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Aspergillus sensitisation in bidi smokers with and without chronic obstructive lung disease

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Email: agarwal.ritesh@outlook.in**Summary**

Recent studies have described fungal sensitisation in patients with chronic obstructive pulmonary disease (COPD). However, no study has evaluated fungal sensitisation specifically in bidi smokers. Herein, we evaluate the prevalence of *Aspergillus* sensitisation in bidi smokers. Bidi smokers with and without COPD underwent chest radiography, spirometry, *Aspergillus* skin test, *A. fumigatus* precipitins, *A. fumigatus*-specific IgE and total IgE. *Aspergillus* sensitisation was defined as the presence of either immediate cutaneous hyperreactivity to *Aspergillus* antigen or raised *A. fumigatus*-specific IgE level >0.35 kUA/L. Bidis were obtained from a subset of cases and controls and cultured for the growth of any fungus. Two hundred subjects with COPD and 72 chronic bidi smokers without COPD were included in the study (258 men; mean age, 56.8 years). *Aspergillus* sensitisation was found to be significantly higher in bidi smokers without COPD (27.8%) compared to the COPD cases (16%). Age, COPD, lung function, severity of smoking and current smoking were not associated with *Aspergillus* sensitisation, on a multivariate logistic regression analysis. We found a high prevalence of *Aspergillus* sensitisation in bidi-smoking subjects. More studies are required to confirm the findings of our study.

KEYWORDS*Aspergillus*, bidi, chronic obstructive pulmonary disease, fungal sensitisation, obstructive airway disease, tobacco smoking

1 | INTRODUCTION

Aspergillus fumigatus can cause a variety of pulmonary disorders depending on the immune status of the host. Traditionally, *Aspergillus*-related lung diseases are classified into three different forms as saprophytic (aspergilloma), allergic (asthma and allergic bronchopulmonary aspergillosis) and invasive (acute, subacute and chronic pulmonary aspergillosis).¹ Allergic bronchopulmonary aspergillosis (ABPA) is a complex pulmonary disorder caused by immune reactions mounted against *A. fumigatus* colonising the tracheobronchial tree.² The disorder usually complicates the course of patients with asthma and cystic fibrosis, both of which are airway disorders characterised by mucus hypersecretion. In addition, patients with cystic fibrosis also have defects in mucociliary clearance. These abnormalities allow persistence of *A. fumigatus* in the airways allowing

them to germinate into hyphae.³ In susceptible individuals, colonisation is followed by *Aspergillus* sensitisation, which is defined as immune-mediated response to *A. fumigatus*, without evidence of inflammation or tissue damage.⁴ *Aspergillus* sensitisation is currently believed to be the first step in the pathogenesis of ABPA.⁵ Recently, *Aspergillus* sensitisation (and ABPA) has also been described in several pulmonary disorders other than asthma and cystic fibrosis.^{6–9}

Chronic obstructive pulmonary disease (COPD) is another airway disorder characterised by progressive, poorly reversible airflow limitation secondary to tobacco smoke and/or other noxious inhalational exposures.¹⁰ Although asthma and COPD are distinct clinical entities, they share certain features including the presence of airway inflammation, airflow obstruction and mucus hypersecretion. Akin to asthma, airways of COPD patients may also get colonised with *A. fumigatus*. One study found the growth of *A. fumigatus* in sputum of 37% of the

COPD subjects at baseline.¹¹ Recent studies have also described the presence of *Aspergillus* sensitisation in COPD patients.¹¹⁻¹³ We had previously described the presence of *Aspergillus* sensitisation in 8.5% of subjects with COPD.¹² However, all cases in this study were bidi-smoking COPD patients, whereas the controls were non-smoking healthy volunteers. Whether bidi smoking could directly contribute to fungal sensitisation secondary to fungal contamination, could not be ascertained from this study. Bidi is a form of smoking tobacco made of 0.2-0.5 g of raw, dried and crushed tobacco flakes rolled by hand in a leaf of the Indian tendu (*Diospyros melanoxylon*) plant and held together by a cotton thread. In this study, we evaluate the prevalence of *Aspergillus* sensitisation in bidi smokers with and without COPD.

2 | MATERIAL AND METHODS

This was a prospective case-control study conducted between January 2013 and December 2014 in the Chest Clinic of this Institute. The study protocol was approved by the Institute Ethics Committee and a written informed consent was obtained from all the study participants.

The cases were consecutive subjects with COPD related to bidi smoking, whereas the controls were otherwise healthy volunteers who were smoking bidis. COPD was diagnosed when the subject met all the following criteria: (i) age more than 40 years; (ii) symptoms of progressive breathlessness and cough with minimal mucoid expectoration for at least three months in a year for two successive years; (iii) history of bidi smoking defined by smoking index greater than 100 (smoking index is the number of bidis smoked per day multiplied by the number of years) and, (iv) evidence of obstructive defect on spirometry defined as a ratio of forced expiratory volume in the first second (FEV1) to forced vital capacity (FVC) less than the lower limit of normal with no bronchodilator reversibility (increment of FEV1 and/or FVC <12% and 200 mL after 400 µg of inhaled salbutamol).¹⁴ The severity of COPD was further classified based on postbronchodilator FEV1 values as mild (>80%), moderate (50-80%), severe (30-50%) and very severe (<30%). Subjects with any of the following were excluded: (i) age >75 years; (ii) any form of tobacco smoking other than bidi; (iii) subjects receiving systemic glucocorticoids (≥10 mg/day for more than seven days of prednisolone or equivalent in the preceding one month); (iv) patients on any other form of immunosuppressive therapy; (v) other comorbid illnesses with potential for immunosuppression including uncontrolled diabetes mellitus, chronic renal failure, chronic liver disease, active malignancy and others and (vi) failure to provide informed consent.

The controls were otherwise asymptomatic healthy volunteers who met all the following criteria: (i) age more than 40 years; (ii) bidi smokers with smoking index >100; (iii) normal physical examination and chest radiograph and, (iv) no evidence of obstructive pattern on spirometry (FEV1/FVC more than the lower limit of normal and FEV1 >80% predicted). The controls were recruited from the attendants accompanying the patients in the outpatient departments of our Institute. Subjects with any of the following were excluded: (i) age >75 years; (ii) any form of tobacco smoking other than bidi and, (iii) failure to provide informed consent.

All cases and controls meeting the inclusion criteria were subjected to detailed clinical history and physical examination. Furthermore, all

patients underwent the following investigations: *Aspergillus* skin test, *A. fumigatus* precipitins, *A. fumigatus*-specific IgE and total IgE. Bidis were also obtained from a subset of cases and controls and cultured for the growth of any fungus.

2.1 | *Aspergillus* skin test

Aspergillus skin test was carried out by injecting 0.2 mL of 100 protein nitrogen units (PNU)/mL of the *Aspergillus* antigen (1 PNU=0.00001 mg/mL) intradermally in the forearm, as previously described.¹⁵

2.2 | *Aspergillus fumigatus*-specific IgE levels

Aspergillus fumigatus-specific IgE was assayed using the M3 ImmunoCap with the commercial ImmunoCAP system (Phadia 100; Phadia, Uppsala, Sweden) that utilises the automated fluorescent enzyme immunoassay technology. The antigen used is a crude extract derived from the conidia and mycelia of *A. fumigatus*. The measuring range of *A. fumigatus*-specific IgE for an undiluted sample is 0.1-100 kUA/L.

2.3 | Total IgE levels

The serum total IgE was also estimated using the automated fluorescent enzyme immunoassay (Phadia 100; Phadia) using the ImmunoCAP technology.

2.4 | *Aspergillus fumigatus* precipitins

Aspergillus fumigatus precipitins were detected using the Ouchterlony gel double-diffusion technique as per the method described by Longbottom and Pepys.¹⁶

2.5 | Spirometry

Spirometry was performed on a dry rolling seal spirometer (Spiro RS-232; P.K. Morgan Limited, Kent, UK). The lung function values including FEV1, FVC and FEV1/FVC ratio were measured before and after administration of 400 µg of inhaled salbutamol.¹⁷ The highest values from among the three technically acceptable and reproducible manoeuvres were expressed at body temperature and pressure saturated with water vapour. Age, gender, height and spirometric data were recorded for all patients using software previously developed at our laboratory.¹⁸ Predicted values for FVC, FEV1 and FEV1/FVC ratio were generated using previously defined prediction equations.¹⁹

2.6 | Culture of bidi samples

Bidi samples from some of the cases and controls were collected and cultured for growth of any fungus. Bidis were surface disinfected in 0.5% sodium hypochlorite for one minute and rinsed in sterile distilled water for five times. The bidi was then cut into small pieces and crushed using a sterile mortar to get a uniform suspension of tobacco and its flakes. The suspension was spread plated on potato dextrose

TABLE 1 Baseline characteristics of the study population

	Bidi smokers with COPD (n=200)	Bidi smokers without COPD (n=72)	P value
Age (y)	58.5 (57.4-59.8)	51.9 (49.6-54.3)	.0001
Male gender	186 (93, 88.6-95.8)	72 (100, 94.9-100)	.02
Rural residence	152 (76, 69.6-81.4)	59 (81.9, 71.5-89.1)	.29
Body mass index (kg/m ²)	22.1 (21.5-22.8)	23.3 (22.3-24.2)	.07
Respiratory symptoms			
Duration of cough (years)	2.6 (2.2-2.9)	–	
Duration of wheeze (years)	0.36 (0.21-0.50)	–	
Duration of expectoration (years)	1.3 (1.0-1.6)	–	
Duration of dyspnoea (years)	3.3 (2.9-3.6)	–	
Smoking Index	521.7 (484.4-558.9)	445.6 (394.3-496.9)	.03
Current smoking	68 (34, 27.8-40.8)	43 (59.7, 48.2-70.3)	.0001
Spirometry			
FEV1 (% predicted)	52.0 (49.5-54.5)	99.2 (96.5-101.9)	.0001
FVC (% predicted)	72.6 (70.2-75.0)	98.7 (95.2-102.2)	.0001
Severity of obstruction			
Mild obstruction	10 (5, 2.7-8.9)	–	
Moderate obstruction	100 (50, 43.1-56.9)	–	
Severe obstruction	69 (34.5, 28.3-41.3)	–	
Very severe obstruction	21 (10.5, 6.9-15.5)	–	

All the values are expressed as mean (95% confidence intervals) or number (percentage, 95% confidence intervals) unless otherwise stated.

COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in the first second; FVC, forced vital capacity.

agar and Czapek Dox agar with streptomycin 0.03 g/L added to the media. The plates were incubated at 25 and 37°C, the growth was observed after 3 days of incubation. The fungi were identified based on phenotypic characteristics.

For the purpose of the study, *Aspergillus* sensitisation was defined as either the presence of immediate cutaneous hyperreactivity to the *Aspergillus* antigen or raised *A. fumigatus*-specific IgE level >0.35 kUA/L.

2.7 | Statistical analysis

Statistical analysis was performed using the statistical package spss for MS Windows (version 22; IBM SPSS Inc., Chicago, IL, USA). Data are presented in a descriptive fashion as mean with 95% confidence intervals (CI) or number (percentage with 95% CI). Differences between categorical variables and continuous variables were analysed using chi-square test and Mann-Whitney U test respectively. A multivariate logistic regression analysis was performed to determine the factors associated with *Aspergillus* sensitisation. Statistical significance was assumed at *P* value <.05.

3 | RESULTS

During the study period, 200 subjects with COPD and 72 otherwise healthy chronic bidi smokers without COPD were enrolled as

controls. The study comprised of 258 (94.9%) men with a mean (SD) age of 56.8 (9.4) years. Majority of the study population resided in rural areas. The baseline characteristics are shown in Table 1. The control subjects were relatively younger and all were men. The smoking index was also significantly lower in the control group (Table 1). The mean FEV1 of the subjects with COPD was 52% of the predicted value; 45% of the COPD cases had FEV1 below 50% predicted.

Aspergillus sensitisation was identified to be significantly higher (Table 2) in controls (n=20, 27.8%) compared to the COPD cases (n=32, 16%). *Aspergillus fumigatus*-specific IgE was positive in 13% cases and 25% controls, whereas *Aspergillus* skin test was positive in 9.5% cases and 19.4% controls (Figure 1). The concordance between *Aspergillus* skin test and *A. fumigatus*-specific IgE for the classification of *Aspergillus* sensitisation was moderate (κ statistic=.74). The frequency of total IgE value >1000 IU/mL and *A. fumigatus* precipitins were similar in the two groups (Table 2). The frequency of subjects with *Aspergillus* sensitisation and total IgE levels >1000 IU/mL was significantly lower when compared with the prevalence of *Aspergillus* sensitisation (7.8% vs 19.1%); however, this proportion was not different in cases and controls (Table 2). *Aspergillus* sensitisation was higher in subjects with total IgE >1000 IU/mL (cases: 12/28 [42.9%]; controls: 9/11 [81.8%]) compared to those with total IgE <1000 IU/mL (cases: 20/172 [11.6%]; controls: 11/61 [18%]). In patients with COPD, the lung function (FEV1, % predicted) was not significantly

TABLE 2 Immunological characteristics in bidi smokers with and without chronic obstructive pulmonary disease (COPD)

	Bidi smokers with COPD (n=200)	Bidi smokers without COPD (n=72)	P value
Type 1 <i>Aspergillus</i> skin test	19 (9.5, 6.2-14.4)	14 (19.4, 11.9-30.0)	.02
Af-specific IgE >0.35 kUA/L	26 (13, 9.0-18.4)	18 (25, 16.4-36.1)	.01
Type 1 cutaneous hypersensitivity or Af IgE >0.35 kUA/L	32 (16, 11.6-21.7)	20 (27.8, 18.8-39.1)	.03
Af-specific IgE levels, kUA/L	0.33 (0.12-0.54)	0.142 (0.11-0.73)	.20
Total IgE levels, IU/mL	568 (450-685)	786 (324-1248)	.66
Total IgE >1000 IU/mL	28 (14, 9.9-19.5)	11 (15.3, 8.8-25.3)	.07
Type 1 cutaneous hypersensitivity or Af IgE >0.35 kUA/L and total IgE >1000 IU/mL	12 (6, 3.1-10.3)	9 (12.5, 5.9-22.4)	.08
<i>Aspergillus</i> precipitins positivity	6 (3, 1.4-6.4)	4 (5.6, 2.2-13.4)	.32

All the values are expressed as mean (95% confidence intervals) or number (percentage, 95% confidence intervals) unless otherwise stated.

Af, *Aspergillus fumigatus*; kUA, kilo unit of antibody.

different in those with (mean [SD], 48.1 [15.8]) and without (mean [SD], 52.8 [18.3]) *Aspergillus* sensitisation ($P=.18$).

A multivariate logistic regression analysis was performed to determine the risk factors for *Aspergillus* sensitisation. We found no relationship between *Aspergillus* sensitisation and age, COPD, smoking index, current smoking and lung function (FEV1) on spirometry (Table 3).

Seventy-two bidi samples were cultured and studied on the fourth day of the growth. Of these, 11 cultured plates showed growth of fungus identified phenotypically as *Aspergillus niger* (compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads). Rest of the plates showed contamination and growth of saprophytic fungi.

4 | DISCUSSION

The results of this study suggest a high prevalence (16%) of *Aspergillus* sensitisation in bidi-smoking patients with COPD. We found an even higher prevalence of *Aspergillus* sensitisation in bidi smokers without COPD (28%) that was significantly higher than the bidi-smoking COPD population.

In an earlier study, we found the prevalence of *Aspergillus* sensitisation of about 8.5% in patients with COPD using *Aspergillus* skin test.¹² Subsequently, the prevalence of *Aspergillus* sensitisation was found ranging from 13 to 15% in two different studies using *A. fumigatus*-specific IgE.^{11,13} Recently, Agarwal *et al.* reported sensitisation to non-*Aspergillus* fungi in three of the 55 (5.5%) COPD subjects studied.²⁰ The results of these studies suggest that fungal sensitisation does occur in COPD and the differences in prevalence could be related to the method used for the diagnosis of fungal sensitisation (skin testing or fungal-specific IgE or both). We have previously demonstrated that *A. fumigatus*-specific IgE is more sensitive than *Aspergillus* skin test for the diagnosis of ABPA.²¹ This was also seen in this study where the prevalence of *Aspergillus* sensitisation was 9% and 13% using *Aspergillus* skin test and *A. fumigatus*-specific IgE respectively.

Bidis are a crude form of smoking tobacco widely available in India, and bidi smoking is the most prevalent form of tobacco smoking in India.^{14,22} Not only are the concentrations of nicotine and tar higher in bidi smoke than in cigarette smoke, but also a higher nicotine and

tar inhalation occurs during bidi smoking due to poor combustibility of the bidi leaf, which requires more frequent and deeper puffs for maintaining the bidi alight.²³ Also, bidi manufacturing does not require sophisticated technology and this process is often outsourced to smaller home-based factories.²⁴ Thus, it is possible that bidis may be contaminated by the presence of fungus. In fact, home-made marijuana has been shown to be contaminated with *Aspergillus* spores,²⁵ and has been linked to causing chronic pulmonary aspergillosis.²⁶

Previously, it has been demonstrated that tobacco could be adulterated by fungus especially *A. fumigatus*.²⁷ Recently, in a study that explored the bacterial metagenome, it was shown that cigarettes contained a wide range of potentially pathogenic microbes.²⁸ Moreover, ergosterol, a marker of fungal biomass, has been demonstrated both in tobacco as well as tobacco smoke.²⁹⁻³¹ This suggests that tobacco smoke is a bio-aerosol containing potentially harmful fungal component. Even in our study, 11 of the 72 bidi samples grew *A. niger*. However, on a multivariate analysis, current smoking was not independently associated with *Aspergillus* sensitisation. This suggests that bidi smoking is a probable risk factor for *Aspergillus* sensitisation but there are likely other causes that were not explored in this study.

An intriguing aspect of the study was that *Aspergillus* sensitisation was lower in bidi smoking COPD subjects compared to those without. The exact reason for this finding remains unclear. Several reasons may be proposed for this finding. It is likely that patients with COPD decrease or quit smoking as they seek medical help for their symptoms, which decreases the exposure to the fungal antigens. Even in this study, the proportion of current smokers was significantly lower in the COPD. Another reason could be that due to dyspnoea, patients decrease their outdoor activities that further reduce the exposure to fungal spores.

Recently, it has been shown that there exists an "allergic phenotype" of COPD and these patients have frequent and severe respiratory symptoms and more exacerbations.³² The total IgE has been proposed as a holistic marker of the underlying allergic condition. In fact, a previous study found *Aspergillus* sensitisation only in subjects with raised total IgE.¹³ Although, we also observed fungal sensitisation in those without raised total IgE (<1000 IU/mL), the prevalence of *Aspergillus* sensitisation was significantly higher in those with raised total IgE, similar to the previous study.

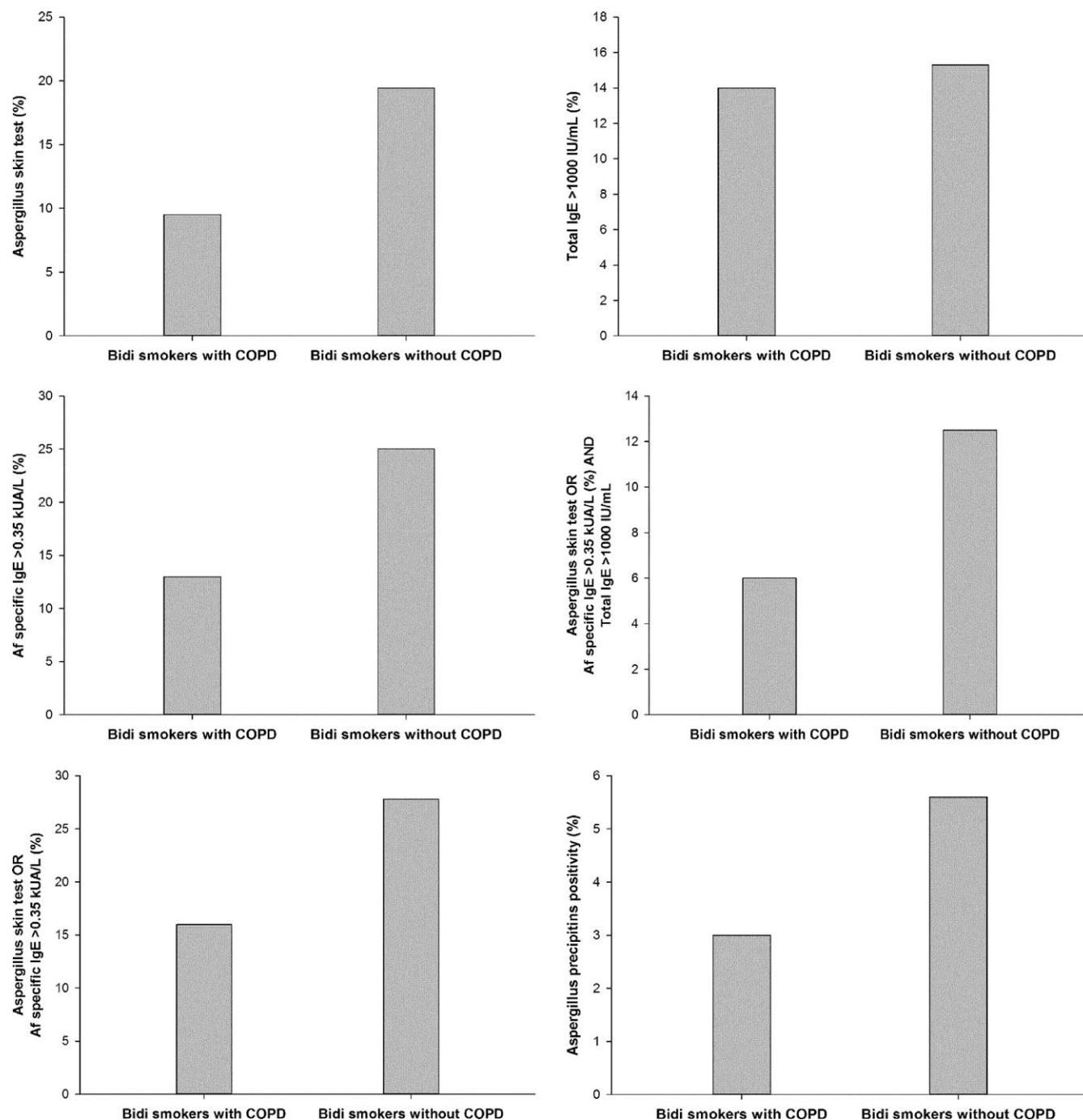


FIGURE 1 Prevalence of *Aspergillus* skin test positivity, *A. fumigatus*-specific IgE >0.35 kUA/L, *Aspergillus* skin test positivity or *A. fumigatus*-specific IgE >0.35 kUA/L, total IgE >1000 IU/mL, *Aspergillus* skin test positivity or *A. fumigatus*-specific IgE >0.35 kUA/L and total IgE >1000 IU/mL, and positive *Aspergillus* precipitins in bidi smokers with and without chronic obstructive pulmonary disease (COPD)

Finally, our study has several limitations. As this was a pilot study, no sample size was calculated. Also, the study is a single centre study conducted in a tertiary care referral hospital. Thus, the prevalence of *Aspergillus* sensitisation observed in the COPD patients does not reflect the true prevalence of *Aspergillus* sensitisation in COPD patients in the community. Although there was no difference in lung function in patients with COPD with and without *Aspergillus* sensitisation, we did not follow up the patients over time. Thus, the clinical significance of *Aspergillus* sensitisation in COPD remains unclear. Furthermore, we

investigated only for sensitisation against *A. fumigatus* without looking for other prevalent aeroallergens and moulds. We also did not analyse any fungal biomarker in bidi smoke which would have further strengthened the hypothesis of bidi smoking directly contributing to fungal sensitisation. It is possible that bidi smoking only alters the airway milieu, which predisposes to fungal colonisation and subsequent sensitisation. Future studies should evaluate fungal sensitisation in three groups namely cigarette and bidi smokers and normal healthy volunteers with matched cases and controls for smoking index, gender

TABLE 3 Multivariate logistic regression analysis of predictors for *Aspergillus* sensitisation in bidi smokers

	Adjusted odds ratio (95% confidence intervals)	P value
Age (y)	0.99 (0.95-1.02)	.52
COPD	0.32 (0.09-1.05)	.06
Smoking index	0.99 (0.99-1.00)	.46
Current smoking	0.97 (0.51-1.86)	.82
FEV1 (% predicted)	0.99 (0.97-1.01)	.27

COPD, chronic obstructive pulmonary disease; FEV1, force expiratory volume in the first second.

and age. Finally, due to the small sample size, our findings require confirmation in a larger sample.

In conclusion, the results of this study suggest a high prevalence of *Aspergillus* sensitisation in bidi-smoking subjects with and without COPD. More studies are required to confirm our findings and ascertain the clinical significance of *Aspergillus* sensitisation in bidi smokers.

CONFLICT OF INTEREST

The authors declare that they have no relevant conflict of interest.

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