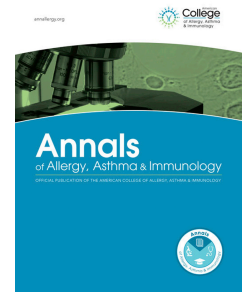


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Development Of A Progesterone-Specific IgE Assay For Diagnosing Patients With Suspected Progestogen Hypersensitivity

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Key words: Progesterone-BSA, Progestogen hypersensitivity, specific IgE, ELISA inhibition, beta-hexosaminidase mediator release

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INTRODUCTION

Progestogen hypersensitivity (PH; a.k.a autoimmune progesterone dermatitis) was first reported 50 years ago in a woman whose symptoms correlated with the luteal phase of her menstrual cycle.¹ Of note, her symptoms were partially resolved with estrogen administration. Since this case, PH cases manifesting as a spectrum of dermatologic eruptions in the presence or absence of anaphylactic symptoms have been reported. The classic presentation of PH symptoms is an onset beginning during the second half of the cycle when progesterone levels begin to increase. Typically, eruptions resolve a few days into the menstrual flow when progesterone levels decline but can persist until completion of the cycle. An important clinical characteristic of PH is that these reactions cyclically reoccur monthly.

A variety of skin conditions have been reported to occur with PH although urticarial lesions tend to be the most common presenting condition. Nguyen et al reviewed 89 cases of PH from 66 publications up until 2016.² In this case series, the median age of onset was 27.3 years (range 12–47) with skin symptoms developing between 3-10 days before the onset of menstruation and typically disappearing after cessation of menstruation. Urticaria was the most common cutaneous symptom but other rashes observed included erythema multiforme, folliculitis, purpura, stomatitis, eczema, angioedema, papulovesicular eruptions, and vulvovaginal pruritus. In addition, a few cases of progesterone anaphylaxis have been reported.³

With the extensive use of oral contraceptives and increased use of supra-physiologic doses of progesterone to support pregnancy by *in vitro* fertilization, there is likely to be a higher prevalence of PH in the future than currently recognized. Although the cause of PH is not fully understood, it has been suggested that sensitization to progesterone likely occurs from using progesterone-estrogen oral birth control pills resulting in subsequent cross-sensitization to

endogenous progesterone due to their structural similarity. An IgE mediated mechanism is believed to be the most common cause of PH but a positive skin-test response to methanol extracts of progesterone or 5b-pregnanediol has not always been observed due to progesterone's low molecular weight and inability to cross-link specific IgE (sIgE) on cutaneous mast cells.

Current diagnosis of PH is made based on clinical history and progesterone skin testing. However, since progesterone is water insoluble, use of progesterone-oil emulsions have been required for skin testing but are wrought with false positive irritant responses making it difficult to determine if the patient is truly sensitized to progesterone. Thus, clinical improvement in response to treatment that shuts off ovulation has still been frequently used as a confirmatory diagnostic criterion for PH. Once PH diagnosis is confirmed, there are a wide range of treatment options that have been proposed for PH ranging from simple use of H1-antagonists to the most extreme decision, oophorectomy.^{4-6,7} Oophorectomy and exogenous ovulation suppressors, previously described as definitive therapies for PH, are not practical options especially for premenopausal women wishing to have children or due to the increased risk of osteoporosis.^{4,6-8} More recently, progesterone desensitization has emerged as the safest and most effective long-term treatment option for PH and is particularly indicated for conditions with uncontrolled symptoms despite receiving first line treatments (i.e. H1-antagonists), or for subjects undergoing IVF requiring supra-physiological doses of progesterone for successful embryo implantation.⁷ Available oral, intramuscular (IM), and intravaginal suppository protocols have previously been reviewed in detail.⁹ Therefore, the allergist-immunologist will be required to collaborate with gynecologists and reproductive endocrinologists to diagnose and treat this condition.

A diagnostic algorithm for PH recently proposed by Li et. al.⁷ emphasized an unmet need for a more reliable *in vivo* or *in vitro* test to improve upon the current options for confirming

progesterone sIgE sensitization. Thus, to address this gap in knowledge, we developed a specific IgE direct ELISA method for reliably identifying progesterone sensitization in women with suspected PH.

METHODS

Study populations:

Serum samples were collected for this study from female patients presenting to our clinic with classic symptoms suggestive of PH. All patients experienced dermal eruptions beginning 3-4 days before the onset of menses which resolved a few days into the menstrual cycle or after completion. All subjects signed an informed consent approved by the University local IRB. These serum samples were used to optimize the serologic assay and to determine positive and negative cut-points. Samples sent by referring physicians over four years for suspected serum progesterone-specific IgE (sIgE) testing were re-analyzed and categorized as being high positive, low positive or negative based on the established cut points. All sera were aliquoted and stored at -20°C until use. To conserve serum, after performing an initial specific IgE-ELISA with each individual's serum, equal volumes of sera was pooled from five patients with high positive specific IgE reactivity for use in all subsequent experiments (i.e. inhibition ELISAs). For controls, sera from age-matched nonatopic healthy female subjects were used for each experiment.

Antibodies and reagents:

Goat anti-human IgE, alkaline phosphatase-conjugated rabbit anti-goat IgG were purchased from KPL (Gaithersburg, MD). ELISA microtiter plates were obtained from Corning (Corning, NY). Eagle's Minimum Essential Medium (EMEM) was obtained from ATCC

(Manassas, VA), while G418 and pNPP substrate were obtained from Sigma-Aldrich (St. Louis, MO).

Analysis of progesterone-BSA conjugate using mass spectrometry:

Progesterone-BSA conjugate was purchased from AbD Serotec (Catalog # 7720-0750). A 5mg/ml BSA standard in 0.1% Formic Acid was made from the supplied BSA. A 2 ul aliquot of the BSA standard and BSA-progesterone conjugate were diluted to 1:50 and 1:100 in MALDI matrix (5 mg/mL sinapic acid in 60% Acetonitrile/0.1% formic acid). All spectra were acquired in Linear Positive Ion Mode on an ABSciex 4800 MALDI-TOF/TOF Instrument. Apomyoglobin and BSA were detected in their singly charged forms labeled $[M+H]^+$. The Apomyoglobin peak and the BSA peak of the cal 3 standard mixture were used for calibration.

IgE-specific ELISA:

Initial optimization of the direct ELISA was achieved using checker board dilutions of positive serum samples and the progesterone-BSA conjugate used for coating ELISA wells. The optimal serum dilution (1:10) and progesterone conjugate concentration (20ug/ml) were determined and used for all analyses. Presence of progesterone-specific serum IgE was determined by coating 96 well micro-titer plates with Progesterone-BSA conjugate in triplicate followed by blocking with 5% BSA in PBS 0.05% Tween 20 buffer (PBST) and incubation with diluted patient serum. Subsequently, the wells were washed to prevent non-specific binding and incubated with goat anti-human IgE. They were washed again, and anti-goat IgG-alkaline phosphatase conjugate was added to detect bound sIgE.

Sera collected from three consented healthy non-atopic female subjects (N=4) were used as negative controls. Sera previously collected from patients (N=3) who showed very high progesterone-sIgE associated with cyclic urticaria were included as positive controls. A positive test was defined as an OD value greater than the average negative control OD value + 3SD.

ELISA inhibition:

For ELISA inhibition, control and patient sera (1:5 diluted in PBST) were preincubated with equal volume of Progesterone-BSA in varying concentrations (5, 1, 0.1, 0.2, 0.01 and 0 ug/ml in BSA-PBST) as an inhibitor. The inhibited serum was then analyzed by ELISA using anti-IgE Alkaline Phosphatase conjugate. The percent inhibition was calculated by:

$$\text{Inhibition (\%)} = [(OD \text{ of test sample}) - (OD \text{ of inhibited sample}) \times 100] \div OD \text{ of test sample}$$

The 0% inhibition was obtained by incubating serum samples using BSA-PBST controls. A dose-response curve was obtained by plotting the percent inhibition against the log of protein concentrations.

Rat Basophil beta hexosaminidase release assay:

This functional assay was performed as previously described, with minor modifications.¹⁰ Briefly, Rat Basophil Leukemia (RBL) cells stably expressing human FcER1(RBL-SX38 cell line obtained from Dr J. P. Kinet, Harvard Medical School) were cultured in complete Eagle's minimum essential medium supplemented with 100 mg/mL G-418 and their FcER1 expression was verified using flow cytometry. For all experiments, RBL cells were cultured in the absence of G-418. Confluent monolayers of cells were incubated overnight with 1:5 diluted (in pre-

warmed EMEM medium) sera obtained from PH patients and control subjects. Cells were then washed in Tyrod buffer and treated with 0.1 mM NECA (5'-N-ethylcarboxamide; Sigma-Aldrich, USA) at 37°C for 5 minutes to increase the antigen sensitivity of the cells, washed again and then exposed to progesterone-BSA conjugate in Tyrod buffer for one hour.^{11,12} Released beta-hexosaminidase was detected by incubating with equal volume of pNAG solution (1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide in citrate 0.05 M, pH 4.5) for 1 hour at 37°C. Total release was determined by lysing the cells with 1% Triton X-100. The reaction was stopped by adding 150 μL of 0.2M glycine, pH 10.7.¹² The absorbance was measured with a microplate reader (Thermo scientific, Madison, WI) at 405 nm and the percentage of total β-hexosaminidase was calculated by: % Degranulation = $\text{OD supernatant} \div (\text{OD supernatant} + \text{OD Triton X-100}) \times 100$.

The mediator release assay was performed using high positive (N=10), low positive (N=5) and ELISA-negative (N=9) samples, which includes ELISA-high, ELISA-low and ELISA-negative sera from our center (5/group; used in the single-batch ELISA) as well as additional 5 ELISA-high and 4 ELISA-negative sera from the 64 samples sent from referring physicians. These additional 5 ELISA-high and 4 ELISA-negative samples were used in the multiple batch runs as controls.

Statistical Analysis:

Age data of PH patients are described in mean ± SD and median values. To analyze ELISA results, first OD values were tested for normality distribution using Shapiro and Wilk's normality test.¹³ In order to statistically discriminate between positive and negative serum samples, Shewhart's "three-sigma limits" was used, which refers to data within three standard deviations (SD) from the mean, to establish a negative cut-point.¹⁴ Any OD value higher than the negative cut-point, defined as > the average control OD + 3 SD, was considered positive. For

this analysis, if values are normally distributed, 99.7% of the control data-points should be within ± 3 SDs from the mean control value. The mean control OD + 3 SD (upper limit of the distribution) was used to establish the cut-point, above which values were considered positive. For further stratification, a serum sample was considered 'high-positive' when the OD value was $\geq 2 \times$ mean negative control OD +3SD and as 'low positive' when it was higher than the negative cut-point, but lower than the high positive cut point ($>1 \times$ OD+3SD to $< 2 \times$ OD+3SD). The frequency of high, low and negative samples was recorded in percent values. An unpaired t-test was used to compare between groups (high vs. control, low vs. control and negative vs. control) for ELISA and mediator release data. The paired t-test, was used to determine whether there was a correlation between OD values from high and low progesterone sIgE subjects and percent mediator release. Finally, to determine if the results of the mediator release assay are reliable for indicating the functional activity of progesterone sIgE, Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the direct ELISA assay were calculated. All statistical analyses and plots were generated using Excel (Windows 10) and JMP SAS (SAS Institute, Cary, NC).

Results:

Characterization of progesterone BSA conjugate:

Type-2 hypersensitivity reactions are augmented by the crosslinking of two effector cell-bound IgE molecules by multivalent allergens. Therefore, the commercially available progesterone-BSA conjugate was analyzed by mass spectrometry to assess whether multiple progesterone molecules were conjugated to the BSA carrier protein thereby representing a

multivalent allergen. Our mass spectrometry data indicated a shift of approximately 4,444 Da for the progesterone-BSA conjugate compared to unconjugated BSA (Figure-1). The conjugated peak was much wider than the BSA standard peak, indicating more heterogeneity in the progesterone-BSA protein which is consistent with BSA being conjugated with varying numbers of progesterone molecules attached. This mass shift represents an approximate average of 14 progesterone molecules (progesterone molecular weight = 314.47) bound to the BSA carrier protein.

Patient population:

The 17 patients evaluated for PH from our clinic used to establish cut points for the direct ELISA had a median age of 37 years (mean 37.5 years; SD = 10.65 years). The time between symptom onset and diagnosis of PH as a possible cause ranged between 1-10 years. Demographic and clinical characteristics of these subject are summarized in Table 1. In addition, 64 serum samples from females with suspected PH obtained from their referring allergy specialist or primary care physician were analyzed. All patients had a history of symptoms that began shortly before the onset of their menstrual cycle and resolved after menses began or ended suggestive of PH. However, complete demographic information was not available for these patients.

Approximately one-third of patients (6/17; 35%) were atopic. Of these individuals, comorbidities included allergic rhinitis (n=7), food allergy (n=6), drug allergy (n=2), atopic dermatitis (n=2), anaphylaxis (n=2) and asthma (n=1). All patients presented with cyclical urticaria, angioedema, pruritic rash or anaphylaxis (Table 1). Information about progesterone exposure (either exogenous or from multiple child-birth) or total serum IgE levels for those patients whose sera were collected by other physicians and sent to our center for analysis was not

available. However, for the patients from our clinic, the total serum IgE values were not significantly different (a) between sIgE negative vs. sIgE low ($p = 0.38$) groups (b) between sIgE negative vs. sIgE high ($p=0.92$) groups or (c) between sIgE low vs. sIgE high ($p=0.44$) groups indicating that the direct sIgE ELISA test is specific and not significantly influenced by total serum IgE. In addition, available information related to previous IVF, use of progesterone-based contraceptives or multiple child-bearing obtained from our study subjects did not indicate any direct association between these factors and PH (Table 1). External exposure of patients included in Table 1 occurred primarily through oral contraceptive. Interestingly, one of our patients not included in single-batch direct ELISA data presented in Table 1 previously underwent multiple IVF attempts and did manifest a high progesterone sIgE response. A larger prospective study has been planned to investigate the associations between these probable risk factors (i.e. exogenous progesterone exposure, multiple vs. single child-birth, IVF) and sIgE values in PH patients.

IgE-specific ELISA:

The OD results of the 17 patients analyzed simultaneously by direct ELISA are summarized in Table 1. Progesterone sIgE was high positive in 6 patients (35%), low positive in 6 patients (35%) and negative in 5 patients (29%) compared to the healthy non-atopic control sera ($n=4$). The ELISA OD values were found to be normally distributed and therefore, suitable for utilizing the 'three sigma limit' to discriminate positive from negative serum samples.¹³ Six (35%) patients had sIgE values defined as high positive ($\geq 2x OD+3SD$), 6 (35%) were low positive ($>1x OD+3SD$ to $< 2x OD+3SD$) and 5 (29%) were negative ($\leq 1 OD+3SD$) (Figure 2). These cut points were applied to the sIgE OD values of the 64 additional patient serum samples which were similarly distributed between high (39%), low (31%) and negative (30%) cut points.

Sera from these three groups (high, low and negative) were used for all subsequent serologic and mediator release assays.

ELISA inhibition:

ELISA inhibition experiments were performed to determine antigen specificity using pooled serum from five high positive progesterone sIgE patients (blue line) which was preabsorbed for 10 hours at room temperature, with increasing microgram amounts of Progesterone-BSA conjugate (Figure 3A). Similarly, an individual patient with high positive progesterone sIgE serum (red line) and serum obtained from a non-atopic control subject (green line) were used. These results indicate a dose-dependent inhibition of IgE binding to surface bound Progesterone-BSA conjugated antigen confirming the presence of Progesterone-specific serum IgE in PH patients. The negative control sera and BSA did not exhibit dose-dependent inhibition. Figure 3B (right panel) illustrates the percent inhibition using pooled sera from PH subjects when either Progesterone-BSA (blue line) or unconjugated BSA (red line) were added as inhibitors. Percent inhibition of a normal control serum (ELISA negative for progesterone sIgE and negative for significant mediator release from IgE-sensitized RBL cells) pre-adsorbed with increasing amounts of Progesterone-BSA conjugate as the inhibitor (green line) is also shown, which does not show dose-dependent inhibition confirming that inhibition of IgE binding to the plate-bound antigen is not occurring in case of control non-atopic serum.

Rat basophil beta-hexosaminidase release assay:

Figure-4 shows the results of a beta-hexosaminidase mediator release assay using serum from patients with high positive, low positive and negative OD values compared to healthy non-atopic controls and spontaneous release. Mediator release using each serum sample was

calculated as a fraction of total cellular mediator obtained after cell lysis.¹¹ Eight out of 10 sera with high positive progesterone-sIgE demonstrated significant mediator release ($p < 0.01$), which was approximately 17% (median value) of the total cellular mediator content. Mediator release from cells using sera from five subjects with low positive levels of serum progesterone sIgE was also statistically significant ($\sim 7.5\%$ median value; $p=0.009$). Progesterone sIgE negative sera as well as sera from non-atopic healthy controls did not demonstrate significant beta-hexosaminidase release.

Finally, correlation between ELISA OD values and percent mediator release was performed using high and low reacting sera ($N = 5$ per group) from the single batch direct ELISA experiment (direct ELISA Figure 2) that were also analyzed by the mediator release assay. The ELISA OD values of High (Figure 5A) and Low (Figure 5B) groups plotted against percent mediator release revealed significant correlation for the High ($p = 0.0001$) and Low ($p = 0.00046$) sample groups.

Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were calculated for the direct assay using 15 ELISA-positive (10 high reacting, and 5 low reacting) sera of which 13 showed positive mediator release (ELISA-high = 8, ELISA-low = 5) and 9 ELISA-negative sera. The two high ELISA-positive sera that did not induce considerable mediator release (5.78 % and 5.69%, respectively), were comparable to the average percent mediator release value of the non-atopic control group (5.4%) and were detected as 'outliers' by JMP SAS package. Therefore, 13 sera were considered "True positive" and two as "False positive". All of the ELISA-negative serum samples ($n = 9$) were considered "True negative" as they didn't demonstrate significant mediator release and therefore, no "False negative" samples were identified. If the result of our mediator release assay is considered to be a reliable test for

indicating the functional activity of progesterone sIgE, then our direct ELISA for progesterone sIgE demonstrated a Specificity ($T^-/T^- + F^+$) of 82 %, a Sensitivity ($T^+/T^+ + F^-$) of 100%, a Positive Predictive Value ($T^+/T^+ + F^+$) greater than 86% and a Negative Predictive Value ($T^-/T^- + F^-$) of 100%.

Discussion:

Although the pathomechanism(s) of PH are still not entirely clear, an IgE mediated immune response is the most probable pathway resulting in symptoms manifested by patients suspected with this condition. It has been speculated that PH occurs as the result of prior progesterone exposure resulting in sIgE sensitization to synthetic progesterone or its cross-reactive analogues.^{7,15,2,9,16-18} Other mechanisms that have been proposed include a role for CD8⁺ cells, immune-complex formation, direct mast cell activation by endogenous progesterone *via* progesterone receptors located on their cell membranes and a specific IgG progesterone response, however, the later proposed mechanism has not been adequately elucidated.^{9,18 15,19,20}

Diagnostic algorithms and available treatment options for PH have recently been reviewed by several authors.^{7,9,21,22} All have emphasized that diagnosis of this condition may be challenging due to the heterogeneous clinical presentation of these patients. Therefore, to establish an IgE-mediated cause of PH, a careful review of the patients' history of symptoms that demonstrate a temporal relationship to the peri-menstrual progesterone surge in conjunction with evidence of an increased sIgE response to progesterone is necessary.

Historically, skin prick and intracutaneous testing with progesterone have been used to confirm PH. However, since progesterone is not water soluble, oil or ethanol has been used as a

diluent. Therefore, when interpreting skin tests, it is important to recognize that these diluents can result in an irritant induced reaction, thereby limiting the positive predictive value of this test. For this reason and because positive and negative predictive values of skin testing with synthetic progestins are difficult to ascertain, there are no standardized progesterone skin test reagents^{7,9,21}. Therefore for this study, we did not correlate the serologic progesterone sIgE results with progesterone skin testing given the well-recognized irritant nature of progesterone skin tests would have made it difficult to interpret a positive or negative response.

The results of our direct ELISA-based sIgE study indicate that the sera of patients with suspected PH can be stratified into high positive *versus* low positive and negative categories and that these responses are specific and functionally relevant based on ELISA inhibition and beta-hexosaminidase assay results, respectively. Furthermore, the results of the mediator release study suggest that the progesterone sIgE direct ELISA has very good sensitivity and negative predictive value in addition to a good specificity and positive predictive value. Therefore, this assay can be very useful for helping the clinician to establish a diagnosis of PH.

Potential limitations of this analysis may include the presence of cross reacting progesterone sIgG. This is less likely as the goat anti-human IgE (KPL Cat. No. 01-10-04) used in our experiments was cross-adsorbed to eliminate binding to human IgG. In addition, differences in the menstrual time period when serum samples were handled and shipped could also have potentially impacted whether the patient exhibited a high *versus* low positive progesterone specific IgE. The collection of these serum samples, which were shipped overnight on wet ice, reflect a real-world experience of obtaining serum samples for analyses of this type. Since these variables would potentially affect all samples collected equally it is unlikely they had

a significant effect on our results. Finally, even though an identical ELISA protocol was used for each batch of sera analyzed as they were randomly received over a four-year period, we considered that the raw OD values of the 64 serum samples analyzed in this study may not be directly comparable, since the results were obtained from multiple independent experiments using different non-atopic negative control sera. To account for this possible discrepancy, additional 17 sera obtained from PH patients in our center and healthy non-atopic female negative controls were analyzed in a single batch ELISA experiment to validate the cut points used to interpret the results of this assay.

A strength of this study is that most of the high and low-positive sera were able to induce mediator release indicating the functional relevance of the progesterone-sIgE direct ELISA test.

As summarized in Table 1, there are no obvious differences in presenting symptoms between high and low sIgE or negative groups. Therefore, clinical characteristics alone appear to be insufficient for diagnosis of PH further emphasizing the need for a reliable diagnostic assay. Larger population studies will be required to confirm this observation. A detailed positive clinical history for PH which requires onset of allergic symptoms 2-3 days before menstruation with resolution midway or by completion of the menstrual flow in the absence of other underlying clinical conditions (e.g., chronic urticaria/angioedema, food or drug allergy...) is the current gold standard for diagnosis. However, all 17 patients' sera (with additional 3 positive and 4 negative controls sera) included in this single batch analysis which was used to identify threshold sIgE responses (i.e., cutpoints) exhibited this clinical history whereas only 12/17 of these subjects had positive sIgE to progesterone and of those with positive sIgE to progesterone only 10/12 had positive mediator release. Therefore, this indicates that the clinical history gold standard is too sensitive and using the functional assay as a gold standard for determining the

relevance of the sIgE progesterone response appears to be a more reasonable approach. Future studies involving a larger population of suspected PH patients might address the pathomechanism in cases showing negative sIgE results. The gold standard of any assay is the ultimate treatment response of the disease once it is diagnosed as this ultimately helps to confirm the positive predictive value of the test. Unfortunately, we were not able to report on the treatment outcomes of all the patients from which serum samples were analyzed. However, among the 17 patients from our center with either high or low positive progesterone sIgE, all responded to some form of treatment ranging from H1/H2 antagonists to progesterone desensitization with or without omalizumab. These results will be reported elsewhere.

In summary, this present study demonstrates the utility of a serum progesterone sIgE direct ELISA test in patients with suspected PH. Given the limitations of intracutaneous skin testing, this assay should be a preferred choice for screening patients with suspected IgE-mediated PH.

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Figure-1: Mass-spectrometry based analysis of the progesterone-BSA conjugate (lower panel) showing a shift of approximately 4444 Da compared to the unconjugated BSA (upper panel). This shift in mass which manifests as a wide peak, is due to a variable number of progesterone molecules bound to BSA carrier protein (average number = 14 progesterone molecules per BSA molecule).

Figure-2: Single batch progesterone-specific IgE ELISA OD results using sera from patients with suspected PH in Table 1 demonstrating distribution of high, low and negative cut points for the assay. (High positive = $OD > 2 \times OD \text{ cut-off}$; low positive = $>1 \times OD + 3SD$ to $< 2 \times OD + 3SD$; negative = $\leq 1 \times OD + 3SD$). Four serum samples collected from healthy non-atopic subjects were used as negative controls.

Figure-3. ELISA inhibition using serum samples from high positive PH patients and healthy non-atopic controls. (A) Left panel: Progesterone-BSA conjugate was added to either a pooled serum sample of high positive progesterone sensitized patients ($N=5$; P-Pool, blue line) or to an individual high positive patient serum sample (P1; red line) *versus* an individual negative control serum (Control; green line). Dose-dependent inhibition of IgE reactivity was observed for patients' sera (pooled and individual), but not for negative control serum or unconjugated BSA (BSA; pink line). (B) Right panel: Plot shows the calculated percent inhibition for the pooled sera indicating dose-dependent inhibition of IgE binding by progesterone-BSA (P-pool, blue line; approximately 10 ng Progesterone-BSA was enough for 50% inhibition, red arrow), while non-atopic control sera supplemented with progesterone-BSA (Control; green line) or pooled PH sera supplemented with unconjugated BSA (BSA; purple line) does not show significant inhibition.

Figure-4. Results of a beta-hexosaminidase mediator release assays using patient sera with suspected PH previously found to be high positive, low positive and negative for progesterone sIgE by direct ELISA. Spontaneous release is defined as mediator release without progesterone-BSA exposure. Two ELISA-high sera failed to induce significant mediator release. ELISA-high and ELISA-low groups induced significantly higher mediator release compared to the non-atopic control group.

Figure 5: Percent histamine release plotted against high (figure 5A) and low reacting (figure 5B) sera ($N = 5$ per group) from the single batch experiment showing correlation.

Patient	Age	Symptoms	Comorbidities	Progesterone	Total	# of	Progesterone
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Table 1. Demographics of patients with suspected PH patients used to establish ELISA cut-points (n=17): Patient age, presenting symptoms and comorbidities are summarized. The results of serum sIgE-specific direct ELISA, categorized as high positive, low positive and negative, total IgE, number of children and previous exposure to progesterone are also included.

				sIgE	IgE (IU/ml)	children	exposure (+/-)
1	48	Cyclical pruritic rash	Asthma, atopic dermatitis, food allergy	High	1163	1	No
2	34	Cyclical angioedema	Seasonal allergic rhinitis, food allergy	Negative	51.8	3	No
3	40	Cyclical pruritic rash, diarrhea	Irritable bowel syndrome, vasomotor rhinitis, chronic interstitial cystitis	High	84	1	NA
4	58	Cyclical urticaria, dermatitis, pruritus	Seasonal allergic rhinitis, headache, osteoporosis, hyperlipidemia	Low	NA	NA	NA
5	38	Cyclical urticaria, angioedema	Food allergy	Low	NA	NA	NA
6	48	Cyclical angioedema and/or urticaria, pruritic rash	Allergic rhinitis, drug allergy	Negative	167	0	NA
7	42	Cyclical urticaria	Seasonal allergic rhinitis, food allergy	Low	NA	1	OCP
8	46	Cyclical urticaria	Drug allergy	High	31.1	NA	NA
9	23	Cyclical urticaria, pruritus	None	High	NA	1	OCP
10	26	Cyclical urticaria	None	Low	34	5	NA
11	47	Cyclical urticaria	Alopecia, depression	Negative	NA	NA	OCP
12	30	Cyclical pruritus and rash	Allergic rhinitis	Low	12	2	OCP
13	14	Cyclical urticaria, Anaphylaxis	Food allergy, seasonal allergic rhinitis, headache, mast cell disorder	Negative	1258	0	No
14	37	Cyclical urticaria, Anaphylaxis	Atopic dermatitis, polycystic ovaries	Negative	31	2	OCP
15	36	Cyclical urticaria	Gastroesophageal reflux disease, chronic rhinitis, iron deficiency anemia, hypertension	Low	NA	NA	No
16	33	Cyclical urticaria, pruritus	None	Low	10	NA	No
17	37	Cyclical urticaria, chronic dermatitis	Food allergy, allergic rhinitis, narcolepsy	High	NA	NA	No

6 NA stands for 'Not Available'. External exposure occurred through oral contraceptive pills
7 (OCPs).

Unconjugated BSA compared to progesterone conjugated BSA

