

ORIGINAL ARTICLE

Spectrum of autoantibodies other than anti-desmoglein in pemphigus patients

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Abstract

Background Pemphigus is a life-threatening autoimmune blistering disease mediated by autoantibodies against adhesion molecule of the skin. Its concurrence with systemic and organ-specific autoimmune disease was described in case reports.

Objectives To evaluate the presence of a broad spectrum of organ-specific and non-organ-specific autoantibodies other than anti-desmoglein antibodies in pemphigus patients.

Patients and methods Serum samples were obtained from 105 pemphigus foliaceus (PF) patients, 51 pemphigus vulgaris (PV) patients and 50 controls. Both indirect immunofluorescence assay and ELISA were used to assess the presence of autoantibodies related to connective tissue diseases, autoimmune hepatitis, vasculitis, rheumatoid arthritis, coeliac disease, diabetes and thyroiditis.

Results Significant difference was observed between the three groups for anti-thyroglobulin antibodies in the pemphigus foliaceus group (18% vs. 4%, $P = 0.03$). A significantly higher occurrence of IgM anti-cardiolipin ($P = 0.03$), IgG anti-reticulon ($P = 0.01$) and IgG anti-gliadin antibodies ($P = 0.008$) were observed in the PV group. Cases with more than four autoantibodies were frequently positives for both anti-desmoglein 1 and anti-desmoglein 3.

Conclusion Autoantibodies other than anti-desmoglein antibodies are not rare in pemphigus patients. Clinical and serological follow-up of pemphigus patients with positive autoantibodies are needed to clarify their impact in disease evolution.

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Keywords

autoantibodies, autoimmune disease, autoimmunity, desmoglein, pemphigus

Conflict of interest

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Introduction

Pemphigus is an autoimmune intra-epidermal blistering disease characterized by the presence of pathogen autoantibodies (auto-Abs) against desmosomal transmembrane glycoproteins: desmoglein 1 (Dsg1) in pemphigus foliaceus (PF) and desmoglein 3 (Dsg3) in pemphigus vulgaris (PV). The deposit of these autoAbs

in the intercellular space mediates acantholytic blisters in the skin and/or mucosa depending on the form of pemphigus.

As all autoimmune diseases (AID), the onset and course of pemphigus depend on both exogenous inducing and genetic predisposing factors. Epidemiological studies suggest potential environmental agents: the black fly *Simulium pruinosum* in endemic

PF (*fogo selvagem*), some parasites such as leishmania and *echinococcus granulosis* in Tunisia and viral infection in many reports.^{1–5} A genetic basis, involving several genes mainly those encoding HLA, cytokines and adhesion molecules has also been involved in the disruption of immune tolerance to skin autoantigens (reviewed in Tron *et al.*).⁶ Accordingly, *IL6*, *CTLA4*, *Dsg1*, *Dsg3* polymorphisms were significantly associated with the disease.^{7–9} Some of these SNP (single nucleotide polymorphism) are also shared by several organ-specific and non-organ-specific AID that may explain the presence of two or more AID in the same patient. However, few AID are observed in association with pemphigus: lupus is the mainly described (reviewed in Malik *et al.*).¹⁰ The concurrence of pemphigoid,¹¹ rheumatoid arthritis,¹² thyroiditis^{13–15} and multiple autoimmune syndrome^{16,17} was also described and mainly reported as case reports.

It was commonly known that the presence of specific autoAbs is the main immunological marker of AID.¹⁸ Thus, to assess the link between pemphigus and the other AID, we carried out a serological analysis to study a broad spectrum of autoAbs, other than pemphigus specific antibodies (anti-Dsg1 and anti-Dsg3 autoAbs), and also to investigate whether the presence of these autoAbs is related to latent (asymptomatic) or clinically symptomatic AID.

Patients and methods

The study included 105 PF patients and 51 PV patients. All patients fulfilled clinical and immunological typical criteria for each form of pemphigus. Their clinical and biological features are summarized in Table 1. Fifty healthy persons matched by age, gender and origin were studied as controls.

Methods

Indirect immunofluorescence Indirect immunofluorescence was performed for the detection of anti-nuclear Abs (ANA), anti-

smooth muscle Abs (SMA), anti-reticulin Abs (ARA), anti-mitochondrial Abs (AMA) and anti-parietal cells Abs on cryo-cut rat tissue sections (liver, kidney and stomach) or on Hep2 cells (BMD[®], Croissy Beaubourg, France) using a fluorescein-labelled anti-GAM globulin (Biorad[®], Marnes la coquette, France). IgA anti-endothelial Abs (AEA) were tested on human umbilical cord section and IgG anti-islet cell Abs (ICA) on monkey pancreas sections (The Binding Site[®], Birmingham, UK).

Enzyme-linked immunosorbent assay ELISA was used for measuring IgG and IgM anti-cardiolipin Abs (ACL), IgA anti-transglutaminase Abs (ATG), IgG and IgA anti-gliadin Abs (AGD), [a home-made ELISA as previously described¹⁹], IgG anti-phospholipids Abs (APL) (BMD[®]), IgG anti- β 2 glycoprotein I Abs (AP2GI), IgG anti-thyroperoxidase Abs (ATPO), IgG anti-thyroglobulin Abs (ATH), IgG anti-myeloperoxidase Abs (AMPO), and IgG anti-proteinase 3 Abs (APR₃) and (IgG, IgA, IgM) rheumatoid factors (RF) (Orgentec[®], Mains, Germany). Results were considered positive according to the manufacturers' cut-off values.

Radio-immuno-assay This test was performed for screening IgG anti-glutamic acid decarboxylase Abs (AGAD) (Immuno-tech[®], Marseille, France) and IgG anti-islet antigen-2 from tyrosine phosphatase family Abs (AIA2) (Immuno-tech[®]). These assays were performed for only 50 PF and 50 PV patients.

Other autoAbs tests According to autoAbs screening by the initial tests, patients were also tested for others specificities as follows:

Subjects with high titres of ANA were assessed for (i) anti-ds-DNA Abs by both IIF (Diasorin[®], Stillwater, MN, USA) and ELISA (Orgentec[®]) and (ii) anti-extractable nuclear antigens (ENA) Abs by ELISA (Orgentec[®]). When these later autoAbs were positive, an ENA profile test (Profile Innolia, Orgentec[®]) using separated antigens was performed. AMA positive sera by IIF were also tested by ELISA using M2 antigen (pyruvate dehydrogenase E2 subunit) (Orgentec[®]). When a tubular fluorescence was observed, suggesting the presence of anti-LKM1, serum was tested on a liver dot kit (Alphadia diagnostic products[®], Wavre, Belgium).

The positivity of parietal cell Abs when observed was confirmed by immunodot analysis dot kit (Biermer-atrophic gastritis, Alphadia diagnostic products[®]).

Patients positive for only IgG AGD were diagnosed for IgA deficiency, whereas those with dual positivity (IgG and IgA) for AGD and/or IgA ATG were assessed by IIF for IgA AEA.

Subjects positive for ATH, ATPO were screened by IIF on monkey thyroid for ATM (anti-thyroid microsomal Abs), and have been evaluated for TSH levels.

Statistical analyses The comparison of autoAbs' prevalence between patients and controls was made using chi-squared test and Yates correction or Fischer's test if necessary.

Table 1 Clinical and biological features of pemphigus patients

	PF (n = 105)	PV (n = 51)
Gender ratio (F : M):	4 : 1 (84/21)	3 : 1 (39/12)
Mean age (range)	46.4 (20–80)	45.3 (19–80)
Clinical status: relapses	38 (36.2%)	32 (62.8%)
Clinical status: remission	67 (63.8%)	19 (37.3%)
Under treatment	35 (33.3%)	30 (58.8%)
Anti-Dsg1	78 (74.3%)	36 (70.6%)
Anti-Dsg3	12 (11.4%)	45 (88.3%)
Anti-Dsg1 and anti-Dsg3	9 (8.6%)	32 (62.8%)
Associated AID	4 (2 thyroiditis, 1 diabetes, 1 psoriasis)	9 (2 diabetes, 3 Sjögren Syndrome, 2 thyroiditis, 2 anti-phospholipid syndrome)

AID, autoimmune disease; Dsg, desmoglein; PF, pemphigus foliaceus; PV, pemphigus vulgaris.

Results

AutoAbs prevalence

AutoAbs prevalence obtained in pemphigus patients and in controls is summarized in Table 2 and Fig. 1.

ANA antibodies Similar frequencies of ANA were detected among pemphigus patients (PF or PV) and controls. A non-significant difference was observed.

- 1 In PF patients, ANA were detected in six (5.7%) patients. One patient (woman aged 47 years) had high level of ANA (1 : 400) with a homogeneous pattern. This patient showed no positivity for either anti-ENA Abs or for anti-ds-DNA Abs. The patient did not show any symptoms of other AID. Five other patients were positive at 1 : 100,

two with a nucleolar pattern and three patients had speckled pattern. All these patients were women.

- 2 In the PV group, ANA were present in seven patients (13.7%). Two patients were positive at high levels: one patient (women aged 79 years) was positive at 1 : 800. She had anti-ds-DNA Abs detected by ELISA with a titre of 223 UI/mL (Normal value <50) and was weakly positive for anti-ENA Abs. ENA profile test showed a weak positivity for anti-SmD Abs. The other patient (woman aged 51 years) had ANA at 1 : 400 with a nucleolar pattern. The screening for both anti-ENA and anti-DNA Abs was negative. The remaining patients were positive at 1 : 100 with a speckled pattern, among them one woman with a cytoplasmic fluorescence. All these patients were negative for both anti-ENA and anti-ds-DNA Abs.

Table 2 AutoAbs prevalence in PF, PV patients and controls

AutoAbs	Cut-off	PF		PV		HC		P-value
		Tested	AAb+	Tested	AAb+	Tested	AAb+	
ANA	1 : 100	105	6 (5.7%)	51	7 (13.7%)	50	6 (12%)	ns
Anti-ENA Abs	25 UI/mL	6	0 (0%)	7	1 (1.9%)	6	0 (0%)	ns
Anti-ds-DNA Abs	50 UI/mL	6	0 (0%)	7	1 (1.9%)	6	0 (0%)	ns
AMA	1 : 100	105	3 (2.8%)	51	1 (7.8%)	50	1 (2%)	ns
AMA (ELISA)	10 UI/mL	3	1 (0.9%)	1	0 (0%)	1	0 (0%)	ns
LKM1 (dot)	1 : 100	3	0 (0%)	4	1 (1.9%)	2	1 (2%)	ns
SMA	1 : 100	105	3 (2.8%)	51	3 (5.8%)	50	1 (2%)	ns
AMPO	5 UI/mL	105	1 (0.9%)	51	1 (1.9%)	50	0 (0%)	ns
APR3	10 UI/mL	105	2 (1.9%)	51	1 (1.9%)	50	1 (2%)	ns
RF (ELISA)	60 UI/mL	53	3 (2.8%)	51	6 (11.72%)	50	5 (10%)	ns
Anti-keratin	1 : 20	3	0 (0%)	7	1 (1.9%)	7	1 (2%)	ns
ARA	1 : 100	105	11 (10.4%)	51	13 (25.49%)*	50	5 (10%)	0.04
ATG	10 UI/mL	105	1 (0.9%)	51	0 (0%)	50	0 (0%)	ns
AGD (IgG)	10 UI/mL	105	7 (6.66%)	51	16 (31.37%)*	50	5 (10%)	0.008
AGD (IgA)	5 UI/mL	105	4 (3.8%)	51	4 (7.8%)	50	4 (8%)	ns
AEA	1 : 20	4	0 (0%)	4	1 (1.9%)	4	0 (0%)	ns
ICA	1 : 20	105	9 (8.57%)	51	7 (13.7%)	50	2 (4%)	ns
AGAD	2 UI/mL	50	1 (2%)	50	0 (0%)	0	nd	ns
AIA2	2 UI/mL	50	0 (0%)	50	0 (0%)	0	nd	ns
ATPO	75 UI/mL	105	3 (2.85)	51	1 (1.9%)	50	3 (6%)	ns
ATH	100 UI/mL	105	19 (18.09%)*	51	7 (13.72%)	50	2 (4%)	0.031
APL	15 UI/mL	105	8 (7.6%)	51	8 (15.6%)	50	6 (12%)	ns
ACL (IgG)	13 UI/mL	53	1 (1.9%)	51	0 (0%)	50	1 (2%)	ns
ACL (IgM)	18 UI/mL	53	0 (0%)	51	6 (11.76%)*	50	0 (0%)	0.03
Aβ2GPI	8 UI/mL	53	2 (3.77)	51	6 (11.7%)	50	6 (12%)	ns
Anti-parietal cells Abs	1 : 100	105	0 (0%)	51	1 (1.9%)	50	0 (0%)	ns

Abs, antibodies; ACL, anti-cardiolipin; AEA, anti-endomysium Abs; AGD, anti-gliadin Abs; AGAD, anti-glutamic acid decarboxylase Abs; AIA2, anti-islet antigen-2 from tyrosine phosphatase family Abs; AMA, anti-mitochondrial Abs; AMPO, anti-myeloperoxidase Abs; ANA, anti-nuclear Abs; APL, anti-phospholipids Abs; APR₃, anti-proteinase 3 Abs; ARA, anti-reticulon Abs; ATG, anti-transglutaminase Abs; ATH, anti-thyroglobulin Abs; ATPO, anti-thyroperoxidase Abs; Aβ2GPI, anti-β2 glycoprotein 1 Abs; ds-DNA, double stranded DNA; ENA, extractable nuclear antigen; HC, healthy controls; ICA, islet cell Abs; nd, not determined; ns, not significant; PF, pemphigus foliaceus; PV, pemphigus vulgaris; RF, rheumatoid factors; SMA, anti-smooth muscle Abs.

*It indicates to which group (PF or PV) the P-value is significant.

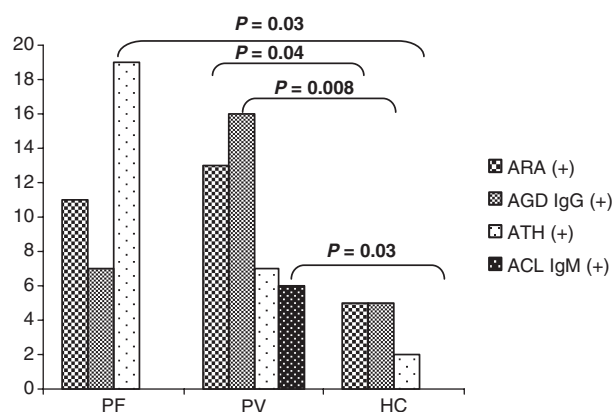


Figure 1 Prevalence of ARA, AGD IgG, ATH and ACL IgM in PF, PV patients and controls. ACL, anti-cardiolipin Abs; AGD, anti-gliadin Abs; ARA, anti-reticulin Abs; ATH, anti-thyroglobulin Abs; HC, healthy controls; PF, pemphigus foliaceus; PV, pemphigus vulgaris.

Rheumatoid arthritis antibodies High titres of RF were noticed in three PF, seven PV and seven controls. These samples were also assessed for anti-keratin Abs. Only one PV patient and one control were positive.

Coeliac disease antibodies The assessment of coeliac disease Abs revealed that ARA were positive in 11 PF patients (10.4%), 13 PV patients (25.49%) and in five controls (10%). A weak significant difference was observed with the PV group ($P = 0.04$) and also when comparing the PV to the PF group. Only one female PF patient was positive for ATG. She was also positive for AEA, but negative for AGD. IgG AGD were frequently detected in the PV group with a significant difference ($P = 0.0081$) that was observed also when comparing PV to PF group ($P < 0.0001$).

IgG and IgA AGD positive sera [five PF (4.7%), four (7.8%) PV and four (8%) controls] were tested for IgA AEA. Among them, only one PV patient showed positivity. No coeliac disease related symptoms were noted in this patient. However, intestinal biopsy was not performed to confirm CD. IgA levels were measured for IgG AGD positive sera: five (4.7%) PF, 11 (21.5%) PV and five (10%) controls. No IgA deficiency was observed in all tested sera.

Diabetes antibodies Nine (8.5%) PF patients, seven (13.7%) PV patients and two (4%) controls were positive for ICA. Among them, one PV patient was known as diabetics (unknown type). Only one PF patient showed positivity for AGAD in association with ICA. No symptoms of diabetes were observed in this patient.

Thyroiditis antibodies All PF and PV patients who showed positivity for ATPO were ATH positive. In PV patients, the frequency of both ATPO and ATH showed no significant difference with the control group. ATH were detected in 19 PF (18.09%), with statistical significant difference when compared with controls

($P = 0.031$). The presence of ATH was confirmed by IIF in eight PF and two PV patients. Among positive patients, for either ATH or ATPO, the diagnosis of autoimmune thyroiditis was already known in one PF patient and in one PV patient. Autoimmune thyroiditis was not diagnosed in the remaining patients. Hormonal analysis was performed in order to confirm a latent thyroiditis. However, TSH levels were normal in all cases.

Anti-phospholipids syndrome antibodies The prevalence of ACL (IgG), APL and A β 2GI was similar among patients and controls with no significant difference. However, in PV patients a significant difference with controls was observed for ACL (IgM) ($P = 0.03$). The presence of at least one autoAb (APL, ACL, A β 2GI) was frequently observed in PF and PV patients (19% and 31% respectively) with a P -value close to the significance zone (0.06). The concomitant presence of ACL, APL and A β 2GI was not observed in any patient. However, the presence of both APL and A β 2GI was noticed in one PF patient and in one PV patient. The latter had a history of fetal loss. Besides, the concomitant presence of APL and ACL was depicted only in two PV patients, among them one patient with a history of fetal loss. All the remaining patients had no history of thrombosis, fetal loss and/or thrombopenia.

Autoimmune gastritis antibodies Anti-parietal cells Abs were observed in one PF patient. This positivity was also confirmed by immunodot analysis kit. The patient had no history of megaloblastic anaemia.

Association between autoAbs

More than one autoAb were observed in almost the quarter of each group. In the PF group, 28% of patients had one autoAb other than anti-Dsg1, whereas 20.9% of the patients have two or more autoAbs. Only one patient had six different autoAbs (ANA, AMA, ICA, ATPO, ATH, IgG AGD).

In the PV group, 31% of patients had two autoAbs. The remaining patients were with more than two autoAbs (21%), among them one patient had a total of six autoAbs.

Clinical and biological features of pemphigus patients with four and more autoAbs ($n = 7$) are summarized in Table 3.

We also noticed that patients with these autoAbs were frequently positive for both anti-Dsg1 and anti-Dsg3 (5 of 7: 71%). When we focused on the PF group, we noticed that only three (2.8%) patients had more than four Abs, among them two with dual positivity for anti-Dsg1 and Dsg3. Similarly in the PV group, four (7.84%) patients had four and more autoAbs other than anti-Dsg3.

AutoAbs, disease severity and treatment

The comparison of autoAbs prevalence in patients under treatment and those who did not start their therapy showed a decrease

Table 3 Pemphigus patients with a broad spectrum of autoAbs

Patients	Age	Gender	Abs	Treatment	Positive autoAbs: non-specific markers	Symptoms other than pemphigus	Anti-Dsg1/Dsg3: specific markers
PF1	76	F	6	1 month	ANA, AMA, ICA, ATPO, ATH, AGD (IgG)	Thyroiditis	Anti-Dsg1
PF2	77	F	4	None	APL, ATH, RF, Aβ2GPI	–	Anti-Dsg1, Anti-Dsg3
PF3	35	M	4	None	ANA, APL, AGD (IgG and IgA)	–	Anti-Dsg1, anti-Dsg3
PV1	65	F	4	3 months	ICA, ATH, ATPO, anti-keratin	–	Anti-Dsg1, Anti-Dsg3
PV2	47	M	7	3 months	ANA, SMA, LKM1, ICA, APL, AGD (IgG), ACL (IgM)	–	Anti-Dsg1, Anti-Dsg3
PV3	30	H	4	2 months	APL, ATPO, ATH, APR3	–	Anti-Dsg3
PV4	79	F	4	1 month	ANA, Anti-ds-DNA, APL, ACL	Anti-phospholipid syndrome	Anti-Dsg1 Anti-Dsg3

Abs, antibodies; ACL, anti-cardiolipin; AGD, anti-gliadin Abs; AGAD, anti-glutamic acid decarboxylase Abs; AMA, anti-mitochondrial Abs; ANA, anti-nuclear Abs; APL, anti-phospholipids Abs; APR₃, anti-proteinase 3 Abs; ATH, anti-thyroglobulin Abs; ATPO, anti-thyroperoxidase Abs; Aβ2GPI, anti-β2 glycoprotein 1 Abs; ds-DNA, double stranded DNA; ICA, islet cell Abs; PF, pemphigus foliaceus; PV, pemphigus vulgaris; RF, rheumatoid factors; SMA, anti-smooth muscle Abs.

Table 4 AutoAbs and pemphigus associated disease

Patients	Age	Gender	Treatment	AID	Non-specific markers: autoAbs+	Specific markers: anti-Dsg1/Dsg3
PF1	76	F	1 month	Thyroiditis	ANA, AMA M2, ICA, ATPO, ATH, AGD (IgG)	Dsg1
PF4	69	F	None	Thyroiditis	None	Dsg1
PF5	76	M	4 years	Diabetes	ICA	Dsg1
PF6	27	F	9 years	Psoriasis	None	Dsg1
PV5	26	F	8 years	Diabetes	None	Dsg1, Dsg3
PV6	52	F	None	Diabetes	ICA	Dsg1
PV7	81	F	None	Sjögren syndrome	AGD (IgG)	Dsg1, Dsg3
PV8	45	F	None	Sjögren syndrome	None	Dsg1, Dsg3
PV9	46	M	None	Sjögren syndrome	AGD (IgG and IgA), MPO	Dsg1, Dsg3
PV10	60	M	None	Thyroiditis	None	Dsg1, Dsg3
PV11	51	F	None	Thyroiditis	ANA, ATH, AGD (IgG)	Dsg1, Dsg3
PV4	79	F	1 month	APL syndrome	ANA, Anti-ds-DNA, APL, ACL	Dsg1, Dsg3
PV12	47	F	None	APL syndrome	APL, Aβ2GPI	Dsg1

Abs, antibodies; ACL, anti-cardiolipin Abs; AGD, anti-gliadin Abs; AMA, anti-mitochondrial Abs; AMPO, anti-myeloperoxidase Abs; ANA, anti-nuclear Abs; APL, anti-phospholipids Abs; ATH, anti-thyroglobulin Abs; ATPO, anti-thyroperoxidase Abs; Aβ2GPI, anti-β2 glycoprotein 1 Abs; ds-DNA, double stranded DNA; ICA, islet cell Abs; PF, pemphigus foliaceus; PV, pemphigus vulgaris.

in Abs frequency in patients under treatment (oral corticosteroids and immunosuppressive drugs); however, the difference was non-significant. We also noticed that the shorter is the duration of treatment, the higher is the immune prevalence of autoAbs (patients with high numbers of autoAbs were either not treated or under recent treatment).

Moreover, we found no correlation between the number of positive autoAbs, consolidation phase duration and number of relapses.

AutoAbs profile in patients with AID other than pemphigus

There are several diseases that were observed in association with pemphigus. The autoAbs profile of these patients is presented in Table 4. Surprisingly, autoAbs that characterize these AID were not observed in some patients even though the presence of anti-Dsg1 and/or anti-Dsg3 was detected.

Discussion

In this study we investigated the prevalence of a large panel of circulating autoAbs in pemphigus patients. These autoAbs could be useful for the assessment of some silent or concomitant associated AID. All PV, PF patients included in this study did not present any symptoms of AID, (other than those related to pemphigus) except those reported in Table 4. Nevertheless, some of them presented autoAbs known as specific markers of AID. Moreover, we mention that for these patients we have no information about chronic infection affecting them, such as hepatitis or tuberculosis, which could be involved in the appearance of these autoAbs.^{20,21}

Only few studies screened a so large panel of organ-specific and non-organ-specific autoAbs in pemphigus patients.^{22–26} Our study seems to be the first to analyse such a large panel of autoAbs that covers almost all AID reported in all these studies. Furthermore, we surpassed this panel by investigating autoimmune diabetes and

Table 5 AutoAbs prevalence and review of literature

AutoAbs	Mendes <i>et al.</i> ²¹	Squiquera <i>et al.</i> ²²	Nisihara <i>et al.</i> ²³		Blondin <i>et al.</i> ²⁴		Our study		
	PF: 28	EPF 196, Relatives (138 + 13) HC: 38, PF: (38)	PF: 120	HC: 200	59 PV	50 HC	105 PF	51 PV	50 HC
ANA	0	0	3 (2.5%)	1 (0.5%)	22 (37%)	4 (8%)	6 (5.7%)	7 (13.7%)	6 (12%)
Anti-ds-DNA	nd	0	nd	nd	0	0	0	1 (1.9%)	0
SMA	1 (3.5%)	nd	1 (0.8%)	4 (2%)	nd	nd	3 (2.8%)	3 (5.8%)	1 (2%)
AMA	2 (7.1%)	nd	0	0	nd	nd	1 (0.9%)	0	0
Anti-ENA	nd	nd	nd	nd	1 (1.6%)	0	0	1 (1.9%)	0
Anti-SSA	nd	0	1	0	1 (1.6%)	0	0	0	0
Anti-SSB	nd	0	nd	nd	1 (1.6%)	0	0	0	0
Anti-Sm	nd	0	nd	nd	0	0	0	1 (1.9%)	0
Anti-RNP	nd	0	nd	nd	0	0	0	0	0
ATPO	0	nd	nd	nd	nd	nd	3 (2.8%)	1 (1.9%)	3 (6%)
ATH	0	nd	2 (1.6%)	3 (1.5%)	nd	nd	19 (18%)	7 (13.7%)	2 (4%)
Anti-parietal cells	6 (21.4%)	nd	0	1 (0.5%)	nd	nd	0	1 (1.9%)	0
RF	0	nd	nd	nd	nd	nd	3 (2.8%)	6 (11.7%)	5 (10%)
ANCA	nd	nd	0	0	nd	nd	3 (2.8%)	2 (3.8%)	1 (2%)

AMA, anti-mitochondrial Abs; ANA, anti-nuclear Abs; ANCA, anti-neutrophil cytoplasmic Abs; ATH, anti-thyroglobulines Abs; ATPO, anti-thyroperoxidase Abs; Epf, endemic PF; HC, healthy controls; nd, not determined; PF, pemphigus foliaceus; PV, pemphigus vulgaris; RF, rheumatoid factor; SMA, anti-smooth muscle Abs.

coeliac disease Abs. Table 5 summarizes autoAbs prevalence reported in literature, as well as those found in our series.

Our study revealed the presence of ANA in PF and PV patients, varying from 7% in PF to 13% in PV patients. No significant difference was observed when compared with the control group. ANA were seen sometimes with high titre (1 : 400 and 1 : 800). Controversial frequencies were reported for these autoAbs in literature. Many screening studies^{22–24} reported a weak frequency with a prevalence of 0.8%.²⁴ Some others reported higher frequencies. In fact, using the multiplexed laser bead assay, Blondin *et al.*²⁵ have reported that ANA were present in 22 (37.3%) PV patients compared with 4 (8%) healthy controls with a significant difference ($P < 0.001$). In these 22 PV positive sera, only one (1.7%) patient showed the presence of anti-ENA Abs with a dual positivity for anti-SSA and anti-SSB Abs. Similar results were reported by Nisihara *et al.*²⁴ where only one patient with anti-SSA Abs had no symptom of concomitant AID. In our study, ANA were present in 13% of PV patients and the screening for anti-ENA Abs was positive in only one PV patient who showed weak positivity for SmD Abs. This PV patient, a 79-year-old woman suffering from a high blood pressure, was highly positive for anti-ds-DNA Abs, whereas no lupus or other associated AID was clinically reported. Nevertheless, as this patient is 79 years old, the presence of ds-DNA Abs might be due to its 'advanced' age.²⁷ Strikingly, these autoAbs have not been found in any of the above mentioned studies.

The presence of anti-ds-DNA Abs was reported as predictors to lupus manifestation. They were observed in 55% of patients before the clinical onset of lupus. With a mean onset of 2.2 years before the diagnosis, they were found later than ANA and earlier than

anti-nuclear ribonucleoprotein Abs.²⁸ Thus, our finding suggests a clinical follow-up to assess lupus settlement. Moreover, the presence of anti-ds-DNA Abs in pemphigus patients was frequently reported in cases with dual concomitant diagnosis of pemphigus and lupus.^{29–32}

As ANA, the assessment of AMA revealed that they were rarely depicted in our screening; they were detected only in one (0.83%) PF patient. The presence of these Abs was also reported by Mendes *et al.*²² in two (7.1%) of 28 endemic PF patients. These autoAbs, however, were not detected by Nisihara *et al.*²⁴ in a sample of 120 endemic PF patients. It is noteworthy that the patient with AMA did not show any symptoms of primary biliary cirrhosis despite that their being the markers of this disease.

SMA were also rarely detected in our study; they were present in three (2.8%) PF patients, three (6%) PV patients and in one (2%) healthy control. With the absence of significant difference with the healthy control group, our results were in agreement with two previously published data.^{22,24} The presence of anti-LKM1 was observed in only one PV patient and one healthy control. Similarly, we could not report any decisive diagnosis of autoimmune hepatitis associated with the presence of these antibodies. These data confirm that the association between pemphigus and autoimmune hepatitis is rare, even exceptional.

We also investigated the presence of vasculitis using AMPO and APR3. We found that these Abs were scarcely detected among PF and PV patients. Although they were observed in one healthy control, the positivity of the test was within the borderline. Our results are in agreement with data published by Nisihara *et al.*,²⁴ who did not detect the presence of these Abs in pemphigus patients.

The assessment of a probable associated arthritis was first analysed by looking for RF Abs using ELISA test. These Abs were detected in three (2.8%) PF patients, in seven (13.7%) PV patients and in seven (14%) controls however with no significant difference. In the PV group a unique positivity was observed for anti-keratin Abs in a patient known to be suffering from a concomitant Sjögren Syndrome disease. Moreover, it could be noticed that RF were scarcely reported in serological studies. In fact, before our study, only Mendes *et al.*²² and some case reports described their presence in pemphigus patients.³⁰

Celiac disease (CD) related Abs (ATG, ARA, AGD, AEA) were searched in this study. Although AEA were scarcely observed in this study, ARA and AGD (IgG) were frequently detected among PV patients with a significant difference. This difference was also observed when comparing PV to PF patients. Such association may be due to the clinical involvement of mucosa in PV, but not in PF.^{33,34} Despite the higher occurrence of these Abs, the presence of CD was not proven. Classically, the association of CD and bullous autoimmune diseases is herpetiformis dermatitis. In fact, both diseases target the same antigen (transglutaminase), but with different clinical manifestation. The association of pemphigus and CD was not reported; nevertheless the improvement of pemphigus under the use of gluten-free diet was reported in one case.³⁵

Our study is the first that focused on the presence of diabetes-specific autoAbs such as ICA, AIA2 and AGAD Abs. The presence of ICA in both cohorts of patients was not significantly different from the control group. It has to be noted that among positive ICA cases, there was a patient suffering from diabetes in each group of patients, which suggests a probable settlement of autoimmune diabetes in seropositive patients. The association of autoimmune diabetes and pemphigus was rarely reported in the literature. However, in Tunisian patients the appearance of corticoid-therapy-induced-diabetes was frequently observed among pemphigus patients when compared with other AID such as lupus, pemphigoid, sarcoidosis (unpublished data). The absence of diabetes-specific autoAbs suggests that the high frequency of the concomitant presence of both diseases is not related to latent autoimmune diabetes.

The association of thyroiditis and pemphigus was reported in few case reports or serological studies.^{15,36} Pitoia *et al.*¹⁵ found a higher prevalence of thyroid autoimmunity (46%) in patients with PV. This may be due to the small number of patients investigated in the study (15 PV patients). In a recent Iranian study, Ansar *et al.*³⁶ revealed the presence of a significant association of ATPO in pemphigus patients. These patients, however, did not present symptoms of thyroiditis. Our findings revealed also that ATPO were frequent among patients and controls as well as ATH. However, a significant difference was observed only with ATH ($P < 0.05$). As ATH titres were frequently within the border line, positive sera were furthermore tested by IIF on monkey thyroiditis. Using this test, positivity was confirmed in eight PF patients and two PV cases. When assessing clinical issues of these patients (19 PF patients, 7 PV patients) we found that two patients had thyroid

alteration (one PV and one PF patient). The analysis of hormonal levels revealed that most of the subjects have normal TSH levels.

Recent data investigated the prevalence of APL in bullous diseases.²⁶ They reported that A β 2GP1 were frequently detected among patients with bullous diseases (14.3% vs. 0% ($P < 0.05$)). The same study revealed that not only ACL but also anti-phosphatidyl serine/prothrombin Abs were significantly detected among PV (18.2% $P < 0.05$) and PF (23.8% $P < 0.05$) patients. The authors also reported that this humoral response was clinically associated with the presence of thromboembolism. In this study, sera were assessed for APL, ACL and A β 2GP1. A significant difference with the control group was observed only with ACL (IgM) in the PV group. Interestingly, when considering the positivity for at least one autoAbs (APL, ACL, A β 2GP1) the difference was close to the significance zone in the PV group. Two patients with APL had already APL syndrome; however, we did not notice the presence of any symptom of anti-phospholipid syndrome in the remaining positive patients. Usually when detected, these Abs are controlled after 3 months. However, these data are missing in our series as it is a retrospective study.

We also focused on the presence of more than four autoAbs in PF and PV patients; we noticed that this situation was mainly observed in patients with dual positivity for anti-Dsg1 and anti-Dsg3 (Table 3). This finding suggested that the presence of disease-specific markers (anti-Dsg) and non-specific markers (other autoAbs) is related to a polyclonal autoimmune response.

To explain the immunogenetic mechanisms for the coexistence of both organ-specific and systemic autoimmune diseases, Fridkiss-Hareli suggested three hypotheses (i) patients carrying two disease-susceptible HLA genes produce two types of autoreactive B cells that cooperate with autoreactive T cells that recognize autoantigen epitopes presented by susceptible HLA molecule; (ii) an epitope derives from either autoantigen and binds HLA determinant resulting in the induction of autoimmune disease by cross-presentation; and (iii) an epitope originating from the same autoantigen may react with two HLA molecules inducing thus the production of both types of autoAbs.³⁷

In conclusion, our results demonstrated that autoAbs other than anti-Dsg1 or anti-Dsg3 could be present in pemphigus patients and with higher occurrence for some specificities more than others. Almost all positive patients did not show any symptoms related to a concomitant AID and have similar disease prognostic. Moreover, cases with more than four autoAbs were frequently positive for both anti-Dsg1 and anti-Dsg3 suggesting a polyclonal B-cell activation or a dysregulation that affects different levels of the immune response. The prevalence of autoAbs observed in our series suggests that epitope spreading may concern not only desmoglein but also other ubiquitous and/or organ-specific antigens. The presence of autoAbs preceded clinical manifestations as reported in many studies.^{27,38,39} Thus, clinical and serological follow-up are needed and a systematic screening for autoAbs highly observed in this study would be recommended.

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