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Circulating total and IgG4^{+} plasmablasts for the diagnosis of type 1 Autoimmune Pancreatitis

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SOMMARIO

OBIETTIVI: La pancreatite autoimmune (AIP) di tipo 1 rappresenta una possibile manifestazione della malattia IgG4-relata (IgG4-RD), la cui diagnosi è a volte ancora difficile da formulare. I plasmablasti circolanti sono stati proposti come possibile marcatore sierico sensibile e specifico, anche se non è chiaro se possano differenziare tra AIP di tipo 1, di tipo 2 e altre condizioni pancreatiche. Obiettivo dello studio è stato valutare il valore diagnostico dei livelli di plasmablasti totali e di $IgG4^+$ circolanti nell'AIP e in altre malattie del pancreas.

METODI: Sono stati arruolati prospetticamente da Gennaio 2018 a Maggio 2020 pazienti con diagnosi di AIP di tipo 1 attiva (Gruppo AIP-1, n = 19) secondo i criteri diagnostici internazionali, insieme a pazienti affetti da AIP attiva di tipo 2 (AIP 2) o non altrimenti specificata (AIP NOS) (Gruppo AIP 2/NOS, n=10) adenocarcinoma pancreatico (Gruppo PDAC, n=17), pancreatite cronica (Gruppo CP, n=20), neoplasia mucinosa papillare intraduttale (IPMN) o iperenzimemia pancreatica cronica asintomatica (CAPH) (Gruppo IPMN-CAPH, n=21) come gruppi di controllo. La citometria a flusso è stata utilizzata per misurare la conta dei plasmablasti totali e dei plasmablasti $IgG4^+$ nel sangue periferico utilizzando i seguenti anticorpi per le cellule $CD45^+CD19^+CD38^{hi}CD20^-CD24^-CD27^+$ e $CD45^+CD19^+CD38^{hi}CD20^-CD24^-CD27^+$ $IgG4^+$. Inoltre, queste cellule sono state misurate nei pazienti con AIP dopo un mese di terapia, dopo 2-4 mesi dalla fine del trattamento e dopo un anno dall'arruolamento.

RISULTATI: Il gruppo AIP-1 ha mostrato livelli significativamente più alti di plasmablasti totali (media 6365, SD 5522 cellule/mL) rispetto sia al gruppo PDAC (media 3216, SD 1228 cellule/mL) ($p=0.0067$) che al gruppo IPMN-CAPH (media 1065, SD 781 cellule/mL)($p<0.0001$). Tuttavia, la conta dei plasmablasti totali non era significativamente differente nel gruppo AIP 1 rispetto a AIP 2/NOS. I plasmablasti $IgG4^+$ hanno invece distinto il tipo 1 da tutti gli altri gruppi, incluso l'AIP di tipo 2, con una sensibilità dell'80% e una specificità del 97% utilizzando un cut-off di 210 cellule $IgG4^+$/mL. Inoltre, i livelli di plasmablasti $IgG4^+$ diminuiscono significativamente dopo la terapia.

CONCLUSIONI: Solo i plasmablasti $IgG4^+$ sembrano essere un biomarcatore potenzialmente utile per differenziare l'AIP di tipo 1 dall'AIP di tipo 2/NOS e da altre condizioni pancreatiche, in particolare il PDAC.

ABSTRACT

OBJECTIVES: Type 1 autoimmune pancreatitis (AIP) is a manifestation of IgG4-related disease (IgG4-RD) whose diagnosis is still challenging. Circulating plasmablasts seem to be a useful tool in this setting. Whether they may differentiate type 1, type 2 AIP, and other pancreatic conditions is still unknown. The aim of the study was to investigate the diagnostic value of circulating total and $IgG4^+$ plasmablasts levels in AIP and other pancreatic diseases.

METHODS: Patients diagnosed with active type 1 AIP (Group AIP-1, n=19) according to International Consensus Diagnostic Criteria from January 2018 to May 2020, were prospectively enrolled together with patients suffering from active type 2 or not otherwise specified AIP (Group AIP 2/NOS, n=10) pancreatic adenocarcinoma (Group PDAC, n=17), chronic pancreatitis (Group CP, n=20), intraductal papillary mucinous neoplasia (IPMN) or chronic asymptomatic pancreatic hyperenzymemia (CAPH) (Group IPMN-CAPH, n=21) as control groups. Flow cytometry was used to measure total plasmablasts' and $IgG4^+$ plasmablasts' counts by gating peripheral blood for $CD45^+CD19^+CD38^{hi}CD20^-CD24^-CD27^+$ cells and $CD45^+CD19^+CD38^{hi}$ $CD20^-CD24^-CD27^+IgG4^+$ cells. Moreover, these cells were measured after one month of therapy, after 2-4 months from the end of treatment, and after one year from the enrollment in AIP patients groups only.

RESULTS: Group AIP-1 showed significantly higher levels of total plasmablasts (mean 6365, SD 5522 cells/mL) compared to both Group PDAC (mean 3216, SD 1228 cells/mL)($p=0.0067$) and Group IPMN-CAPH (mean 1065, SD 781 cells/mL)($p<0.0001$). However, plasmablasts count was not significantly higher in Group AIP 1 compared to AIP 2/NOS. $IgG4^+$ plasmablasts distinguished type 1 AIP from all other pancreatic disorders with a sensitivity of 80% and a specificity of 97% using a cut-off of 210 $IgG4^+$ cells/mL. $IgG4^+$ plasmablasts significantly decrease after steroids.

CONCLUSIONS: Only $IgG4^+$ plasmablasts may be a potentially useful biomarker to differentiate type 1 AIP from type 2 or NOS AIP and other pancreatic conditions, especially PDAC.

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1.INTRODUCTION

1.1 Autoimmune pancreatitis

Autoimmune pancreatitis (AIP) is a chronic inflammatory disease affecting a portion or the whole organ that responds dramatically to systemic steroids and may relapse unpredictably (1).

According to the histological features, two forms of the disease can be identified: type 1 and type 2. Although both forms display similar clinical and imaging characteristics, only type 1 AIP shows elevated serum IgG4 levels and abundant $IgG4^+$ plasma cells infiltration at histopathology as hallmarks of the disease(2). However, since the histology is neither always feasible nor diagnostic, the differentiation between the two forms may be achieved following the International Consensus Diagnostic Criteria (ICDC) (3), based on a combination of different variables (imaging, serum levels of IgG4, involvement of other organs, histology and response to steroids). When a diagnosis of type 1 or 2 cannot be achieved, the disease can be classified as not otherwise specified (NOS)(3).

The disease can begin with obstructive jaundice with or without a pancreatic mass. Other symptoms include acute pancreatitis, weight loss, abdominal pain, steatorrhea, and diabetes(4-6).

According to the amount of pancreas involved, a diffuse and a focal form may be identified (5, 7). The focal form may mimic a pancreatic ductal adenocarcinoma (PDAC), and in this case, a biopsy is highly recommended to rule out a diagnosis of pancreatic cancer(8).

A dramatic response to steroid therapy has been reported in AIP. Therefore, in a clinical, histological, and radiological scenario suggesting AIP, treatment with steroids

is indicated and represents a diagnostic criterion of the disease (3, 9, 10).

1.1.1 Terminology

AIP has been differently named over time:

- *primary chronic pancreatitis* (11);
- *non-alcoholic duct destructive chronic pancreatitis*(12);
- *sclerosing pancreato-cholangitis*(13);
- *granulomatous pancreatitis*(14, 15);
- *sclerosing pancreatitis*(16);
- *lymphoplasmacytic sclerosing pancreatitis*(17);
- *idiopathic fibrosing pancreatitis*(18).

Currently, the name universally accepted is *autoimmune pancreatitis*. Japanese authors proposed this term in the mid-nineties to emphasize the rapid and important response of this disease to steroid therapy(19). However, no organ-specific auto-antibodies have been identified to allow to definitely classified autoimmune pancreatitis as an immune-mediated disease.

1.1.2 Epidemiology

The incidence and prevalence of AIP are still unknown. Data from a nationwide survey in Japan, where the disease is much more common than in western countries, show a prevalence of 2.2/100.000 and an incidence of 0.9/100.000 people(20).

AIP accounts for 4-6% of all chronic pancreatitis(21, 22). Moreover, approximately 5% of pancreatic resections performed in the suspicion of pancreatic cancer showed a final diagnosis of pancreatic cancer (23, 24).

AIP usually affects male patients more than 50 years old, with an M/F ratio of 2:1(25), even though peculiar epidemiological differences are detected between subtypes of the disease as described later.

1.1.3 Histology

According to histology features, two histopathologic subtypes of AIP can be identified(2, 25-27):

- *Type 1 AIP*, also called *lymphoplasmacytic sclerosing pancreatitis* (*LPSP*), is characterized histologically by lymphoplasmacytic infiltration affecting the tissue either diffusely or in a patchy manner, storiform fibrosis, obliterative phlebitis, and increased numbers of $IgG4^+$ plasma cells at immunochemistry. Type 1 AIP is included in IgG4- related diseases(28), with possible involvement of other organs (more often biliary tree, kidney, salivary and lachrymal glands, retroperitoneum).
- *Type 2 AIP*, also called *idiopathic duct-centric chronic pancreatitis* (*IDCP*), is characterized by the presence of few or no $IgG4^+$ plasma cells in pancreatic specimens, combined with the presence of granulocytic epithelial lesions (GELs). GELs are characterized by the infiltration of neutrophilic granulocytes in the duct epithelial lining, causing degenerative epithelial changes, often including epithelial detachment. Association with inflammatory bowel diseases, in particular ulcerative colitis, has been described.

Table 1 shows a comparison between the histological findings of the two subtypes of AIP (from Song TJ et al.(29)

Table 1. Histological findings of type 1 and type 2 AIP.

Histological features	Type 1 AIP	Type 2 AIP
Periductal lymphoplasmacytic infiltrate	Present	Present
Storiform fibrosis	Prominent	Occasional
Obliterative phlebitis	Present	Rare
IgG4$^+$ plasma cell infiltration	Marked	Scant or absent
GEL	Absent	Present
Lobular neutrophilic infiltrate	Absent	Present
Inflammation of peripancreatic fat	Possible	Rare

1.1.4 Serology

Elevation in serum total IgG, IgG4, and antinuclear antibody titers is commonly seen in AIP. However, serum IgG4 elevation is the single best marker of type 1 AIP. A twofold increase in serum IgG4 levels using a cut-off value of 135 mg/dL is strongly suggestive of type 1 AIP with a sensitivity of 65% and a specificity of 98%(30). Serum IgG4 levels occasionally slightly increased in type 2 AIP. In addition, a mild increase of IgG4 in approximately 7% of patients with pancreatic cancer and can lead to a misdiagnosis of AIP(31, 32).

Therefore, elevation in serological markers is not sufficient to diagnose AIP unless seen in the setting of typical imaging findings.

1.1.5 Imaging

The classical imaging features of AIP involve both the pancreatic parenchyma and the pancreatic duct system. Parenchymal involvement may be diffuse (diffuse form, 40-

50% of cases) or focal (focal form, 50-60% of cases), but it can also be highly variable(7).

International Consensus Diagnostic Criteria (ICDC)(3) and European Guidelines on IgG4-related digestive disease(33) report the following parenchymal changes as suggestive of AIP:

(i) Diffuse or (multi-) focal enlargement with loss of the normal multilobulated pattern ('sausage-like' shape)

(ii) Altered imaging characteristics, such as lower signal intensity (SI)/echogenicity on unenhanced T1-w MRI/(E)US, respectively, moderately higher SI on T2-w MRI, impeded diffusion on MRI and increased 18 F-fluorodeoxyglucose (FDG)-uptake on PET-CT compared with normal parenchyma. After contrast media injection, there is dotted/patchy enhancement in the late arterial/pancreatic phase that progressively increases towards the later vascular phases.

(iii) Rectangular shape of the tail ('cut-tail sign').

(iv) Thin peripancreatic edematous rim or progressively enhancing true capsule.

Ductal changes suggestive of AIP are:

(i) Long-segment (i.e.,>1/3 of the length) or multifocal main pancreatic duct (MPD) involvement (narrowing or vanishing) without upstream dilatation or other signs of obstructive pancreatitis.

(ii) Skip lesions, i.e. >2 involved MPD-segments separated by a normal MPD-segment.

(iii) 'Duct-penetrating' (i.e., visible MPD- and/or common bile duct (CBD)-lumen) and 'icicle' (i.e., a progressive decrease of MPD-diameter) signs within an enlarged parenchymal area.

In diffuse form, the diagnosis is generally easy with imaging. Potential differential diagnoses are pancreatic lymphoma, extremely rare, and acute pancreatitis, which has a different clinical presentation and imaging features(34). The real issue in clinical practice is the diagnosis of focal AIP because the risk of misdiagnosing a PDAC is high. A correct diagnosis is crucial to avoid pancreatic resections in inflammatory masses responsive to steroids and the use of steroids in PDAC patients. The differential diagnosis is extremely challenging in the absence of extrapancreatic involvement or serum IgG4 elevation. Therefore, new serological markers and/or pancreatic biopsy are necessary for a correct diagnosis(35).

1.1.6 Diagnosis

The diagnosis of AIP is based on the International Diagnostic Consensus Criteria (ICDC), elaborated in 2011 by an international consensus(3). These criteria make it possible to diagnose a subtype of pancreatitis even in the absence of the histological specimen, not always feasible nor diagnostic. These criteria are based on the combination of one or more of the five cardinal features of AIP:

- Parenchymal and ductal imaging;
- Serology;
- Other Organ Involvement (OOI);
- Histology;
- Response to steroid therapy

These criteria are divided into *level 1* (more reliable) and *level 2* (less reliable). **Table 2** shows the diagnostic criteria for type 1 AIP, and their combination allows a *definitive* or *probable* diagnosis of type 1 AIP (**Table 3**).

Table 2. Level 1 and Level 2 Criteria for type 1 AIP according to ICDC (from Shimosegawa T et al.(3)

	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 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/HPF) IgG4-positive cells	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/HPF) 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	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/HPF) 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/HPF) IgG4-positive cells
		Diagnostic steroid trial	
	Response to steroid (Rt)*	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.

Table 3. Diagnosis of definitive or probable type 1 AIP according to ICDC (from Shimosegawa T et al.(3)

Diagnosis	Primary Basis for Diagnosis	Imaging Evidence	Collateral Evidence
Definitive type 1 AIP	Histology	Typical/indeterminate	Histologically confirmed LPSP (level 1 H)
	Imaging	Typical	Any non-D level 1/level 2
		Indeterminate	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.

When a diagnosis of type 1 AIP is not definitive nor probable, criteria for the diagnosis of type 2 AIP may be applied. These criteria are the same for imaging and the response to steroid therapy, but they differ in histology and OOI and without the serological criterion (**Table 4**). Combining these criteria allows a *definitive* or *probable* diagnosis of type 2 AIP (**Table 5**).

Table 4. Level 1 and Level 2 Criteria for type 1 AIP (from Shimosegawa T et al.(3)

	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)
OOI	Other organ involvement		Clinically diagnosed inflammatory bowel disease
H	Histology of the pancreas (core biopsy/resection)	IDCP: Both of the following: (1) Granulocytic infiltration of duct wall (GEL) with or without granulocytic acinar inflammation (2) Absent or scant (0–10 cells/HPF) IgG4-positive cells	 Both of the following: (1) Granulocytic and lymphoplasmacytic acinar infiltrate (2) Absent or scant (0–10 cells/HPF) IgG4-positive cells
		Diagnostic steroid trial	
Response to steroid (Rt)*		Rapid (≤2 wk) radiologically demonstrable resolution or marked improvement in 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).

Table 5. Diagnosis of definitive or probable type 1 AIP according to ICDC (from Shimosegawa T et al.(3)

Diagnosis	Imaging Evidence	Collateral Evidence
Definitive type 2 AIP	Typical/indeterminate	Histologically confirmed IDCP (level 1 H) or clinical inflammatory bowel disease + level 2 H + Rt
Probable type 2 AIP	Typical/indeterminate	Level 2 H/clinical inflammatory bowel disease + Rt

When a diagnosis of type 2 AIP cannot be achieved, criteria for the diagnosis of *not otherwise specified* (NOS) AIP have been identified. Therefore, this disease subtype does not correspond to a histological entity but is reserved for those patients who cannot be classified as type 1 or 2 (**Table 6**).

Table 6. Diagnosis of Not Otherwise Specified (NOS) AIP according to ICDC (from Shimosegawa T et al.(3)

Diagnosis	Imaging Evidence	Collateral Evidence (Case With Only D1/2)
AIP–not otherwise specified	Typical/indeterminate	D1/2 + Rt

1.1.7 *Type 1 versus type 2 AIP*

The main clinical and histopathological features of Type 1 and type 2 AIP are summarized in **Table 7** and reported below.

Table 7. Clinical and histological features of type 1 and type 2 AIP.

Histological and clinical findings	***TYPE 1 AIP***	***TYPE 2 AIP***
Prevalence	Asia>EU, US	EU>US>Asia
Mean age at diagnosis	7th decade	5th decade
Male sex	75%	50%
Serum IgG4 elevation	66%	25%
Obstructive jaundice	Often	Often
Abdominal pain	Rare	Common
Pancreas swelling	Common	Common
OOI	50%	No
Lymphoplasmacytic infiltration	++	++
Periductal inflammation	++	++
Storiform fibrosis	++	+
Obliterative phlebitis	++	+
GEL	-	+++
IgG4 tissue staining	>10 cells/hpf	<10 cells/hpf
Response to steroid	100%	100%

Risk of relapse	20-60%	<10%
Associated with IgG4-RD	Yes	No
Ulcerative colitis	Rare	Often

Type 1 AIP is the most common subtype of AIP (60-90% of cases)(29). It occurs more frequently between the sixth and seventh decade of age, mainly affecting the male sex (M/F ratio of 3:1). Sudden asymptomatic jaundice represents a typical sign of disease onset. It can exist as an isolated form or, more often, in the context of IgG4-related disease (IgG4-RD), with inflammatory/fibrosing involvement of various organs and tissues (such as lung, liver, kidney, salivary glands, pancreas)(28). In this setting, the pancreatic involvement is mostly associated with the biliary involvement in the so called "Pancreato-Hepato-Biliary phenotype" of IgG4-RD(36). It is characterized by $IgG4^+$ plasma cells within the involved organs, and it is associated with high circulating levels of serum IgG4(37). Type 1 AIP is a more aggressive disease in recurrences and extrapancreatic organ involvement(38).

Type 2 AIP is less common (5-20% of cases) and almost completely absent in Asian populations. It affects younger patients (typically, third and fourth decades of life) with an equal distribution by sex (M / F ratio of 1:1)(39, 40). Acute pancreatitis is the main clinical symptom. It does not correlate with an increase in serum IgG4, and it is frequently associated with chronic inflammatory bowel disease, in particular ulcerative colitis (present in 20-30% of cases) and Crohn's disease (in a lower percentage of cases)(27). It occurs more frequently as a pseudotumor form and with fewer stigmata of autoimmune disease and relapses after steroids are less common compared to type 1 AIP(41).

1.1.8 Therapy

The therapy of AIP includes an *induction of remission* treatment and a *maintenance* treatment(38). The *induction of remission* therapy is obtained with steroids in near all patients(42).

Following the International consensus for the treatment of AIP (41), indications for treatment are:

1. Symptomatic patients due to pancreatic involvement (e.g., obstructive jaundice, abdominal pain) or involvement of other organs (e.g., jaundice due to biliary strictures).
2. Asymptomatic patients with a persistent pancreatic mass on imaging or patients with liver test abnormalities due to biliary involvement (e.g., IgG4-related sclerosing cholangitis).

Different therapeutic strategies have been proposed to induce remission, from *"low dose"* prednisone (0.2 mg/kg/day)(43), to *"medium dose"* 0.6 mg/kg/day(44), up to *"high dose"* (1 mg/kg/day)(45) for 2-4 weeks, to be subsequently tapered by 5 mg every 1-2 week. However, the standard dosage of steroids has not been established yet because prospective randomized controlled trials are still lacking. The International consensus for the treatment of AIP recommends 0-6-1 mg/kg/day(42). The use of the steroid may be associated with the onset of diabetes, which may require insulin treatment, especially in elderly patients, with a history of glucose intolerance and with extensive inflammatory involvement of the pancreatic gland. Careful control of blood sugar levels, especially post-prandial, a few days after the start of steroid therapy is therefore strongly suggested.

Steroid treatment achieved high remission rates in a Japanese national study (98% in steroid-treated AIP patients vs. 88% in untreated)(44) and the international multicenter

study for AIP (99.6% in type 1 AIP and 92.3% in type 2 AIP)(44). Evaluations of steroid effects by imaging and serological examinations are recommended within two weeks after starting steroid treatment(46). The prospective Korean study suggested estimating "a two-week steroid trial" in selected cases to differentiate AIP from pancreas cancer in difficult cases after a non-conclusive complete workup(47). In cases of poor response to steroids, reevaluation of the diagnosis, including PDAC, is needed. When the diagnosis is confirmed and complete response with a steroid course is achieved, possible approaches are to follow-up patient for relapse or to introduce a maintenance therapy(42).

Disease relapse can occur in about 30% of cases (25). However, this frequency is higher in patients with type 1 than in type 2 AIP.

Disease relapse can also be observed only morphologically on radiological investigations (CT or MRI abdomen) in the absence of specific clinical symptoms or signs. An annual radiological follow-up with an MRI of the abdomen can be therefore suggested. In the presence of disease recurrence, retreatment with steroids inducing remission and maintenance therapy should be started.

Although there is no indication yet for introducing a maintenance therapy, some patients may benefit from maintenance therapy as the disease could relapse(46, 48). Risk factors for relapse have not yet been identified. However, some hypothesized predictors of relapse are(38):

- Remarkably high serum IgG4 levels (such as > x4 UNL) before treatment.
- Persistently high serum IgG4 levels after steroid treatment.
- Diffuse enlargement of the pancreas.
- Proximal type of IgG4-SC.
- Extensive multi-organ involvement (> 2xOOI).

Different pharmacological approaches have been proposed for maintenance therapy: low-dose and long-term steroid, immunomodulatory drugs, and rituximab (38). Since type 1 AIP is the one at greatest risk of recurrence, maintenance therapy has been used mainly in this subgroup of patients.

Japanese experts recommend using low-dose (2.5-7.5 mg/day) long-term (up to 3 years) glucocorticoid maintenance therapy. Stop of maintenance therapy should be planned in cases of radiological and serological improvement, although the duration of maintenance therapy is still debated (46). Several cohort studies seem to justify this treatment, particularly in elderly patients, at greater risk of cancer due to age, and therefore, with contraindications to the use of immunomodulators or biologics(38, 44).

Among the immunomodulators, azathioprine at a 2-2.5 mg/kg/day dosage is the most studied in the cohort group of patients (49, 50). Studies on AIP and IgG4-associated cholangitis reported a 33% risk of relapse three years after starting treatment with azathioprine(49). However, some patients are intolerant to this therapy (about 10%) or cannot be treated with it for history of malignancy within the previous five years(49).

Some studies on type 1 AIP and IgG4-Related disease seem to demonstrate the clinical efficacy of rituximab, an anti-CD20 monoclonal antibody, able to induce depletion of B lymphocytes at a dose of 1 g intravenously. Different therapeutic schedules have been proposed:

1. *Hematological schedule*, which consists of administration of 1 g of rituximab per week for four weeks and then one administration every two months up to 6 months(51, 52).
2. *Rheumatological schedule*, which consists of 2 administrations of 1 g of rituximab 15 days apart (53, 54).

Rituximab is commonly used in case of failure of maintenance therapy with

azathioprine, and it can also be used in cases of steroid intolerance as induction therapy for type 1 AIP(42).

1.2 IgG4-Related Disease (IgG4-RD)

Type 1 AIP can be considered part of the IgG4-related disease, a chronic and relapsing fibro-inflammatory condition characterized by(37, 55):

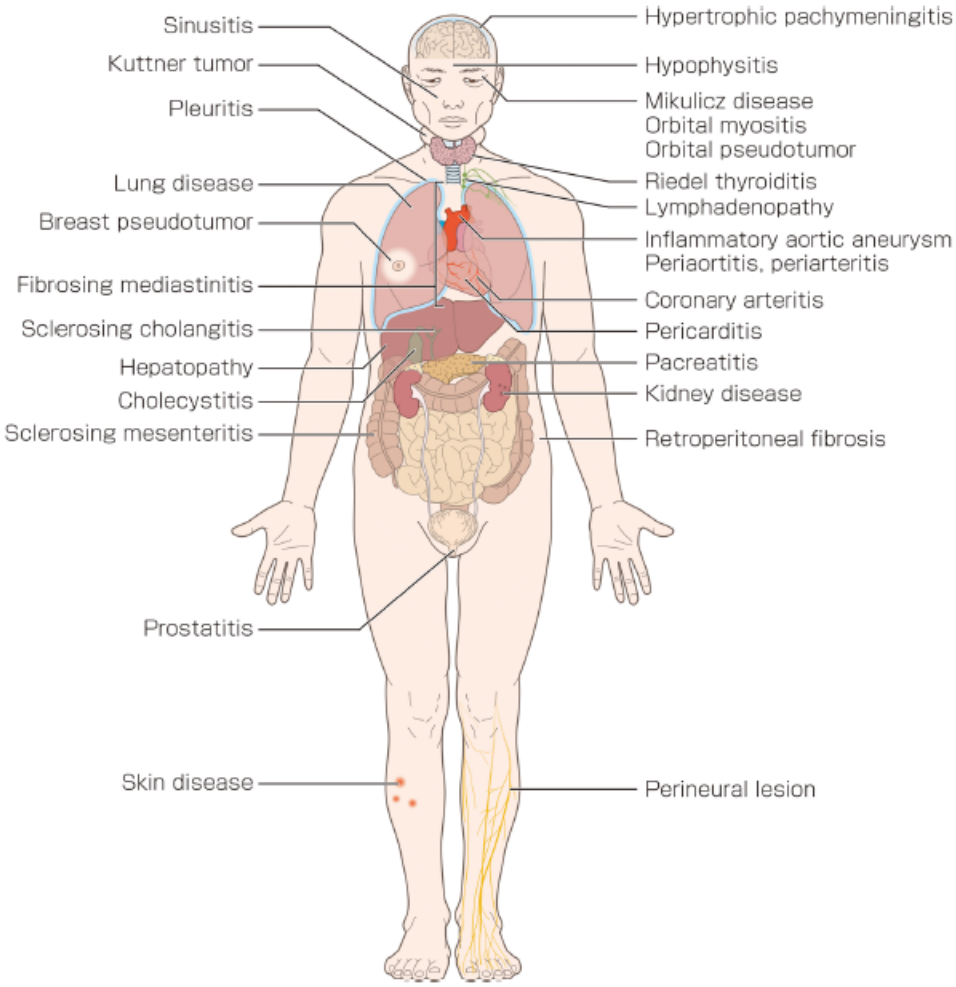
- Single or multiple organs involved by diffuse or localized swelling, masses, nodules, and/or hypertrophic lesions in a wide spectrum of anatomical sites;
- Elevated serum IgG4 levels(≥135 mg/dL)
- Histopathological features that include marked lymphocytic and plasma cell infiltration and storiform fibrosis, $IgG4^+$ plasma cell infiltration ($IgG4/IgG^+$ cell ratio ≥40 % and $IgG4^+$ plasma cells exceed 10/HPF
- Prompt response to steroid therapy.

The sites most frequently affected by this disease are the pancreas, biliary tree, salivary and lacrimal glands, retroperitoneum, central nervous system, thyroid, lungs, liver, kidney, prostate, lymph nodes, and vessels(55-57), but almost every organ can be involved, as showed in **Figure 1**.

Affected patients may present with single nodular lesions or thickening or global swelling of involved organs. The symptoms are extremely variable according to the site involved.

Organomegaly can cause obstruction symptoms or signs (e.g., obstructive jaundice in AIP or IgG4 related sclerosing cholangitis). Persistent inflammation that is not adequately treated leads to progressive fibrosis of the parenchyma with subsequent organ failure(48).

Figure 1. The spectrum of IgG4-Related Disease.



1.2.1 IgG4-Related Disease Responder Index

A score to evaluate the disease activity in IgG4-RD is the "IgG4-Related Disease Responder Index" (IgG4-RD RI). It was introduced in 2012 to obtain an objective confirmation of the disease trend over time (58), particularly in clinical trials.

The basis of the IgG4-RD RI is the evaluation of each affected organ by assigning a score of 0-4, evaluating the presence or absence of symptoms, the need for urgent treatment, and the presence or absence of organ damage, as shown in **Figure 2**.

The attribution of the organ/site score is stratified as follows:

0) Absence of activity

1) Disease activity decreasing but persisting in some degrees

2) Persistent or unchanged disease activity since the last check

3) Presence of a new site or activity of recurrent disease

4) Disease activity worsening despite treatment

As for the symptoms, the IgG4-RD RI simply evaluates the presence (yes) or absence (no) of these.

Urgency is also assessed in terms of presence (yes) or absence (no), where urgency means the need for immediate treatment. In particular, when an organ is defined as positive for urgency and therapy is initiated to reduce disease activity, the score awarded is doubled.

In addition to evaluating the individual organs and sites involved, to calculate the final IgG4-RD RI score, it is necessary to detect the serum levels of IgG4. Serum IgG4 can correlate with disease activity(59, 60), but it is possible to find normal serum IgG4 even in the presence of active disease(61). Also, for serum IgG4 levels, a score from 0 to 4 is assigned as follows:

0) Normal

1) Reduced

2) Persistent

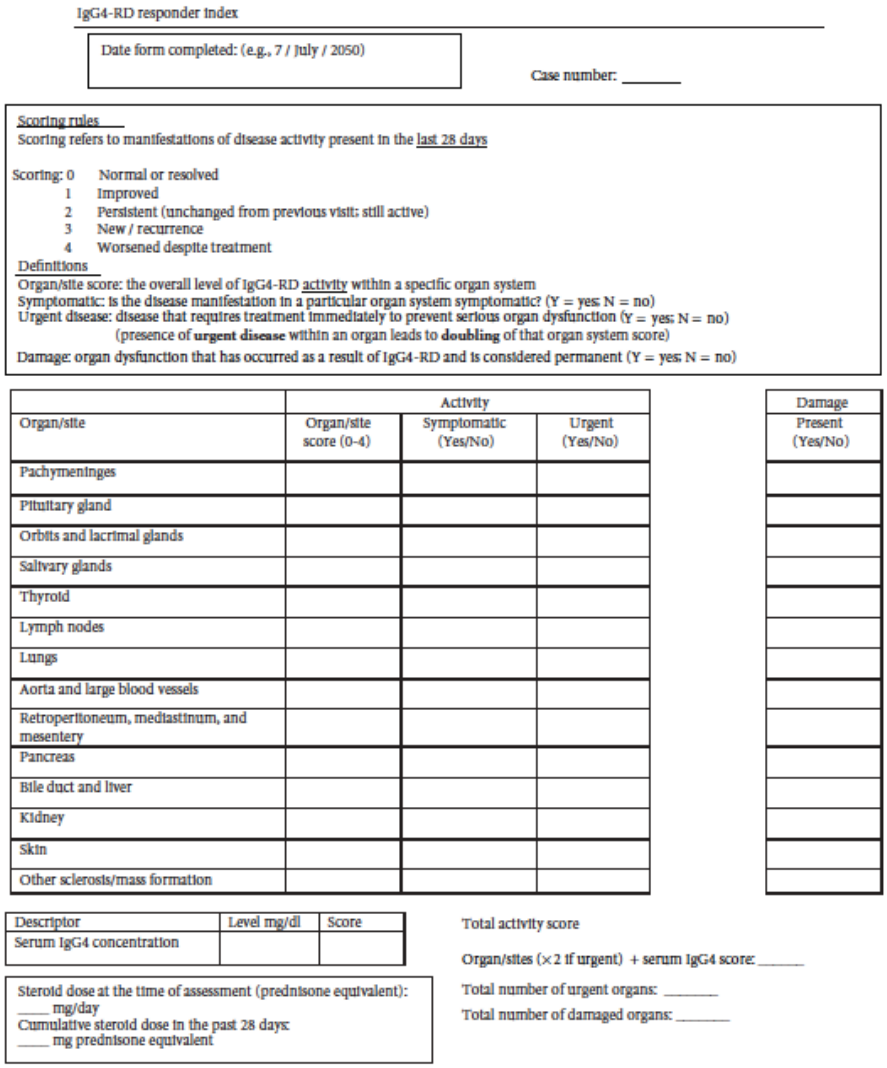
3) New

4) Recurring or worsening despite treatment

The total score of the Responder Index is obtained by adding the scores of the organ evaluations to the score attributed to the serum levels of IgG4, with doubling organ score in case of urgency. The sum of scores can thus be used to compare and evaluate the course of the disease in subsequent temporal checks and becomes a useful tool in

clinical studies. Damage control over time is also important for predictive purposes.

Figure 2. IgG4-Related Disease Responder Index (IgG4-RD RI) Scoring Sheet



IgG4-RD responder index

Date form completed: (e.g., 7 / July / 2050)

Case number: _____

Scoring rules

Scoring refers to manifestations of disease activity present in the last 28 days

Scoring: 0 Normal or resolved
1 Improved
2 Persistent (unchanged from previous visit; still active)
3 New / recurrence
4 Worsened despite treatment

Definitions

Organ/site score: the overall level of IgG4-RD activity within a specific organ system
Symptomatic: is the disease manifestation in a particular organ system symptomatic? (Y = yes; N = no)
Urgent disease: disease that requires treatment immediately to prevent serious organ dysfunction (Y = yes; N = no)
(presence of **urgent disease** within an organ leads to **doubling** of that organ system score)
Damage: organ dysfunction that has occurred as a result of IgG4-RD and is considered permanent (Y = yes; N = no)

	Activity			Damage
Organ/site	Organ/site score (0-4)	Symptomatic (Yes/No)	Urgent (Yes/No)	Present (Yes/No)
Pachymeninges				
Pituitary gland				
Orbits and lacrimal glands				
Salivary glands				
Thyroid				
Lymph nodes				
Lungs				
Aorta and large blood vessels				
Retroperitoneum, mediastinum, and mesentery				
Pancreas				
Bile duct and liver				
Kidney				
Skin				
Other sclerosis/mass formation				

Descriptor	Level mg/dl	Score
Serum IgG4 concentration		

Steroid dose at the time of assessment (prednisone equivalent):
____ mg/day
Cumulative steroid dose in the past 28 days:
____ mg prednisone equivalent

Total activity score

Organ/sites (×2 if urgent) + serum IgG4 score: _____

Total number of urgent organs: _____

Total number of damaged organs: _____

1.2.2 "Lights and shadows" in the type 1 AIP pathophysiology and role of plasmablasts

Recent studies have suggested that the pathogenesis of type 1 AIP is multifactorial, like other immune-mediated disorders. Predisposing genetic factors, disease-specific antigens, and an altered response of innate and cell-mediated immunity are the possible players in the pathogenesis of the disease. Recent reports have showed how

the depletion of the circulating B lymphocytes is effective in the treatment of patients suffering from IgG4-RD, and how this depletion correlates with a reduction in circulating levels of IgG4 but also of IgG1, IgG2, and IgG3(51, 59).

In 2013, some researchers identified the expansion of a subpopulation of B lymphocytes, which expressed $IgG4^{+}$ receptors, both in the blood and in the tissues of patients with IgG4-related cholangitis. This clone was no longer detected after steroid treatment of affected patients(62). One year later, Sumimoto K et al. showed that elevated levels of $CD19^{+}CD24^{high}CD38^{high}$ regulatory B lymphocytes could reduce disease activity and, conversely, elevated circulating levels of $CD19^{+}CD24^{high}CD27^{+}$ regulatory B lymphocytes had a triggering role in the onset of type 1 AIP(63).

These results suggest that B lymphocytes are pivotal actors in the development of the IgG4-RD.

Some Authors have recently focused their attention on the study of plasmablasts. Plasmablasts, a cell population that belongs to the B-cell lineage, are an intermediate stage between activated B-cells and plasma cells and are characterized by the expression of the $CD19^{low}CD38^{+}CD20^{-}CD27^{+}$ surface molecules(64). Their peripheral blood rate is generally very low in healthy people (65, 66), while it increases transiently during infections or vaccination (67). A long-lasting elevation instead occurs in autoimmune diseases, such as inflammatory bowel diseases, rheumatoid arthritis, systemic lupus erythematosus (65, 66, 68-71), and also in the setting of IgG4-RD where treatment with steroids or rituximab has been shown to cause almost disappearance of their circulating levels that may re-emerge after suspension (58, 72, 73). Furthermore, they appear to correlate with serum IgG4 levels and the IgG4-RD RI(58, 72).

Circulating plasmablasts levels of 900 cells/mL showed a sensitivity of 95%, a specificity of 82%, a positive predictive value of 86%, and a negative predictive value of 97% for the diagnosis of IgG4-RD. A cut-off of 2000 cells/mL had a sensitivity of 87%, a specificity of 91%, a positive predictive value of 91% and a negative predictive value of 87%(64) for the diagnosis of IgG4-RD. In addition, $IgG4^+$ serum plasmablasts were measured in a subgroup of IgG4-RD patients. They were found to be approximately 61% of total plasmablasts and appeared to have a close correlation with circulating IgG4 levels (64). Serum levels of plasmablasts could be a promising biomarker for diagnosis, but also for the evaluation of treatment response as well as an early index of possible relapse in IgG4-RD (64, 72, 74-76). However, in all these studies, only patients suffering from IgG4-RD were enrolled, with the pancreas being only seldomly involved.

A common clinical challenge in clinical practice is to differentiate AIP from PDAC because of overlapping clinical and radiological findings. Another clinical need is to differentiate type 1 AIP from type 2/type NOS AIP, since clinical outcome in terms of relapse and maintenance treatment is different.

So far, the only available biomarker is the dosage of serum IgG4 levels that has a 65% sensitivity and a 98% specificity in diagnosing type 1 AIP with a cut-off of 135 mg/dL (30). However, its diagnostic value is limited because it does not apply to the type 2 and NOS forms. Moreover, a mild increase of serum IgG4 levels can be observed in patients with pancreatic adenocarcinoma (PDAC) (31, 32). Furthermore, an increase of Ca 19-9 can be found in some AIP patients (77, 78). In this complex scenario, the availability of a specific and sensitive biomarker helping to differentiate type 1 AIP from either type 2 AIP and PDAC would be of utmost utility, especially considering that misdiagnosis may lead to unnecessary surgery for AIP (79) or to the harmful

administration of high-dosage steroids to PDAC patients.

1.3 Rationale of the study

This study aimed at investigating the diagnostic value of total and $IgG4^+$ circulating plasmablasts levels in the setting of AIP compared with other pathological conditions affecting the pancreas. Secondary endpoint was to evaluate its potential utility as a biomarker of treatment response and/or disease relapse in type 1 AIP.

2.PATIENTS AND METHODS

2.1 Study population

A total of 87 adult patients were prospectively recruited at the Gastroenterology Unit - Pancreas Institute, University of Verona (Verona, Italy) from January 1st, 2018 to May 30th, 2020. They included 19 patients with type 1 AIP (Group AIP-1) as index population; 10 patients with type 2 or NOS AIP (Group AIP-2/NOS), 17 patients affected by PDAC (Group PDAC), 20 cases with chronic pancreatitis (CP) (Group CP), and 21 subjects with intraductal papillary mucinous neoplasia (IPMN) or chronic asymptomatic pancreatic hyperenzymemia (CAPH) (Group IPMN-CAPH) as control groups. Data on the clinical history, alcohol consumption, and smoking habit were collected for each patient, together with a full physical examination and body mass index calculation. The demographic and clinical features of all recruited cases are shown in **Table 8** where, as expected, older age and a prevalence of male gender are evident in Groups AIP-1 and PDAC, while a prevalence of active smokers appears in Groups PDAC and CP.

Table 8. Demographic and clinical features of the study population

	Group AIP-1	**Group AIP-2/NOS**	**Group PDAC**	**Group CP**	**Group IPMN-CAPH**
Number of cases	19	6/4	17	20	21
Mean age in years (SD)	61 (14)	43 (18)	66 (9.1)	43 (15.3)	43 (18)
Male/female ratio (male %)	18:1 (95)	6:4 (60)	13:4 (76.5)	15:5 (75)	12:9 (57)
Mean BMI (SD)	26 (10)	23 (3.9)	21 (3.1)	22 (2.9)	22 (2.9)
Number of active smokers (%)	6 (31)	4 (40)	10 (59)	13 (65)	8 (38)
Number of active drinkers (%)	3 (16)	0 (0)	4 (23)	7 (35)	5 (23)
Median disease duration in months (range)	23.8 (2-192)	4 (2-25)	4 (2-36)	36 (3-216)	24 (6-180)

Abbreviations. *AIP: autoimmune pancreatitis; BMI: body mass index; CAPH: chronic pancreatic hyperenzymemia; CP: chronic pancreatitis; IPMN: intraductal papillary mucinous neoplasia; NOS: not otherwise specified; PDAC: pancreatic adenocarcinoma; SD: standard deviation.*

Diagnosis of AIP was based on ICDC (3). Only patients naïve to or free from any immunosuppressive treatment (steroids, biologics or immunosuppressant drugs) in the last six months were enrolled. All AIP patients underwent treatment with prednisone at a daily dosage of 0.6-1 mg/kg body weight p.o. for one month, tapered by 5 mg/week. As protocol in our Center, AIP patients underwent abdominal magnetic resonance imaging (MRI) at baseline (timepoint 0), after one month of therapy (timepoint 1), after 2-4 months from the end of treatment (timepoint 2), and after one year from the enrollment (timepoint 3). Two expert clinicians (LF and AA) re-evaluated MRI at these timepoints, to assess whether the disease was stable, in remission or relapsed. In addition, disease activity in Group AIP-1 was assessed at each time point by applying the IgG4-RD RI(58). Patients with a disease relapse needing for Rituximab or long-term steroids were considered drop-out at timepoints 1 and 2,

respectively. Moreover, those enrolled during a second relapse of the disease who underwent long-term maintenance treatment with a low dosage of steroids because of contraindication or intolerance to Azathioprine were considered drop-out at timepoint 2. As far as the Group PDAC is regarded, only patients with a biopsy-proven (performed percutaneously or by fine-needle aspiration during endoscopic ultrasound exam) diagnosis were enrolled. Furthermore, only cases diagnosed with CP according to the UEG evidence-based guidelines(33), independently from the etiology and the clinical, radiological, and functional findings, were included in Group CP. Finally, patients with a diagnosis of IPMN of the main pancreatic duct, branch ducts, or mixed, as assessed by MRI cholangiopancreatography, were recruited in Group IPMN-CAPH together with subjects with CAPH, defined as a benign condition characterized by persistent levels of serum amylase and lipase above the normal upper limits for more than six months in the absence of pancreatic disease(80).

AIP patients underwent peripheral blood harvesting at each timepoint to count both total and $IgG4^+$ plasmablasts, and IgG4 serum levels. Control groups were tested only once to assess total and $IgG4^+$ plasmablasts at timepoint 0. Patients were excluded if they had undergone blood or bone marrow donation/transfusion within two months from the screening, if they had a history of hematological disorders, or if they had undergone bone marrow transplantation, pancreatic surgery or had received any immunosuppressant therapy within six months before enrollment.

The study was approved by the local Ethics Committee (protocol number 59133, 30/11/2017), and each enrolled case provided written informed consent to participate.

2.2 Flow cytometric analysis

To assess the number of circulating $CD45^+CD19^+CD38^{hi}CD20^-CD24^-CD27^+$ plasmablasts, a total amount of 100 μl of peripheral blood was incubated with optimized concentrations of the following anti-human fluorochrome-conjugated antibodies: CD19-FITC, CD20-PerCP, CD24-PECy7, CD27-APC, CD38-V450, and CD45-V500 (all by Miltenyi Biotec; Bergisch-Gladbach, Germany) following the manufacturer's instructions. An aliquot of the cell suspension was also stained with anti-human IgG4 Fc-PE (clone HP6025, Southern Biotech; Birmingham, AL, USA) upon fixation and permeabilization steps were carried out with the FIX&PERM kit (Nordic MUbio, Susteren, The Netherlands) in order to assess the number of $IgG4^+$ plasmablasts. Both $CD45^+CD19^+CD38^{hi}CD20^-CD24^-CD27^+$ and $IgG4^+$ plasmablasts were detected by using a BD FACSCanto II system (BD Biosciences; San Jose, CA, USA) and analyzed by the FlowJo software (TreeStar Inc; Ashland, OR, USA). During the hierarchical gating strategy, $CD45^+$ cells were firstly selected in order to include nucleated single cells simultaneously and exclude the presence of erythrocytes from the analysis. Then, $IgG4^+$ cells were detected on $CD27^+$ events derived from $CD19^+CD38^{hi}CD20^-CD24^-$ cells. The absolute count (number of cells/mL) of the two cell populations was calculated by referring to the white blood cell count concomitantly assessed.

2.3 Statistical analysis

Baseline demographic and disease features are presented by using descriptive statistics. As such, continuous variables are described as range and mean ± standard deviation (SD). Both total and $IgG4^+$ plasmablasts rates were compared between groups using the Mann-Whitney test or Wilcoxon test, as appropriate. The count of both total and

$IgG4^+$ plasmablasts was correlated with clinical parameters by applying the Spearman's rank-order or Pearson product-moment correlation when discrete or continuous variables were involved, respectively. The receiver operating characteristic (ROC) analysis was used to identify plasmablasts value (cut-off value) for discrimination between type 1 AIP and all the other control groups. A value of $p<0.05$ was considered statistically significant. Statistical analyses were performed using Prism software version 8.0 (GraphPad software, La Jolla, CA, USA).

3. RESULTS

3.1 Clinical findings of the index population

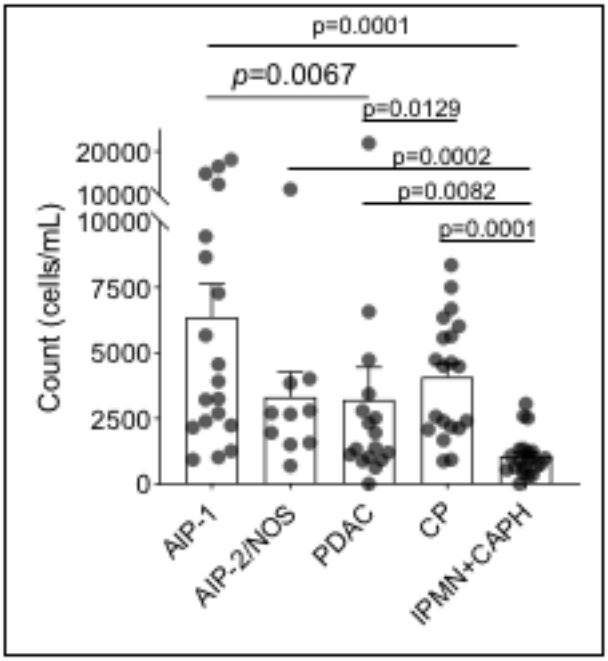
All patients enrolled in Group AIP-1 displayed elevated serum levels of IgG4 at timepoint 0 [mean value: 624.2 (SD 485) mg/dL; normal values <135], that decreased over time [timepoint 1: 277.2 (SD 208), timepoint 2: 379 (SD 295) with one patient excluded because of relapse; timepoint 3: 297 (SD 198), with three cases excluded because of relapse]. The pancreas was the only organ affected in nine patients (47%), whereas a combination of multi-organ involvement was evident in 10 cases (53%), as follows: biliary tree in 9, kidneys in 4, lymph nodes in 2, salivary glands in 2, and aorta in 2. Moreover, six patients (32%) showed a focal pancreatic disease, whereas 13 patients (68%) presented with a diffuse organ disease at imaging (MRI). Nine cases (47%) underwent endoscopic ultrasound with fine-needle aspiration during their diagnostic work-up. The examination evidenced type 1 AIP in five cases, was negative for cancer in three cases and highly suspicious for cancer in one case. This latter patient had undergone pancreaticoduodenectomy and the pathologic examination of the surgical specimen posed a final diagnosis of type 1 AIP. As regards therapy, at enrollment, 14 patients (73%) were naïve to treatment, three cases had been treated with a course of steroid therapy and were at their first relapse, one patient was enrolled during a relapse that occurred one year after the end of treatment of Azathioprine (2.0 mg/kg body weight) and one patient was enrolled during a relapse occurred two years from the end of a cycle with Rituximab (1000 mg iv at day 0 and 15, repeated after 6 months). As regards disease activity, the median value of the IgG4-RD RI at baseline was 10 (IQR 6), which dropped to 2 (IQR 1), 3 (IQR 4), 2 (IQR 2) at timepoints 1, 2, 3, respectively. Finally, 11 out of 19 patients did not complete the follow-up because

three dropped out at time point 1 (two underwent treatment with Rituximab and one patient preferred to decline the steroid therapy), and eight at timepoints 2 or 3 (six were lost at the follow-up, one underwent long-term low-dosage steroid therapy, one showed a relapse and started a new cycle of steroid therapy).

3.2 Total plasmablasts counts at baseline

As evident in **Figure 3** showing the rates of circulating $CD45^{+}CD19^{+}CD38^{hi}CD20^{-}CD24^{-}CD27^{+}$ plasmablasts at baseline across the groups, the mean level in Group AIP-1 was higher than in Group PDAC (mean 6365, SD 5522 cells/mL *versus* mean 3216, SD 1228 cells/mL, respectively; p=0.0067), despite the presence of an outlier in the Group PDAC displaying a very high value (21900 cells/mL). By contrast, if dissecting the data of Group AIP-1 among those with focal (mean 6992, SD 7569 cells/mL) and diffuse (mean 6077, SD 4650 cells/mL) pancreatic involvement, the statistically significant difference was evident only when comparing the latter subgroup with PDAC patients (p=0.006). Even the Group IPMN-CAPH displayed a mean rate of circulating plasmablasts lower than Group AIP-1 (mean 1065, SD 781 cells/mL; p<0.0001), whereas no statistically significant difference was found between Group AIP-1 and Group AIP-2/NOS (mean 3318, SD 3025 cells/mL), possibly due to the presence of a significant outlier in Group AIP-2/NOS (11410 cells/mL). Also, no statistical difference was found between Group AIP-1 and Group CP (mean 4084, SD 2272 cells/mL). Notably, the Group IPMN-CAPH showed the lowest plasmablasts frequency that, indeed, resulted even significantly lower than both PDAC and CP Groups (p=0.0082 and 0.0001, respectively).

Figure 3. Plasmablasts count (cells/mL) detected in AIP-1 (n=19), AIP-2/NOS (n=10), PDAC (n=17), CP (n=20), IPMN and CAPH (n=21) at baseline



Worth of note, the plasmablasts rate at baseline in Group AIP-1 showed a strong positive correlation with both the IgG4-RD RI (rho=0.6, p=0.007; **Figure 4A**), the grade of multi-organ involvement (rho=0.7, p=0.025; **Figure 4B**), and also with serum levels of IgG4 (rho=0.6, p=0.007; **Figure 4C**).

The ROC curve analysis for the differential diagnosis of type 1 AIP and all the other Groups according to total plasmablasts counts is reported in **Figure 5**. A cut-off of 4500 cells/mL had a sensitivity of 47% and specificity of 81% for differentiating type 1 AIP and all the other groups (AUC = 0.738).

Figure 4. Correlation between baseline plasmablasts serum level and basal IgG4-RD-RI **(A)**, multi-organ involvement **(B)**, and serum IgG4 **(C)** in Group AIP-1. Data are represented as mean ± SEM. Statistical analyses were performed by the Mann-Whitney test. Correlations were analyzed by Spearman's rank-order or Pearson product-moment correlation when discrete or continuous variables were involved, respectively.

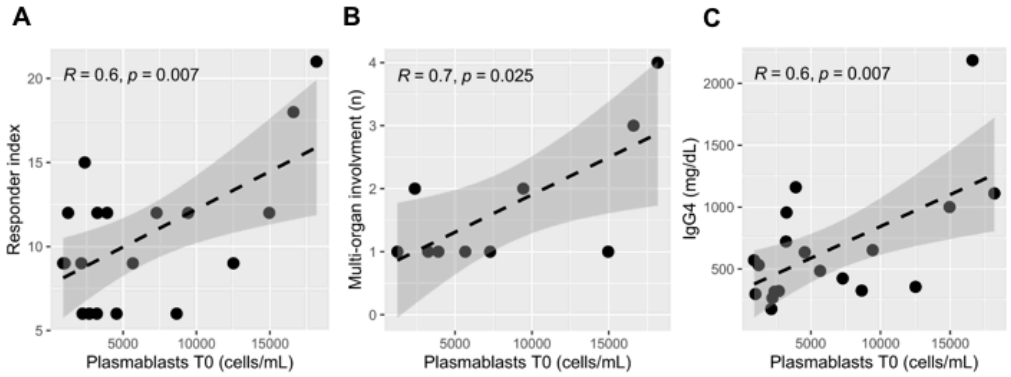
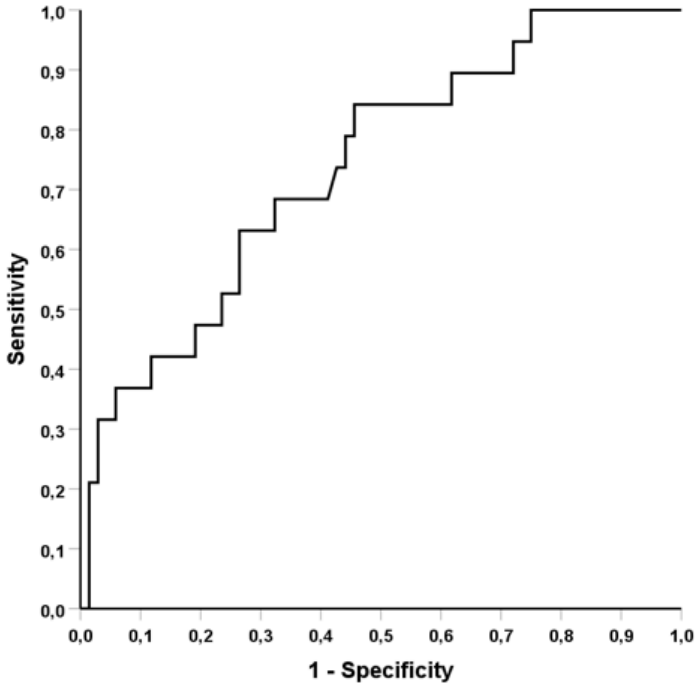


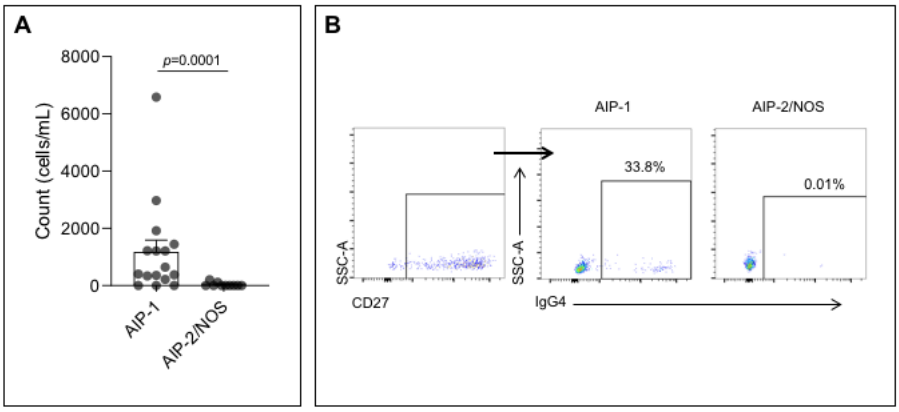
Figure 5. ROC curve analysis for the differential diagnosis of type 1 AIP and all the other Groups according to total plasmablasts counts (Type 1 AIP n=19, Type 2/NOS n=10, PDAC n=17, CP n=20, IPMN+CAPH n=21). ROC, receiver operating characteristic.



3.3 IgG4$^+$ plasmablasts counts at baseline

When selecting the plasmablasts according to the expression of IgG4, again the number of circulating IgG4$^+$ cells resulted significantly higher in Group AIP-1 (mean 1177, SD 1712 cells/mL) in comparison with Group AIP-2/NOS (mean 35, SD 73 cells/mL) (p=0.0001), as clearly evident in **Figure 6A**, with a representative case of both conditions showed in **Figure 6B**.

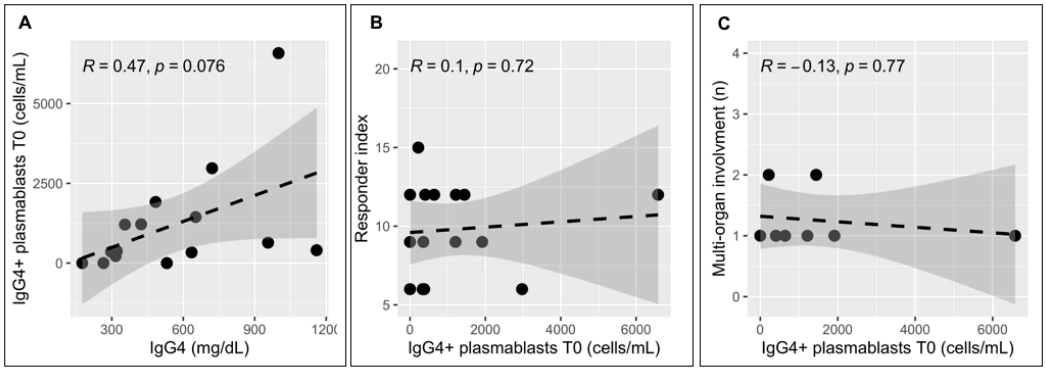
Figure 6: Circulating total IgG4$^+$ plasmablasts in AIP-1 and AIP-2/NOS patients. IgG4$^+$ plasmablasts count (cell/mL) detected in AIP-1 (n=15) and AIP-2/NOS (n=9) groups at baseline **(A)**. Representative percentages of IgG4$^+$ plasmablasts (out of total plasmablasts) detected in AIP-1 and AIP-2 **(B)**. Data are reported as mean ± SEM (statistical analyses were performed by Mann-Whitney text).



However, it should be noted that three out 19 patients with type 1 AIP did not show detectable levels of IgG4$^+$ plasmablasts, and that two out 10 patients with type 2/NOS AIP displayed a detectable number of circulating IgG4$^+$ plasmablasts, although with values (200 and 119 cells/mL) consistently lower than the mean level observed in type 1 AIP patients. Moreover, no correlation was found between the IgG4$^+$ plasmablasts count and either serum IgG4 levels (rho=0.47, p=0.076; **Figure 7A**), IgG4-RD RI

(rho=0.1, p=0.72; **Figure 7B**), and multi-organ involvement (rho=-0.13, p=0.77; **Figure 7C**) in the Group AIP-1.

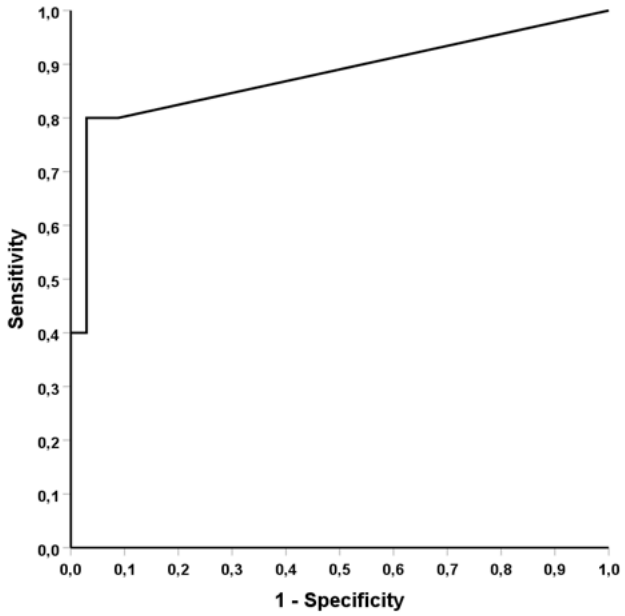
Figure 7. Correlation between IgG4$^+$ plasmablasts and serum IgG4 levels **(A)**, IgG4-responder index **(B)**, and multi-organ involvement **(C)**, in type 1 AIP.



Finally, also one case belonging to the Group CP showed a detectable value of IgG4$^+$ plasmablasts (732 cells/ml), whilst no case suffering from PDAC, IPMN and CAPH displayed detectable frequency of this cell population in peripheral blood.

At the ROC curve analysis **(Figure 8)**, a cut-off of 210 cells/mL had a sensitivity of 80% and specificity of 97% for differentiating AIP1 from all the other groups (AUC = 0.879), according to serum IgG4$^+$ plasmablasts counts.

Figure 8. ROC curve analysis for the differential diagnosis of type 1 AIP and all the other Groups according to serum IgG4$^+$ plasmablasts counts. (Type 1 AIP n=15, Type 2/NOS n=9, PDAC n=7, CP n=8, IPMN+CAPH n=10). ROC, receiver operating characteristic.

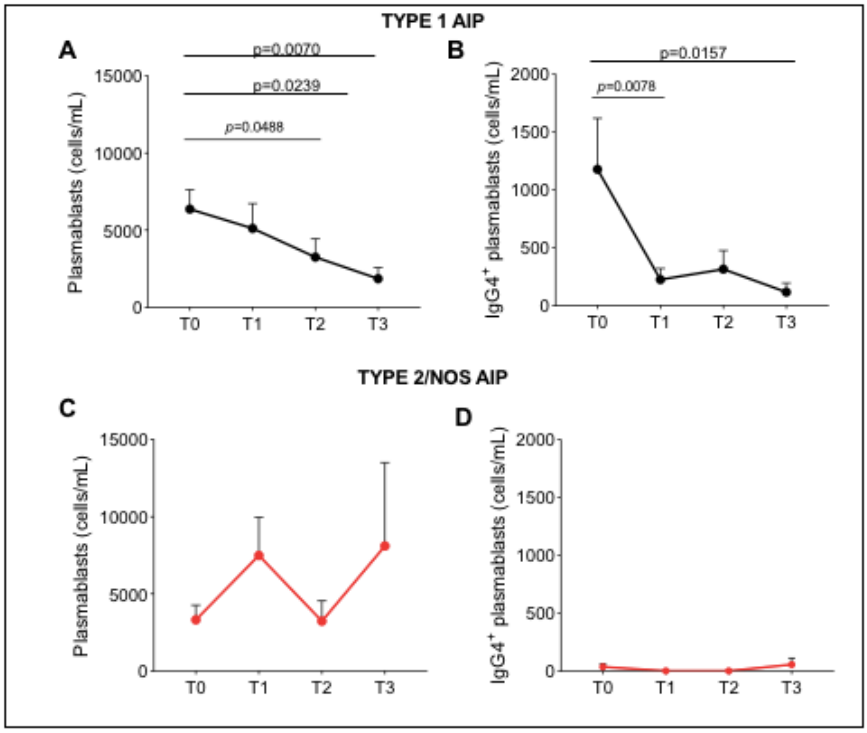


3.4 Total and IgG4$^+$ plasmablasts counts over time

During the follow-up period, we observed a progressive decrease of circulating plasmablasts rates in AIP-1 patients (timepoint 1: mean 5124, SD 5790 cells/mL) that achieved the statistically significance at time point 2 (mean 3259, SD 3520 cells/mL; p=0.0239) as well as at time point 3 (mean 1856, SD 1751 cells/mL; p=0.0070) (**Figure 9A**). At variance with total plasmablasts, the mean of the IgG4$^+$ subset showed a critical reduction as early as at time point 1 (mean 224, SD 321 cells/mL; p=0.0149) and remained substantially constant over time (mean 314, SD 429 cells/mL; p=ns at time point 2 and mean 115, SD 191 cells/mL; p=0.0157 at time point 3) (**Figure 9B**). Remarkably, those three patients who experienced a flare of the disease (one patient at time point 2 and two patients at time point 3) and that hence were not included in this analysis, displayed at the time of relapse high values of circulating

plasmablasts (i.e., 4878, 4868 and 1148 cells/mL), of IgG4$^+$ plasmablasts (i.e., 652, 295 and 220 cells/mL) as well as elevated serum IgG4 levels (i.e., 840, 227 and 167 mg/dL). By contrast, no significant modification in the Group AIP-2/NOS was observed of both total plasmablasts (mean 7492, SD 7000 cells/mL at timepoint 1; mean 3230, SD 3203 cells/mL at timepoint 2; mean 8111, SD 13239 cells/mL at time point 3) (**Figure 9C**), and IgG4$^+$ plasmablasts (not detected at time points 2 and 3; mean 56 cells/mL, range 0-282 cells/mL, SD 126 cells/mL at time point 3) (**Figure 9D**) despite a treatment was established in all cases.

Figure 9. Total and IgG4$^+$ plasmablasts in AIP patients and disease activity. Total (**A**) and IgG4$^+$ (**B**) circulating plasmablasts count (cells/mL) detected in AIP-1 at T0 (plasmablasts n=19, IgG4$^+$ plasmablasts n=15), T1 (plasmablasts n=13, IgG4$^+$ plasmablasts n=11), T2 (plasmablasts n=9, IgG4$^+$ plasmablasts n=7), and T3 (plasmablasts n=6, IgG4$^+$ plasmablasts n=6). Total (**C**) and IgG4$^+$ (**D**) circulating plasmablasts count (cells/mL) detected in AIP-2 at T0 (plasmablasts n=10, IgG4$^+$ plasmablasts n=9), T1 (plasmablasts n=8, IgG4$^+$ plasmablasts n=7), T2 (plasmablasts n=6, IgG4$^+$ plasmablasts n=4), and T3 (plasmablasts n=6, IgG4$^+$ plasmablasts n=5). Data are represented as mean ± SEM. Statistical analyses were performed by Mann-Whitney test.



4. DISCUSSION

AIP represents a clinical challenge due to both diagnostic difficulty and high relapse rate, particularly in type 1. Indeed, the diagnostic algorithm includes invasive procedures, such as percutaneously or endoscopic ultrasound-guided fine-needle aspiration. In a limited proportion of cases, the final diagnosis cannot be reached and is achieved only on the surgical specimen. In this peculiar clinical context, the availability of a non-invasive biomarker with a high predictive value to differentiate AIP from PDAC, is still an unmet need. In addition, we also cannot predict the relapse of the disease whose frequency is high during the first two years from the onset(25). Recently, a few studies reported the clinical usefulness of plasmablasts dosage in the setting of IgG4-RD. In particular, Wallace et al. showed that high levels of both total plasmablasts (median value 4698/mL) and $IgG4^+$ plasmablasts (median value 2808/mL) were detectable in patients suffering from active and naïve IgG4-RD, as compared to both healthy subjects and patients with other autoimmune diseases, and a total plasmablasts count >2000/mL was suggested as a highly specific predictor of IgG4-RD(64). Remarkably, both values underwent a significant decrease following a cycle of treatment with Rituximab. However, in this cohort, pancreatic involvement was present only in eight out of 37 cases. Similarly, Mattoo et al. demonstrated the presence of significantly higher levels of circulating CD19+CD27+CD38hi plasmablasts in 84 patients with IgG4-RD than 16 healthy controls, which decreased upon Rituximab-mediated B-cell depletion(74). Furthermore, Akiyama et al. reported a high mean number of total plasmablasts (3294 cell/mL, SD 1483 cell/mL) in a series of 15 active, untreated patients with IgG4-RD (only one with pancreatic involvement) that, again, significantly decreased after steroid therapy(81). These data were subsequently confirmed, including the ability of steroids to induce a reduction of

plasmablasts levels(82). Following these interesting results, we aimed to explore whether this analysis was suitable in the specific setting of AIP, and to validate it with respect to other pancreatic disorders.

From a clinical standpoint, our results show that the total plasmablasts count may help to distinguish type 1 AIP from other pancreatic diseases, but with an unsatisfactory sensitivity (47%) and specificity (81%) using the best cut-off (4500 cells/mL) in ROC curve from the present study. Even applying the Wallace cut-off of 2000 cells/mL (64) to our population, the sensitivity was increased (84%) but with a very low specificity of 51%. Furthermore, no difference was found when comparing the total plasmablasts count of PDAC Group with focal type 1 AIP, possibly due to the few cases enrolled in this sub-group (n=6) and considering that this was not an aim of the present study. Therefore, total plasmablasts seem to be not useful for the diagnosis of type 1 AIP.

Notwithstanding, it should be emphasized the robust positive correlation between the total plasmablasts counts at baseline in Group AIP-1 with all parameters routinely evaluated, such as serum levels of IgG4, multi-organ involvement, IgG4-RD RI. This is a remarkable point since Wallace et al. did not find any correlation between the median values of peripheral plasmablasts in active IgG4-RD and IgG4 serum levels(64), thus highlighting that pancreatic involvement of the IgG4 disease may display different features compared to other phenotypes of IgG4-RD.

To search for more valuable diagnostic tools and biomarker predictors of disease course, as performed by others (63,71,73), we postulated that by performing a deeper characterization of circulating plasmablasts with the class of immunoglobulin that is the hallmark of type 1 AIP, a better result would have been achieved. Indeed, a cut-off of 210 $IgG4^+$ plasmablasts/mL seems to distinguish type 1 AIP from all other

pancreatic disorders with a sensitivity of 80% and a specificity of 97%. In fact, in almost all PDAC, CP, IPMN, and CAPH patients, undetectable values of this cell subset in peripheral blood were found. A mild increase of $IgG4^+$ plasmablasts <210 mg/mL was observed in 2 patients, one type 2 and 1 type NOS AIP at time 0, whereas on CP patient with multiple pseudocysts and diffuse inflammatory thickening of the gastric and duodenal showed an increase over the cut-off used (732 cells/mL). We do not have an explanation for these findings. Of note, also three cases of type 1 AIP, all serum $IgG4^+$, were negative for $IgG4^+$ plasmablasts. We cannot exclude sample processing errors.

Regarding the three cases that relapsed during the follow-up, our analyses did not demonstrate any clinical utility of total plasmablasts since they showed heterogeneous expression at the time of disease recurrence. In contrast, all relapsers had values of $IgG4^+$ plasmablasts over the cut-off proposed of 210 cells/mL. This result may suggest introducing the $IgG4^+$ plasmablasts when suspecting a disease recurrence. However, this data needs to be confirmed in a prospective study with this specific aim. The main limitation of this study is the sample size. It should be recalled that AIP is a rare condition. A strength is that the study is prospective, monocentric, with many control groups including the most common pancreatic conditions. Furthermore, the experience of our center, the interpretation of the data, the clear definition and classification of the study groups, and the established time points for disease evaluation allowed us to achieve reliable results. One further strength is the ability to fine-tuning a multiparametric flow cytometric analysis to evaluate the frequency of $IgG4^+$ plasmablasts that deserves further and wider investigation not only in type 1 AIP but also in additional pathological conditions. All these considerations, together with the low invasiveness needed to harvest the blood sample, the high reproducibility

and feasibility of the technique, and the affordability of its costs, make this biomarker suitable for application in daily clinical practice.

These results need to be confirmed through further prospective and multicenter studies.

5. CONCLUSION

Our data suggest that circulating $IgG4^+$ and not total plasmablasts might represent an important diagnostic tool for diagnosis of type 1 AIP, particularly for differential diagnosis with type 2 AIP/NOS and from PDAC. $IgG4^+$ plasmablasts may be helpful for monitoring therapy and to confirm type 1 AIP disease relapse.

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