

Detection of IgG and IgE reactivity to BP180 using the ISAC[®] microarray system

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Summary

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Accepted for publication

26 November 2012

Funding sources

The study was funded by the Italian Ministry of Health, Current Research Program 2008–2010.

Conflicts of interest

None declared.

The first two authors contributed equally to the study.

DOI 10.1111/bjd.12161

Background Bullous pemphigoid (BP) is an autoimmune skin disease in which patient autoantibodies react with BP180 and BP230 proteins. In addition to IgG, IgE has been shown to play a role in the disease.

Objectives To evaluate the feasibility of detecting IgE and IgG against the immunodominant BP180 NC16A domain (BP180) using a microarray system.

Methods BP180 was immobilized on an experimental version of the ISAC[®] microarray (Exp96). The BP study group and the controls were all tested on the commercial ISAC 103 version and on the Exp96. IgG and IgE were measured in a single run. BP180 IgG and IgE results were compared with those using an enzyme-linked immunosorbent assay (ELISA).

Results All results obtained using the IgG ELISA on the 31 patients with BP were replicated with the ISAC IgG. Five of eight BP sera tested by ELISA showed similar results with ISAC IgE. Twenty-nine (94%) and 19 (61%) of the 31 patients with BP were IgG and IgE positive to BP180, respectively, whereas four (3%) and six (4%) of 138 normal donors were IgG and IgE positive, respectively. Interestingly, the levels of IgG against BP180 detected using the ISAC system were related to the disease severity. Patients with BP showed a peculiar profile of IgE recognition toward some groups of allergens, which was absent in a group of allergic individuals. A significant, higher prevalence of hen's egg recognition was observed in patients with BP who had specific IgE to BP180.

Conclusions The present preliminary study indicates that the ISAC microarray system is suitable for detecting IgG and IgE autoantibodies in patients with BP. Notably, this system allows the assessment of IgE and IgG autoantibodies at the same time, could be employed for the detection of autoantibodies to other autoantigens, and allows profiling for specific IgE to allergens.

Bullous pemphigoid (BP) is the most striking autoimmune subepidermal blistering disease; the cutaneous manifestations are polymorphic, typically affecting the elderly after the seventh decade. BP is characterized by circulating and tissue-bound IgG autoantibodies directed against the cutaneous basement membrane zone (BMZ) and autoreactive T cells.¹ The autoimmune response is mainly directed against BP180 (also termed BPAG2 or type XVII collagen) and BP230 (also termed BPAG1-e), components of the hemidesmosome junctional complexes promoting the adhesion of epithelial cells to the BMZ in skin and mucous membranes. BP230 is localized in the intracellular hemidesmosomal plaque, while BP180 is a transmembrane glycoprotein whose extracellular region consists of 15 collagenous domains separated from each other by

16 noncollagenous domains. The vast majority of patients with BP produce IgG autoantibodies directed against an immunodominant region located in the membrane-proximal noncollagenous region 16A (NC16A) of the BP180 ectodomain.² Animal models have provided unequivocal evidence for the pathogenic role of BP180 IgG autoantibodies.^{3,4} In BP, blood eosinophils and IgE levels are increased in most cases.^{5–8} In addition, specific IgE reacting with BP180 and/or BP230 correlates with disease activity,^{9–11} as also reported in the case in patients affected by pemphigus vulgaris who have IgE antidesmoglein 3.¹² Recent studies have provided strong evidence that IgE autoantibodies specific for BP180 are also pathogenic, inducing erythematous plaques in mice similar to those observed in BP.^{13,14}

When the diagnosis of BP is suspected, direct immunofluorescence on punch biopsies should be performed on uninvolved, perilesional skin, in order to disclose the presence along the epidermal basement membrane of fine, linear, continuous deposits of IgG and/or C3. Recently, the development of an enzyme-linked immunosorbent assay (ELISA) using recombinant proteins encompassing various regions of BP180 and BP230 has provided a useful diagnostic tool for detecting the presence of circulating autoantibodies.¹⁵ The commercially available BP180 ELISA (MBL, Naka-ku, Nagoya, Japan), based on the immunodominant domain NC16A, shows high specificity and good sensitivity.^{16,17} ELISAs using BP230 recombinant proteins are less sensitive and specific than the BP180 ELISA but have confirmatory diagnostic value.^{18,19} Two recent retrospective studies indicated that the BP230 ELISA can improve the diagnostic performance of the BP180 ELISA, detecting reactivity in 5–10% of patients with BP who were NC16A negative.^{20,21} Furthermore, an in-house ELISA system useful for the diagnosis of NC16A-negative patients reacting with additional BP180 extracellular epitopes has been developed.²²

In the past 10 years, the use of allergenic molecules spotted on microarrays has represented one of the most exciting innovations for *in vitro* detection of specific IgE,²³ allowing the determination at the same time of the patient's IgE sensitization profile and producing a wealth of data useful for large epidemiological studies.²⁴ The same microarray system allows the evaluation not only of the immune recognition of hundreds of molecules, but also the detection of different immunoglobulin isotypes at the same time in the same patient. All the above features enabled us to perform studies in patients affected by rare and severe skin diseases, such as Sézary and IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndromes,^{25,26} otherwise diagnostically unapproachable using allergy skin testing or single-plex IgE detection.

The aim of our preliminary study was to evaluate the feasibility of a molecule-based, microarray system for the detection of IgG and IgE to BP180 in patients affected by BP, to explore whether they have specific IgE production patterns against environmental antigens to which humans are exposed, and finally to evaluate similar IgG and IgE reactivity to BP180 in a large control group of allergic patients.

Materials and methods

Patients

Serum samples were obtained from 31 patients with well-characterized BP (80.6% male; median age 84 years, range 68–96). Diagnosis of BP was established based on the following criteria: (i) compatible clinical features (excoriated, eczematous, urticaria and/or bullous lesions associated with variable itch intensity); (ii) deposits of IgG and/or C3 along the epidermal BMZ by direct immunofluorescence; and (iii) presence of circulating autoantibodies binding to the epidermal side by indirect immunofluorescence on human salt-split skin. Demographic (age and sex) and clinical data were

recorded for all patients, as well as the date of the first BP diagnosis and the level of total IgE, measured by the ImmunoCAP system (Phadia, Uppsala, Sweden) following the manufacturer's instructions. The total IgE median value was 970 IU L⁻¹ (range 10.7–8470) for the BP group.

A total of 753 subjects (47.7% male; median age 28 years, range 1–81), referred to us for a self-reported history of food allergy, rhinitis, asthma, atopic dermatitis, urticaria and who asked for a consult at the Center for Molecular Allergology (IDI-IRCCS, Rome, Italy) during 2010 and 2011, were evaluated using both the ISAC[®] 103 allergen microarray [Phadia Multiplexing Diagnostics (PMD), Vienna, Austria] and an experimental microarray (ISAC Exp96; PMD)²⁷ in which the immunodominant domain of BP180 [NC16A; amino acids (aa) 490–562] had been immobilized. Among these patients, 111 individuals (44.1% male; median age 82 years, range 70–89 years) acted as allergic age- and total IgE (median value 860 IU L⁻¹, range 15–6790)-matched controls. One hundred and thirty-eight (61.6% male; median age 42 years, range 1–87) donors, lacking any allergy or dermatological problems related to BP, acted as a further control group. Demographic (age and sex) and clinical data (respiratory and ingestion-related symptoms) were recorded for all patients at history-taking by using the InterAll software [Allergy Data Laboratories (ADL), Latina, Italy], a customized allergy electronic record for diagnostic and clinical data storing.

All subjects were enrolled on the basis of study protocols approved by the ethical committee of IDI-IRCCS (63/CE/2007) and informed signed consent was obtained.

Disease severity index

The score of disease severity was calculated as the sum of scores for activity, extent and pruritus. For disease activity assessment, the number of new blisters arising during two consecutive days was counted and scored as: none (0), in the absence of blisters; mild (1), > 3 blisters; moderate (2), 3–10 blisters; and severe (3), > 10 blisters. Disease extent was estimated as involved body surface, calculated according to the rule of nines. Areas were considered affected when erythema, urticaria, papular, eczematous lesions, blisters or erosions/crusts were present. The extent was scored as: none (0), no lesions present; limited involvement (1), areas affected by individual or confluent lesions involved up to 10% of the body surface; moderate involvement (2), 11–36% of body surface area affected by individual or confluent lesions; severe involvement (3), > 36% of body surface involved. In light of a lack of satisfactory methods for quantification of pruritus severity, the patient's subjective impression of itch intensity was recorded. Pruritus was scored as: none (0), absent; moderate (1), present; and severe (2), interfering with sleep.¹⁵

Antigens

The immunodominant domain of BP180 encompassing aa 490–562 (NC16A) was obtained as a glutathione-S-transferase

(GST) fusion protein and expressed in *Escherichia coli* DH5 α by cloning in pGEX-3x vector (Amersham Biosciences, Piscataway, NJ, U.S.A.). *Escherichia coli* GST (Sigma-Aldrich, St Louis, MO, U.S.A.) acted as a control molecule.²² Both BP180 and GST were used for IgG measurements using ELISA, and immobilized on the ISAC microarray (see below) for IgG and IgE measurements. A commercial ELISA used for IgG measurements is based on the immunodominant domain of BP180 encompassing aa 490–566 (MBL).²⁸

The fragment comprising the immunodominant domain of BP180 encompassing aa 467–567 was obtained as GST and 6xHis-tagged protein and produced in a baculovirus expression system as described previously.¹⁹ This molecule was used for IgE measurements with ELISA.

Enzyme-linked immunosorbent assays for IgG and IgE detection

The commercial BP180 NC16A ELISA kit (MBL) was used according to the manufacturer's protocol. The in-house ELISA for detection of IgG and IgE reactivity against the NC16A domain was performed as previously described.^{22,29} Briefly for IgG, immunomicrotitre plates (Corning Inc., Corning, NY, U.S.A.) were coated with 400 ng per well of purified GST-NC16A, or with the molar equivalent of GST, in 50 μ L of 50 mmol L⁻¹ bicarbonate buffer, pH 9.6, for 2 h at room temperature (RT). Wells were washed with PBS and blocked with blocking solution (5% skimmed milk in PBS, 0.1% Tween-20) overnight at 4 °C. Serum samples, diluted 1 : 100 in the blocking solution, were added to each well and the plates were incubated for 2 h at RT. After washing with PBS, horseradish peroxidase (HRP)-conjugated rabbit antihuman IgG (Southern Biotechnology Associates Inc., Birmingham, AL, U.S.A.), diluted 1 : 20 000, was added for 1 h at RT. Colour development was achieved using 100 μ L per well of 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) and the plate read using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). Briefly for IgE, immunomicrotitre plates half area, high binding (Microlon; Greiner Bio-One, Frickenhausen, Germany) were coated with 500 ng per well of purified recombinant NC16A or a molar equivalent amount of the control protein GST/6xHis in 50 μ L of 0.1 mol L⁻¹ bicarbonate buffer, pH 9.8, at 4 °C overnight. After washes, ELISA plates were blocked for 2 h with 100 μ L of blocking solution at RT. Serum samples were diluted at 1 : 10 in blocking buffer, added to the wells in duplicate and incubated overnight at 4 °C. The ELISA plates were washed and wells were reacted with HRP-labelled antihuman IgE at 1 : 1000 (Southern Biotechnology) for 90 min at RT. After another series of washes, colour development was achieved and the plate read as described above.

Each serum sample was assayed in duplicate for reactivity with both GST-NC16A and GST; for each serum sample the mean optical density (OD) reading obtained with GST was subtracted from the mean reading obtained with GST-NC16A. To reduce interassay variability all extinction data were stan-

dardized based on the positive and negative internal standards, which were set to 1 and 0 OD units, respectively, and termed pemphigoid index value (PIV).¹⁵

IgG and IgE detection using the ISAC microarray

The IgE sensitization profile of patients with BP was investigated using the ISAC 103 (PMD) providing a panel of 103 common allergens immobilized onto solid phase on a chemically modified microscopy slide.²³ The customized ISAC Exp96 microarray was also set based on the same technology (PMD), bearing further allergenic molecules including NC16A and the *E. coli* GST (Sigma-Aldrich) as described above. All allergens/antigens were immobilized in triplicates on the two microarray versions.

IgE and IgG testing was performed as previously reported,^{24,27} but a modified protocol was adopted to measure the two isotypes in a single run on the same microarray. Briefly, glass microscopy slides, each bearing four allergen reaction sites, were placed into a humid chamber and 20 μ L of undiluted serum from each patient was applied to the reaction site for 2 h at RT. After incubation with the patient's sera, slides were rinsed and washed with buffer solution in order to remove unbound serum immunoglobulins. After drying, allergen-specific IgE and IgG bound to the single allergens were detected using Alexa Fluor[®] 532-conjugated antihuman IgE and Alexa Fluor 647-conjugated antihuman IgG (PMD) antibodies (Invitrogen, Carlsbad, CA, USA). The two secondary antibodies were mixed together after preliminary experiments to optimize their relative amounts. Control experiments were performed to verify that the newly introduced procedure was able to reproduce results obtained by the two secondary antibodies when tested unmixed (data not shown). After a 1-h incubation at RT protected from light and a second rinsing and washing procedure, fluorescence was measured using a commercial biochip reader (ScanArray Gx Microarray Analysis System; Perkin Elmer Life and Analytical Sciences, Shelton, CT, U.S.A.). Captured images were acquired as tagged image file format (TIFF) and imported into the ISAC software (PMD). IgE and IgG values were expressed as ISAC standardized units (kU L⁻¹) by interpolating the mean fluorescence values of three spots with a previously established reference curve.³⁰ Values > 0.01 were considered positive assay results in the ISAC system. For each serum sample, ISAC values obtained with GST were subtracted from values obtained with GST-NC16A. At the end of each run, data were exported to the InterAll software (ADL) by real-time connectivity, and stored in the patient's record together with demographic and clinical data from patients with BP and all enrolled control patients.

Statistical analysis

Statistical analyses were performed using statistical package SPSS release version 13.0 (SPSS Inc., Chicago, IL, U.S.A.). Differences between categorical groups were evaluated using the nonparametric Mann–Whitney U-test. The degree of relation-

ship between the quantitative variables studied was analysed using Spearman's rank correlation (r) test, and the most commonly used bivariate correlation technique. Pearson's chi-squared test or Fisher's exact test (used for two-by-two contingency tables with fewer than 50 cases) were used to assess if paired observations on two variables expressed in a contingency table were independent of each other. All tests were used with two-sided options and statistical significance level was set at $P < 0.01$. In order to assess test performances, receiver operating characteristic (ROC) curve analyses were performed. Sensitivity and specificity, proving the diagnostic performance of each test, were estimated at the optimal cut-off level, bordering the upper left corner in the ROC curve and optimizing both sensitivity and specificity. Thus, based on the maximization of the Youden index ($J = \text{sensitivity} + \text{specificity} - 1$), ROC curves were used to determine a cut-off value for ELISAs used in this study. Previously established cut-off values were used for the following ELISAs: commercial ELISA cut-off = 9 U mL^{-1} (MBL); in-house NC16A-ELISA for IgG, cut-off = 11 PIV;¹⁵ NC16A-ELISA for IgE, cut-off = 4 PIV.²⁹

Results

IgG testing

In order to verify if the ISAC multiplex system, originally set up to detect IgE to allergenic molecules, can also be considered feasible for the detection of BP180-specific IgG, we tested the 31 patients with BP.

As shown in Figure 1a, positive IgG results obtained using the commercial and in-house ELISA systems were replicated by the ISAC IgG testing; 29/31 (94%) patients with BP were detected positive for IgG towards BP180. Furthermore, when ISAC IgG values were compared with results obtained by means of ELISA, a good relationship was recorded comparing ISAC IgG with both the commercial and the in-house system, with Spearman coefficients ranging from 0.65 to 0.76 (Fig. 1b).

In order to verify the sensitivity and specificity of ISAC testing, we also tested a group of 138 nonallergic controls without any dermatological problems related to BP. Among these controls, four (2.9%) subjects showed an IgG recognition of BP180 by ISAC (Fig. 1c), higher than 4.5 kU L^{-1} , which represents the optimal cut-off level for predicting the presence of IgG toward BP180 by means of the ISAC system (sensitivity 93.5% and specificity 97.1%) (Fig. 1d).

When comparing IgG values found in patients according to the disease severity index, significant differences were measured after IgG ELISA detection. Anti-NC16A values of $63.2 \pm 71.2 \text{ U mL}^{-1}$, obtained using the commercially available ELISA, were found in patients with a severity score index of 2–5, whereas IgG values of $102.7 \pm 71.2 \text{ U mL}^{-1}$ were found in subjects with a severity score > 6 . Similar results were obtained with the ISAC microarray IgG measurement, where values were $27.7 \pm 23.78 \text{ kU L}^{-1}$ in patients with a

low severity score index vs. $54.71 \pm 28.12 \text{ kU L}^{-1}$ in subjects with the highest severity score (Mann–Whitney U-test 29.5, $P < 0.03$).

IgE testing

We investigated by means of the same microarray system the presence of IgE autoantibodies in all BP sera available to us. We observed 19 of 31 (61%) patients with BP had measurable specific IgE to the immunodominant domain of BP180, after ISAC Exp96 testing (Fig. 2c).

We then compared ISAC IgE testing with the in-house ELISA system in eight cases of BP (Fig. 2a). Four cases were ELISA+/ISAC+, one patient ELISA–/ISAC–, one patient ELISA+/ISAC–, and two cases were ISAC+/ELISA– (Fig. 2b). IgE results obtained using two different systems were concordant in five of the eight patients. Because of the low number of observations, statistical evaluation was not applied, but we assumed that the ISAC system, which was originally set for IgE detection was capable of detecting anti-NC16A IgE.

Among nonallergic controls, six of 138 (4.3%) subjects showed IgE recognition of BP180 (Fig. 2c), only one of them also IgG positive toward BP180. The optimal decisional cut-off for predicting the presence of BP180 IgE recognition in this case was $> 0 \text{ kU L}^{-1}$ (61.3 sensitivity, 95.7% specificity) (Fig. 2d).

In the case of ISAC IgE evaluation, no significant differences were observed comparing disease severity score and specific IgE values.

BP180 IgG and IgE reactivity in a large cohort of allergic subjects

The ability of the ISAC system to measure IgG and IgE reactivity at the same time in a large cohort of subjects, the combined IgG and IgE dysregulation observed in BP, and the preliminary finding that some normal controls had detectable IgG and IgE to BP180, prompted us to evaluate both BP180 IgG and IgE reactivity also in a larger group of 753 allergic subjects. In 30 cases (4%), IgG levels higher than the 4.5 cut-off level were found; 723 subjects had IgG under the negative cut-off of the microarray system as such (Fig. 3a); 22 subjects (2.9%) had low levels of specific IgE to BP180 (Fig. 3b). No correlation was found comparing IgG and IgE BP180 recognition (Spearman r value 0.407) (data not shown).

The overall number of BP180-reactive individuals, measuring both IgG and IgE, was comparable in nonallergic and allergic controls (IgG: 2.9% vs. 4.0%; IgE: 4.3% vs. 2.9%). Interestingly, IgG- or IgE BP180-reactive subjects among controls were all aged < 65 years.

Specific IgE allergen recognition prevalence

When we looked at the specific IgE reactivity to environmental and food antigens evaluated by means of the ISAC system,

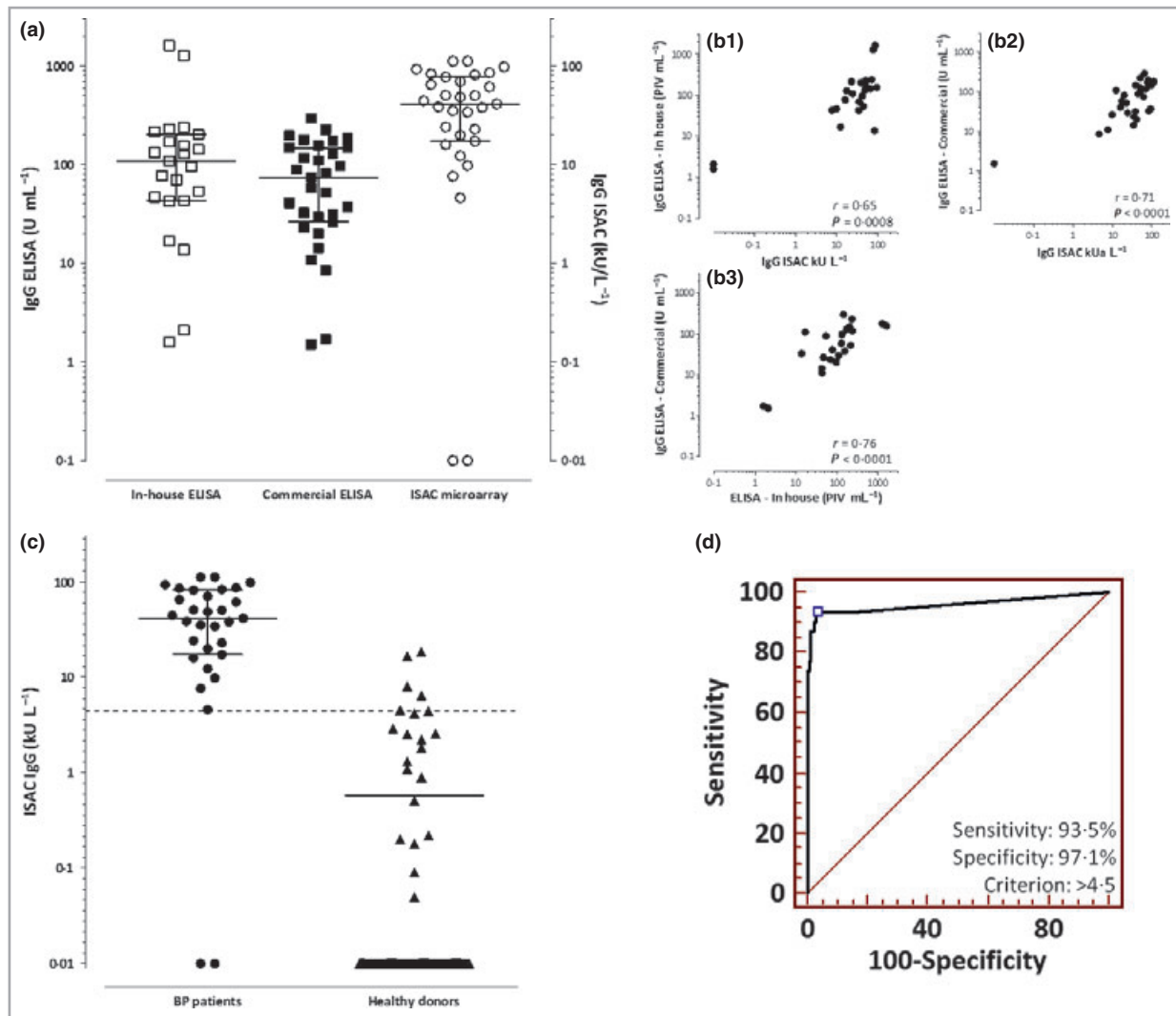


Fig 1. Enzyme-linked immunosorbent assays (ELISAs) and ISAC® BP180 autoantigen IgG determinations. (a) BP180 IgG values, dot-plot representation considering median values with interquartile range; IgG value distributions evaluated using three distinct methods, in-house ELISA (empty squares, left y-axis scale values), commercial ELISA (filled squares, left y-axis scale values) and ISAC BP180 (empty circles, right y-axis scale values), are shown. For graphical visualization needs of log scales, zero values were set at 0.1 (ELISA) and 0.01 (ISAC). (b) Bivariate analysis of reciprocal relationships of IgG to BP180 measured by in-house ELISA, commercial ELISA and ISAC BP180. Correlation and concordance results for each paired tests as in the graph are reported. The Spearman's rank correlation coefficient was used for statistical evaluation. For graphical visualization needs of log scales, zero values were set at 0.1 (ELISA) and 0.01 (ISAC). (c) ISAC BP180 IgG values detected in patients with bullous pemphigoid (BP) (filled circle) and controls (filled triangle) considering median values with interquartile range are shown. For graphical visualization needs of log scales zero values were set at 0.01 (ISAC). (d) A receiver operating characteristic analysis was used to evaluate the diagnostic performance of BP180 ELISA IgG.

there was a very peculiar and uncommon profile of IgE recognition toward some groups of molecules, mainly food allergens, in patients affected by BP.

As shown in Figure 4a, IgE to hen's egg allergenic molecules (Gal d 1, Gal d 2, Gal d 3 and Gal d 5) was observed in > 48% of patients with BP, while > 40% of them had IgE to the kiwi molecule Act d 1 and the *Alternaria major* allergen Alt a 1. Also for seed-derived molecules, namely Ana o 2 from cashew, Ara h 1, Ara h 2, Ara h 3 from peanuts, Ber e 1 from brazil nut, Cor a 9 from hazelnuts, Gly m 5, Gly m 6 from soy, and Ses i 1 from sesame, and cow's milk allergens (Bos d 4, Bos d

5, Bos d 6, Bos d 8 and Bos d lactoferrin), IgE was more frequently found in patients with BP.

Finally, we sought to verify if patients with BP who have specific IgE to BP180 showed a distinct IgE allergen recognition profile. Notably, a significant, higher prevalence of hen's egg recognition was observed in these cases (Fig. 4b).

Discussion

In the present preliminary study we demonstrate, for the first time, that a customized version of the ISAC microarray system

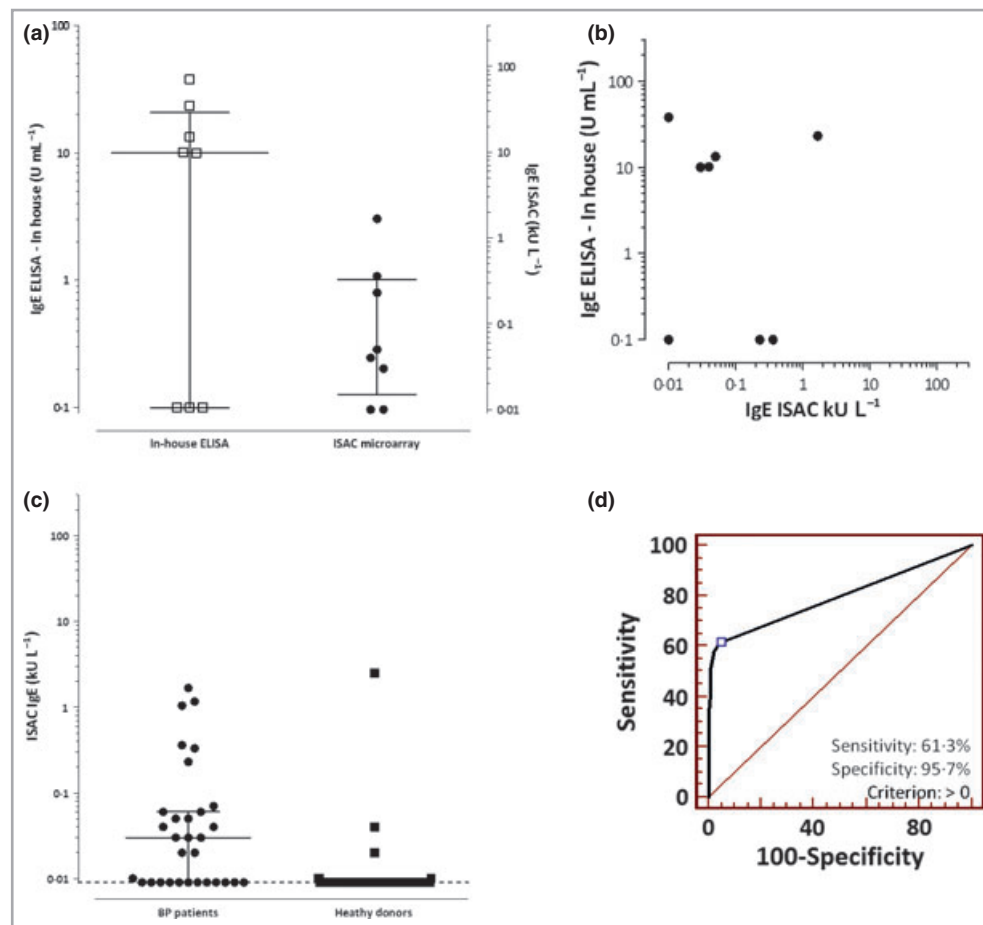


Fig 2. Enzyme-linked immunosorbent assays (ELISAs) and ISAC[®] BP180 autoantigen IgE determinations. (a) BP180 IgE values, dot-plot representation considering median values with interquartile range; IgE to BP180 value distributions evaluated with two distinct methods, in-house ELISA and ISAC, are shown. For graphical visualization needs of log scales, zero values were set at 0.1 (ELISA) and 0.01 (ISAC). (b) Bivariate analysis of reciprocal relationship of IgE to in-house ELISA and ISAC BP180. The figure shows the correlation and concordance results for the paired tests in the graph. For graphical visualization needs of log scales, zero values were set at 0.1 (ELISA) and 0.01 (ISAC). (c) ISAC BP180 IgE values detected in patients with bullous pemphigoid (BP) and controls considering median values with interquartile range are shown. For graphical visualization needs of log scales, zero values were set at 0.01 (ISAC). (d) A receiver operating characteristic analysis was used in order to identify the optimal cut-off level for predicting the presence of IgE toward BP180 in patients affected by BP.

is suitable for detecting IgG and IgE autoantibodies in patients affected by BP. Data analysis showed a very good linear correlation of IgG detected using ISAC testing and the available ELISA tests, with good *r* values (Fig. 2).

The ISAC system based on NC16A showed a sensitivity and a specificity for IgG (93.5% and 97.1%, respectively) and IgE (61.3% and 95.7%, respectively) that are in line with previous reported diagnostic performance for NC16A ELISAs.^{9,16,22} These findings show that the microarray system could be a possible alternative for the serological diagnosis of BP. However, the low levels of IgE reactivity against BP180 detected by the ISAC system, possibly due to the very low amount of protein coated on the chip (100 pg), suggest that the IgE detection against autoantigen needs further improvements. Of note, this system allows the evaluation of the reactivity to other autoantigens immobilized on the microarray in the same test and this could represent a possible added value, both for

differential diagnosis purposes within distinct skin bullous diseases or in the field of other autoimmune diseases. In addition, a customized microarray system based on different antigens will be cheaper than multiple ELISA kits analysis. The ELISA kit cost is roughly €25.00 per test (only one antigen tested), whereas the cost of a microarray is three times (€75.00 per test) as much as the ELISA but it currently tests reactivity to 112 different antigens!

A major achievement is also the possibility of evaluating at the same time in the same test, both IgG and IgE recognition of the BP180 immunodominant epitope (NC16A). In addition, the detection of IgE and IgG towards other antigens spotted on the array could allow an understanding of patients' clinical and allergy profiles and suggest possible disease immune mechanisms. Interestingly, the levels of circulating IgG autoantibodies against NC16A detected by the ISAC system were related to disease severity, suggesting a place for this

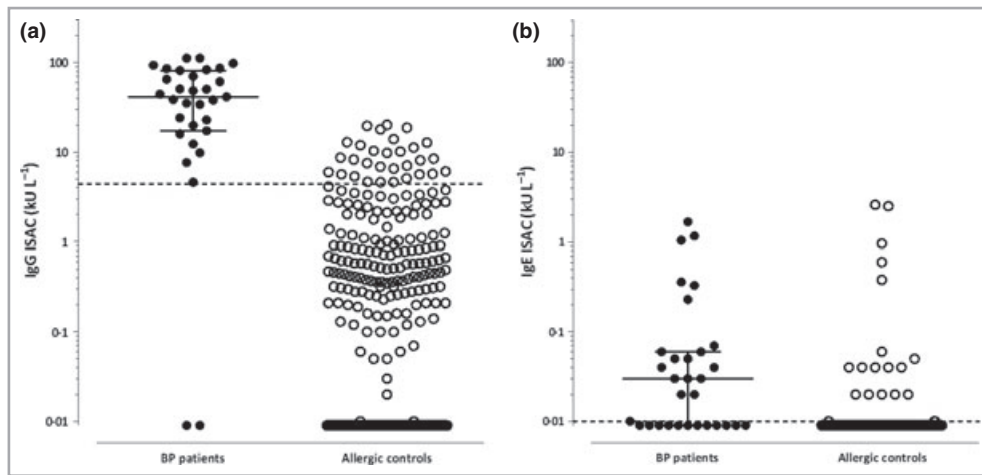


Fig 3. ISAC BP180 IgG (a) and IgE (b) reactivity in patients with bullous pemphigoid (BP) (filled circles) and in a group of 753 allergic individuals (empty circles).

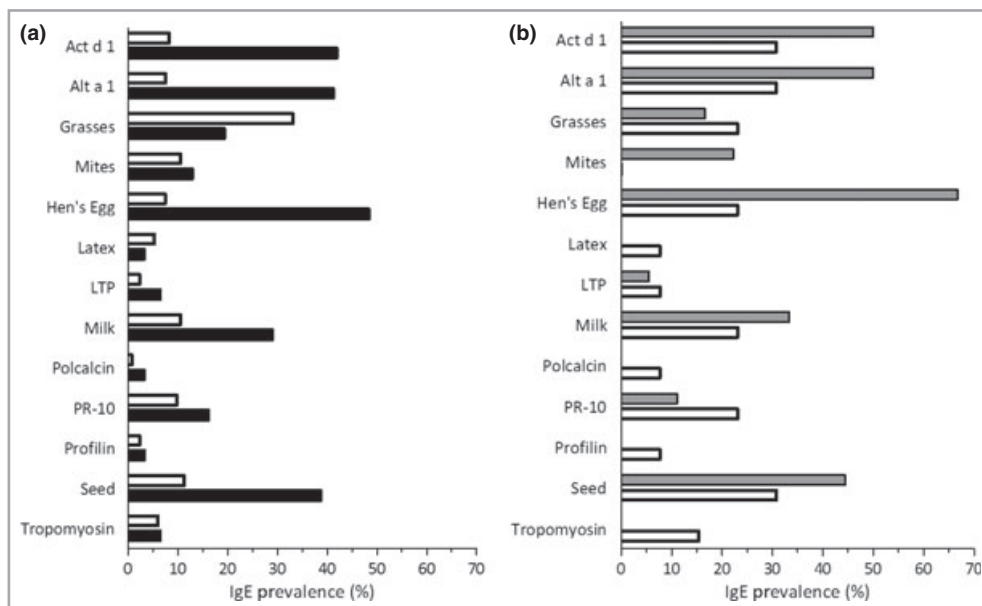


Fig 4. IgE testing by ISAC® 103 microarray for available allergenic molecules. (a) Additional allergen sensitizations detecting IgE using the ISAC 103 microarray system in patients with bullous pemphigoid (BP) (black bars) and an age-matched group of allergic patients (white bars). At least one positive IgE result for one of the following allergens was evaluated for allergenic molecule groups and allergenic sources. Pearson's chi-squared test was applied to define significantly different prevalence. Mites = Der f 1, Der f 2, Der p 1, Der p 2, Eur m 2; Hen's egg = Gal d 1, Gal d 2, Gal d 3, Gal d 5 ($\chi^2 = 49.9$; $P < 0.0001$); Latex = Hev b 1, Hev b 3, Hev b 5, Hev b 6.02; LTP = Pru p 3, Cor a 8, Art v 3; Milk = Bos d 4, Bos d 5, Bos d 6, Bos d 8, Bos d lactoferrin ($\chi^2 = 9.5$; $P < 0.002$); Grasses = Cyn d 1, Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6, Phl p 11; Polcalcin = Bet v 4, Phl p 7; PR10 = Act d 11, Api g 1, Aln g 1, Ara h 8, Bet v 1, Cor a 1.0101, Cor a 1.0401, Dau c 1, Gly m 4, Mal d 1, Pru p 1; Profilin = Bet v 2, Hev b 8, Ole e 2, Mer a 1, Phl p 12; Seed = Ana o 2, Ara h 1, Ara h 2, Ara h 3, Ber e 1, Cor a 9, Gly m 5, Gly m 6, Ses i 1 ($\chi^2 = 24.2$; $P < 0.0001$); Tropomyosin = Ani s 3, Bla g 7, Der p 10, Pen a 1, Pen i 1, Pen m 1. Detailed information about the nature of additional IgE positive allergens detected using the ISAC microarray system can be obtained from <http://www.allergome.org>. (b) IgE sensitization profile of patients with BP IgE reactive to BP180 detected by ISAC (grey bars) in comparison with BP180 IgE-negative patients (white bars). Statistically significant difference was observed only in the case of hen's egg allergens ($\chi^2 = 7.88$; $P < 0.005$).

microarray system as a tool for monitoring disease in BP. In parallel, the lack of a relationship between IgE reactivity and the disease severity of BP could account for a contradictory situation in which anti-BP180 IgE reactivity relates with disease

severity in several, but not in all, patients, as also reported in allergic patients. Although the above reported results pave the way for a new approach to BP diagnosis, further multicentre studies are required to expand the tested BP cohort.

Blood or tissue eosinophilia, together with increased serum IgE levels, are a prominent feature of patients with BP and also of allergic subjects. In BP, subepidermal vesiculation is often preceded by the development of urticaria, plaque and itch; in addition several authors have demonstrated the role of IgE autoantibodies against BP180 antigen in disease pathogenesis.^{14,31} On the other hand, it has been reported that the majority of patients (approximately 60%) with severe and chronic allergic symptoms could exhibit IgE reactivity against self-antigens.³² Interestingly, patients with atopic dermatitis possess IgE autoantibodies binding epithelial antigens and in some cases cell membrane-associated proteins.³² In this context, to study further the relation between IgE antibodies and BP we investigated both BP180 reactivity in allergic subjects and environmental or food antigen IgE recognition in patients with BP.

The ability of the ISAC system to measure IgG and IgE reactivity at the same time in a large cohort of subjects has allowed us to evaluate BP180 reactivity in the largest cohort of allergic subjects and, in general, in the largest cohort of individuals studied to date. Thirty of 753 subjects (4%) possessed circulating IgG reactive for BP180, perfectly in line with the recognition frequency detected using ELISAs in 25 allergic individuals recently studied³³ and a large cohort of normal individuals.^{34,35} In addition, 22 subjects (3%) had specific IgE to BP180, such as previously obtained using ELISA in a normal population⁹ and less than previously detected in a very limited cohort of allergic individuals.²⁹ Altogether, these findings further validate the ISAC system in detecting BP180 IgG and IgE reactivity and demonstrate that the allergic condition does not increase the frequency of IgEs, or IgGs, recognition of BP180 in subjects without BP. As the allergen sensitization profiles are related to environmental exposure, a future multicentre study will allow us to explore further the immune response of patients with BP in the local environment.

On the other hand, we have found in BP a peculiar and uncommon profile of IgE recognition toward some groups of molecules, mainly food allergens. In particular, our patients with BP showed IgE reactivity, mainly directed to cow's milk and hen's eggs allergens, rarely observed in age-matched allergic subjects (111 subjects). Interestingly, in patients with BP there was a trend of association between this unusual reactivity profile and BP180 IgE recognition. In particular, IgE recognition of hen's egg allergens was significantly related to BP180 reactivity. To assess the possible cross-reactivity between anti-NC16A IgE and environmental antigens and its biological significance further studies are needed.

Two studies recently showed that patients with pruritic dermatoses with specific BP180 and/or BP230 IgG did not develop BP during a follow-up period of 4–31 months.^{33,36} In addition, we found IgE and IgG reactivity to BP180 in normal and allergic individuals was not related to age, suggesting that BP180 positivity is not necessarily a sufficient condition for the development of BP.³⁴ In the present study we showed that the allergen profile typically present in patients with BP is not found in BP180 IgE-positive allergic individuals, suggest-

ing a role for the occurrence of this immunological feature in the development of the BP condition.

A common cause of the concomitant IgE reactivity against BP autoantigens (BP180) and environmental and food antigens could be an elderly-associated state of dysregulated immune function.³⁷ Several studies have emphasized the critical role of CD4+ CD25+ FOXP3+ regulatory T cells (Tregs) in maintaining immune homeostasis and in preventing reactivity to both autoantigens and allergens.^{26,38,39} In line with this possible explanation, a significant reduction of the number of FOXP3+ Treg cells was found in BP.^{40,41} Moreover, according to reports in the literature, two possible links between anti-BP180 response and IgE sensitization to environmental allergens can be postulated: (i) tissue damage induced by IgE to environmental allergens leads to denaturation of different proteins (BP180) and recognition of novel autoepitopes such as reported for patients with atopic dermatitis;^{42,43} and/or (ii) IgE cross-reactivity between environmental allergens and auto-antigen (BP180) could induce an autoimmune response such as suggested for allergy to the profilin allergen.⁴⁴

In conclusion, in the present preliminary study we demonstrate the feasibility of the ISAC system in the evaluation of both BP180-specific IgG and IgE detection, and the presence of a peculiar IgE sensitization profile, mainly to food allergens, in elderly subjects affected by BP. The reported results on a limited number of patients with BP and in a single geographical area require further studies involving a larger number of patients with BP from different centres worldwide.

What's already known about this topic?

- Bullous pemphigoid (BP) is a severe autoimmune blistering disease.
- IgG and IgE autoantibodies to skin BP180 and BP230 autoantigens are detected by enzyme-linked immunosorbent assay (ELISA) in patients with BP.
- IgE seem to play a role in a subset of patients with more severe disease.

What does this study add?

- IgG and IgE autoantibodies in BP sera were detected using the immunodominant portion of BP180 immobilized on a microarray along with allergenic molecules, with almost overlapping results compared with ELISAs.
- Specific IgE allergen profiles differ in patients with BP and control groups.
- Specific BP180 IgG and IgE are rarely detected in an allergic population.

Acknowledgments

The use of the Allergome-InterAll application was generously made available for free by Allergy Data Laboratories s.c., Lati-

na, Italy. We are grateful to Christian Harwanegg for helping us in developing the customized ISAC Exp96 at the PMD facilities in Vienna, Austria, and for building up the IT infrastructure to connect the ISAC system and the InterAll e-record, allowing us to manage easily the data produced by the extensive microarray testing.

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