ORIGINAL ARTICLE

Cutoff values for serum mold-specific IgG levels for hypersensitivity pneumonitis: A diagnostic test accuracy study of 219 subjects

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ABSTRACT

Background: The American Thoracic Society guidelines suggest serum specific IgG (sIgG) testing targeting potential antigens associated with hypersensitivity pneumonitis (HP). Their diagnostic cutoffs remain undefined.
Methods: We conducted a diagnostic test accuracy study using a case-control design. We enrolled subjects with HP (cases), other interstitial lung diseases (ILDs, diseased controls), and healthy controls with/without mold exposure. We measured sIgG against *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Cladosporium herbarum*, and *Micropolyspora faeni* using an automated, fluorescent enzyme immunoassay. Cutoffs were derived from the 95th percentile values among non-exposed healthy controls for individual sIgGs and Youden's J-statistic for pooled sIgG (sum of all four sIgG levels). We evaluated the performance characteristics of sIgG levels for HP diagnosis among subjects with ILDs.
Results: We included 219 subjects: 105 HP, 64 non-HP ILDs, and 50 healthy controls (25 non-exposed, 25 exposed). Cutoffs (mgA/L) were 33, 22, 34, 8, and 53, for *A. fumigatus*, *P. chrysogenum*, *C. herbarum*, *M. faeni*, and pooled sIgG levels, respectively. The 4-mold panel (any sIgG exceeding cutoff) showed 57.1% sensitivity and 78.1% specificity for HP diagnosis. The pooled sIgG test shifted post-test probability of HP diagnosis to 44% for

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a negative test and 84% for a positive test. A positive 4-mold panel (adjusted odds ratio, 4.03) was independently associated with a diagnosis of HP in multivariable regression analysis.
Conclusion: Mold-specific IgGs at the proposed cutoffs may assist in refining a multi-component diagnosis of HP; their performance characteristics do not justify their use in isolation. Larger, geographically diverse studies are needed.

Key words: hypersensitivity pneumonitis, interstitial lung disease, sarcoidosis, molds, exposure, biomarker, precipitins, idiopathic pulmonary fibrosis

Introduction

Hypersensitivity pneumonitis (HP) is an interstitial lung disease (ILD) characterized by T cell-mediated inflammation and granuloma formation in the lung interstitium (1). The inflammatory response is triggered by inhaled organic antigens in individuals who may be genetically predisposed (1, 2). If the exposure and inflammation are long-standing, lung fibrosis may ensue. HP is diagnosed by integrating clinical history of relevant exposures with characteristic chest computed tomography (CT) findings (3). In some cases, bronchoalveolar lavage fluid analysis and/or lung biopsy may be needed to establish a confident diagnosis (4). The antigens that provoke the immune response in HP include specific organic chemicals or proteins derived from microbes (bacteria or molds), plants, birds, or animals (5). Exposure is determined by detailed clinical history taking or targeted questionnaires (6-8). Another approach involves testing the serum for specific immunoglobulin G (sIgG) antibodies against protein components of commonly implicated antigens (9). The 2020 American Thoracic Society (ATS) guideline on HP diagnosis suggests measuring serum IgG against HP-related antigens in patients with newly diagnosed ILD, but with very low confidence in the estimated effects (2). This is because sIgG tests have suboptimal diagnostic performance, and their threshold values remain undefined. A separate ATS workshop report has described a conceptual framework in which exposure identification tools should not be used as standalone diagnostic tests. Rather, their impact on the *post hoc* probability of HP should be assessed based on likelihood ratios (9). Such an approach has not been systematically evaluated. In this study, we sought to establish cutoff values for serum sIgG against four molds—*Aspergillus fumigatus*, *Penicillium chrysogenum*, *Cladosporium herbarum*, and *Micropolyspora faeni*—to identify exposure to these antigens. We assessed the performance characteristics of these sIgGs (index tests) in differentiating HP from other ILDs using our proposed thresholds, with the multidisciplinary discussion (MDD) diagnosis as the reference standard. We then assessed the impact of positive or negative sIgG tests on the post-test probability of HP diagnosis.

Methods

We conducted a diagnostic test accuracy study using a case-control design at our institute's Chest Clinic. The study protocol was approved by the Institutional Ethics Committee. We report the study results according to the Standards for Reporting of Diagnostic Accuracy Studies (STARD) guidelines (10).

Study subjects

We screened consecutive ILD subjects recorded in our clinic database from December 2018 to July 2024 with available data on sIgG against mold antigens. The ILD subtypes were diagnosed based on current clinical guidelines (11-20). For HP cases diagnosed before 2020, we updated diagnoses as needed to align with the 2020 ATS guidelines (2). The multidisciplinary

team adjudicating the diagnosis integrated findings on clinical evaluation, exposure history, chest CT, bronchoalveolar lavage cell counts, and lung biopsy, when available. Index test results (i.e., serum sIgG values) were not disclosed to the MDD team.

Cases

We included subjects with a MDD diagnosis of HP as cases if they had available data on mold-specific serum IgG levels. We excluded subjects with (i) bird-related HP either diagnosed by a clear exposure history or by elevated pigeon-specific IgG levels in the serum; (ii) clear exposures to other non-mold-related antigens; or, (iii) co-existing mold-related lung disorders such as chronic pulmonary aspergillosis (CPA) or allergic bronchopulmonary aspergillosis (ABPA).

Diseased controls

We included subjects from our ILD database as diseased controls if they (i) were diagnosed to have a non-HP ILD on MDD; (ii) had available data on mold-specific serum IgG levels; and, (iii) did not have a co-existing mold-related lung disorder.

Healthy controls

We included healthy subjects from the accompanying attendants of non-ILD patients in our clinic or from hospital staff if they had no history suggesting chronic respiratory disease. Exposure history to potential HP-related antigens including molds (Table S1) was obtained, based on which they were designated as exposed or non-exposed healthy controls.

Study procedures

Data collection

We extracted the following data from our database: (1) demographic and clinical details (age, sex, smoking history, biomass smoke exposure, and comorbidities), (2) detailed exposure history relevant to HP collected via a questionnaire (Table S1) adapted from a previous study,(21) and (3) additional diagnostic information for subjects with HP and other ILDs including radiologic patterns and biopsy results.

Specific IgG determination

We measured specific IgG levels in the sera of subjects using an automated, cartridge-based, fluorescent enzyme immunoassay method (FEIA, Phadia 250, Phadia, Uppsala, Sweden). Crude antigen extracts from the conidia and mycelia of four prevalent molds—*A. fumigatus*, *P. chrysogenum*, *C. herbarum*, and *M. faeni*—available in separate cartridges, were used in the assay. A technician, blinded to other study details, performed the assay. Results were reported as milligrams of antigen-specific antibodies per liter (mgA/L).

Calculating sIgG cutoff values

We evaluated three approaches for determining sIgG cutoffs. First, we set the 95th percentile value from the non-exposed control group as the cutoff for each sIgG. Second, we calculated the mean and standard deviation (SD) of each sIgG and used mean + (2 x SD) in the non-exposed group as the cutoff. In the third method, we generated the receiver operating characteristic (ROC) curves for each sIgG level among HP cases and non-HP ILD controls, applying Youden's J-statistic to identify optimal thresholds. We calculated a 'pooled sIgG' level by adding all four serum sIgG values. We then calculated various cutoffs for the pooled sIgG level using the above methods. A separate '4-mold panel' was deemed positive, if a subject had elevated serum sIgG against any of the four molds.

Testing diagnostic performance at the proposed thresholds

We selected the 95th percentile method as the primary method for cutoff determination. Considering the MDD diagnosis as the reference standard, we assessed the following performance characteristics of the index tests (serum sIgG levels) for diagnosing HP: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), likelihood ratio for a positive result (LR+), and the likelihood ratio for a

negative test result (LR-). We also evaluated diagnostic performance of sIgG levels at the same thresholds for detecting exposure among healthy controls.

Calculating post-test probabilities

We calculated the post-test probabilities for positive and negative tests at different pre-test probabilities (prevalence) of HP among ILD subjects. Post-test probabilities were derived from pre-test probabilities using likelihood ratios (LRs) based on Bayes' theorem. The pre-test probability was first converted into pre-test odds using the formula: pre-test odds = pre-test probability / (1 – pre-test probability). Post-test odds were then calculated by multiplying the pre-test odds by the relevant likelihood ratio: post-test odds = pre-test odds × LR. For a positive test result, LR+ was used, whereas for a negative test result, LR– was applied. Finally, post-test odds were converted back to post-test probability using post-test probability = post-test odds / (1 + post-test odds).

Assessing characteristics associated with a diagnosis of HP

We explored whether the presence of a positive 4-mold panel (statistically termed the primary exposure) predicted a diagnosis of HP. The following covariates were selected in view of their clinical relevance to HP diagnosis: age, sex, smoke exposure, and an HP pattern (typical or compatible) on chest CT. We performed a binary logistic regression analysis to assess whether the primary exposure (positive 4-mold panel) or any of the covariates was independently associated with a diagnosis of HP amongst all subjects with ILDs.

Statistical analysis

We analyzed data using a commercial statistical package (IBM SPSS Statistics, version 22; IBM Corporation, Armonk, NY). Descriptive data are reported as numbers and percentages or mean ± SD, unless specified otherwise. We plotted receiver operating characteristic (ROC) curves for the serum sIgG levels against each of the four molds and the pooled sIgG levels in subjects with HP and non-HP ILDs. We calculated the area under the curve (AUC) and calculated the Youden's J-statistic (sensitivity + specificity - 1) to identify optimal thresholds. We calculated the odds ratios (ORs) with 95% confidence intervals (CI) for the primary exposure and covariates in the logistic regression analysis. Significance was set at a p-value <0.05 for all analyses.

Sample size calculation

We determined sample size based on the 2020 ATS guidelines estimates of sensitivity (85%) and specificity (70%) of HP-related serum sIgG tests.(2) With prevalence set at 0.6, precision at 0.11, and 95% confidence intervals, we needed 167 subjects with HP and non-HP ILDs.

Results

We screened 264 subjects (Figure 1) and included 219 (mean age, 51.4 years; 50.2% women). This included 105 cases with HP (Table 1), 64 diseased controls (non-HP ILDs) and 50 healthy controls (25 exposed, 25 non-exposed). The most prevalent non-HP diagnoses were sarcoidosis (n=21, 32.8%) and idiopathic pulmonary fibrosis (IPF, n=19, 29.7%). Hypertension (23.1%) and diabetes (17.2%) were the most frequent comorbidities among ILD subjects. Lung biopsies were performed in 67 subjects; 54 (80.6%) were diagnostic. Serum sIgG levels against *A.fumigatus*, *P.chrysogenum*, *C.herbarum* were elevated significantly in exposed vs. non-exposed controls (Table S2). The diagnostic thresholds (rounded to whole numbers) derived using the 95th percentile method for serum sIgG levels against *A.fumigatus*, *P.chrysogenum*, *C.herbarum*, *M.faeni*, and the pooled sIgG levels (Table 2) were 33 mgA/L, 22 mgA/L, 34 mgA/L, 8 mgA/L, and 87 mgA/L, respectively. Using these cutoffs, the 4-mold panel showed sensitivity and specificity of 44% and 84%, respectively, for identifying exposures amongst healthy controls (Table S3).

The respective performance characteristics for diagnosing HP among ILD subjects were 57.1% and 78.1% (Table 3). Among 59 HP subjects with a positive 4-mold panel, 43 (72.8%) showed reactivity

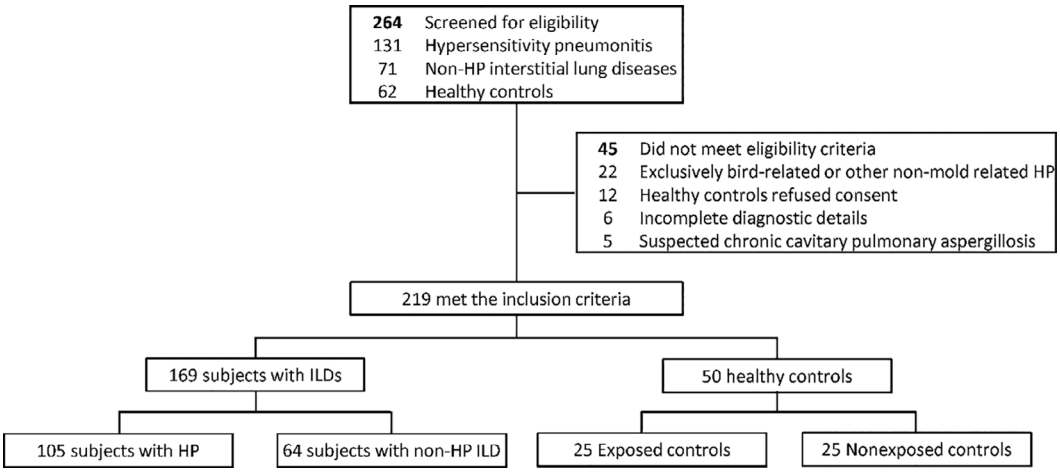

Figure 1. Participant flow. HP-hypersensitivity pneumonitis, ILD-interstitial lung disease.

Table 1. Baseline characteristics of subjects with hypersensitivity pneumonitis (HP) and non-HP interstitial lung diseases

Parameter	HP (N=105)	Non-HP ILD (N=64)	p-value
Age	57.0 ± 12.8	54.4 ± 11.6	0.17
Female sex	67 (63.8%)	18 (28.1%)	<0.001
Smoke exposure*	59 (56.2%)	35 (54.7%)	0.85
Comorbidity			
Diabetes mellitus	13 (12.4%)	16 (25%)	0.04
Hypertension	26 (24.8%)	13 (20.3%)	0.51
Coronary artery disease	5 (4.8%)	5 (7.8%)	0.51
Hypothyroidism	13 (12.4%)	7 (10.9%)	0.78
Others†	1 (1%)	2 (3.1%)	0.56
Exposures			
Farm	52 (49.5%)	35 (54.7%)	0.51
Cattle	49 (46.7%)	25 (39.1%)	0.33
Visible molds in house	45 (42.9%)	27 (42.2%)	0.93
Birds	33 (31.4%)	12 (18.8%)	0.07
Predominant pattern on chest CT			
Typical HP	50 (47.6%)	2 (3.1%)	
Compatible with HP	21 (20%)	1 (1.6%)	
Indeterminate	4 (3.8%)	9 (14.1%)	
Nonspecific interstitial pneumonia	13 (12.4%)	12 (18.8%)	
Definite usual interstitial pneumonia	0 (0%)	9 (14.1%)	
Probable usual interstitial pneumonia	1 (1%)	9 (14.1%)	
Perilymphatic nodules	0 (0%)	18 (28.1%)	
Upper lobe fibrosis	9 (8.6%)	2 (3.1%)	

Table 1 (*continued*)

Parameter	HP (N=105)	Non-HP ILD (N=64)	p-value
Diffuse fibrosis	5 (4.8%)	2 (3.1%)	
Airway-centered fibrosis	2 (1.9%)	0 (0%)	
Lung biopsy performed			
Performed	39 (37.1%)	28 (43.8%)	0.39
Diagnostic	28/39 (71.8%)	26/28 (92.9%)	0.03
Diagnoses (for non-HP ILDs)			
Sarcoidosis		21 (12.4%)	
Idiopathic pulmonary fibrosis		19 (11.2%)	
Unclassifiable ILD		11 (6.5%)	
Idiopathic NSIP		4 (2.4%)	
Undifferentiated CTD-ILD		4 (2.4%)	
Rheumatoid arthritis-associated-ILD		1 (0.6%)	
Myositis-associated ILD		1 (0.6%)	
Cryptogenic organizing pneumonia		1 (0.6%)	
Desquamative Interstitial Pneumonia		1 (0.6%)	
Smoking-related interstitial fibrosis		1 (0.6%)	

Abbreviations: CT-computed tomography, CTD-connective tissue disease, HP-hypersensitivity pneumonitis, ILD-interstitial lung disease, NSIP-nonspecific interstitial pneumonia. All values represent number (percentage) or mean ± standard deviation. *Smoke exposure included tobacco smoke (18 non-HP-ILD and 16 HP subjects) and biomass smoke (21 non-HP ILD and 44 HP subjects) exposures. †Other comorbidities included chronic liver disease, chronic kidney disease, and breast cancer in one subject each.

Table 2. Cutoff values for mold-specific immunoglobulin G levels in the serum using different methods

	Method of determining cutoffs			
Mold	**95th percentile**	**Mean + (2 x SD)**	**Youden's J statistic**	**Manufacturer's cutoff (for India)**
Aspergillus fumigatus, mgA/L	**33.0**	34.6	12.3	27.0
Penicillium chrysogenum, mgA/L	**21.8**	23.2	20.1	30.0
Cladosporium herbarum, mgA/L	**34.4**	33.3	14.3	30.0
Micropolyspora faeni, mgA/L	**8.4**	6.5	3.2	10.0
Pooled sIgG, mgA/L	**86.8**	89.1	53.3	-

Abbreviations: SD-standard deviation, sIgG-specific immunoglobulin G. The 95th percentile and the mean + (2 x SD) were applied in the non-exposed controls. The Youden's J statistics were derived from the receiver operating curve plotted between the cases with hypersensitivity pneumonitis (HP) and non-HP interstitial lung diseases.

to multiple molds. Cutoffs from the mean + 2xSD method matched 95th percentile values, without significantly enhancing diagnostic performance (data not shown). The ROC curve analysis between HP and non-HP ILD subjects demonstrated AUC values ranging from 0.647 to 0.716 for individual and pooled sIgG tests (Figure 2). The diagnostic sensitivity of the 4-mold panel (Table 3) was significantly ($p<0.001$) better when Youden's J-statistic cutoffs were used than with the 95th percentile cutoffs (83.8% vs. 57.1%), but specificity was substantially lower (43.8% vs. 78.1%; $p<0.001$).

At Youden's J-statistic cutoff of 53 mgA/L, the sensitivity and specificity of the pooled sIgG level were

Table 3. Performance characteristics of the mold-specific immunoglobulin G levels at the proposed cutoffs (using the 95th centile method unless otherwise specified) in differentiating hypersensitivity pneumonitis from other interstitial lung diseases (n=169)

Mold	Sensitivity	Specificity	PPV	NPV	LR+	LR-
Aspergillus fumigatus	42.9%	85.9%	83.3%	47.8%	3.05	0.66
Penicillium chrysogenum	48.6%	82.8%	82.3%	49.5%	2.83	0.62
Cladosporium herbarum	26.7%	90.6%	82.4%	43.0%	2.84	0.81
Micropolyspora faeni	19.8%	92.2%	81.0%	40.5%	2.52	0.87
4-mold panel	57.1%	78.1%	81.1%	52.6%	2.61	0.55
4-mold panel (Youden's cutoff)	83.8%	43.8%	71%	62.2%	1.49	0.37
Pooled sIgG	37.2%	90.2%	86.5%	46.0%	3.80	0.7
Pooled sIgG (Youden's cutoff)	58.1%	82.4%	84.7%	53.8%	3.29	0.51

Abbreviations: LR+: likelihood ratio for a positive test; LR-: likelihood ratio for a negative test; NPV: negative predictive value; PPV: positive predictive value; sIgG-specific immunoglobulin G. The 4-mold panel was considered positive if any of the four mold-specific IgG level exceeded the cutoff. The pooled sIgG value was derived for each subjects by adding all the sIgG level values.

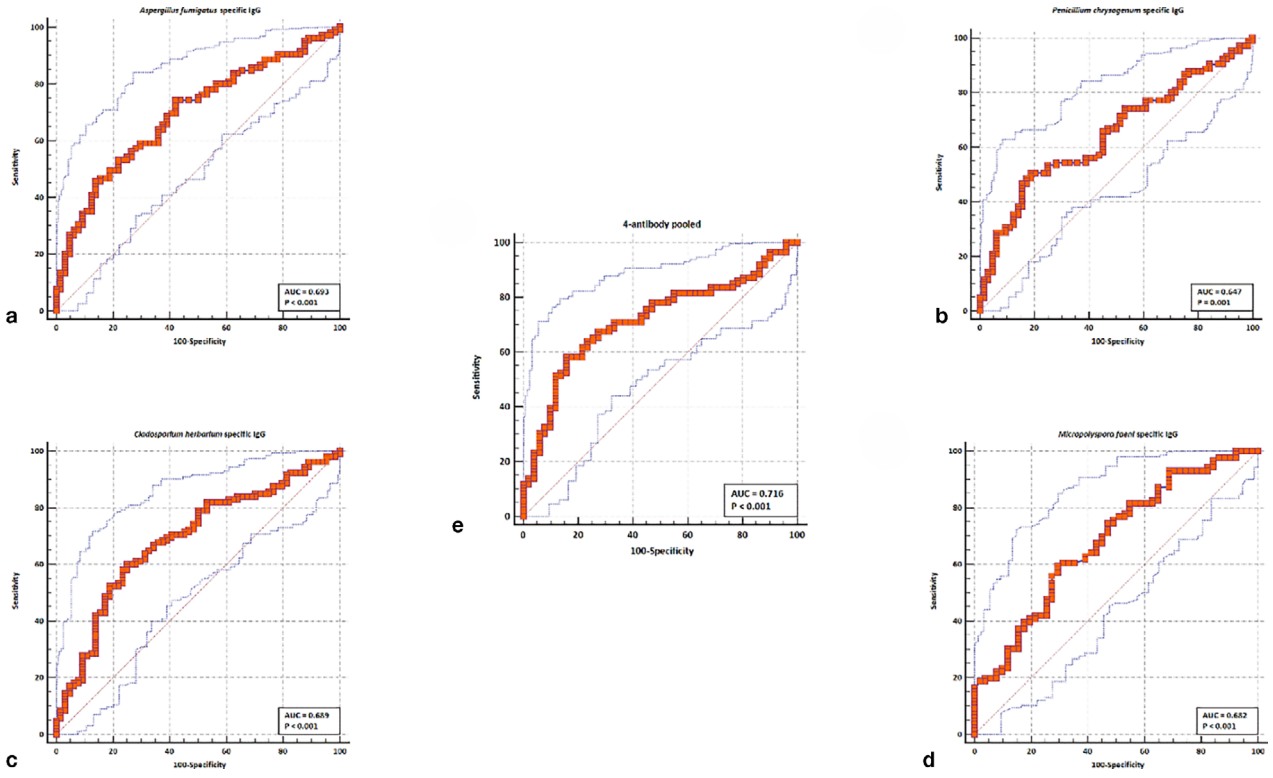

Figure 2. Receiver operating characteristics curves plotted for the specific IgG levels in the serum against each of the four molds and for the (4-antibody) pooled levels amongst subjects with hypersensitivity pneumonitis (HP, n=105) and non-HP interstitial lung diseases (n=64).

58.1% (vs. 37.2% with 95th percentile method cutoff; p<0.001) and 82.4% (vs. 90.2% with 95th percentile method cutoff; p=0.25), respectively. At this cutoff, the likelihood ratios for positive (LR+) and negative (LR-) tests were 3.29 and 0.51 (Table 3). The pre-test probability (i.e., the prevalence) of HP in the ILD cohort in this study is 61%. Based on the above likelihood ratios, a positive test for the pooled sIgG levels

would increase the probability of an HP diagnosis to a post-test probability of 84% (Table 4). A negative test would reduce the probability of HP from 61% to 44%.

We found that female sex, an HP pattern (typical or compatible) on chest CT, and a positive 4-mold panel (adjusted OR, 4.03; 95% CI, 1.57-10.34; p=0.004) were independently associated with a diagnosis of HP among all ILD subjects (Table 5). Smoke exposure had an inverse association with a diagnosis of HP.

Discussion

Our study establishes practical threshold values for sIgG levels against four prevalent molds for the diagnostic evaluation of HP. As diagnostic tools, the 4-mold panel (sensitivity, 57%, specificity, 78%) and the pooled sIgG level (sensitivity, 58%, specificity, 82%) demonstrated suboptimal performance. Nevertheless, these tests may provide useful clues regarding inciting antigens and relevant exposures, thereby

Table 4. Post-test probability of hypersensitivity pneumonitis in case of a negative or positive specific Immunoglobulin G test

Mold	LR+	LR-	Pre-test Probability	Post-test Probability (Positive Test)	Post-test Probability (Negative Test)
Aspergillus fumigatus	3.05	0.66	0.05	0.14	0.03
			0.1	0.25	0.07
			0.2	0.43	0.14
			0.3	0.57	0.22
			0.4	0.67	0.31
			0.5	0.75	0.40
			0.6	0.82	0.50
			0.7	0.88	0.61
			0.8	0.92	0.73
			0.9	0.96	0.86
Penicillium chrysogenum	2.83	0.62	0.05	0.13	0.03
			0.1	0.24	0.06
			0.2	0.41	0.13
			0.3	0.55	0.21
			0.4	0.65	0.29
			0.5	0.74	0.38
			0.6	0.81	0.48
			0.7	0.87	0.59
			0.8	0.92	0.71
			0.9	0.96	0.85
Cladosporium herbarum	2.84	0.81	0.05	0.13	0.04
			0.1	0.24	0.08
			0.2	0.42	0.17
			0.3	0.55	0.26
			0.4	0.65	0.35
			0.5	0.74	0.45
			0.6	0.81	0.55
			0.7	0.87	0.65

Mold	LR+	LR-	Pre-test Probability	Post-test Probability (Positive Test)	Post-test Probability (Negative Test)
			0.8	0.92	0.76
			0.9	0.96	0.88
Micropolyspora faeni	2.52	0.87	0.05	0.12	0.04
			0.1	0.22	0.09
			0.2	0.39	0.18
			0.3	0.52	0.27
			0.4	0.63	0.37
			0.5	0.72	0.47
			0.6	0.79	0.57
			0.7	0.85	0.67
			0.8	0.91	0.78
			0.9	0.96	0.89
4-mold panel	2.61	0.55	0.05	0.12	0.03
			0.1	0.22	0.06
			0.2	0.39	0.12
			0.3	0.53	0.19
			0.4	0.64	0.27
			0.5	0.72	0.35
			0.6	0.80	0.45
			0.7	0.86	0.56
			0.8	0.91	0.69
			0.9	0.96	0.83
4-mold panel (Youden's cutoff)	1.49	0.37	0.05	0.07	0.02
			0.1	0.14	0.04
			0.2	0.27	0.08
			0.3	0.39	0.14
			0.4	0.50	0.20
			0.5	0.60	0.27
			0.6	0.69	0.36
			0.7	0.78	0.46
			0.8	0.86	0.60
			0.9	0.93	0.77
Pooled sIgG	3.80	0.7	0.05	0.17	0.04
			0.1	0.30	0.07
			0.2	0.49	0.15
			0.3	0.62	0.23
			0.4	0.72	0.32
			0.5	0.79	0.41

Table 4 (*continued*)

Mold	LR+	LR-	Pre-test Probability	Post-test Probability (Positive Test)	Post-test Probability (Negative Test)
			0.6	0.85	0.51
			0.7	0.90	0.62
			0.8	0.94	0.74
			0.9	0.97	0.86
Pooled sIgG (Youden's cutoff)	3.29	0.51	0.05	0.15	0.03
			0.1	0.27	0.05
			0.2	0.45	0.11
			0.3	0.59	0.18
			0.4	0.69	0.25
			0.5	0.77	0.34
			0.6	0.83	0.43
			0.7	0.88	0.54
			0.8	0.93	0.67
			0.9	0.97	0.82

Abbreviations: LR+: likelihood ratio for a positive test; LR-: likelihood ratio for a negative test; sIgG-specific immunoglobulin G.

Table 5. Univariable and multivariable logistic regression analyses of factors predicting a diagnosis of hypersensitivity pneumonitis among subjects with interstitial lung disease (n=169)

Parameter	OR (95% CI)	p-value	Adjusted OR (95% CI)	p-value
Age	0.98 (0.96-1.01)	0.17	1 (0.96-1.03)	0.85
Female sex	4.51 (2.29-8.85)	<0.001	5.35 (2.08-13.77)	**0.001**
Smoke exposure	1.06 (0.57-1.99)	0.85	0.38 (0.15-0.97)	**0.04**
Typical or compatible with HP pattern on chest CT	42.46 (12.42-145.13)	<0.001	54.91 (13.94-216.31)	<**0.001**
Positive 4-mold panel*	4.76 (2.35-9.66)	<0.001	4.03 (1.57-10.34)	**0.004**

*Elevated specific IgG levels to any of the four mold antigens. *Abbreviations:* CI-confidence intervals, CT-computed tomography, HP- hypersensitivity pneumonitis, IgG-immunoglobulin G, OR-odds ratio

increasing the post-test probability of an HP diagnosis. To our knowledge, this is the first study to systematically establish cutoff values for HP diagnosis.

We selected four common molds linked to HP (5, 7). Previous studies have demonstrated serum IgG antibodies against various molds in individuals with mold dust exposures and in patients with mold-related HP (22-30), though their diagnostic utility remains uncertain in the absence of standardized thresholds (2). Most of these studies relied on in-house and laboratory-specific methods, lacking reproducibility or broad applicability. In contrast, we used an automated, cartridge-based, fluorescent enzyme immunoassay, with culture filtrate-derived antigens (9). This platform has shown reliability in other mold-related disorders such as ABPA and CPA (31, 32). We used the 95th percentile values among non-exposed healthy controls as the primary cutoffs to achieve high (≥95%) specificity for identifying mold exposure (22). This is important because sIgG assays are fundamentally tests of exposure rather than standalone diagnostic tests for HP. However, their clinical utility can be defined only after evaluating their performance characteristics in the diagnostic framework of HP. Given the limitations of

a percentile-based approach, we additionally evaluated ROC-derived thresholds. Using Youden's J statistic theoretically optimized the performance for individual molds; however, combining them in a 4-mold panel drastically reduced specificity from 78.1% (for the 95th percentile cutoffs) to 43.8% (for the Youden's cutoffs). Therefore, we did not adopt Youden-derived cutoffs for individual sIgGs. In contrast, the pooled sIgG using a Youden-derived cutoff of 53 mgA/L yielded a specificity (82.4%) similar to the specificity (90.2%) obtained with the respective 95th percentile method cutoff, but a much better sensitivity (58.1% vs. 37.2%).

Few studies have proposed thresholds for HP-related antibodies, and these appear inconsistent and often arbitrary (22, 23). A recent study by Demirkol, et al. provided reference ranges for certain mold-specific IgG tests used in Turkey without providing a scientific source for the suggested thresholds (23). Our cutoff for *A. fumigatus* (33 mgA/L) is lower than that described in the above study (46 mgA/L), but for *M. faeni*, it is higher (our study, 8 mgA/L; Demirkol study, 5 mgA/L). For *P. chrysogenum* , our cutoff matched the Demirkol study threshold (22 mgA/L). Another study among blood donors reported a 97.5th centile value of 136 mgA/L for sIgG levels against *A. fumigatus* (22).

The four-mold panel in our study lacked sufficient accuracy to confidently rule in or exclude HP, as it missed more than two-fifths of HP cases and yielded a false-positive rate >20%. The AUC values (0.647–0.716) suggest only fair diagnostic performance. However, likelihood ratios using the thresholds in our study support clinical utility within a probabilistic framework as suggested by the ATS workshop report on exposure assessment tools for HP (9). With a LR+ of 3.29, a positive test for the pooled sIgG levels increases the probability of an HP diagnosis from a pretest value of 61% (i.e., the HP prevalence in our study cohort) to a post-test probability of 84%. A negative test (LR-, 0.51), on the other hand, lowers it to 44%. Thus, a positive pooled test nearly doubles the possibility of an HP diagnosis compared to a negative test. Additionally, in HP cases confidently diagnosed on chest CT or histologic findings, the sIgG levels hint at the inciting antigen, potentially facilitating antigen avoidance, which is an important component of managing HP (9).

On multivariable analysis, a positive 4-mold panel was independently associated with a diagnosis of HP (adjusted OR, 4.03), in addition to female sex, an HP pattern on chest CT, and the absence of smoke exposure. This finding supports the relevance of the derived thresholds. The 2025 European Respiratory Society/ATS statement on updated classification of interstitial pneumonias has introduced the term bronchiolocentric interstitial pneumonia (BIP) to denote an airway-centered ILD (33). While HP, connective tissue disorders, aspiration, and inhalational injury may be the underlying causes of secondary BIP, the term "idiopathic BIP" has been proposed for use when no clear cause of this pattern can be identified. Identification of an exposure relevant to HP thus becomes more critical than ever to diagnose HP rather than idiopathic BIP. Refined cutoffs for various serum specific IgG antibodies against HP-relevant antigens will help in strengthening the exposure-identification part of the HP diagnostic pathway.

The average sIgG levels against *M. faeni* were not significantly different between the exposed and non-exposed healthy controls. With a sensitivity <20%, the *M. faeni* sIgG test offers little utility as a standalone test, but as part of the 4-mold panel and the pooled sIgG level, it might still add value. We observed that among 59 HP subjects with a positive 4-mold panel, 43 (73%) exceeded sIgG thresholds for multiple molds. This may either reflect true exposure to multiple molds triggering reactive antibodies, or cross-reactivity due to shared epitopes in crude mold-derived antigens. All the four molds grow outdoors in the soil and on decaying organic matter. Indoors, *Aspergillus spp* are known to grow in water and air-conditioning systems, *Cladosporium spp* in refrigerated food, and *Penicillium spp* in low ambient humidity, while all three can grow on water-damaged building materials (wood, insulation), textiles, and carpeting. Thus an individual may be exposed to several different molds at the same or different locations. Alternatively, there may be an actual exposure only to one mold, but the reactive antibodies might cross-react with the antigens derived from other molds, as the antigens used in the index tests are crude and have several epitopes. Our methods cannot distinguish between multiple exposures and cross-reactivity. The use of highly specific recombinant antigens could

clarify these factors and improve specificity by pinpointing the actual inciting mold antigens (34).

Our study is limited by a small sample size. Our entire cohort originates from a single center in the northern region of the country, with local antigen exposure patterns. Also, we have used a particular laboratory platform. This limits the generalizability of the proposed cutoffs to other geographic or ethnic populations, and other testing platforms. Even within the same region or across regions with similar exposure patterns, these findings should be confirmed in independent derivation and validation cohorts. We selected cases with a clear diagnosis of HP or a non-HP ILD, rather than including cases with a more circumspect diagnosis, as expected in real-life clinical practice. This may have led to an overestimation of sensitivity and specificity. We omitted measuring sIgG against other molds and thermophilic actinomycetes. Our methods for estimating thresholds are by no means perfect, but they offer a practical way to define cutoffs. With the given set of likelihood ratios, they can be used to alter the post-test probabilities in a multi-component diagnosis of HP.

In conclusion, as standalone tests, the sIgG levels against mold antigens have suboptimal performance in diagnosing mold-related HP. The cutoffs derived in this study, when used in clinical practice may help in refining a MDD diagnosis of HP. Larger multicenter studies across different geographic regions are needed to refine these cutoffs based on the corresponding population and region-specific exposures.

Authors Contribution: SD: Conceptualization, Guarantor; SD, RA, ISS, VM, KTP, ANA: Patient management, Data curation; SMR, MSM, MKS: Laboratory experiments, Data curation, Writing – original draft, Writing – review and editing; NG: Cytopathological examination, Data curation, Writing – original draft, Writing – review and editing; AB: Histopathological examination, Data curation, Writing – original draft, Writing – review and editing; MG: Radiologic assessments, Data curation, Writing – review and editing. SD takes responsibility for the integrity of the work from inception to published article.

Ethical Clearance: The Institutional Ethics Committee, PGIMER, Chandigarh cleared the study protocol (Pulm-471/2025).

Conflict of Interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

Declaration on the Use of AI: The software Grammarly was used for language editing.

Consent for Publication: Written informed consent was obtained from all study subjects for all prospective data collection, sample collection, and other study procedures.

Abbreviations

A. fumigatus: *Aspergillus fumigatus*
C. herbarum: *Cladosporium herbarum*
CT: computed tomography
HP: hypersensitivity pneumonitis
IgG: immunogolubulin G
ILD: interstitial lung disease
IPF: idiopathic pulmonary fibrosis
LR+: likelihood ratio for a positive test
LR-: likelihood ratio for a negative test
M. faeni: *Micropolyspora faeni*
P. chrysogenum: *Penicillium chrysogenum*
sIgG: specific immunoglobulin G

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Annex

Supplementary Tables

Table S1. Questionnaire for identifying exposures relevant to hypersensitivity pneumonitis modified from Barnes, et al.[1]

A.	Have you been exposed to any of the following at home, workplace, or any other place you regularly spend time?
1.	Farm dust, cattle work, or similar organic matter
2.	Visible or significant mold on the walls or other places
3.	Water damage, moisture or leaks
4.	Air coolers, air conditioners with water reservoir and visible molds
5.	Musty smells
6.	Wood work or wood dust
7.	Birds (pets, hobby, other), bird droppings, or feathers
8.	Bird feather products (comforters, pillows, furniture)
9.	Significant vapors or gases or fumes
10.	Anything else at home or workplace, which you think is important
B.	When were you exposed?
C.	How long have you been exposed?
D.	What was the daily average exposure duration?
E.	Is the exposure ongoing or cessation has occurred
F.	Did the symptoms get worse during periods you were exposed and got better when you were unexposed?

Reference: Barnes H, Morisset J, Molyneaux P, Westall G, Glaspole I, Collard HR. A Systematically Derived Exposure Assessment Instrument for Chronic Hypersensitivity Pneumonitis. *Chest.* 2020;157(6):1506-1512.

Table S2. Levels of specific IgG in the serum against four common molds implicated in causing hypersensitivity pneumonitis

Mold	Non-exposed controls (N=25)	Exposed controls (N=25)	p-value	Non-HP ILD (N=64)	HP (N=105)	p-value
Aspergillus fumigatus	15.6 ± 9.5	35.8 ± 32	0.004	15.7 ± 14.4	34.6 ± 48.5	<0.001
Penicillium chrysogenum	10.8 ± 6.2	22.8 ± 23.5	0.02	14.9 ± 14.5	28.2 ± 29.8	<0.001
Cladosporium herbarum	13.7 ± 9.8	34.5 ± 35.8	0.007	14.5 ± 15.3	26.4 ± 23.3	<0.001
*Micropolyspora faeni**	2.7 ± 1.9	3.6 ± 2	0.09	3.2 ± 2.6	8 ± 11.6	<0.001
Pooled sIgG	42.7 ± 23.2	96.8 ± 89.9	0.007	41.4 ± 36	85.7 ± 70.1	<0.001

Abbreviations: HP-hypersensitivity pneumonitis, ILD-interstitial lung disease, sIgG-specific immunoglobulin G. All values represent mean ± standard deviation. *The specific IgG levels in the serum against *Micropolyspora faeni* and for the pooled levels were available for 86 HP cases and 51 non-HP ILD controls.

Table S3. Sensitivity and specificity of mold-specific immunoglobulin G levels at the proposed cutoffs (using the 95th percentile method) in identifying mold exposures amongst healthy controls (n=50)

	Sensitivity	**Specificity**
Aspergillus fumigatus	44%	96%
Penicillium chrysogenum	36%	96%
Cladosporium herbarum	32%	96%
Micropolyspora faeni	4%	96%
4-mold panel	44%	84%
Pooled sIgG	40%	96%

The 4-mold panel was considered positive if any of the four mold-specific IgG level exceeded the cutoff. The pooled sIgG level was derived for each subject by adding all 4 sIgG levels.