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Desmoglein antibody titers and loss of keratinocyte adhesion *in vitro* are associated with disease severity in patients with Pemphigus Vulgaris

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An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
In partial fulfillment of the requirements for the degree of Master of Science
in Clinical Research
2019

ABSTRACT

Desmoglein antibody titers and loss of keratinocyte adhesion *in vitro* are associated with disease severity in patients with Pemphigus Vulgaris

By Maxine F. Warren

Pemphigus vulgaris (PV) is an autoimmune bullous disease characterized by severe epidermal blistering and mucous membrane erosions. PV is caused by antibodies directed against the desmosomal cadherin desmoglein 3 (Dsg3), and in cases involving the skin, both Dsg3 and Dsg1. PV can be assessed clinically using the Pemphigus Disease Area Index (PDAI) and by ELISA for anti-Dsg IgG titers in patient sera. *In vitro*, PV IgG activity can be investigated by incubating normal human keratinocytes with PV patient IgG and monitoring changes in strength of cell-cell adhesion caused by desmosome disruption using a dispase-based cell-cell dissociation assay. However, the relationship between clinical PV assessments and *in vitro* activity of patient IgG have not been systematically evaluated. In the present study, we compared the relationship between the *in vitro* pathogenicity of PV IgG from 23 patients to their PDAI score and ELISA titers. Overall, Dsg1 ELISA values showed a stronger correlation with PDAI scores ($r=0.56$, $p<0.001$) when compared to Dsg3 ELISA ($r=0.45$, $p<0.02$). However, the sum of Dsg3 and Dsg1 ELISA values exhibited the strongest correlation to PDAI ($r=0.67$, $p<0.0001$). Additionally, the loss of cell-cell adhesion strength as assessed using *in vitro* dispase cell dissociation assays exhibited a positive correlation with Dsg3 ELISA titers ($r=0.54$, $p<0.005$), supporting the validity of the dispase assay as a measure of PV pathogenicity. These findings confirm the association of Dsg ELISA values with PDAI and establish a relationship between Dsg IgG titers in patients and loss of adhesion in cultured keratinocytes.

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INTRODUCTION

Pemphigus vulgaris (PV) is a devastating autoimmune skin disease characterized by severe epidermal blistering and mucosal membrane erosions. Its incidence is estimated to be 0.1-0.5 cases per 100,000 people per year, with an average age of onset between 40 and 60 years (1). Although rare, PV can be associated with significant morbidity and was considered almost universally fatal before the advent of immunosuppressive agents, which are currently the mainstay of treatment (2). PV is caused by IgG autoantibodies directed against desmosomal cadherins, a class of cell-cell adhesion proteins with critical roles in skin biology and pathophysiology; specifically, desmogleins 3 (Dsg3, located primarily just above the basal layer of epidermis) and 1 (Dsg1, located primarily in the superficial epidermis) are targeted (Figure 1) (3). This attack on desmosomal integrity results in loss of keratinocyte cohesion, a phenomenon termed acantholysis, which translates clinically into intraepidermal blistering that can be extensive and debilitating.

In the present study, we sought to determine if these anti-desmoglein antibodies cause alterations in desmosomal adhesion *in vitro* that are directly related to and indicative of disease severity as measured both clinically and serologically. To that end, we isolated IgG from PV patients and used the purified antibodies to perform *in vitro* experiments and compare the data to the current gold standard clinical measures of disease activity, hypothesizing that direct correlations between these measurements would be observed. We hope that these studies may form a foundation for developing new ways to assess PV patient IgG activity, and thus guide dermatologists in their use of aggressive immunosuppressive agents – or better still, to aid in the development of more targeted treatment options for PV.

BACKGROUND

There are two clinical variants of PV, mucosal dominant and mucocutaneous (3). Mucosal dominant patients present with mucosal erosions and primarily express autoantibodies against Dsg3, while mucocutaneous patients have erosions on both mucosal and epidermal surfaces and produce antibodies against both Dsg1 and Dsg3 (3, 4). A "desmoglein compensation hypothesis" was proposed in 1999 to explain the variation seen in blister locations depending upon the autoantibody profile (4). This theory postulates that in mucous membranes, there is enough Dsg3 in the superficial epidermis to compensate for loss of Dsg1 such that even if all of the Dsg1 is knocked out by autoantibody attack, the integrity of the desmosomes and therefore of cell-cell adhesion is maintained and blisters do not form in these sites. This differentiates PV from pemphigus foliaceous, in which Dsg1 is the only autoantigen and mucous membranes remain intact due to Dsg3 compensation. In the mucocutaneous form of PV, by contrast, both skin and mucous membranes are affected due to antibodies presents against both desmogleins.

For both mucocutaneous and mucosal dominant disease, there are currently two gold standard measurement tools for monitoring disease severity. The first is the Pemphigus Disease Area Index (PDAI), a validated scoring system that standardizes the clinical evaluation of PV across institutions and physicians with a high inter-rater reliability (ICC~0.98) (5). A patient may be given up to 120 points for skin disease, 120 points for mucosal disease, 10 points for scalp involvement, and an additional 13 points for skin damage (such as post-inflammatory hyperpigmentation), for a total possible PDAI score of 263. The second is enzyme-linked immunosorbent assay (ELISA), which is the standard method for measuring the level of antibodies

present in patient sera (6). Generally, higher antibody titers are associated with a more severe clinical presentation of a given autoimmune disease; with PV, however, several studies have demonstrated that this direct correlation is not always observed (7, 8). Indeed, even in cases of complete clinical remission for more than 6 months, anti-Dsg titers often remain elevated and it is unclear whether this represents true active disease (9). Some studies report that anti-Dsg1 ELISA levels correlate better with PV disease severity, while others find that anti-Dsg3 ELISA levels are more strongly correlated, particularly for mucosal dominant PV patients (10-12). A recent international study revealed that both anti-Dsg1 and anti-Dsg3 ELISA scores correlated with PDAI at diagnosis, but that these correlations, particularly Dsg3 ELISA, were no longer significant after treatment (13). Perhaps this is reflective of the fact that some PV treatments (such as systemic corticosteroids) are able to rapidly suppress inflammation before other treatments (such as rituximab) are able to suppress the production of antigen-specific antibodies, such that clinical improvement is achieved faster than decrease in anti-desmoglein ELISA titers. Alternatively, perhaps while some Dsg3 antibodies are still able to bind to the protein, they are somehow less pathogenic. In either case, this conflicting data leads to uncertainty about how aggressively to treat PV patients based on either PDAI or ELISA values alone.

The precise mechanism by which PV IgG causes loss of cellular cohesion in patient skin has also not been fully elucidated. This knowledge gap is largely because our current understanding of PV pathomechanisms is derived almost exclusively from in vitro models of disease. PV lends itself well to being studied using the “disease in a dish” approach, because the purified autoantibodies from patients can be added to cultured skin cells to replicate the disease in vitro. Such experiments in the past have demonstrated that exposure of cultured keratinocytes to

pathogenic PV antibodies triggers a reproducible sequence of events culminating in acantholysis: clustering of cell surface Dsg3, endocytosis of these clusters, and their disassembly within the cell (14-17). Recently, we confirmed with super-resolution microscopy that mislocalization of desmosome proteins also occurs within actual PV patient skin (18). However, much is still unknown about how the processes observed *in vitro* correlate with disease progression *in vivo*. Specifically, a direct comparison of *in vitro* and clinical assessments of disease severity has not been performed to date.

To address this, we designed a cross-sectional study of 23 patients diagnosed with PV at the Emory Clinic between 2012-2018 and compared ELISA scores to PDAI. We then directly tested the pathogenicity of patient IgG using an *in vitro* assay of cell-cell adhesion strength to determine whether this quantitative assessment of desmosomal mechanical disorganization correlated with either the clinical (PDAI) or molecular (ELISA) measures of disease severity. The overarching hypothesis underpinning these studies is that anti-desmoglein antibodies in PV patients cause alterations in desmosomal adhesion that are directly related to and indicative of disease severity. In comparing the *in vitro* assay to these existing gold standard clinical measurements, we hope to validate its future applications as a model of disease to continue to study PV pathomechanisms, as an additional tool for clinical assessment, and as a testing medium for drug screens to be able to reduce dependence on immunosuppressive therapy.

MATERIALS AND METHODS

Specific Aim

In this study, we sought to determine whether there is a correlation between clinical disease severity (as measured by PDAI score), anti-Dsg1 and anti-Dsg3 antibody levels (as measured by ELISA), and the degree of loss of skin cell-cell adhesion (as measured by dispase fragmentation assay) among patients with pemphigus vulgaris treated at Emory Clinic between 2012-2018. We hypothesized that a direct positive correlation would be observed between all three data points for each patient.

Study design and population

Study subjects were recruited for enrollment in this cross-sectional study if they were seen at the Emory Dermatology Clinic between 2012-2018 and carried a diagnosis of pemphigus vulgaris, confirmed by both intraepidermal anti-Dsg3 staining on direct immunofluorescence and by anti-Dsg3 antibody titer of >0 by ELISA. Relevant clinical information including sex, date of birth, PDAI scores, ELISA levels of Dsg1 and Dsg3 autoantibodies, and type and duration of treatment were obtained either from chart review or at the time of enrollment and recorded. Patients with other autoimmune blistering diseases, such as pemphigus foliaceous or paraneoplastic pemphigus, were excluded. Permission for use of all stored samples was obtained from Emory University's Institutional Review Board. We then purified each patient's IgG antibody from a serum sample drawn as close to the date of the clinical assessment as possible (within a maximum of 2 weeks)

and performed dispase assays on human keratinocytes using each patient's purified antibody as detailed below.

Purification of IgG

All blood specimens were drawn in the Emory Dermatology Clinic by a certified phlebotomist. Specimens were collected from enrolled subjects into five red top vacutainer tubes and allowed to clot for 30 minutes in a vertical position. Tubes were then centrifuged for 15 minutes, and sera was pipetted off from the top into a single plastic transfer tube labeled with the patients study ID number and stored at -80°C. Samples were later thawed to room temperature, and IgG was purified using the Melon purification kit (Thermofisher 45206). Concentration of the total recovered IgG was measured using a Nano drop lite from thermofisher.

Cells and culture conditions

Primary human keratinocytes (HKs) were isolated as described by Calkins *et al* and cultured in KBM-Gold basal medium (100 μM calcium) supplemented with KGM-Gold Single-Quot Kit (Lonza, Walkersville, MD) (16). To make low calcium media, calcium was removed from standard keratinocyte growth media using BT Chelex 100 resin (Bio-rad #143-2832) and supplemented with $CaCl_2$ to reach 30mM.

Dispase-based fragmentation assay

HKs were cultured to 100% confluence in 24-well tissue culture plates and switched to 50 μM calcium to prevent any junction assembly for 16-18 hrs prior to switching to 550 μM calcium for 3 hrs to allow for junction assembly (19, 20). HKs were then exposed to NH or PV IgG for 6hrs at 37°C. Monolayers were removed from the cell culture substrate using the enzyme dispase and gently washed twice with PBS+ before transfer to an epindorf tube. Monolayers were exposed to mechanical force by being taped to an orbital shaker to for 1-2 minutes to induce fragmentation (Figure 5B). Fragments were fixed in paraformaldehyde and stained with Methelyne blue prior to imaging using (CTL, cellular technologies). The number of monolayer fragments was then counted. The following formula was used to normalize fragments between replicates and generate the disassociation index score with AK23 as the positive (pathogenic) control and Normal Human (NH) treatment as the negative control (19).

$$\frac{\#Fragments_{sample} - \#Fragments_{negative\ control}}{\#Fragments_{positive\ control} - \#Fragments_{negative\ control}}$$

For each IgG specimen, this process was repeated 3 times for a total of 4 disassociation index scores per sample, and an average was taken.

Data Analysis and Statistical Methods

One tailed, Non-parametric Spearman tests were used to identify correlations between PDAI, ELISA, and dissociation index scores, and p-values are reported (Table 3) (21). We also used linear

regression to explain the variation in PDAI score explained by the covariates Dsg1 and Dsg3 ELISA levels, and R^2 is reported (Table 4). All statistical tests were performed using GraphPad Prism 8.2.1 Software.

RESULTS

Epidemiology and clinical phenotypes

Twenty-five samples from 23 patients were included in this study. Samples from the same patient were taken at different points during their treatment and disease progression. The ages ranged from 25 to 68 years old with a mean of 47.3 years and a standard deviation of 10.5 years (Table 1). Nine patients (39.1%) were female and 14 (60.9%) were male. Ten (43.5%) patients and 11 (44%) samples were mucosal dominant while 14 (56.5%) patients and 15 (56%) samples were mucocutaneous. Disease severity varied by patient as determined by PDAI and ELISA scores (Figure 2). Most patients were already on at least one immunosuppressive agent at the time of PDAI and ELISA scoring, including prednisone (n=18), rituximab (n=7), or azathioprine, mycophenolate, dapsone, or doxycycline (n=1 each). Each patient was assigned a unique symbol to facilitate tracking across graphs, with mucosal dominant patients depicted as squares and mucocutaneous patients are depicted with circles (Figure 3).

Total ELISA titer correlates with PDAI

When comparing all 25 samples regardless of clinical subtype, both Dsg3 ($r=0.45$, $p<0.012$) and Dsg1 ELISA titers ($r=0.56$, $p<0.002$) demonstrated a moderate correlation with PDAI (Table 3, Figure 4). Interestingly, cumulative Dsg1 and Dsg3 ELISA values were more strongly associated with high PDAI scores ($r=0.67$, $p<0.0001$) than either titer alone. Similarly, a linear regression model showed that total ELISA explained more of the variation in PDAI than either

Dsg1 or Dsg3 alone (36% vs 31% and 17%, respectively) (Table 4). Adding an interaction term between Dsg1 and Dsg3 to the model did not yield a significant result.

Looking at the two variants of PV separately, we found that for mucosal dominant (mPV) patients, PDAI strongly correlates with Dsg3 ELISA ($r=0.61$, $p<0.05$) and total ELISA ($r=0.61$, $p<0.05$) but does not correlate significantly with Dsg1 ELISA. Conversely, for mucocutaneous (mcPV) patients, we found that PDAI demonstrated a strong correlation with Dsg1 ($r=0.73$, $p<0.001$) and total ELISA ($r=0.68$, $p<0.003$) but did not correlate with Dsg3 ELISA.

DSG3 ELISA correlates with disassociation index

We found that for mPV patients, Dsg3 titers demonstrate a moderate correlation to the degree of keratinocyte fragmentation as measured by the disassociation index ($r=0.68$, $p<0.03$). For the total patient pool, Dsg3 titers also demonstrate a moderate but statistically significant correlation to the degree of fragmentation ($r=0.54$, $p<0.003$). In contrast, Dsg1 ELISAs did not correlate to disassociation index in any of the patient groups.

PDAI correlates to the disassociation index for mucosal dominant patients

We found that for mPV patients, PDAI strongly correlates with disassociation index ($r=0.78$, $p<0.01$); however, for mcPV patients as well as for the total patient pool, no statistically significant relationship between PDAI and the disassociation index was identified.

DISCUSSION AND CONCLUSIONS

Total ELISA titer correlates with PDAI

While the PDAI score has been validated in a number of studies, there has yet to be a clear consensus regarding the relationship between anti-desmoglein ELISA titers and clinical activity of PV (5-9). It has been shown that generally, mucosal dominant patients mainly express anti-Dsg3 antibodies, while mucocutaneous patients produce antibodies against both Dsg3 and Dsg1, and that generally, these anti-desmoglein autoantibodies increase with increased disease severity (3, 4). However, if ELISA values are going to be used by clinicians to gauge disease activity and thereby guide treatment decisions, there is a need to more precisely understand how these scores correlate with disease progression as measured by PDAI because the clinical impact and potential morbidity and mortality associated with immunosuppressive therapy is significant (22).

In our study, both Dsg1 and Dsg3 titers demonstrated a moderate correlation with PDAI individually across all patients, consistent with previous reports. Interestingly, cumulative anti-desmoglein ELISA values were more strongly associated with PDAI score than either titer alone, and a linear regression model similarly revealed that total ELISA explained more of the variation in PDAI than either titer alone. These results suggest that perhaps the total antibody burden may be playing a synergistic role biologically that contributes to clinical disease severity more than either specific antibody individually.

When analyzing the two clinical subtypes of PV separately, we found that anti-Dsg1 ELISA levels correlate more strongly with PDAI than anti-Dsg3 ELISAs in patients suffering from primarily cutaneous disease, whereas the converse held true for patients with mucosal dominant

disease. These results are consistent with prior studies that suggest that anti-Dsg1 autoantibody titers correlate more strongly with cutaneous PV, while anti-Dsg3 titers correlate with mucosal PV. These findings also make sense within the context of the desmoglein compensation theory discussed earlier, which postulates that there is enough Dsg1 present in skin—but not in mucosal tissue—to compensate for the loss of Dsg3 alone, so a patient needs only antibodies against Dsg3 to have mucosal involvement, whereas loss of both Dsg1 and Dsg3 is needed for the integrity of the skin to be compromised as well.

DSG3 ELISA correlates with disassociation index

Keratinocyte monolayer fragmentation assays have been used to study the pathogenicity of PV autoantibodies. In this dispase-based assay, higher levels of keratinocyte monolayer fragmentation after treatment with PV IgG indicates greater loss of cell-cell adhesion strength (19). However, to our knowledge, no prior studies have correlated the degree of fragmentation seen *in vitro* to clinical measures of disease severity or to autoantibody titers. After applying purified PV IgG from 25 samples to cultured human keratinocytes and comparing the degree of cell-cell disassociation to clinical severity scores, we found a moderate correlation between the degree of fragmentation and anti-Dsg3 titers for mPV patients as well as for the total patient pool. In contrast, Dsg1 ELISAs did not correlate to disassociation index in any of the patient groups. These results are consistent with what we would expect, since the keratinocyte culture method used in this assay forms desmosomes predominantly containing Dsg3 rather than Dsg1 due to the basal

differentiation status of the cells (23). Overall, these data confirm a relationship between Dsg3 autoantibody titers and loss of cell-cell adhesion in keratinocyte cell culture.

PDAI correlates to the disassociation index for mucosal dominant patients

We found that for mPV patients, clinical disease severity as measured by PDAI strongly correlates with loss of cell-cell adhesion as measured by the disassociation index; however, for mcPV patients as well as for the total patient pool, no significant relationship between PDAI and the disassociation index was identified. Again, given the correlation observed in this study between anti-Dsg3 titers and PDAI in mPV patients and given that the dispase assay is Dsg3-based, it is likely that the correlation seen between PDAI and disassociation index in this subgroup is Dsg3-mediated. These findings validate the disassociation assay as an assessment tool for mPV patient clinical disease severity.

Concluding remarks

Together, these findings confirm the association of anti-desmoglein ELISA values with PDAI and establish a relationship between desmoglein autoantibody titers in patients and loss of adhesion in cultured keratinocytes. In order to explore the observation found in this and other studies that anti-Dsg1 levels may indicate cutaneous disease activity while anti-Dsg3 levels may indicate mucosal disease activity, we plan to stratify the PDAI score into its cutaneous and mucosal components and perform similar correlation tests to see if the results remain consistent.

It is also possible that stronger or more statistically significant correlations would have been observed between our 3 metrics had our patients not already been treated with immunosuppressive therapy, as clinical symptoms can be alleviated before a decrease in autoantibody titer is observed, and the effects of treatment on patient IgG activity *in vitro* is unknown (9,11). To investigate this further, we plan to perform additional retrospective analyses to test whether the types and doses of the various treatments received by patients had any effect on the disassociation assay.

In the future, we and others hope to continue using our *in vitro* model of PV as a reliable way to study disease pathophysiology in the laboratory and as a platform for drug discovery screens in order to reduce dependency on immunosuppressive therapy. We also hope to explore other *in vitro* assays that measure degree of morphological alterations in desmosome architecture to determine if those changes correlate with clinical markers as well.

REFERENCES

1. Kridin K. Pemphigus group: overview, epidemiology, mortality, and comorbidities. Immunol Res. 2018;66(2):255-70.

2. Bystryn JC, Steinman NM. The adjuvant therapy of pemphigus. An update. Arch Dermatol. 1996;132(2):203-12.

3. Amagai M, Tsunoda K, Zillikens D, et al. The clinical phenotype of pemphigus is defined by the anti-desmoglein autoantibody profile. J Am Acad Dermatol. 1999;40(2 Pt 1):167-70.

4. Amagai M, Komai A, Hashimoto T, Shirakata Y, et al. Usefulness of enzyme-linked immunosorbent assay using recombinant desmogleins 1 and 3 for serodiagnosis of pemphigus. Br J Dermatol. 1999;140(2):351-7.

5. Rahbar Z, Daneshpazhooh M, Mirshams-Shahshahani M, et al. Pemphigus disease activity measurements: pemphigus disease area index, autoimmune bullous skin disorder intensity score, and pemphigus vulgaris activity score. JAMA Dermatol. 2014;150(3):266-72.

6. Harman KE, Seed PT, Gratian MJ, et al. The severity of cutaneous and oral pemphigus is related to desmoglein 1 and 3 antibody levels. Br J Dermatol. 2001;144(4):775-80.

7. Cozzani E, Di Zenzo G, Riva S, et al. Are clinical phenotype and autoantibody profile always concordant in pemphigus? A study in a cohort of pemphigus patients. Eur J Dermatol 2013; 23(1):40-8

8. Herrero-Gonzalez JE, Mascaro JM Jr, Iranzo P, Herrero C. Atypical pemphigus: discordance between clinicopathological findings and the antigenic profile in four cases. J Cutan Pathol 2006;33:502-7

9. Russo I, Paolo De Siena F, Saponeri A, et al. Evaluation of anti-desmoglein-1 and anti-desmoglein-3 autoantibody titers in pemphigus patients at the time of the initial diagnosis and after clinical remission. Medicine 2017 Nov; 96(46): e8801

10. Patsatsi A, Kyriakou A, Giannakou A, et al. Clinical significance of anti-desmoglein-1 and -3 circulating autoantibodies in Pemphigus Patients Measured by Area Index and Intensity Score. Acta Derm Venereol. 2014;94(2):203-6.

11. Zhao CY, Murrell DF. Outcome measures for autoimmune blistering diseases. J Dermatol. 2015;42(1):31-6.

12. Sharma VK, Prasad HR, Khandpur S, Kumar A. Evaluation of desmoglein enzyme-linked immunosorbent assay (ELISA) in Indian patients with pemphigus vulgaris. Int J Dermatol. 2006;45(5):518-22.

13. Hebert V, Boulard C, Houivet E, et al. French Study Group on Autoimmune Bullous Skin D, Autoimmune Bullous Skin Disease Task Force of the European Academy of D, Venereology. Large International Validation of ABSIS and PDAI Pemphigus Severity Scores. J Invest Dermatol. 2019;139(1):31-7.

14. Jennings JM, Tucker DK, Kottke MD, et al. Desmosome disassembly in response to pemphigus vulgaris IgG occurs in distinct phases and can be reversed by expression of exogenous Dsg3. J Invest Dermatol. 2011;131(3):706-8.

15. Delva E, Jennings JM, Calkins CC, et al. Pemphigus vulgaris IgG-induced desmoglein-3 endocytosis and desmosomal disassembly are mediated by a clathrin- and dynamin-independent mechanism. The Journal of biological chemistry. 2008;283(26):18303-13.

16. Calkins CC, Setzer SV, Jennings JM, et al. Desmoglein endocytosis and desmosome disassembly are coordinated responses to pemphigus autoantibodies. The Journal of biological chemistry. 2006;281(11):7623-34.

17. Saito M, Stahley SN, Caughman CY, et al. Signaling Dependent and Independent Mechanisms in Pemphigus Vulgaris Blister Formation. PLoS ONE 2012; 7(12):e50696

18. Stahley SN, Warren MF, Feldman RJ, et al. Super-Resolution Microscopy Reveals Altered Desmosomal Protein Organization in Tissue from Patients with Pemphigus Vulgaris. J Invest Dermatol. 2016;136(1):59-66.

19. Ishii K, Harada R, Matsuo I, Shirakata Y, Hashimoto K, Amagai M. In vitro keratinocyte dissociation assay for evaluation of the pathogenicity of anti-desmoglein 3 IgG autoantibodies in pemphigus vulgaris. J Invest Dermatol. 2005;124(5):939-46.

20. Spindler V, Endlich A, Hartlieb E, Vielmuth F, Schmidt E, Waschke J. The extent of desmoglein 3 depletion in pemphigus vulgaris is dependent on Ca(2+)-induced differentiation: a role in suprabasal epidermal skin splitting? Am J Pathol. 2011;179(4):1905-16.

21. Mukaka MM. Statistics corner: A guide to appropriate use of correlation coefficient in medical research. Malawi Med J. 2012;24(3):69-71

22. Curtis JR, Westfall AO, Allison J, et al. Population-based assessment of adverse events associated with long-term glucocorticoid use. Arthritis Rheum. 2006; 55(3):420

23. Denning MF, Guy SG, Ellerbroek SM, et al. The expression of desmoglein isoforms in cultured human keratinocytes is regulated by calcium, serum, and protein kinase C. Exp Cell Res. 1998;239(1):50-9.

TABLES AND FIGURES

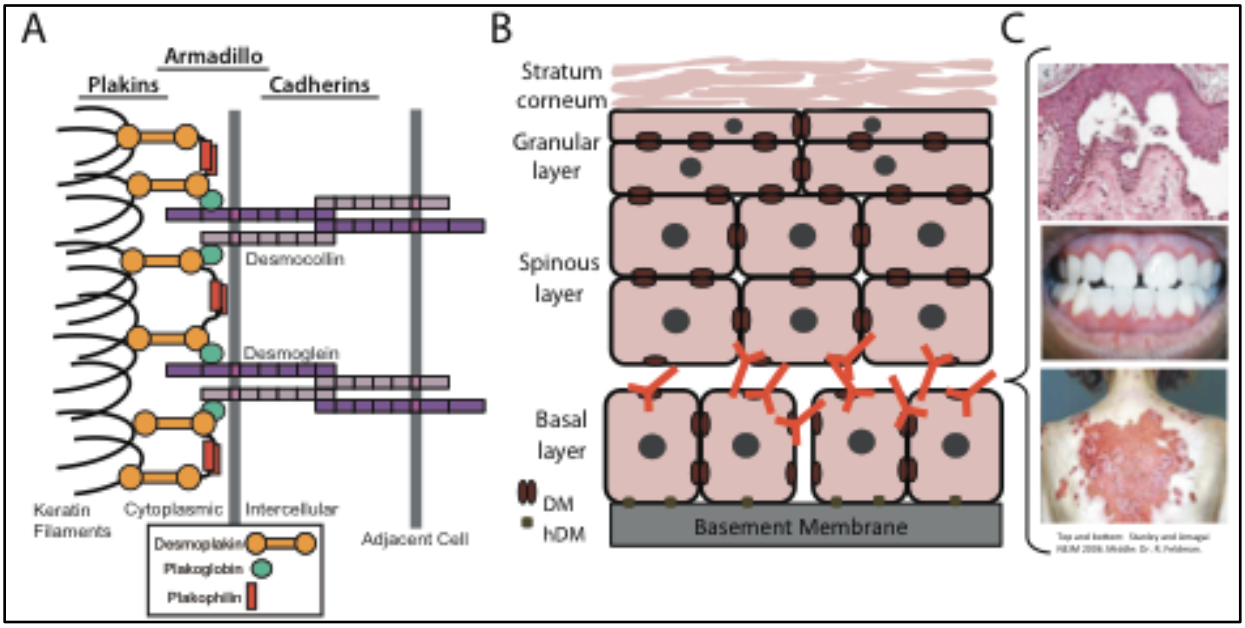

Figure 1: **The role of desmogleins in PV pathophysiology.** A) The desmoglein protein is a member of the cadherin family and spans the intracellular space between two skin cells, also known as keratinocytes. B) Desmosomes are spot-weld adhesive junctions that link keratinocytes to one another. Antibodies against Dsg3 are shown, revealing that Dsg3 is primarily located just above the deepest basal layer of epidermal cells. C) Histologic (top panel) and clinical (middle and bottom panels) findings in PV.

Table 1. Demographic and clinical characteristics for patients (n = 23)	
Age	
Mean age +/-SD[1]	47.26 +/- 10.51
Age range (years)	25-68
Sex n(%)	
Male	14 (60.9%)
Female	9 (39.1%)
Clinical Characterization n(%)	
Mucosal Dominant	9 (39.1%)
Mucocutaneous	14 (60.9%)
Severity[2] n(%)	
Mild <12	8 (34.8%)
Moderate 12<x<25	5 (21.7%)
Severe >25	9 (39.1%)
Unknown	1 (4.3%)
[1]Standard deviation, [2]PDAI score	

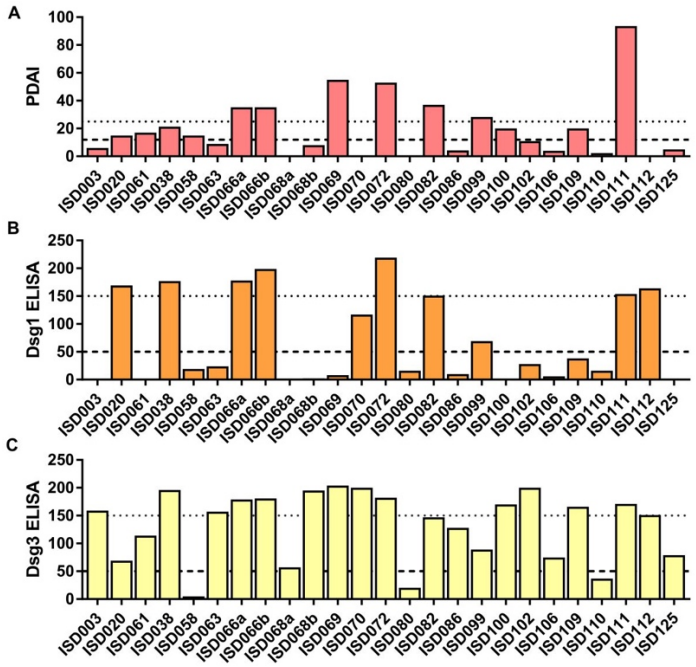

Figure 2: PDAI, ELISA levels, and Disassociation Index scores of 25 PV patients. A) Patient PDAI scores range from 0 to 93.6. Patient ISD112 has no recorded PDAI. B) Dsg1 ELISA scores range from 0 to 219. C) Dsg3 ELISA scores range from 5 to 204. Below the dashed line represents mild or low scores, between the two lines represents moderate scores, and above the dotted line represents high or severe scores.

Sample	Mucosal Dominant	Shape
ISD003	Yes	
ISD020	No	
ISD025	Yes	
ISD038	No	
ISD058	Yes	
ISD063	No	
ISD066a	No	
ISD066b	No	
ISD068a	Yes	
ISD068b	Yes	
ISD069	Yes	
ISD070	Yes	
ISD072	No	
ISD080	Yes	
ISD082	No	
ISD086	No	
ISD099	No	
ISD100	Yes	
ISD102	No	
ISD106	No	
ISD109	No	
ISD110	No	
ISD111	No	
ISD112	No	
ISD125	Yes	

Figure 3: Patients have unique symbol assigned. 25 samples were used throughout this study. Mucosal dominant patients are coded as squares of varying colors and patterns while mucocutaneous patients are circles with varying colors and patterns. This allows a single patient to be tracked throughout the figures.

Table 2. Clinical and serological data for patients (n = 25)

Patient ID#	Mucosal Dominant	Muco-cutaneous	Dsg1	Dsg3	Total ELISA	PDAI
1	Yes	No	0	159	165	6
2	No	Yes	169	69	84	15
3	No	Yes	177	196	217.3	21.3
4	Yes	No	19	5	20	15
5	Yes	No	0	114	131	17
6	No	Yes	24	157	165.9	8.9
7	No	Yes	178	179	214.3	35.3
8	No	Yes	199	181	216.3	35.3
9	Yes	No	0	57	57	0
10	Yes	No	2	195	203	8
11	Yes	No	8	204	259	55
12	No	Yes	117	200	200	0
13	No	Yes	219	182	235	53
14	Yes	No	16.1	20.5	20.5	0
15	No	Yes	151	147	184	37
16	No	Yes	10	128	132.3	4.3
17	No	Yes	69	89	117.3	28.3
18	Yes	No	0	170	190	20
19	No	Yes	28	200	211	11
20	No	Yes	6	75	79	4
21	No	Yes	38	166	186	20
22	No	Yes	16	37	39.3	2.3
23	No	Yes	154	171	264.6	93.6
24	No	Yes	164	151	180.6	29.6
25	Yes	No	0	79	84	5

Table 3. Spearman correlation coefficients (r) between PDAI, ELISAs and Disassociation Index Scores with 95% confidence intervals (CI) and p–values reported.

Spearman correlation tests	r	95% CI	p
All Patients			
PDAI vs Dsg1 ELISA	0.5614	0.2018 to 0.7876	0.0018
PDAI vs Dsg3 ELISA	0.4526	0.05771 to 0.7250	0.0115
PDAI vs Total ELISA	0.669	0.3616 to 0.8452	0.0001
PDAI vs Disassociation Index	0.2446	-0.1787 to 0.5914	0.1194
Dsg3 ELISA vs Disassociation Index	0.5428	0.1760 to 0.7772	0.0025
Mucosal dominant patients			
PDAI vs Dsg1 ELISA	0.0825		0.417
PDAI vs Dsg3 ELISA	0.6109		0.0439
PDAI vs Total ELISA	0.6109		0.0439
PDAI vs Disassociation Index	0.7782		0.0091
Dsg3 ELISA vs Disassociation Index	0.6833		0.0252
Mucocutanous patients			
PDAI vs Dsg1 ELISA	0.7285	0.3503 to 0.9024	0.001
PDAI vs Dsg3 ELISA	0.2327	-0.3119 to 0.6622	0.1912
PDAI vs Total ELISA	0.677	0.2579 to 0.8817	0.0025
PDAI vs Disassociation Index	-0.001472	-0.5088 to 0.5066	0.499
Dsg3 ELISA vs Disassociation Index	0.4224	-0.1087 to 0.7659	0.052

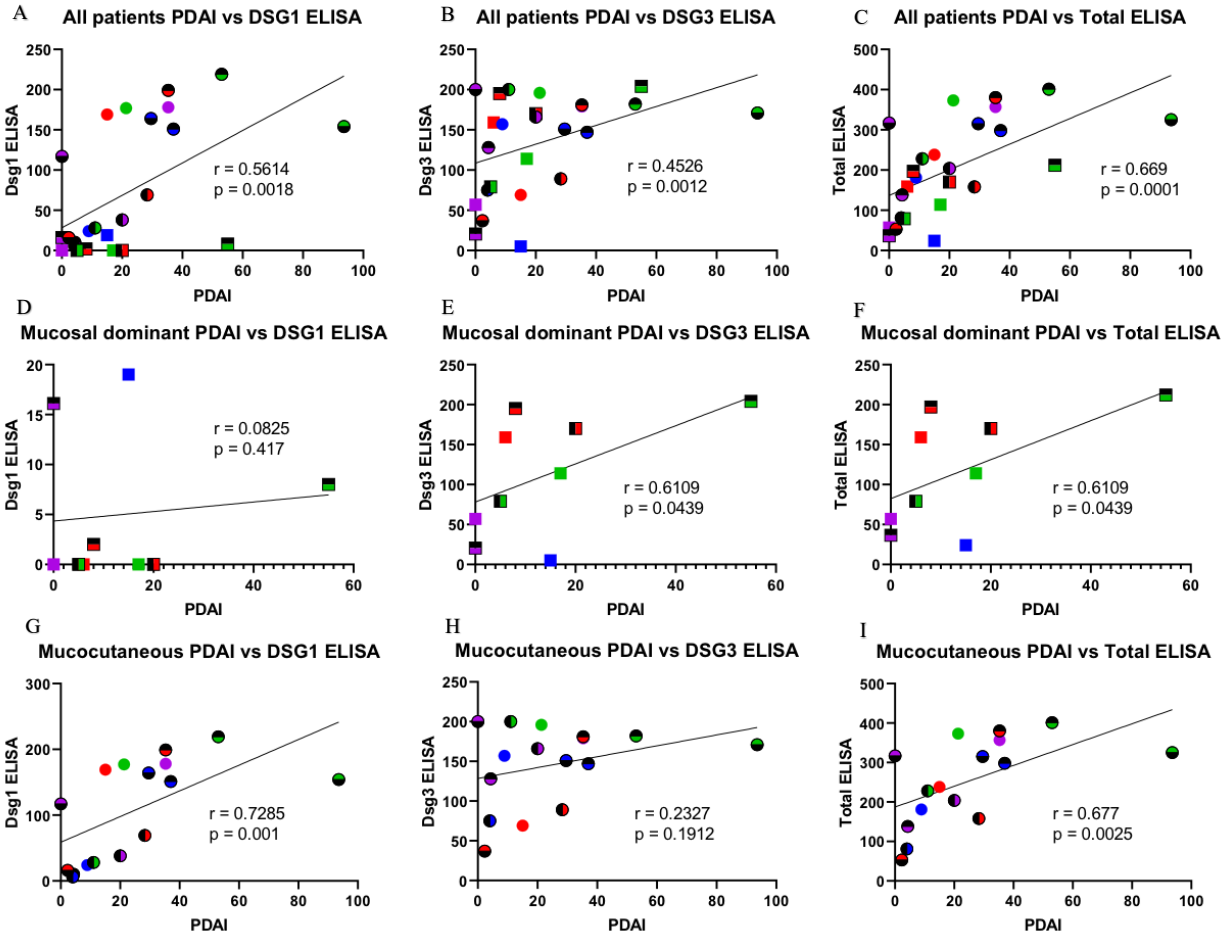

Figure 4: Total ELISA correlates with PDAI. Both Dsg1 ELISA (A) and Dsg3 ELISA (B) showed a significant correlation with PDAI for all patients. C) The sum of Dsg3 and Dsg1 ELISA scores to generate a total ELISA score yielded the most significant correlation with PDAI. D) Dsg1 ELISA does not correlate with PDAI for mPV patients. Dsg3 ELISA (E) and Total ELISA (F) correlate with PDAI for mPV patients. G) PDAI shows a statistically significant correlation to Dsg1 ELISA for mcPV patients. H) Dsg3 ELISA does not correlate with PDAI for mcPV patients. I) The sum of Dsg3 and Dsg1 ELISA scores correlates with PDAI for mcPV patients.

Table 4. Linear regression analysis (R^2) for PDAI and ELISAs with 95% confidence intervals (CI) and p-values reported.				
Model 1	**PDAI = β0 + β1*(Total ELISA)**			
Parameters	**Variable**	**Estimate**	**Standard error**	**p**
β0	Intercept	-1.859	7.3255	0.8019
β1	Total ELISA	0.1121	0.03135	0.0016
Rsquared =	**0.3574**			
Model 2	**PDAI = β0 + β1*(Dsg1)**			
Parameters	**Variable**	**Estimate**	**Standard error**	**p**
β0	Intercept	10.15	5.017	0.0548
β1	Dsg1 ELISA	0.1537	0.04784	0.0039
Rsquared =	**0.3097**			
Model 3	**PDAI = β0 + β1*(Dsg3)**			
Parameter	**Variable**	**Estimate**	**Standard error**	**p**
β0	Intercept	1.322	9.845	0.8944
β1	Dsg3 ELISA	0.1476	0.0673	0.0386
Rsquared =	**0.173**			

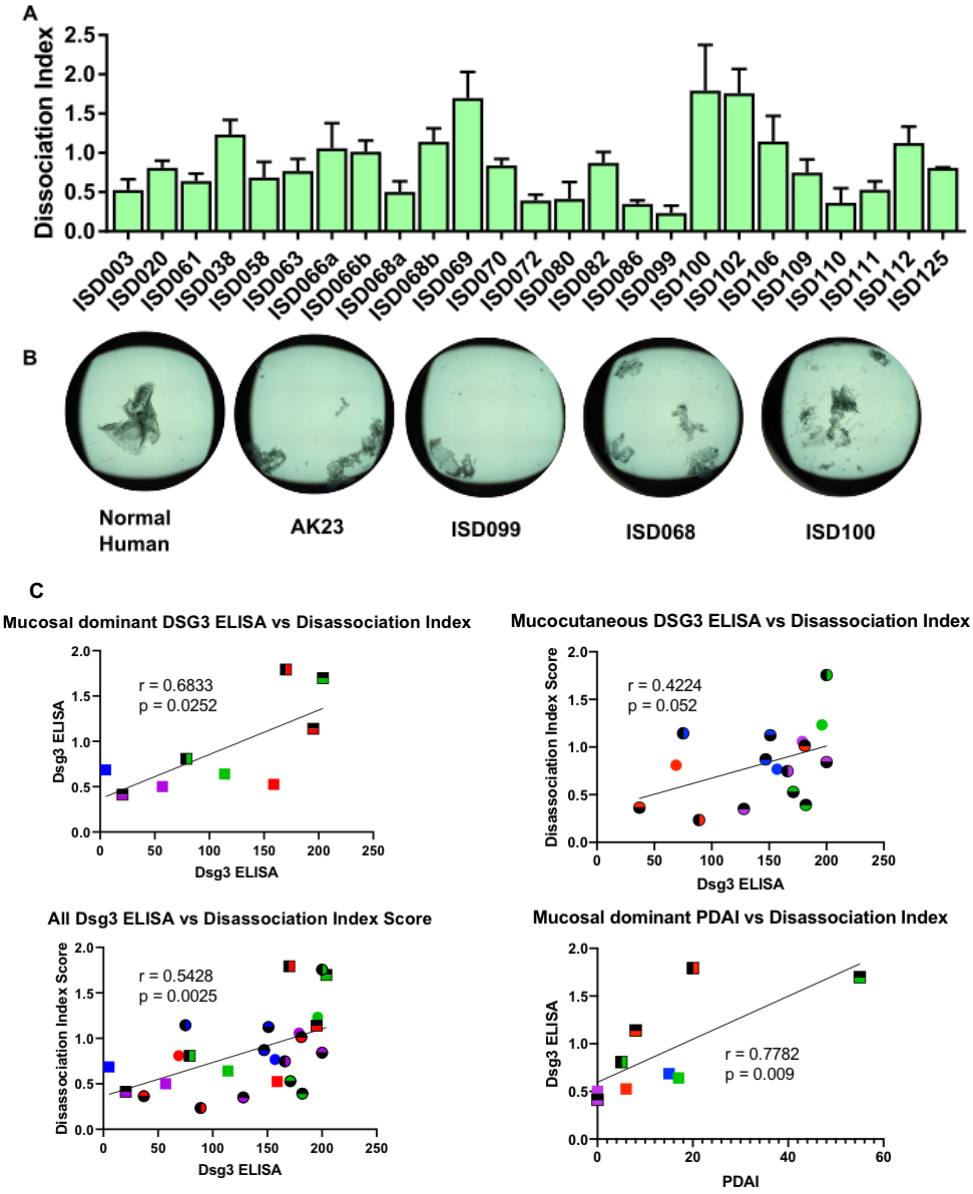

Figure 5: Disassociation Index correlates with Dsg3 ELISA. A) The 25 patients demonstrate a range of pathogenicity as determined by the disassociation index. Four replicates are represented and SEM is shown. B) Representative images of monolayers after the application of mechanical force. Monolayers treated with normal human serum remain largely intact while monolayers treated with AK23 (positive control) or Patient IgG fragment. C) Dsg3 ELISA correlates with disassociation index score in mucosal dominant patients and the total patient pool, but not for mucocutaneous patients. Mucosal dominant patients also demonstrate a correlation between PDAI and disassociation index score.

ACKNOWLEDGEMENTS

We would like to thank Sue Manos and Bridget Bradley for collecting samples from patients. Supported by the National Center for Advancing Translational Sciences of the National Institutes of Health under Award Number UL1TR002378. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This work was also supported by grants (TL1TR002382 to M.F.W. and R01AR048266 to A.P.K.). Additional support was provided by the Emory Flow Cytometry Core.