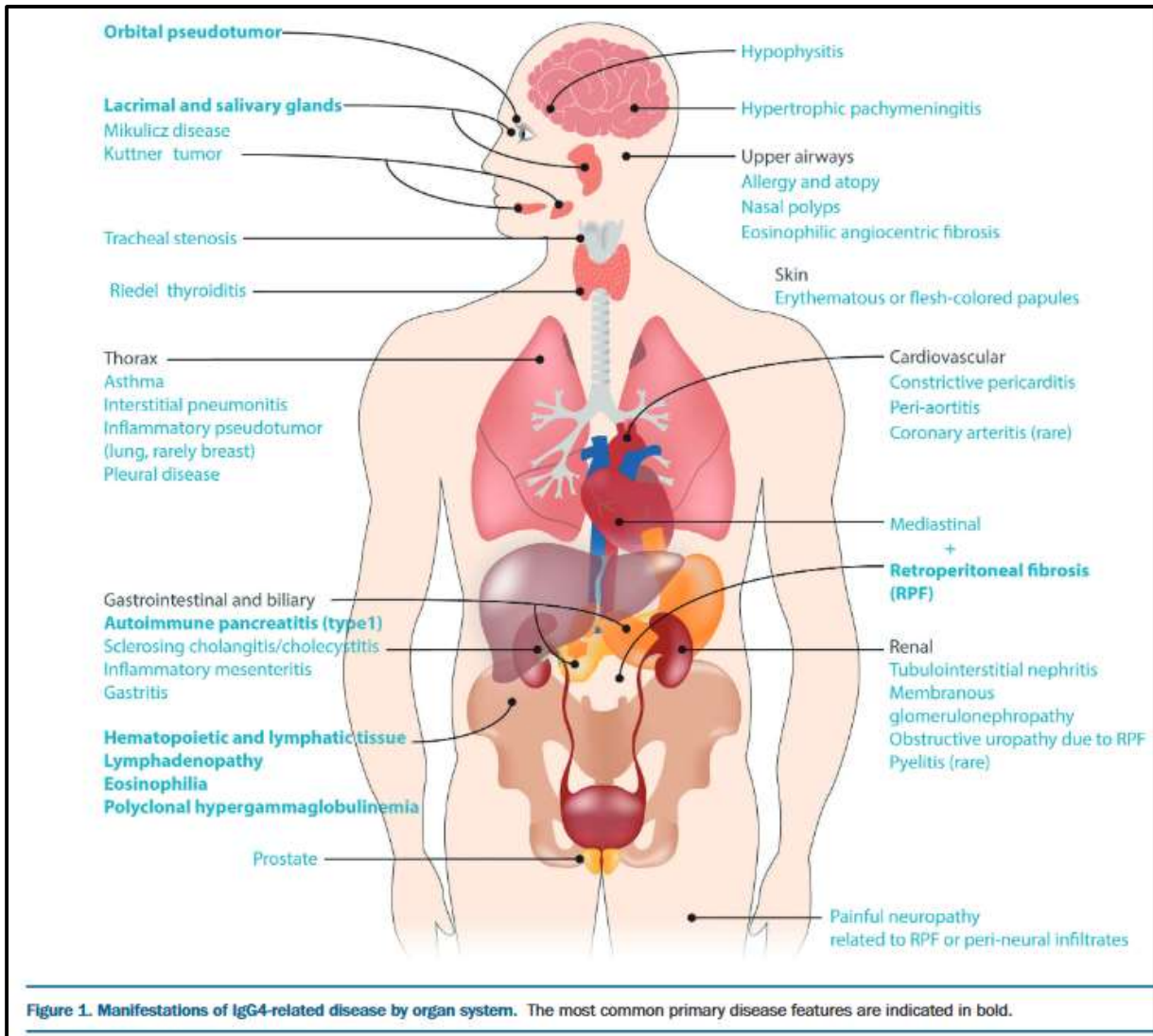
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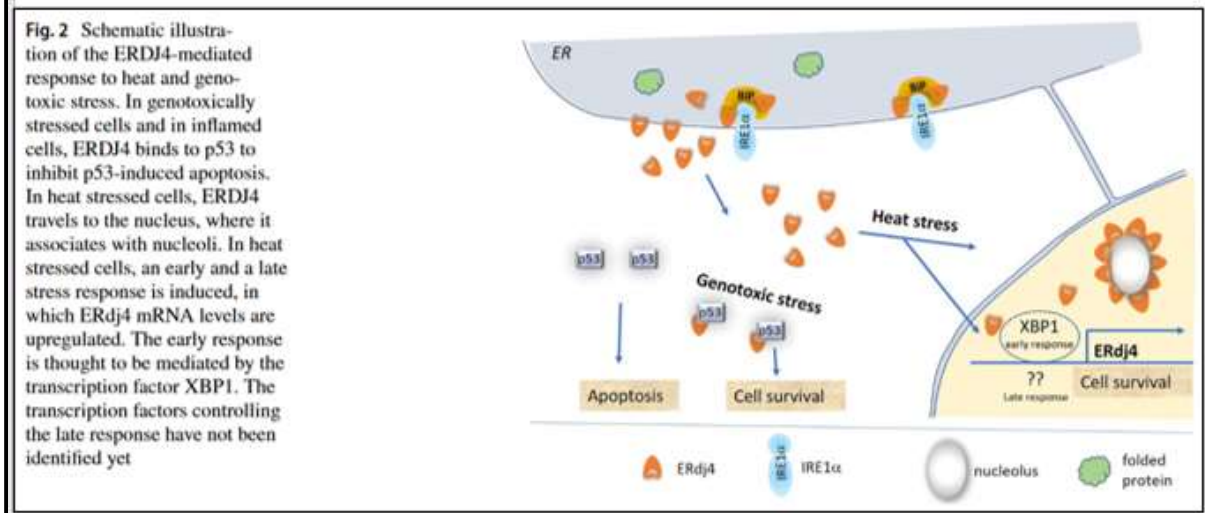
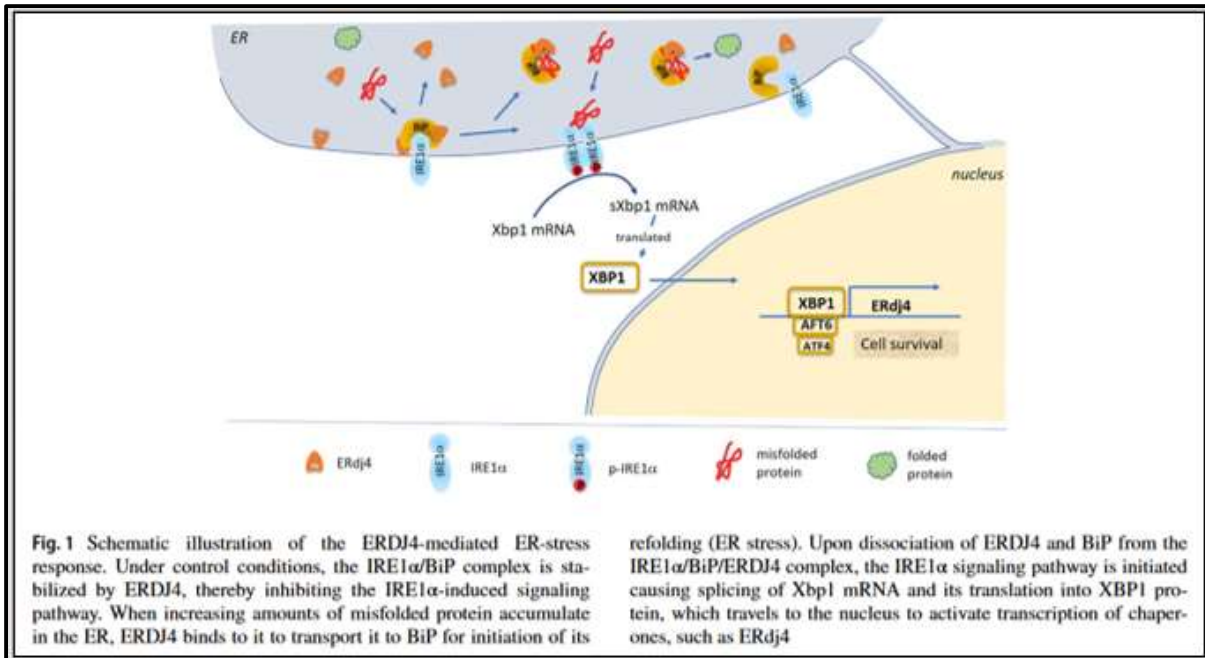
ACKNOWLEDGEMENTS

The authors wish to thank the Immunology Department of *Gabriel Montpied* Teaching Hospital, Clermont-Ferrand, particularly Prof. B. Evrard and Dr. J. Cosme for helping with data collection. We thank also the Internal Medicine Departments of *Gabriel Montpied* and *Estaing* Teaching Hospitals, particularly Prof. M. André, Prof. M. Ruivard, Dr. V. Rieu, Dr. P. Smets, Dr. V. Grobost, Dr. G. Le Guenno, Dr. L. Olagne, Dr. H. Lobbes and Dr. M. Clément for their help with patient recruitment. We thank the Pathology Departments of *Gabriel Montpied* and *Estaing* Teaching Hospitals, Clermont-Ferrand, for their support, particularly Mr. W. Glaziou for his much-appreciated help with sample screening, Mrs. M. Pirol and Mrs. M-P. Terras for their work with sample processing, Dr. C. Darcha for supporting this study and Junior Dr. V. Mattutzu for her help with pathology slides reading. Finally, we thank M. B. Pereira for his invaluable help with statistics.

APPENDICES



Appendix I: Manifestations of IgG4-related disease(22)



Appendix II: Illustration of DNAJB9 functions in cell survival. Implications of DNAJB9 in (Fig.1) ER-stress response and (Fig.2) heat and genotoxic stress response(23).

Table 2. The 2020 Revised comprehensive diagnostic (RCD) criteria for IgG4-RD.

| |
|---|
| <p>[Item 1] clinical and radiological features One or more organs show diffuse or localized swelling or a mass or nodule characteristic of IgG4-RD. In single organ involvement, lymph node swelling is omitted.</p> <p>[Item 2] serological diagnosis Serum IgG4 levels greater than 135 mg/dl.</p> <p>[Item 3] pathological diagnosis Positivity for two of the following three criteria: ① Dense lymphocyte and plasma cell infiltration with fibrosis. ② Ratio of IgG4-positive plasma cells /IgG-positive cells greater than 40% and the number of IgG4-positive plasma cells greater than 10 per high powered field ③ Typical tissue fibrosis, particularly storiform fibrosis, or obliterative phlebitis</p> <p>Diagnosis: Definite: 1) +2) +3) Probable: 1) +3): Possible: 1) +2)</p> |
|---|

Explanatory note 1: Combination of organ-specific diagnostic criteria*.

Patients with a possible or probable diagnosis by comprehensive diagnostic criteria who fulfill the organ-specific criteria for IgG4-RD are regarded as being definite for IgG4-RD.

*Diagnostic criteria according to the IgG4-related organ:

① International consensus diagnostic criteria for autoimmune pancreatitis⁷⁾, ② IgG4-related lacrimal gland, saliva adenitis diagnostic criteria⁸⁾, ③ Diagnostic criteria for IgG4-related kidney disease⁹⁾, ④ Clinical diagnostic criteria of IgG4-related sclerosing cholangitis 2012¹⁰⁾, ⑤ Diagnostic criteria for IgG4-related ophthalmic disease¹¹⁾, ⑥ Diagnostic criteria for IgG4-related respiratory disease¹²⁾, ⑦ Diagnostic criteria for IgG4-related large periarteritis/periarteritis and retroperitoneal fibrosis¹³⁾.

Explanatory note 2: exclusion diagnosis.

1) It is important to acquire tissue samples from each involved organ to distinguish malignant tumors (e.g. cancer, malignant lymphoma) and similar benign conditions (e.g. Sjögren syndrome, primary sclerosing cholangitis, multicentric Castleman's disease, secondary retroperitoneal fibrosis, granulomatosis with polyangiitis, sarcoidosis, eosinophilic granulomatosis with polyangiitis).

2) It is important to exclude an infectious- or inflammation-related disease in patients with high fever, highly elevated CRP and neutrophilia.

Explanatory note 3: pathologic diagnosis.

1) The numbers of IgG4-positive cells are usually more abundant in resected organs and partially enucleated tissue than in tissue samples obtained by needle biopsy or endoscopic biopsy. Thus, it is important to not be too particular about cell number and to provide a precise judgment.

2) Storiform fibrosis is defined as spindle-shaped cells, inflammatory cells and fine collagen fibers forming a flowing arrangement. Obliterative phlebitis is defined as fibrous venous obliteration with inflammatory cells. Both are helpful for a diagnosis of IgG4-RD. ① and ③ without ② can only be applied in a case with poor IgG4 and/or IgG staining.

Explanatory note 4: steroid reactivity.

Steroid trial is not recommended. However, if patients do not respond to initial steroid therapy, the diagnosis should be reconsidered.

Appendix III: The 2020 Revised Comprehensive Diagnostic criteria for IgG4-related disease(24)

Table 4. The 2019 American College of Rheumatology/European League Against Rheumatism classification criteria for IgG4-related disease

| Step | Categorical assessment or numeric weight |
|---|--|
| Step 1. Entry criteria | Yes† or No |
| Characteristic* clinical or radiologic involvement of a typical organ (e.g., pancreas, salivary glands, bile ducts, orbits, kidney, lung, aorta, retroperitoneum, pachymeninges, or thyroid gland [Riedel's thyroiditis]) OR pathologic evidence of an inflammatory process accompanied by a lymphoplasmacytic infiltrate of uncertain etiology in one of these same organs | |
| Step 2. Exclusion criteria: domains and items‡ | Yes or No§ |
| <i>Clinical</i> | |
| Fever | |
| No objective response to glucocorticoids | |
| <i>Serologic</i> | |
| Leukopenia and thrombocytopenia with no explanation | |
| Peripheral eosinophilia | |
| Positive antineutrophil cytoplasmic antibody (specifically against proteinase 3 or myeloperoxidase) | |
| Positive SSA/Ro or SSB/La antibody | |
| Positive double-stranded DNA, RNP, or Sm antibody | |
| Other disease-specific autoantibody | |
| Cryoglobulinemia | |
| <i>Radiologic</i> | |
| Known radiologic findings suspicious for malignancy or infection that have not been sufficiently investigated | |
| Rapid radiologic progression | |
| Long bone abnormalities consistent with Erdheim-Chester disease | |
| Splenomegaly | |
| <i>Pathologic</i> | |
| Cellular infiltrates suggesting malignancy that have not been sufficiently evaluated | |
| Markers consistent with inflammatory myofibroblastic tumor | |
| Prominent neutrophilic inflammation | |
| Necrotizing vasculitis | |
| Prominent necrosis | |
| Primarily granulomatous inflammation | |
| Pathologic features of macrophage/histiocytic disorder | |
| <i>Known diagnosis of the following:</i> | |
| Multicentric Castleman's disease | |
| Crohn's disease or ulcerative colitis (if only pancreaticobiliary disease is present) | |
| Hashimoto thyroiditis (if only the thyroid is affected) | |
| If case meets entry criteria and does not meet any exclusion criteria, proceed to step 3. | |
| Step 3. Inclusion criteria: domains and items¶ | |
| <i>Histopathology</i> | |
| Uninformative biopsy | 0 |
| Dense lymphocytic infiltrate | +4 |
| Dense lymphocytic infiltrate and obliterative phlebitis | +6 |
| Dense lymphocytic infiltrate and storiform fibrosis with or without obliterative phlebitis | +13 |
| <i>Immunostaining#</i> | 0–16, as follows: |
| | Assigned weight is 0 if the IgG4+ : IgG+ ratio is 0–40% or indeterminate and the number of IgG4+ cells/hpf is 0–9.** |
| | Assigned weight is 7 if 1) the IgG4+ : IgG+ ratio is ≥41% and the number of IgG4+ cells/hpf is 0–9 or indeterminate; or 2) the IgG4+ : IgG+ ratio is 0–40% or indeterminate and the number of IgG4+ cells/hpf is ≥10 or indeterminate. |
| | Assigned weight is 14 if 1) the IgG4+ : IgG+ ratio is 41–70% and the number of IgG4+ cells/hpf is ≥10; or 2) the IgG4+ : IgG+ ratio is ≥71% and the number of IgG4+ cells/hpf is 10–50. |
| | Assigned weight is 16 if the IgG4+ : IgG+ ratio is ≥71% and the number of IgG4+ cells/hpf is ≥51. |

(Continued)

*Appendix IV: The 2019 ACR/EULAR classification criteria for IgG4-related disease(25)
(continued on the next page)*

| Step | Categorical assessment or numeric weight |
|--|--|
| Serum IgG4 concentration | |
| Normal or not checked | 0 |
| > Normal but <2x upper limit of normal | +4 |
| 2–5x upper limit of normal | +6 |
| >5x upper limit of normal | +11 |
| Bilateral lacrimal, parotid, sublingual, and submandibular glands | |
| No set of glands involved | 0 |
| One set of glands involved | +6 |
| Two or more sets of glands involved | +14 |
| Chest | |
| Not checked or neither of the items listed is present | 0 |
| Peribronchovascular and septal thickening | +4 |
| Paravertebral band-like soft tissue in the thorax | +10 |
| Pancreas and biliary tree | |
| Not checked or none of the items listed is present | 0 |
| Diffuse pancreas enlargement (loss of lobulations) | +8 |
| Diffuse pancreas enlargement and capsule-like rim with decreased enhancement | +11 |
| Pancreas (either of above) and biliary tree involvement | +19 |
| Kidney | |
| Not checked or none of the items listed is present | 0 |
| Hypocomplementemia | +6 |
| Renal pelvis thickening/soft tissue | +8 |
| Bilateral renal cortex low-density areas | +10 |
| Retroperitoneum | |
| Not checked or neither of the items listed is present | 0 |
| Diffuse thickening of the abdominal aortic wall | +4 |
| Circumferential or anterolateral soft tissue around the infrarenal aorta or iliac arteries | +8 |
| Step 4: Total inclusion points | |
| A case meets the classification criteria for IgG4-RD if the entry criteria are met, no exclusion criteria are present, and the total points is ≥20. | |
| * Refers to enlargement or tumor-like mass in an affected organ except in 1) the bile ducts, where narrowing tends to occur, 2) the aorta, where wall thickening or aneurysmal dilatation is typical, and 3) the lungs, where thickening of the bronchovascular bundles is common. | |
| † If entry criteria are not fulfilled, the patient cannot be further considered for classification as having IgG4-related disease (IgG4-RD). | |
| ‡ Assessment for the presence of exclusion criteria should be individualized depending on a patient's clinical scenario. | |
| § If exclusion criteria are met, the patient cannot be further considered for classification as having IgG4-RD. | |
| ¶ Only the highest-weighted item in each domain is scored. | |
| # Biopsies from lymph nodes, mucosal surfaces of the gastrointestinal tract, and skin are not acceptable for use in weighting the immunostaining domain. | |
| ** "Indeterminate" refers to a situation in which the pathologist is unable to clearly quantify the number of positively staining cells within an infiltrate yet can still ascertain that the number of cells is at least 10/high-power field (hpf). For a number of reasons, most often pertaining to the quality of the immunostain, pathologists are sometimes unable to count the number of IgG4+ plasma cells with precision yet even so, can be confident in grouping cases into the appropriate immunostaining result category. | |

Appendix IV: The 2019 ACR/EULAR classification criteria for IgG4-related disease(25)

TABLE 1. Diagnosis of Definitive and Probable Type 1 AIP Using ICDC

| Diagnosis | Primary Basis for Diagnosis | Imaging Evidence | Collateral Evidence |
|-----------------------|-----------------------------|--------------------------|---|
| Definitive type 1 AIP | Histology | Typical/indeterminate | Histologically confirmed LPSP (level 1 H) |
| | Imaging | Typical Indeterminate | Any non-D level 1/level 2 Two or more from level 1 (+level 2 D*) |
| | Response to steroid | Indeterminate | Level 1 S/OOI + Rt or level 1 D + level 2 S/OOI/H + Rt |
| Probable type 1 AIP | | Indeterminate | Level 2 S/OOI/H + Rt |

*Level 2 D is counted as level 1 in this setting.

TABLE 2. Level 1 and Level 2 Criteria for Type 1 AIP

| Criterion | Level 1 | Level 2 |
|-----------------------------|--|--|
| P Parenchymal imaging | Typical; Diffuse enlargement with delayed enhancement (sometimes associated with rim-like enhancement) | Indeterminate (including atypical [†]); Segmental/focal enlargement with delayed enhancement |
| D Ductal imaging (ERP) | Long (>1/3 length of the main pancreatic duct) or multiple strictures without marked upstream dilatation | Segmental/focal narrowing without marked upstream dilatation (duct size, <5 mm) |
| S Serology | IgG4, >2× upper limit of normal value | IgG4, 1–2× upper limit of normal value |
| OOI Other organ involvement | a or b a. Histology of extrapancreatic organs Any three of the following: (1) Marked lymphoplasmacytic infiltration with fibrosis and without granulocytic infiltration (2) Storiform fibrosis (3) Obliterative phlebitis (4) Abundant (>10 cells/HIPF) IgG4-positive cells b. Typical radiological evidence At least one of the following: (1) Segmental/multiple proximal (hilar/intrahepatic) or proximal and distal bile duct stricture (2) Retroperitoneal fibrosis | a or b a. Histology of extrapancreatic organs including endoscopic biopsies of bile duct [‡] : Both of the following: (1) Marked lymphoplasmacytic infiltration without granulocytic infiltration (2) Abundant (>10 cells/HIPF) IgG4-positive cells b. Physical or radiological evidence At least one of the following: (1) Symmetrically enlarged salivary/lachrymal glands (2) Radiological evidence of renal involvement described in association with AIP |
| H Histology of the pancreas | LPSP (core biopsy/resection) At least 3 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (>10 cells/HIPF) IgG4-positive cells | LPSP (core biopsy) Any 2 of the following: (1) Periductal lymphoplasmacytic infiltrate without granulocytic infiltration (2) Obliterative phlebitis (3) Storiform fibrosis (4) Abundant (>10 cells/HIPF) IgG4-positive cells |
| Response to steroid (Rt)* | Diagnostic steroid trial Rapid (≤2 wk) radiologically demonstrable resolution or marked improvement in pancreatic/extrapancreatic manifestations | |

*Diagnostic steroid trial should be conducted carefully by pancreatologists with caveats (see text) only after negative workup for cancer including endoscopic ultrasound-guided fine needle aspiration.

[†]Atypical: Some AIP cases may show low-density mass, pancreatic ductal dilatation, or distal atrophy. Such atypical imaging findings in patients with obstructive jaundice and/or pancreatic mass are highly suggestive of pancreatic cancer. Such patients should be managed as pancreatic cancer unless there is strong collateral evidence for AIP, and a thorough workup for cancer is negative (see algorithm).

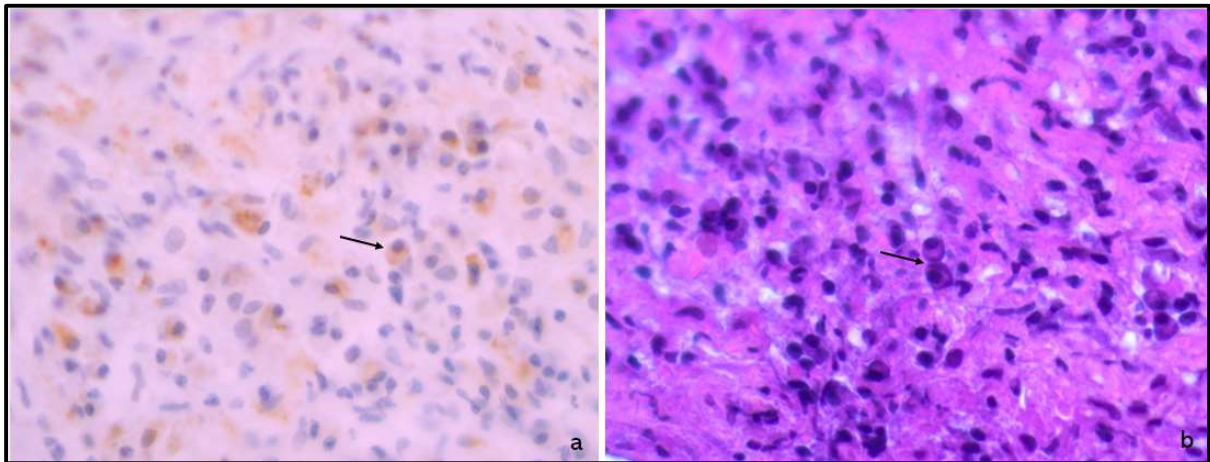
[‡]Endoscopic biopsy of duodenal papilla is a useful adjunctive method because ampulla often is involved pathologically in AIP.

Appendix V: International Consensus Diagnostic Criteria for Autoimmune Pancreatitis(26)

Table 2. Immunohistochemical expression of DNAJB9 in normal tissue

| Organ system | Location of DNAJB9 positivity |
|------------------------------------|--|
| Central nervous system | |
| Cerebral cortex | Neurons (variably fine to coarsely granular CS) |
| Digestive system | |
| Stomach, small and large intestine | Entero-endocrine cells (coarsely granular CS); Epithelial cells (finely granular CS) |
| Liver | Sinusoidal endothelial cells (variably fine to coarsely granular CS) |
| Pancreas | Acinar cells (variably fine to coarsely granular CS) |
| Respiratory system | |
| Lung | Epithelial cells (finely granular CS) |
| Endocrine system | |
| Pituitary (adenohypophysis) | Subset of adenohypophyseal cells (variably finely to coarsely granular CS) |
| Thyroid | Epithelial cells (finely granular CS) |
| Parathyroid | Endothelial cells (finely granular CS) |
| Adrenal | Secretory cells of medulla (variably fine to coarsely granular CS) |
| Lymphoid system | |
| Spleen | Splenic cord (variably finely granular CS) |
| Tonsil | Small lymphocytes (variably finely granular CS) |
| Lymph node | Small lymphocytes of paracortex (variably finely granular CS) |
| Thymus | Small lymphocytes in the medulla (finely granular CS) |
| Female reproductive system | |
| Endometrium, Fallopian tube | Epithelial cells (finely granular CS) |
| Ovary | Endothelial cells (finely granular CS) |
| Placenta (term) | Syncytiotrophoblasts (variably finely granular CS) |
| Mammary glands | Alveolar and ductal epithelial cells and myoepithelial cells (finely granular CS) |
| Male reproductive system | |
| Testis | Primary spermatocytes and spermatids (finely granular CS) |
| Prostate | Fibromuscular stroma (finely granular CS) |
| Urinary System | |
| Kidney | Tubular epithelium and podocytes and mesangial cells and endothelial cells (variably finely granular CS), vascular smooth muscle cells |
| Ureter | Smooth muscle cells and urothelium (finely granular CS) |
| Skeletal muscle | (finely granular CS) |

CS, cytoplasmic staining.



Appendix VII: Plasma cells. (a) immunohistochemistry and (b) hematoxylin and eosin stain showing plasma cells (arrows) in a pleural sample from a patient with IgG4-related pleural involvement. (a,b, magnification x400.)

SERMENT D'HIPPOCRATE

Au moment d'être admis(e) à exercer la médecine, je promets et je jure d'être fidèle aux lois de l'honneur et de la probité.

Mon premier souci sera de rétablir, de préserver ou de promouvoir la santé dans tous ses éléments, physiques et mentaux, individuels et sociaux.

Je respecterai toutes les personnes, leur autonomie et leur volonté, sans aucune discrimination selon leur état ou leurs convictions. J'interviendrai pour les protéger si elles sont affaiblies, vulnérables ou menacées dans leur intégrité ou leur dignité. Même sous la contrainte, je ne ferai pas usage de mes connaissances contre les lois de l'humanité.

J'informerai les patients des décisions envisagées, de leurs raisons et de leurs conséquences.

Je ne tromperai jamais leur confiance et n'exploiterai pas le pouvoir hérité des circonstances pour forcer les consciences.

Je donnerai mes soins à l'indigent et à quiconque me les demandera. Je ne me laisserai pas influencer par la soif du gain ou la recherche de la gloire.

Admis(e) dans l'intimité des personnes, je tairai les secrets qui me seront confiés. Reçu(e) à l'intérieur des maisons, je respecterai les secrets des foyers et ma conduite ne servira pas à corrompre les mœurs.

Je ferai tout pour soulager les souffrances. Je ne prolongerai pas abusivement les agonies. Je ne provoquerai jamais la mort délibérément.

Je préserverai l'indépendance nécessaire à l'accomplissement de ma mission. Je n'entreprendrai rien qui dépasse mes compétences. Je les entretiendrai et les perfectionnerai pour assurer au mieux les services qui me seront demandés.

J'apporterai mon aide à mes confrères ainsi qu'à leurs familles dans l'adversité.

Que les hommes et mes confrères m'accordent leur estime si je suis fidèle à mes promesses ; que je sois déshonoré(e) et méprisé(e) si j'y manque.

Nom, Prénom

Signature

SERMENT D'HIPPOCRATE

En présence des Maîtres de cette FACULTE et de mes chers CONDISCIPLES, je promets et je jure d'être fidèle aux lois de l'Honneur et de la Probité dans l'exercice de la Médecine.

Je donnerai mes soins gratuits à l'indigent et je n'exigerai jamais un salaire au-dessus de mon travail. Admis dans l'intérieur des maisons, mes yeux ne verront pas ce qui s'y passe, ma langue taira les secrets qui me seront confiés et mon état ne servira pas à corrompre les mœurs ni à favoriser le crime.

Respectueux et reconnaissant envers mes MAÎTRES, je rendrai à leurs enfants l'instruction que j'ai reçue de leurs pères.

Que les HOMMES m'accordent leur estime si je suis fidèle à mes promesses. Que je sois couvert d'OPPROBRE et méprisé de mes confrères si j'y manque.

Nom, Prénom

Signature

Immunohistochemical expression of DNAJB9 in IgG4-related disease

Introduction. Immunoglobulin G4-related disease (IgG4-RD) is a rare immune-mediated condition with expansion of plasmablasts/plasma cells and possible upregulation of *DNAJB9*. *DNAJB9* (a co-chaperone protein that governs the integrity and functions of cells) was described as a specific marker for fibrillary glomerulonephritis, a rare proliferative form of glomerulonephritis displaying histological similarities with IgG4-related kidney disease. *DNAJB9* may be a putative autoantigen. We aimed to study the immunohistochemical expression of *DNAJB9* in IgG4-RD to explore its role in disease pathophysiology.

Methods. We selected patients with definite IgG4-RD (RCD, ACR/EULAR or ICDC classification criteria), non-auto-immune (NAI) controls and Sjögren's syndrome controls, and produced tissue slides for anti-*DNAJB9* immunohistochemistry on which we performed double reading and applied our intensity scoring system ranging from 0 to 4. Our positive control was a renal sample suggestive of fibrillary glomerulonephritis.

Results. We studied the tissue slides of 9 IgG4-RD patients and 15 NAI controls (including salivary gland, pancreatic, pulmonary, pleural, and retroperitoneal fibrotic tissue). Median intensity for endothelial cells was 0 in IgG4-RD and 2 in NAI controls ($p < 0.01$). In each group, it was 3 for ductal epithelial cells ($p = 0.60$), 2 for glandular epithelial cells ($p = 0.26$) and 3 for inflammatory cells ($p = 0.74$). All samples displayed a cytoplasmic and granular immunohistochemical staining pattern, with no difference between the 2 groups.

Conclusions. There was no difference in terms of staining intensity for *DNAJB9* between IgG4-RD patients and NAI controls regarding ductal and glandular epithelial cells and inflammatory cells. Surprisingly, endothelial cells showed significantly weaker staining in IgG4-RD, suggesting loss of *DNAJB9* expression in those cells. The underlying mechanisms are unclear but the endostatin and regulatory T cell pathways could be partially explanatory and raise interest for further exploration.

Key words: IgG4-related disease, *DNAJB9*, endothelial cells, immunohistochemical intensity scoring system