

# Intercellular, Linear Direct Immunofluorescence Staining Pattern of Sweat Glands and Hair Follicles may be Used as a Diagnostic Marker for Pemphigus Vulgaris in Cases where Epidermis is Missing

Orsolya N. HORVÁTH<sup>1</sup>, Michaela MAURER<sup>1</sup>, Vivian WIETZKE<sup>1</sup>, Victoria FISCHER<sup>1</sup>, Lars E. FRENCH<sup>1,2</sup> and Miklós SÁRDY<sup>1,3</sup>

<sup>1</sup>Department of Dermatology and Allergy, LMU University Hospital, Munich, Germany, <sup>2</sup>Dr. Phillip Frost, Department of Dermatology and Cutaneous Surgery, University of Miami Miller School of Medicine, Miami, FL, USA, and <sup>3</sup>Department of Dermatology, Venereology and Dermatocology, Semmelweis University, Budapest, Hungary

**Due to its rarity, it takes months for patients to be correctly diagnosed with pemphigus vulgaris. This delay can be exacerbated by factors such as incorrect biopsy site selection. When the epidermis detaches from the dermis, evaluating fluorescence patterns is impossible. This monocentric, retrospective study aimed to assess the clinical significance of the honeycomb-like staining pattern of adnexal structures in patients with pemphigus vulgaris. The study was conducted in Munich, from 1 January 2012 to 31 December 2020. Forty-four biopsy samples for direct immunofluorescence microscopy from pemphigus patients along with 44 samples from control patients were included. The fluorescence intensity of adnexal structures did not differ significantly from that of the epidermis in pemphigus, except for hair follicles and sweat glands with C3 staining, where the epidermis showed stronger fluorescence. The sensitivity for the characteristic fluorescence pattern of at least 1 adnexal structure was 88.64% ( $n = 39/44$ , 95% CI 75.44% to 96.21%), with 100% specificity. The positive predictive value of adnexal fluorescence was 100%, and the negative predictive value was 89.8%. It was found that the honeycomb-like staining pattern of sweat glands, sweat gland ducts, and hair follicles is a highly specific marker for pemphigus vulgaris.**

**Key words:** direct immunofluorescence; hair follicles; pemphigus; sebaceous glands; sweat glands.

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**Corr:** Orsolya N. Horváth, Department of Dermatology and Allergy, LMU University Hospital, Frauenlobstr. 9-11, DE-80337 Munich, Germany. E-mail: Orsolya.Horvath@med.uni-muenchen.de

**P**emphigus vulgaris (PV) is a rare blistering skin disorder characterized by the formation of fragile blisters on both mucous membranes and the skin. Due to its rarity, the time between the initial onset of symptoms and accurate diagnosis of PV may extend over several months, particularly in cases involving oral manifestations (1). When PV is among the differential diagnoses, diagnostic procedures typically involve obtaining 2 biopsies: 1 for direct immunofluorescence microscopy, ideally taken from the perilesional area, and another for classical HE-stained histology, taken from the lesion's edge. The

## SIGNIFICANCE

Pemphigus vulgaris is a rare blistering skin disorder. For direct immunofluorescence microscopy, a perilesional biopsy should be taken to evaluate the fluorescence of the epidermis. Inaccurate biopsy site selection or technical challenges can lead to absence of the epidermis and a subsequent biopsy will be required. This study aimed to evaluate the fluorescence of sweat glands and hair follicles in the biopsy samples and compare them with that of the epidermis. Our findings indicate that these structures can serve as diagnostic markers, obviating the need for repeat biopsies in cases where the epidermis is missing.

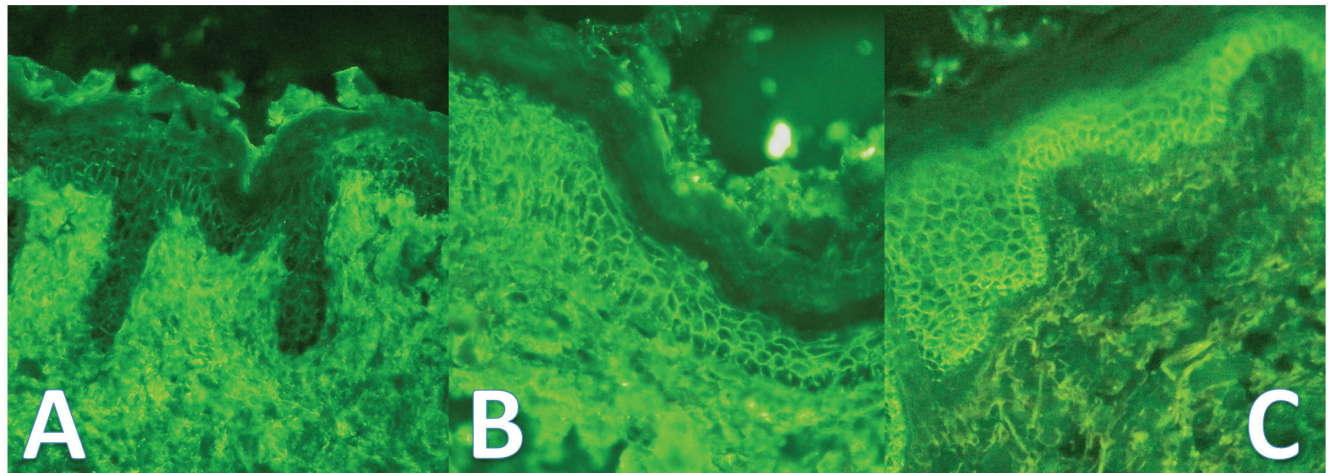
epidermis or epithelium should be histologically evaluated, but they are often missing from specimens due to their fragility, clinicians' potential lack of awareness regarding the optimal biopsy site, or technical difficulties.

Patients with erosions on the skin and mucous membranes often endure severe pain (2, 3) and encounter difficulties performing daily activities (such as eating or putting on clothes). If the initial biopsy cannot be evaluated, repeated surgical intervention is required, further delaying the correct diagnosis. The authors of this study have frequently encountered this scenario. Despite the observation of the characteristic honeycomb-like PV pattern in the walls of adnexal structures, there is a lack of studies clarifying the exact correlation between this fluorescence and that of the epidermis. Here we attempt to fill this gap.

## MATERIALS AND METHODS

### Patients and methods

This monocentric, case-control study included all inpatients and outpatients diagnosed with PV who presented positive findings in direct immunofluorescence (DIF) microscopy at the Department of Dermatology and Allergy of Ludwig Maximilian University Hospital from 1 January 2012, to 31 December 2020. A total of 44 frozen biopsy specimens from PV patients (including 5 mucous membrane and 39 skin samples) and 44 control biopsies from patients with other disorders (including 5 patients with bullous pemphigoid [BP] and 2 with linear IgA dermatosis) were cut, stained, and analysed. From every PV patient, only 1 specimen was included. PF cases or ambiguous cases were excluded. The control patients were not age-matched to the PV cases, we de-



**Fig. 1. Examples for the semiquantitative grading, epidermis.** (A) (1) fluorescence of the epidermis is typical for pemphigus vulgaris, but weaker than the background; (B) (2) fluorescence of the epidermis is approximately equal in intensity to the background; (C) (3) fluorescence of the epidermis is visibly stronger than the background fluorescence (original magnification:  $\times 200$ ).

cided to include 44 consecutive slides received for diagnostics in our laboratory to represent the clinical reality. Only PV, PF cases, and repeated biopsies from the same patients were excluded.

The diagnosis of PV was based on clinical, histological (compatible traditional histology and positive DIF microscopy of the epidermis), and serological (indirect immunofluorescence microscopy and desmoglein 1 and 3 ELISAs) findings. Sweat and salivary glands (SG) and sweat/salivary gland ducts (SGD) were collectively analysed. In cases where identical structures were present multiple times in the samples but exhibited varying fluorescence intensities, only the structure with the strongest fluorescence was analysed. The IgG and C3 complement results were evaluated together for easier interpretation. Any separate presentations of IgG and C3 results are explicitly mentioned in the text.

#### *DIF microscopy (standard method)*

Nine-micrometre-thick cryosections were dried for 30 min, and incubated with fluorescein isothiocyanate-labelled antibodies directed against human IgG (F1641, Sigma-Aldrich, Burlington, MA, USA) and C3 complement (F0201, Dako, Santa Clara, CA, USA) according to the manufacturers' instructions. Following washing, sections were mounted in 2.5% diazabicyclooctane, 0.1% sodium azide and 10% phosphate buffered saline, pH 7.4, with 0.05% Tween-20 in glycerol for visualization. Positivity was defined as deposition of anti-human IgG and anti-human C3 complement in a honeycomb-like pattern within the basal or total epidermis or epithelium. A semi-quantitative grading allowed a more detailed analysis: 0: no fluorescence; 1: visible, but weak fluorescence, weaker than the background fluorescence; 2: visible fluorescence, approximately equal in intensity to the background; 3: strong fluorescence, immediately visible when looking into the microscope, stronger than the background. The typical immunofluorescence pattern observed using our semiquantitative grading is illustrated in **Fig. 1**. The typical immunofluorescence pattern of adnexal structures is visualised in **Fig. 2**.

All slides were independently assessed by 2 authors (ONH and MM). In cases of disagreement between the 2 examiners or in borderline cases (7% of all cases), consensus was reached.

#### *Statistical analysis*

Data analysis was performed using Microsoft Excel version 16.61.1 (Microsoft Corp, Redmond, WA, USA). The Wilcoxon signed-rank test was used to compare fluorescence intensities

within the same slides (paired samples, which included only slides where the compared values were both noted; thus, the number of included slides differs for each variable). Spearman's rank correlation test was used to compare fluorescence intensities and desmoglein values (to measure the linear dependence between the variables). Statistical analysis was performed using Jamovi (version 2.4.14.0; <https://www.jamovi.org/>) and Microsoft Excel (version 16.54). MedCalc ([https://www.medcalc.org/calc/diagnostic\\_test.php](https://www.medcalc.org/calc/diagnostic_test.php)) was used to calculate the sensitivity, specificity, and the positive and negative predictive value. A significant difference was defined as  $p < 0.05$ .

## RESULTS

Forty-four patients with PV were compared with 44 control patients. In the PV group, 5 mucous membrane samples and 39 skin samples were analysed, while all control specimens included skin samples. The epidermis/epithelium showed the characteristic honeycomb-like intercellular fluorescence pattern in all PV but none of the control cases. SG, SGD, or hair follicles (HF) were found in 43 (97.8%) PV and 38 (86.4%) control tissue samples. Patient demographics and the presence of typical fluorescence patterns of adnexal structures are presented in **Table I**.

The fluorescence patterns of the SG, SGD, and HF were compared with those of the epidermis and the epithelium, revealing that the intensity of fluorescence of adnexal structures did not differ significantly from

**Table I. Summary of patient demographics and presence of typical fluorescence patterns (TFP) in adnexal structures**

| Factor                            | Pemphigus vulgaris  | Control patients |
|-----------------------------------|---------------------|------------------|
| Patients, <i>n</i>                | 44                  | 44               |
| - Female                          | 17                  | 20               |
| - Male                            | 27                  | 24               |
| - Mean age, years (range)         | 53.5 (23–78)        | 60.9 (16–92)     |
| Presence of SG/TFP, <i>n</i> (%)  | 37 (84.1)/29 (65.9) | 28 (63.6)/0      |
| Presence of SGD/TFP, <i>n</i> (%) | 33 (75.0)/33 (75.0) | 20 (45.5)/0      |
| Presence of HF/TFP, <i>n</i> (%)  | 30 (68.2)/25 (56.8) | 19 (43.2)/0      |

SG: sweat gland; SGD: sweat gland duct; HF: hair follicle.

Table II. Fluorescence intensities of the epidermis and adnexal structures defined by our system

| Item                 | IgG fluorescence intensities, value (SD) | C3 fluorescence intensities, value (SD) |
|----------------------|------------------------------------------|-----------------------------------------|
| SGD papillary dermis | 2.46 (0.63)                              | 2.47 (0.72)                             |
| SGD mid-dermis       | 2.63 (0.60)                              | 2.67 (0.60)                             |
| SGD deep dermis      | 2.77 (0.52)                              | 2.42 (0.81)                             |
| Sweat gland          | 2.06 (1.17)                              | 1.84 (1.15)                             |
| Hair follicle        | 2.19 (1.12)                              | 1.77 (1.12)                             |
| Epidermis            | 2.43 (0.72)                              | 2.36 (0.93)                             |

The slides were incubated with the same labelled antibody using the same preparation and concentration.  
SD: standard deviation; SGD: sweat gland duct.

that of the epidermis/epithelium (Tables II and III), except for HF and SG with anti-C3 complement staining ( $p=0.002$ , and  $p=0.03$ , respectively). We compared the fluorescence intensities of the same adnexal structures with anti-IgG and anti-C3 complement staining; the sensitivity values are presented in Fig. 3. No significant differences were found between the IgG and C3 values of the adnexal structures, or the epidermis/epithelium ( $p=0.708$ ).

The SGD were further examined based on their depth of localization. A typical fluorescence pattern was observed with both anti-IgG and anti-C3 in PV patients across all dermal levels. The sensitivity of SGD fluorescence was 36.36%, 43.18%, and 54.54% in the papillary, mid-, and reticular dermis, respectively. The sensitivity of SG and HF fluorescence was 65.9% and 70.45%, respectively. The sensitivity of all adnexal structures combined was 88.64% ( $n=39/44$ , 95% confidence interval [CI] 75.44% to 96.21%). The specificity was 100%, as none of the control patients exhibited the typical PV pattern. The positive predictive value of adnexal fluorescence was 100% (95% CI 90.97% to

Table III. Differences between fluorescence intensities of the epidermis and adnexal structures within the same slides (using our grading 0–3)

| Item                 | Epidermis         |                        |
|----------------------|-------------------|------------------------|
|                      | IgG               | C3                     |
| SGD papillary dermis | $w=0.00\ p=1.0$   | $w=3.00\ p=0.120$      |
| SGD mid dermis       | $w=1.00\ p=1.0$   | $w=11.0\ p=0.408$      |
| SGD deep dermis      | $w=10.0\ p=0.072$ | $w=25.0\ p=0.832$      |
| SG                   | $w=33.5\ p=0.134$ | $w=207\ p=0.030^*$     |
| HF                   | $w=15.5\ p=0.233$ | $w=4.50\ p=0.002^{**}$ |

SGD: sweat gland duct; SG: sweat gland; HF: hair follicle; w: Wilcoxon W.

100%), and the negative predictive value was 89.8% (95% CI 79.41% to 95.26%).

The semiquantitative grading of SG, SGD, HF, and epidermal fluorescence was compared with the PV patients' desmoglein 1 and 3 titres (at time of first diagnosis, when the biopsy was taken). In 2 cases, desmoglein ELISA was not performed. The comparison showed only a weak correlation ( $p=0.047$ ) between the desmoglein 3 titres and the fluorescence of the SGD in the papillary dermis, while the strength of fluorescence measured using the grading system (including the epidermal fluorescence) did not correlate with the desmoglein titres.

The 5 biopsies from mucous membranes were analysed separately. In 1 case, a salivary gland showed a weak fluorescence (grade 1) with C3 but not with IgG; in 3 cases, SG were present, but they did not exhibit the typical fluorescence pattern, and in 1 case, no specific structures (SG/SGD) could be identified.

DISCUSSION

Koch et al. (4) reported in 1998 that desmoglein 3 plays an important role in anchoring the telogenic hair to the

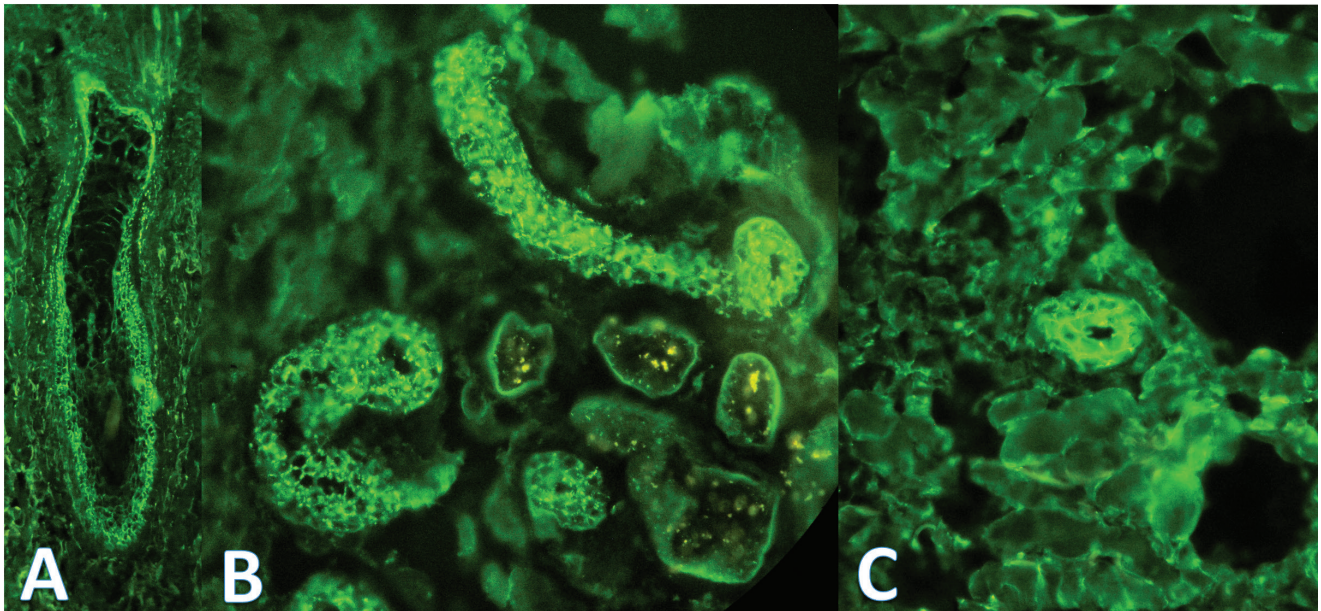
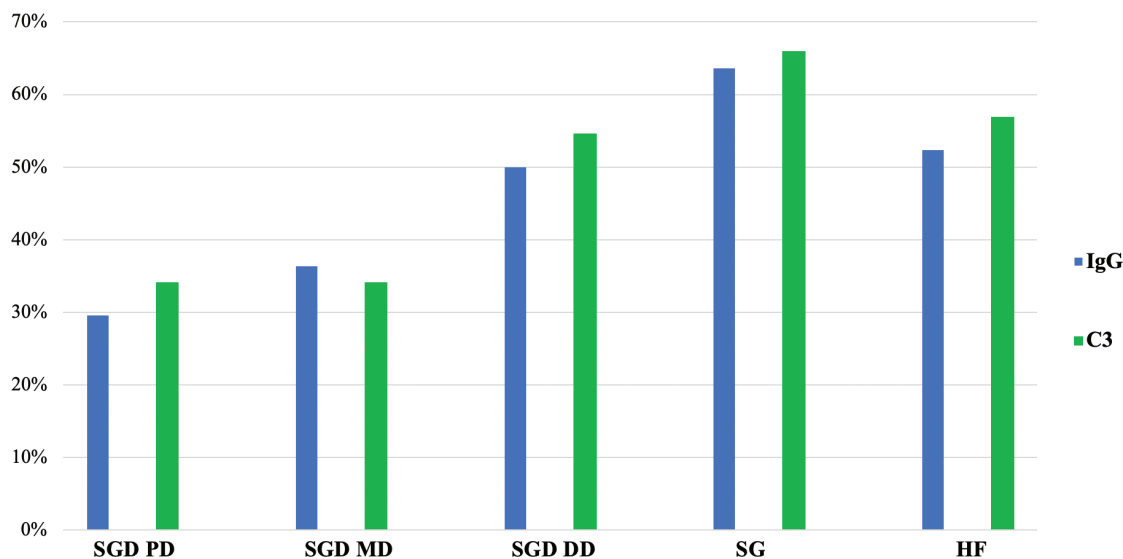


Fig. 2. Fluorescence of adnexal structures. (A) Fluorescence of a hair shaft. (B) Fluorescence of sweat glands including sweat gland ducts. (C) Lone sweat gland duct in the mid-dermis (original magnification: A: x200 B, C: x400).



**Fig. 3. Sensitivity values for adnexal structures.** SGD: sweat gland duct; PD: papillary dermis; DD: deep dermis; SG: sweat gland; HF: hair follicle; IgG: anti-immunoglobulin G; C3: anti-C3 complement.

outer root sheath. Consequently, it was anticipated that anti-desmoglein 3 autoantibodies could be visualized in the outer root sheath in PV. Studies examining the diagnostic utility of the hair shaft and plucked hair in PV have been conducted since 2003 (5). Plucked hair showed 91% specificity in a large sample of different PV phenotypes with oral involvement (6). Another study found that the typical intercellular, reticular fluorescence of hair was present in 85% of patients (on samples collected from the scalp and other regions) (7). Sandhya et al. found that DIF of hair is comparable to that of the skin, and telogen hair fluorescence could potentially predict a relapse (8). A subsequent study highlighted the high diagnostic value of anagen hair (9). According to Rai et al (10), the DIF microscopy findings in the epidermis and the HF correlated with each other (IgG and C3 complement findings were analysed together). They included patients with negative DIF microscopy results as well, and in 6 cases (20%), only the hair showed a positive pattern, whereas the epidermis was negative. In our study, there was no difference between the fluorescence of adnexal structures and the epidermis, except for the fluorescence of SG and HF with C3, where the epidermis exhibited significantly stronger fluorescence.

Lehman et al. (11) reported on DIF microscopy findings in various conditions, including 4 patients with PV, and suggested that evaluating adnexal structures could be beneficial. They proposed that clinicians should preferentially biopsy skin regions with folliculosebaceous structures. Zhou et al. (12) analysed skin biopsy specimens from 88 patients with PV or BP for the presence of eccrine gland fluorescence. They found that 58% of tissue samples contained sweat glands, 42% sweat gland ducts, and 54.5% at least 1 hair follicle. The 40 samples from patients with PV were further analysed using DIF microscopy, and 82.5% and 65% of eccrine

glands were positive for IgG and C3, respectively. Additionally, 91.3% and 78.3% of HF showed positive patterns, respectively. In our cohort, 80% and 78.4% of the SG exhibited a honeycomb-like pattern with IgG and C3, respectively, while 82.2% and 83.3% of the HF were positive, respectively.

Anagen and telogen hairs were also found to have diagnostic value in PF (13), but our analysis included only PV patients. There are reports on the diagnostic value of SGD fluorescence in BP (14), and we also included BP patients as controls, but the fluorescence pattern was linear along the basement membrane without intercellular staining; thus, BP wrongly diagnosed as PV is not expected. In our study, only the typical PV pattern was graded according to our system.

In mucosal PV, we were unable to identify any adnexal or other structures (salivary glands/salivary gland ducts) that could help to diagnose patients if the epithelium is missing. Thus, in such cases, a new biopsy is inevitable.

Further research is needed to clarify the ideal biopsy site and to analyse the clinical outcomes, and the potential use of adnexal fluorescence in therapy planning. A prospective study according to guidelines (15) with the inclusion of PF patients would be useful.

#### Limitations

The limitations of this study were its monocentric, retrospective design, the inclusion of PV patients only with positive DIF microscopy, and the relatively low number of patients. The selection of control patients (almost every patient in a randomly selected month) could also be a limitation, because seasonal differences were therefore not taken into consideration. Further, the clinical presentation and the response to therapy could not be analysed due to the anonymity of the patients. The

exact location of the biopsies had not been documented in several cases; thus, the ideal biopsy site could not be assessed.

### Summary

The honeycomb-pattern fluorescence of adnexal structures serves as a specific marker for PV and can be used to diagnose patients correctly, even in cases where the epidermis is absent. However, the lack of adnexal fluorescence does not rule out PV.

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Data available on request from the authors.

**IRB approval status:** The study was performed with the approval of the Ethics Committee of the Medical Faculty of LMU Munich, Germany (Ref.-No. 20-1159) and in compliance with the Declaration of Helsinki.

*The authors have no conflicts of interest to declare.*

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