# Pemphigus vulgaris is characterized by low IgG reactivities to specific self-antigens along with high IgG reactivity to desmoglein 3

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## Summary

**Pemphigus vulgaris (PV) is an autoimmune skin disease, which has been characterized by IgG autoantibodies to desmoglein 3. Here we studied the antibody signatures of PV patients compared with healthy subjects and with patients with two other autoimmune diseases with skin manifestations (systemic lupus erythematosus and scleroderma), using an antigen microarray and informatics analysis. We now report a previously unobserved phenomenon – patients with PV, compared with the healthy subjects and the two other diseases, show a significant decrease in IgG autoantibodies to a specific set of self-antigens. This novel finding demonstrates that an autoimmune disease may be associated with a loss of specific, healthy IgG autoantibodies and not only with a gain of specific, pathogenic IgG autoantibodies.**

**Keywords:** autoantibodies; pemphigus vulgaris; scleroderma; systemic lupus erythematosus.

## Introduction

Pemphigus vulgaris (PV) is a potentially life-threatening autoimmune skin disease that has been characterized serologically by elevated IgG antibodies to desmoglein 3 (DSG3)[1] and to other self-antigens. The aim of this study was to use an antigen microarray and informatic analysis[2–4] to extend the serological characterization of patients with PV. We compared antibody signatures of patients with PV with those of healthy subjects and patients with two other autoimmune diseases with skin involvement: scleroderma (SSc)[5] and systemic lupus erythematosus (SLE).[4,6] We now report that, in addition to autoantibodies to DSG3, patients with PV can be distinguished from these other groups by significantly lower IgG reactivities to several self-antigen epitopes: actin; fatty acid binding protein 3 (FABP3); a peptide epitope of the 60 000 molecular weight heat-shock protein (hsp 60); a peptide of p53; and proliferating cell nuclear antigen (PCNA). There were no significant differences detected in IgM levels between PV patients and the other subject groups.

## Materials and methods

### *Antigen microarrays and serum testing*

We used an antigen microarray methodology described previously.[2–4] In this study, 50 selected antigens were

 

spotted in triplicates on epoxy-activated glass substrates (ArrayIt SuperEpoxi microarray substrate slides, Sunnyvale, CA) using a 48-pin robot (Microgrid 600; Genomics Solutions, Ann Arbor, MI). These antigens included proteins, synthetic peptides from the sequences of selected proteins, phospholipids, and other self and non-self molecules that have been reported in the literature or have been previously found by us to be associated with autoimmune disease (see list of antigens in Table 1). The microarrays were then blocked for 1 hr at 37° with 1% BSA. Test serum diluted 1 : 10 in 1% BSA blocking buffer (volume ~ 10 μl) was incubated under a cover slip for 1 hr at 37°. The arrays were then washed and incubated for 1 hr at 37° with a 1 : 500 dilution of two detection antibodies mixed together: a goat anti-human IgG Cy3-conjugated antibody and a goat anti-human IgM Cy5-conjugated antibody (both from Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Image acquisition was performed by laser (Agilent Technologies, Santa Clara, CA) and the results were analysed using QUANTARRAY software (Packard BioChip Technologies, Billerica, MA). The quantitative range of signal intensity of binding to each antigen spot was 0–65 000; this range of detection made it possible to obtain reliable data at a 1 : 10 dilution of test samples.

### *Human subjects*

The study was approved by the Institutional Review Boards of each participating clinical unit; informed consent was obtained from all participants. At first we tested sera from 18 patients with PV on slides that contained 50 antigens. Later we performed an additional test set of eight patients with PV on slides that contained 25 of the previously informative antigens and the full DSG3 protein as a positive control (graciously provided by Professor Masayuki Amagai, Keio University, Japan). Overall, 26 samples from 20 patients with PV were studied (see patient clinical data in Table 2). The diagnosis of PV was based upon clinical features, suprabasal separation on histology, positive direct and indirect immunofluorescence microscopy, and/or ELISA detection of anti-desmoglein antibodies.[7] These 20 patients are a representative sampling taken from a cohort of a previous PV genetic study.[8] In this study, significant associations between PV and 69 markers clustered within three peaks across the MHC locus were identified: P1: rs2244839–rs2246618, P2: rs1980495–rs9275312 and P3: rs9275312–rs3916765. No associated HLA alleles were found within the P1 region. P2, which contains the DRB1 and BTNL2 loci, was remarkably associated with the DRB1*0401 allele but not with the DRB1*0402 allele, which has been previously shown to be associated with PV in Jewish patients, supporting an association with BTNL2. And finally, as expected, P3 was found to be associated with the DQB1*0302 allele previously shown to be associated with PV in Jewish populations.

Sera from healthy subjects and from patients of two other autoimmune diseases were studied: 28 samples from 26 healthy subjects (median age = 44·5 years, 3 male and 23 female), 50 samples from 34 patients with SLE (median age = 45 years, 8 male and 26 female), and 15 samples from 15 patients with SSc (median age = 58 years, 1 male and 14 female). It should be noted that there are no noticeable differences of antigen expression between genders.

Patients with SSc or SLE were diagnosed according to clinically accepted criteria.[9,10] Blood samples and clinical data were collected from patients visiting the Department of Dermatology, Tel Aviv Sourasky Medical Centre, Israel; the Rheumatology and Nephrology Units at Rabin Medical Centre, Petach Tikva, Israel; the Rheumatology Unit and the Haematology Department of the Sheba Medical Centre, Israel; and the Dipartimento di ScienzeMediche e Chirurgiche, Sezione di ClinicaMedica, Polo Didattico, Ancona, Italy.

### *Statistical analysis*

We identified antigens with reactivities that were higher or lower in a designated study subgroup compared with the other subgroups using a Student's *t*-test, as described previously.[4] *P*-values below 0·05 were accepted as statistically significant. A Mann–Whitney *U*-test[11] gave similar results. To correct for multiple hypothesis testing, we noted only antigens that also passed a false discovery rate of 5%.[12]

## Results

### PV patients manifest high IgG reactivities to DSG3

As expected, patients with PV had significantly heightened IgG reactivity to DSG3,[1] compared with the healthy subjects as a group (Fig. 1a). This finding confirms that the antigen microarray technology is able to detect an autoantibody hallmark of PV. Note that in confirmation of previous studies, IgG antibodies to DSG3 were also found in some subjects in the healthy group.[13] Other IgG reactivities were similar between patients with PV and healthy subjects, for example reactivity to Epstein–Barr virus (Fig. 1b). Further analysis of the microarray data uncovered an unexpected finding.

### Patients with PV manifest low IgG reactivities to five antigens compared with healthy subjects

Pemphigus vulgaris patients compared with healthy subjects were found to manifest significantly lower IgG binding to the following antigens: actin, FABP3, a peptide of hsp 60p255–275, a peptide of p53p253–272(mouse),


 

**Table 1.** List of antigens

| | | | | |
|---|---|---|---|---|
| Actin – from bovine muscle, A3563, Sigma | DSG1 (desmoglein1) – Human recombinant, H00001828-P01, Abnova | GST (glutathione-*S*-transferase) – G8642, Sigma | La-recombinant, PRO-327, Prospec | PDI (protein disulphide isomerase) recombinant, ENZ-262, Prospec |
| β2GP1 – A2299-77E, US Biological | DSG3 (desmoglein 3) peptide – ab87441, Abcam | HGF (hepatocyte growth factor) – recombinant, CYT-244, Prospec | Lysosomal membrane protein 2 – recombinant, H00003920, Abnova | Pneumococcal capsular polysaccharide type 4 – obtained from the ATCC |
| Bone morphogenic protein 4 – human recombinant, CYT-36, Prospec | dsDNA – D1501, Sigma | Horseradish peroxidase – P6782, Sigma | MPO (myeloperoxidase) – ENZ-074, Prospec | PR3 (proteinase 3) – CSI14825A, Cell Sciences |
| Cardiolipin C0563, Sigma | ssDNA – D8899,Sigma | hsp 60p255-275, QSIVPALEIANAHRKPLVIIA. UniProt:: P10809 | NRMJp206-234 p1 rat (tenascin-R) – LGCSSRGVCVDGQCICDSE, UniProtKB: F1LQ63 | RNA polymerase – recombinant, R0884, Sigma |
| CD99 – human recombinant, PRO-294, Prospec | EBVEA (Epstein–Barr virus early antigen) – recombinant, EBV-272, Prospec | hsp 90p105-121, AGQHLYKDLQPFILLRL, UniProtKB: P07900-2 | p53p14-33 (mouse) – KTCPVQLWVSATPPAGSRVR, UniProtKB: A5JTV6 | RO60 – recombinant, PRO-329, Prospec |
| Centromere A – human recombinant, PRO-389 Prospec | FABP3 (fatty acid binding protein 3) – recombinant, PRO-340, Prospec | Hyaluronic acid human – H1504, Sigma | p53 p29-48 (mouse) – GSRVRAMAIYKKSQHMTEVV, UniProtKB: A5JTV6 | SAP90 p63-82 (Disks large homologue 4) rat – VDVREVTHSAAVEALKEAGS, UniProtKB: K7EKU8 |
| Centromere B – human recombinant, PRO-390 Prospec | Fibrinogen-F4753,Sigma | Hyaluronic acid sodium salt, from *Streptococcus equi* – 53747, Sigma | P53p53-72 – LPQDVEEFFEGPSEALRVSG, UniProtKB: P02340 | Sm (Smith antigen) – CSI14863, Cell Sciences |
| CollagenIII-C4407, Sigma | FOX (Forkhead box) Protein 3-p290-304-TKASSVASSQGPVVP, UniProtKB: B7ZLG1. (60% overlap with the matching human sequence TKASSVASSDKGSCC) | IGFBP1 (insulin growth factor binding protein 1) – recombinant, CRI232B, Cell Sciences | p53 p253-272(mouse) – DSSGNLLGRDSFEVRVCACP, UniProtKB: P02340. (The mouse peptide differs in one amino acid from the human peptide; aspartic acid (p262) in the mouse is aspartate in the human peptide) | SYPH (synaptophysin; rat) p81-100. CVKGGTTKIFLVGDYSSSAE, UniProtKB: P07825 |
| CollagenIV- C7521, Sigma | GLP1 (glucagon like peptide-1) – recombinant, HOR-236, Prospec | IgG – I2511, Sigma | PCNA (proliferating cell nuclear antigen)-recombinant, PRO-303, Prospec | Topoisomerase 1-recombinant, ENZ-306, Prospec |
| Cytomegalovirus – recombinant, CMV-216, Prospec | GROa (growth-regulated protein α) – recombinant, CHM-329, Prospec | IgM-I8260, Sigma | PDGF receptor (platelet-derived growth factor receptor) – recombinant, D0946, Sigma | U1RNP (U1 ribonucleoprotein complex) – recombinant, PRO-445, Prospec |

The peptides whose sequences are shown were synthesized by the Biological Services Department of the Weizmann Institute of Science.

**Table 2.** Clinical details of patients with pemphigus vulgaris

| Number | Gender | Age | Treatment | Activity |
|---|---|---|---|---|
| 1 | M | 58 | P, MTX | H |
| 2 | F | 61 | P | H |
| 3 | F | 43 | P | H |
| 4 | M | 56 | P | H |
| 5 | M | 80 | P, A | M |
| 6 | F | 39 | P, MMF | M |
| 7 | M | 57 | P, MTX | M |
| 8 | F | 56 | P | M |
| 9 | M | 47 | P | M |
| 10 | F | 78 | P | M |
| 11 | M | 72 | P | M |
| 12 | F | 73 | P | M |
| 13 | F | 56 | NR | M |
| 14 | M | 58 | NR | N |
| 15 | F | 57 | NR | N |
| 16 | M | 58 | P | N |
| 17 | M | 67 | P | N |
| 18 | F | 79 | NR | N |
| 19 | M | 88 | NR | N |
| 20 | M | 44 | NR | N |

Treatment data: P, prednisone; MTX, methotrexate; A, azathioprine; MMF, mycophenolate mofetil; NR, not receiving any immunosuppressive drugs. Disease activity: H, highly active; M, moderately active; L, low activity.

and PCNA (Fig. 2, Table 3). These reactivities were characterized by very low mean values and standard deviations – the group of patients with PV showed little variation in these low values (Fig. 2). We did not find significant differences between patients with PV and the healthy subjects in IgM levels to these antigens.

### Patients with SLE or SSc do not manifest these low antibody levels

To test whether lower IgG levels to these self-antigens might be a property of other autoimmune skin diseases, we also compared the patients with PV with patients suffering from two other autoimmune diseases with skin involvement: SSc and SLE (Fig. 2, Table 3). We found that the SLE and SSc patients, like the healthy participants, did not manifest the low levels of IgG autoantibodies to these five self-antigens that characterized the patients with PV. Note, however, the low antibodies binding to hsp 60 peptide p255–275, or to p53 peptide p253–272 were of borderline significance ($P = 0{\cdot}0579$ and $P = 0{\cdot}0547$, respectively, see Table 3). Again, we did not find significant differences between patients with PV and the other groups in IgM levels to these antigens.

## Discussion

Classically, the pathophysiology responsible for PV has been attributed to autoantibodies to DSG3;[14] DSG3 is a member of the desmoglein family of transmembrane glycoproteins that are components of the desmosomes: integral structures that mediate cell-to-cell adhesion. It was previously thought that IgG autoantibodies to DSG3 initiate local inflammation that can advance to the destruction of desmosomes, the loss of adhesion between skin epithelial cells and the formation of blisters.[15] In recent years, however, new findings have challenged this explanation:[14] anti-DSG3 IgG antibody titres do not necessarily correlate with disease activity, and may even be absent in PV patients with active disease. Moreover, some 50 other self antigens were reported to specifically react with IgG auto-antibodies in pemphigus subjects.[14] Furthermore, a recent genetic study indicated that the ST18 gene, which regulates apoptosis and inflammation, can be associated with the disease.[8] These and other findings have supported alternative theories for the PV disease mechanism, summarized in a recent review.[14]

In the present study, we report a previously unobserved phenomenon that may shed new light on PV and other autoimmune diseases – a decrease in specific IgG autoantibodies. The common belief is that a specific clinical autoimmune disease results from large amounts of specific disease-associated autoantibodies or effector T cells; a state of disease emerges from augmented autoimmune agents.[16] However, the present results demonstrate that

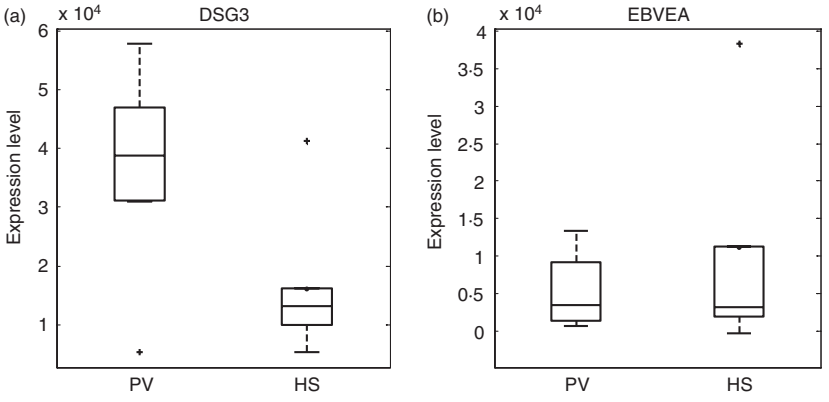


**Figure 1.** IgG autoantibody levels to desmoglein 3 (DSG3) are higher in patients with pemphigus vulgaris (PV). Box plots of the IgG binding to DSG3 in patients with PV and in healthy subjects (HS) are displayed (a). As expected, patients with PV manifested significantly heightened IgG reactivity to DSG3, compared with the healthy subject group. Other IgG reactivities were similar between patients with PV and healthy subjects; we show, for example, reactivity to ENVEA (Epstein–Barr virus early antigen) (b).


 

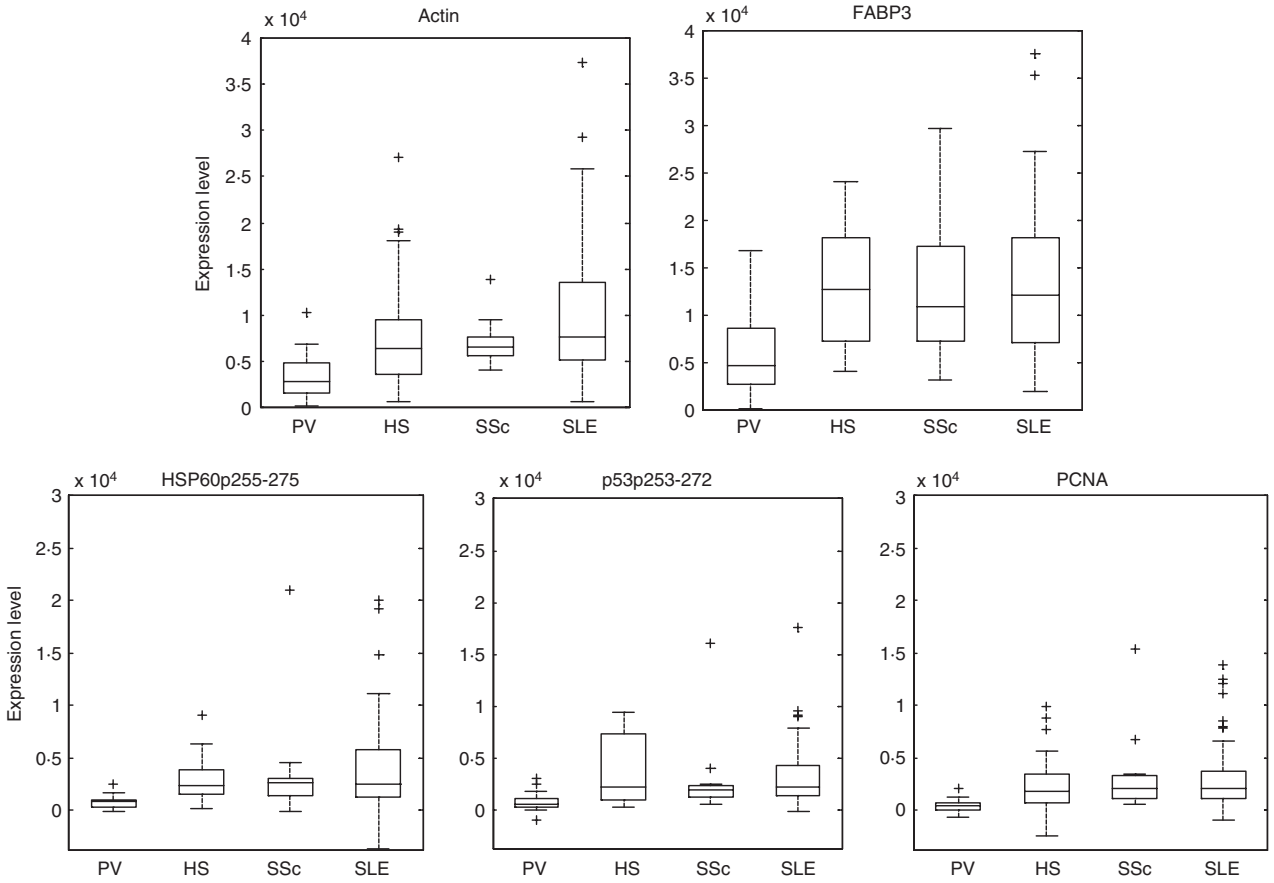


**Figure 2.** Patients with pemphigus vulgaris (PV) can be distinguished from other subjects by significantly lower IgG reactivities to five self-antigen epitopes. Box plots showing five IgG reactivities that were found to be decreased in patients with PV. Note the significant decreases of IgG reactivities in patients with PV to actin, fatty acid binding protein 3 (FABP3), heat-shock protein 60 (hsp 60) p255–275, p53p253–272, and proliferating cell nuclear antigen (PCNA), compared with the healthy subjects and other study groups. PV, pemphigus vulgaris; HS, healthy subjects; SSc, scleroderma; SLE, systemic lupus erythematosus.

**Table 3.** Statistical values of the five low IgG reactivities in patients with pemphigus vulgaris, compared with the other subgroups

| Antigen name | HS | SSc | SLE |
|---|---|---|---|
| Actin | 0·00164 | 0·00009 | 0·00000 |
| FABP3 | 0·00007 | 0·00539 | 0·00001 |
| hsp60p255–275 | 0·00002 | 0·05791 | 0·00001 |
| p53p253–272 | 0·00019 | 0·05470 | 0·00002 |
| PCNA | 0·00084 | 0·00984 | 0·00000 |

Each row displays the *P*-values obtained from Student's *t*-test comparisons of the IgG reactivity of the PV group compared with reactivities in each of the other subgroups.

Two out of the five reactivities are marginal compared with SSc patients. To correct for multiple hypothesis testing, we only marked antigens that also passed a false discovery rate of 5%.

FABP3, fatty acid binding protein 3; HS, healthy subjects; hsp60, heat-shock protein 60; PCNA, proliferating cell nuclear antigen; PV, pemphigus vulgaris; SSc, scleroderma; SLE, systemic lupus erythematosus.

patients with PV can be characterized by low levels of autoantibodies to five self-antigens; IgG autoantibodies to epitopes of these self-antigens are expressed in significantly higher amounts in the sera of healthy subjects and in patients suffering from the other autoimmune diseases – SLE and SSc.

Examining the list of antigens we have identified in this research, it is interesting to note that these molecules perform major functions in cell maintenance and growth, and some of them are also related to cancer development. Actin participates in many important cellular processes, including cell division and cytokinesis, cell motility, cell signalling, and the establishment and maintenance of cell junctions and cell shape.[17] FABP3 belongs to a multi-gene family that is thought to participate in the uptake, intracellular metabolism and/or transport of long-chain fatty acids. FABP3 molecules may also modulate cell growth and proliferation.[18] The FABP3 gene is also a candidate tumour-suppressor gene for human breast cancer.[19]

Heat-shock proteins are generally responsible as chaperones for preventing damage mediated by denatured proteins induced by stresses of all kinds. Heat-shock protein 60 is a chaperone that functions in the transport and refolding of proteins throughout the cell.[20] It has been adopted by the immune system as a biomarker for body maintenance,[20] and studies have linked hsp 60 to the stress response, type 1 diabetes[21] and certain types of immunological disorders,[20] and also to cancer.[22] Indeed, administration of a peptide of hsp 60 has been effective in down-regulating the destruction of β cells in clinical trials in new-onset type 1 diabetes[23] and administration of hsp 60 via plasmid treatment has been found to inhibit a model of autoimmune arthritis;[24] and antibodies to peptides of hsp 60 were found to be associated with resistance to type 1 diabetes in male NOD mice.[25]

The p53 molecule is critical in multicellular organisms, where it regulates the cell cycle and functions as a tumour suppressor by conserving genomic stability and by inducing the apoptosis of transformed cells.[26] The present results suggest that the p53 molecule is a target for autoantibodies found in health, and a decrease in such reactivity could have functional consequences yet to be discovered.

In response to DNA damage, the PCNA protein is ubiquitinated, is involved in the DNA repair pathway, and is associated with different neoplasms.[27]

A state of health has been associated with the presence of so-called 'natural' IgM autoantibodies;[28] and it has been proposed that IgM autoantibodies might protect against autoimmune diseases by blocking their target self-antigens from contact with potential autoimmune effector T cells or from T cells that might otherwise provide help in the production of pathogenic IgG autoantibodies.[29]

At this early stage, we do not yet know the full meaning of our observation regarding the apparent role played, in a 'state of health', by IgG autoantibodies to these self-molecules, and how their specific deficit could be involved in the pathogenesis of PV.

It has already been suggested, however, that cell death in pemphigus may be also triggered by autocrine and paracrine factors released from damaged keratinocytes *in situ*.[14,30] Studies demonstrated that PV IgG binding elicits Fas ligand secretion and elevates cellular expression of factors such as Bax, p53 and c-myc.[14,30] Since autoimmune diseases are associated with deviation from immune regulation, we speculate that autoantibodies may be needed to maintain homeostasis by assisting in the clearing of molecules released from dead or dying cells, as happens regularly in the physiology of the skin. A lack of IgG autoantibodies binding to critical antigens may, in some way, upset immune regulation; but this hypothesis needs to be tested experimentally.

It is also possible to speculate that an association of an autoimmune disease with decreased IgG autoantibodies might explain the benefit of administering normal IgG (IVIg therapy) in so many and diverse autoimmune diseases.[31] Much remains to be done, but we believe that our finding of reduced IgG autoantibodies is an observation that may point towards new directions for research into the pathogenesis of PV in particular and autoimmune diseases in general. Be that as it may, our findings suggest a novel approach to a diagnostic serology for PV.

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## Disclosure

None.

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