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## Level of IgG4 Anti-Desmoglein 3 Antibodies Correlates with Disease Activity in Patients with Pemphigus Vulgaris

José Antonio Delgado García<sup>1</sup>, Carmen Pérez Robles<sup>1</sup>, Juan José Mata Molanes<sup>1</sup>, Laura Moreno Narro<sup>1</sup>, Paula Arana Berganza<sup>1</sup>, Agustín España Alonso<sup>2</sup>, Cristina Moreno Parado<sup>1</sup>, Juana Merino Roncal<sup>1\*\*</sup> and Alfonso Sánchez-Ibarrola<sup>1\*\*</sup>

<sup>1</sup>Department of Immunology, Clínica Universidad de Navarra, Spain

<sup>2</sup>Department of Dermatology, Clínica Universidad de Navarra, Spain

#These authors contributed equally.

**Corresponding author:** Alfonso Sánchez-Ibarrola, Department of Immunology, Clínica Universidad de Navarra, Av. Pío XII, 36, 31008 Pamplona, Spain, Tel: 34-948-255400; Fax: 34-948-296500; E-mail: [asanchezib@unav.es](mailto:asanchezib@unav.es)

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### Abstract

**Background:** Pemphigus vulgaris is an autoimmune blistering disease that involves the skin and mucous membranes. The antigen target is desmoglein, a cadherin-type cell-to-cell adhesion molecule found in desmosomes. The levels of immunoglobulin G anti-desmoglein 3 antibodies correlate positively with disease activity. Furthermore, the immunoglobulin G4 isotype appears to play a predominant role in pathogenesis. However, anti-desmoglein 3 antibodies are present in patients with pemphigus vulgaris during clinical remission. Our objective was to measure and compare the immunoglobulin G4 response to desmoglein 3 in patients with pemphigus vulgaris in clinical remission with those with clinically active disease.

**Methods:** We included sera from 7 male and 10 female caucasian patients with an age range of 35-76 years. Patients were previously diagnosed with pemphigus vulgaris (12 with clinically active disease, 14 in clinical remission) and who were positive for anti-desmoglein 3 autoantibodies by enzyme-linked immunosorbent assay were studied. Anti-desmoglein 3 (total immunoglobulin G, immunoglobulin G1, and immunoglobulin G4 subclasses) antibodies were measured by modified enzyme-linked immunosorbent assay. Also, immunoglobulin G subclasses of anti-skin antibodies were tested by indirect immunofluorescence.

**Results:** The anti-desmoglein 3 specific immunoglobulin G1 and immunoglobulin G4 subclass study revealed that sera from patients with clinically inactive disease have lower anti-desmoglein 3 immunoglobulin G4 subclass antibody levels than sera from those with active disease by enzyme-linked immunosorbent assay ( $p=0.03$ ). However, there was no statistically significant difference for immunoglobulin G1 between the two groups. Similarly, the presence of immunoglobulin G subclasses of anti-skin antibodies by indirect immunofluorescence between the two groups was not statistically significantly different.

**Conclusion:** Levels of anti-desmoglein 3 immunoglobulin G4 subclass autoantibodies look very adequate to compare patients in remission with clinically active patients with pemphigus vulgaris ( $p=0.03$ ).

**Keywords:** Pemphigus vulgaris; Desmoglein; Immunoglobulin G; Anti-skin antibodies; Enzyme-linked immunosorbent assay; Indirect immunofluorescence; Clinical remission; Autoantibodies

### Introduction

Pemphigus vulgaris (PV) is an autoimmune disease that causes blistering of the skin and mucous membranes. The autoimmune target of pemphigus is desmoglein, a cadherin-type cell-to-cell adhesion molecule found in desmosomes [1,2]. The desmoglein 1 (Dsg1) and desmoglein 3 (Dsg3) isotypes are found in the stratified squamous epithelia where blisters form [1]. The development of an enzyme-linked immunosorbent assay (ELISA) for Dsg1 and Dsg3 has led to advancements in the classification of pemphigus [3,4]. Patients with PV have anti-Dsg3 and anti-Dsg1 IgG antibodies, and some patients have only anti-Dsg3 IgG antibodies [5-7].

In addition to the identification of pemphigus subtypes based on the antibody profile, the quantification of desmoglein antibodies correlates positively with the clinical activity of the disease. In patients with PV, quantitation of Dsg3 antibodies by ELISA parallels the clinical activity of the disease [6,8]. Several studies have shown that the participation of the IgG subclasses in the pathogenesis of PV is very different, with IgG4 having a predominant role. Different IgG subclasses associate with different functional properties and may thus determine the pathogenic

potential of IgG antibodies [3,9-11]. In active PV, IgG4 autoantibodies against Dsg3 were found to predominate. An IgG4 response is necessary in order for clinical disease to develop. On this basis, complement activation is not required for blister formation. Moreover, the total amount of serum IgG4 has been shown to increase in pemphigus [12].

These data suggest that IgG4-targeted therapies for pemphigus could have a beneficial effect. Intravenous immunoglobulin therapy lowers the levels of IgG4 anti-Dsg3 antibodies, and this effect correlates with improved clinical activity [13]. Additionally, the good results obtained in pemphigus and other diseases treated with B lymphocyte-depleting therapies are related to decreased levels of serum IgG4 or Dsg3 autoantibodies [14,15].

Despite that the levels of IgG4 anti-Dsg3 autoantibodies correlate with the clinical activity of PV in patients, the presence of anti-Dsg3 autoantibodies has been found in patients in clinical remission [6,11]. These data call the usefulness of these assays in assessing disease activity into question. Also, the meaning of these autoantibodies during clinical remission is a matter of discussion [16].

The aim for the study was to assess the IgG subclass composition of anti-Dsg3 antibodies in two groups of patients, clinical remission patients

compared with patients who have clinically active disease. We evaluated levels of IgG1 and IgG4 anti-Dsg3 antibodies in both groups by ELISA. Sera from patients with PV in clinical remission contained lower IgG4 anti-Dsg3 antibody levels than sera from patients with clinically active disease ( $p=0.03$ ). We also compare these data with the IgG subclass composition of anti-skin antibodies (ASA) by indirect immunofluorescence (IIF).

## Materials and Methods

### Human Sera

We collected sera from 7 male and 10 female caucasian patients with an age range of 35-76 years previously diagnosed with PV by clinical presentation, histology, and positive serological test. We selected 26 serum samples positive for anti-Dsg3 autoantibodies by ELISA. The project was done under the approval of Ethics in Research Committee of Universidad de Navarra (n° 79/2010).

Sera were classified into two groups depending on the activity of each patient's disease (supplementary information). The first group consisted of 12 serum samples taken at a situation of clinical activity, characterized as the presence of several active lesions on the skin and/or mucous membranes prior to any treatment. The second group included 14 serum samples collected in clinical remission, which was defined as an absence of active lesions on the skin and/or mucous membranes while the patient received treatment with low-dose (less than 15 mg per day) corticosteroids as maintenance treatment.

The treatment given to patients in the second group was as follows: high-dose (more than 15 mg per day) corticosteroids (patients P1, P6, P8, P9, P13 and P14), rituximab, 4 weekly intravenous doses of 375 mg/m<sup>2</sup> each (patients P4, P5, P12, P15 and P16), or intravenous cyclophosphamide pulse therapy (patients P2, 7 doses of 1000 mg per dose; P7, 3 doses of 1700 mg per dose; and P10, 10 doses of 1200 mg per dose).

To establish the cutoff values for anti-Dsg3 IgG1 and IgG4 subclasses by ELISA, 20 sera from healthy persons were selected as controls. All sera were stored at -20°C.

### Detection of Dsg3 antibodies by enzyme-linked immunosorbent assay

Total IgG autoantibodies against Dsg3 were quantified using a commercial Dsg3 ELISA Kit (Euroimmun, Luebeck, Germany) according to the manufacturer's instructions. Anti-Dsg3 IgG1 and IgG4 subclasses were quantified using the same commercial Dsg3 ELISA Kit (Euroimmun, Luebeck, Germany), except that the mouse antihuman IgG1 or IgG4 conjugated with horseradish peroxidase (Beckman Coulter Inc, Fullerton, California) was used as the secondary antibody.

Serum samples were serially diluted starting 1:100. The lowest dilution measurable in the optical density (OD) range at a wavelength of 450 nm was determined as the optimal serum dilution. The value for each serum was calculated by multiplying the OD value at the optimal serum dilution by the dilution factor. The IgG1 conjugate was diluted 1:1000 and the IgG4 conjugate was diluted 1:3000. All dilutions were made with sample buffer provided in the commercial Dsg3 ELISA Kit (Euroimmun, Luebeck, Germany).

Assay values were considered positive when the IgG ELISA was greater than or equal to 20 relative units (RU)/mL, the cutoff recommended by the manufacturer (Euroimmun, Luebeck, Germany). The cutoff values for anti-Dsg3 IgG subclasses were 0.643 OD for IgG1 and 0.237 for IgG4 at a wavelength of 450 nm. These cut off points were established based on analysis of values in 20 control serum samples. The values of the cutoffs were equal to the mean plus 2 standard deviations (SD) of the control values.

### Indirect immunofluorescence

Anti-skin antibodies were detected by standard IIF on monkey esophagus substrate (A. Menarini Diagnostics, Florence, Italy). The staining procedure employed was as follows: serial serum dilutions (from 1/10 to 1/640) were applied to sections for 30 minutes (min). The slides were washed in phosphate-buffered saline (PBS) for 10 min using a magnetic stirrer and were incubated for 30 min with a secondary antibody. A final wash of 10 min was performed and mounting medium included. Primate anti-human IgG FITC-conjugate (Inova Diagnostics Inc, San Diego, California) was used as the secondary antibody for ASA analysis. For IgG subclass staining, mouse anti-human IgG1 or mouse anti-human IgG4 FITC-conjugated monoclonal antibodies (Beckman Coulter Inc, Fullerton, California) were used as secondary antibodies.

### Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics v20 software. Normality of the dataset was analyzed with the Shapiro-Wilk test. Based on these results, we performed a non parametric (Mann-Whitney U) test to compare variables. The linear relationship was studied with the Spearman's rank correlation coefficient. In all cases, a p-value <0.05 was considered significant.

## Results

### Quantification of anti-Dsg3 antibodies by ELISA

Sera from patients with clinically active PV had higher IgG anti-Dsg3 values (median=254.5 RU/mL) than serum samples from patients with clinically inactive disease (median=178.0 RU/mL) ( $p=0.04$  by U Mann-Whitney test, Table 1). Also the IgG4 anti-Dsg3 antibody levels were different between the two groups. Patients with clinically active disease had higher values (median=2.28 OD) compared with patients in clinical remission (median=0.68 OD) ( $p=0.03$ , by U Mann-Whitney test, Table 1). However, there was no statistically significant difference in IgG1 levels between the two groups (median=0.75 OD and 0.71 OD, respectively). To summarize, serum samples from patients with clinically inactive PV had lower values of total IgG and IgG4 subclass anti-Dsg3 than sera from patients with clinically active disease (Figure 1).

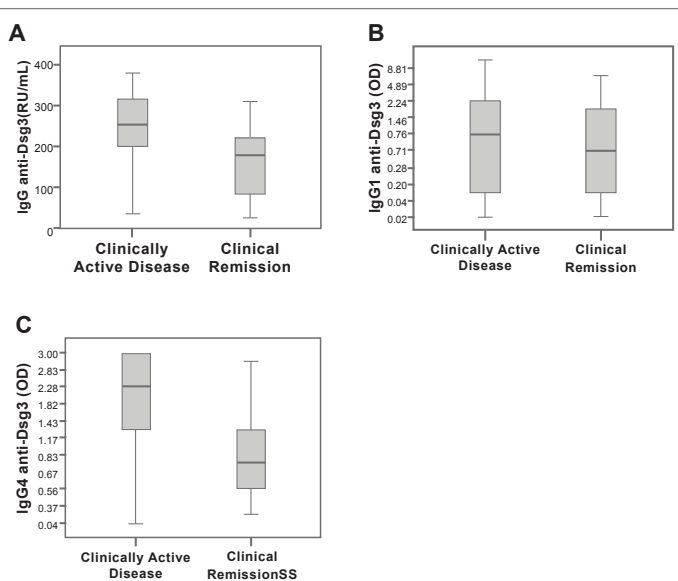
### Detection of IgG total and subclasses of ASA antibodies by IIF

We analyzed data from IIF to detect IgG, IgG1, and IgG4 ASA. The serum dilution with a typical pattern of fluorescence of pemphigus vulgaris (staining of intercellular spaces in monkey esophagus) was determined as positive. We compared data from patients in clinical remission with those experiencing a period of clinically active disease (Figure 2). There were no statistically significant differences between clinical remission patients and clinically active patients. Only ASA-specific IgG4 trended towards significance ( $p=0.05$  by U Mann-Whitney test, Table 1).

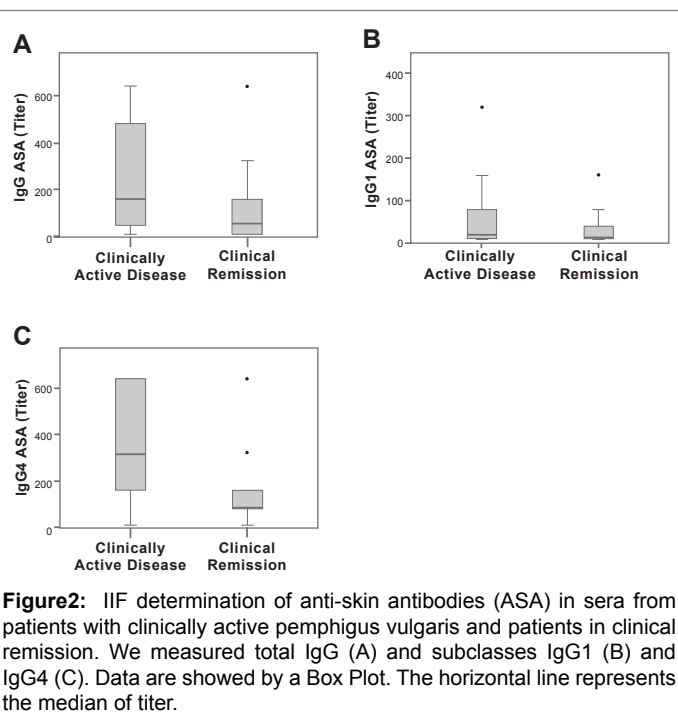
		Active disease	Clinical remission	p-value
ELISA	IgG (RU/mL)	254.5	178.0	0.04
	Anti-Dsg3			
	IgG1 (OD)	0.75	0.71	NS
	IgG4 (OD)	2.28	0.68	0.03
IIF	IgG	160	60	NS
	ASA titer			
	IgG1	20	10	NS
	IgG4	320	80	0.05

**Abbreviations:** ASA: Anti-skin Antibodies; Dsg3: Desmoglein 3; ELISA: Enzyme-linked Immunosorbent Assay; IIF: Indirect Immunofluorescence; NS: No Significant; OD: Optical Density; RU/mL: Relative Units per Milliliter.

**Table 1:** Anti-Desmoglein 3 and Anti-Skin Antibodies in Patients with Pemphigus Vulgaris



**Figure 1:** ELISA determination of anti-desmoglein 3 antibodies (anti-Dsg3) in sera from patients with clinically active pemphigus vulgaris and patients in clinical remission. We measured total IgG (A) and subclasses IgG1 (B) and IgG4 (C). Data are showed by a Box Plot. The horizontal line represents the median of relative units (RU)/mL or optical densities (OD) measured at 450 nm.



**Figure 2:** IIF determination of anti-skin antibodies (ASA) in sera from patients with clinically active pemphigus vulgaris and patients in clinical remission. We measured total IgG (A) and subclasses IgG1 (B) and IgG4 (C). Data are showed by a Box Plot. The horizontal line represents the median of titer.

### Correlation between modified ELISA with conventional IIF

Data from the IIF and ELISA showed a very good correlation. The values of both IgG1 and IgG4 anti-Dsg3 and ASA IgG1 and IgG4 antibodies showed a positive correlation ( $R=0.74$  and  $0.75$  for IgG1 and IgG4 values, respectively).

### Discussion

We compared the levels of IgG1 and IgG4 anti-Dsg 3 antibodies by ELISA in sera from patients with PV in clinical remission with levels

from patients with active disease. We also studied the distribution of IgG subclasses in ASA in the two groups of patients by IIF. The levels of anti-Dsg3 IgG4 subclass antibodies detected by ELISA were lower in patients in clinical remission, differentiating patients with clinically active PV from those with inactive disease. The IgG subclasses of ASA detected by IIF did not reach statistically significant differences between the groups.

The clinical evaluation of patients has been easier since the identification of Dsg3 antibodies detected by ELISA because these data correlate with the clinical activity of the disease [8,17,18]. Several therapeutic approaches are used to treat PV, including intravenous immunoglobulins or B lymphocyte depletion, which have been successful because of their effects on the level of anti-Dsg3 autoantibodies [13-15]. Moreover, the distinction between IgG subclasses provides a better understanding of the pathogenesis of this disease [3,9]. IgG4 has been implicated as the IgG subclass responsible for the tissue damage in PV [19].

However, it has been repeatedly reported that numerous pemphigus patients present with anti-Dsg 3 antibodies during clinical remission [6,11,16,20,21]. These observations raise interesting questions about the value of Dsg3 antibody measurements in the evaluation of clinical activity. The role of these autoantibodies during periods of clinical remission is unknown, although several possibilities are proposed. One is that the autoantibodies remaining in circulation during remission are predominantly non-pathogenic rather than pathogenic [16,22]. Another explanation is that the immunosuppressive treatments used lead to a symptomatic suppression of the disease while also being insufficient to completely block the autoantibody response [16].

The presence of anti-Dsg3 antibodies in patients in clinical remission together with the described pathogenic role of anti-Dsg3 IgG4 subclass antibodies led us to test whether the levels of anti-Dsg3 IgG4 subclass antibodies differ in patients with PV in clinical remission compared with those of patients whose disease is clinically active. We show that the levels of anti-Dsg 3 antibodies are lower during clinical remission with respect to levels during active disease as previously reported [6]. In this report, the authors claimed that monitoring the levels of these autoantibodies in patients with PV during clinical remission on a regular basis could be useful for assessing disease activity. Our study adds information, showing that the levels of IgG4 anti-Dsg3 antibodies are also lower in patients with PV during clinical remission. In a group of heterogeneous PV patients in clinical remission which include anti-Dsg3 negative patients, Dhandha et al. showed that there is not a subclass switch, and that these patients maintain high levels of both IgG4 and IgG1. We found that the levels of IgG4 anti-Dsg3 are lower in patients in clinical remission, but that there was no change in the level of IgG1 in patients in remission with respect to those with clinically active disease. Perhaps these differences occurred because our patients are more homogeneous concerning the positivity of Dsg3 antibodies during clinical remission.

These findings have particular relevance because of the well known pathogenic role attributed to IgG4 antibodies in different pathological conditions [3]. The decisive role of IgG4 antibodies in blister formation has been documented in patients with PV [9,10]. On the other hand, the beneficial effects of new therapeutic measures on the clinical disease activity of PV correlate with the levels of IgG4 autoantibodies. Intravenous immunoglobulin treatment ameliorates PV symptoms, inducing a decrease in anti-Dsg3 IgG4 subclass antibodies [13]. Moreover, B lymphocyte-depleting therapy such as anti-CD20 monoclonal antibodies produces a clinical benefit in patients with PV that is associated with a decrease in anti-Dsg3 antibodies [15].

Interestingly, the effect of anti-CD20 therapy in IgG4-related pathology is linked to decreases in serum IgG4 levels [14]. Our data showing a decrease in IgG4 anti-Dsg3 autoantibodies in patients with low clinical

activity could help to better understand these findings. No statistically significant differences in the IIF data were observed between patients with PV according to the presence or absence of clinical disease activity. Only the titer of ASA-IgG4 subclasses trended towards a significant difference ( $p=0.05$ ). This could be due to the lower sensitivity with respect to ELISA, and the different characteristics of both assays.

Measurements of IgG4 anti-Dsg3 autoantibodies appear to be a good marker when antibody levels are to be correlated with the degree of clinical activity of PV. Furthermore, the use of biological parameters, such as the detection of IgG 4 anti-Dsg 3, look very adequate for interpreting the efficacy of a given new treatment in patients with PV. Additionally, the pathogenic significance of the presence of these antibodies when there is no clinical activity deserves further research.

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