



# Occurrence of Cystic Fibrosis Transmembrane Conductance Regulator Gene Mutations in Patients with Allergic Bronchopulmonary Aspergillosis Complicating Asthma

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## Abstract

**Background** Whether cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations contribute to the high prevalence of allergic bronchopulmonary aspergillosis (ABPA) in India remains unknown. We aimed to evaluate the occurrence of CFTR mutations in subjects with ABPA complicating asthma.

**Methods** We sequenced the CFTR gene using genomic DNA from blood on the Illumina Next-Seq500 platform. Before undertaking zygosity analysis by genome analysis toolkit, the known or novel single nucleotide polymorphisms (SNPs) and indels were called. For rigorous analysis, we included only

high-quality SNPs (scores > 500) and coverage ranging from 30 × 150x.

**Results** We included 18, 12, and eight adult participants of ABPA, asthma, and healthy controls, respectively. The frequency of SNPs was higher in asthmatic subjects than ABPA or healthy controls, albeit not statistically significant (9/12 [75%] vs. 11/18 [61.1%] vs. 3/8 [37.5%],  $p = 0.24$ ). Of the 38 subjects, 23 yielded 50 variants (healthy controls [n = 5], ABPA [n = 22], asthma [n = 23]) corresponding to six SNPs not previously linked with ABPA. Of these, four SNPs (rs213950, rs200735475, rs1800113, and rs1800136) were catalogued in the NCBI database. We identified two novel SNPs (chr7:117250703, chr7:117282655) in four (ABPA [n = 1], asthma [n = 3]) subjects without corresponding reference SNP. Most SNPs (85.5%) were heterozygous. The frequency of SNPs was higher in ABPA subjects with high-attenuation mucus (52.2%) and bronchiectasis (39.1%) than serological ABPA (8.7%).

**Conclusions** Our study suggests the role of CFTR mutations in the pathogenesis of ABPA. The SNPs in the CFTR gene may contribute to disease severity in ABPA. Larger studies are required to confirm our findings.

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## Introduction

The pathogenesis of allergic bronchopulmonary aspergillosis (ABPA), a complex immunological disorder caused by *Aspergillus fumigatus* in asthmatic patients, remains unclear [1–3]. Despite dwelling in the same surroundings, not all asthmatic patients develop ABPA. Thus, environmental factors are not believed as the primary pathogenetic factors in ABPA [4]. The lung involvement in ABPA shares several features with cystic fibrosis (CF) lung disease, including wheezing, mucus plugging, and proximal bronchiectasis [5]. The cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations affect not only the epithelial cells but also the innate and adaptive immune responses [6, 7]. The CFTR is probably important in influencing T cell differentiation and the effectiveness of immune responses against pathogenic organisms. Notably, CFTR is necessary for maintaining the Th1/Th2 balance [8–10]. Allard et al. showed that CFTR-deficient mice develop an enhanced Th2 inflammatory response to *A. fumigatus*, indicated by elevated lung IL-4 and serum IgE [11]. ABPA is frequently (7–11%) encountered in CF [12] and has been suggested as a CFTR-related disease [13, 14].

ABPA is also highly prevalent in the Indian subcontinent, particularly North India [15]. The prevalence of ABPA in chest clinics in India is estimated to be double that of other countries [16]. The global burden of ABPA was estimated at five million cases, [17] with 1.4 million cases in India alone [18]. Interestingly, in a study from India, Shah et al. documented familial occurrence in 4.9% of the 164 ABPA patients raising the possibility of an underlying genetic disorder [19]. CFTR mutations may underlie some patients with ABPA complicating asthma [20]. We hypothesized that the heightened prevalence of ABPA in asthma could be partly contributed by single nucleotide polymorphisms (SNPs) in the CFTR gene of our population. In the present study, we employed next-generation sequencing (NGS) to evaluate the occurrence of CFTR gene variations in subjects with ABPA complicating asthma.

## Material and Methods

We conducted a prospective case-control study between January 2017 and June 2018 in the Chest Clinic of our institute. The Institute Ethics Committee approved the study protocol. We obtained written informed consent from all the study subjects.

### Study Population

#### *Inclusion Criteria*

We included individuals (age > 18 years) with ABPA complicating asthma, asthma without ABPA, and healthy controls, as defined below.

#### *Asthma with ABPA*

We diagnosed ABPA according to the International Society for Human and Animal Mycology (ISHAM)-ABPA working group criteria. The subjects should meet the following: *A. fumigatus*-specific IgE  $\geq$  0.35 kUA/L and total IgE  $\geq$  1,000 IU/mL; and, at least two of the following: *A. fumigatus*-specific IgG  $\geq$  27 mgA/L, peripheral blood eosinophil count  $\geq$  500 cells/ $\mu$ L, and consistent imaging [21]. We further classified the subjects as serological ABPA (ABPA-S, those without bronchiectasis), ABPA-B (ABPA with bronchiectasis), and ABPA-HAM (ABPA with high-attenuation mucus) [21].

#### *Asthma without ABPA*

We included subjects with asthma without ABPA as disease controls. We defined asthma according to the Indian guidelines for bronchial asthma [22]. Briefly, the subject should not meet the criteria for ABPA and must have any three of the following features: (1) History of recurrent episodes of cough, dyspnea, or chest tightness; (2) Wheeze on chest auscultation; (3) Evidence of obstruction on spirometry; and (4) Documented clinical or spirometric response to asthma therapy after 4–8 weeks of treatment. We further categorized the asthmatic subjects into asthma with aspergillus sensitization (SA) and asthma without aspergillus sensitization (UA) based on *A. fumigatus*-specific IgE (cut-off, 0.35 kUA/L).

### Healthy Controls

We included healthy volunteers who were asymptomatic without any history of pulmonary or systemic disease. The healthy controls were randomly recruited from the hospital staff. We ensured that the controls were not family members of asthma or ABPA subjects. All the healthy controls underwent a chest radiograph but did not undergo any immunological testing for ABPA.

### Exclusion Criteria

We excluded subjects with pre-existing cystic fibrosis, pre-existing immunosuppressive illnesses, and failure to provide informed consent.

### Study Procedure

We assayed serum total IgE, *A. fumigatus*-specific IgE, and IgG using the commercial fluorescent enzyme immunoassay (Phadia 100, Thermo Fisher Scientific, Uppsala, Sweden) [23–25]. We used a multiple-detector scanner (LightSpeed plus, GE medical systems, Slough UK) for performing CT of the chest. We performed sweat chloride analysis in all the study participants using the pilocarpine iontophoresis method. Sweat chloride values  $\leq 29$ , 30–59, and  $\geq 60$  mmol/L were considered normal, intermediate, or indicative of CF, respectively.

**DNA extraction from blood samples:** We extracted genomic DNA from peripheral blood using the DNA Mini Kit (Qiagen, Hilden, Germany), as per the manufacturer's protocol. We quantified DNA using the nanodrop 2000c spectrophotometer (Thermo-Fisher Scientific, Wilmington, USA).

**NGS protocol:** The DNA samples were sequenced for the CFTR gene on the Illumina NextSeq500 platform and the Illumina TruSight One panel. We used the GRCh37/hg19 human genome for variant calling. The library preparation was undertaken following the manufacturer's protocols. We used the raw data as paired reads ( $2 \times 150$  bp) for in-house mapping and variant calling. We performed an initial quality check (average read quality per cycle, average read quality, base call percentage per position) using Fast QC ([www.bioinformatics.babraham.ac.uk/projects/fastqc](http://www.bioinformatics.babraham.ac.uk/projects/fastqc)).

We employed Burrows-Wheeler aligner (BWA) (GNU General Public License version 3.0, version), samtools (Sequence Alignment/Map; [www.samtools.sourceforge.net/SAM1.pdf](http://www.samtools.sourceforge.net/SAM1.pdf)), bamtools (Binary Alignment/Map), bedtools (Browser Extensible Data), vcfutils, and IGV (version 2.4.14) ([www.broadinstitute.org/software/igv/home](http://www.broadinstitute.org/software/igv/home)) along with in-house “R” scripts and shell scripts for parsing, filtering, downstream analysis, and visualizations.

We downloaded the hg19 genome from UCSC (University of Santa Cruz, CA; [www.genome.ucsc.edu/cgi-bin/hgGateway](http://www.genome.ucsc.edu/cgi-bin/hgGateway)) and used “bwa mem” (version 0.5.10) piped to samtools to map the raw reads to the reference genome. We used post indexing and sorting “samtools mpileup” to call the variants, including SNPs, and generated the vcf (variant call format) files. The Illumina TruSight One panel incorporated 26 exonic sequences of the CFTR gene, as these were known to harbor hotspots for disease-associated mutations. The coverage for each sample ranged from 30x to 150x, with an average coverage of all the samples of approximately 50x. Post variant calling by “samtools mpileup,” the QUAL scores of various variants ranged from approximately 200 to 1800. We considered only the high-quality SNPs with QUAL values  $> 500$  for analysis. The known SNPs, novel SNPs, and indel variations were analyzed separately. Comparisons with the dbSNP resulted in SNPs being assigned the “rs\_ids.” These were further visualized for conformation using the IGV viewer. We used the genome analysis toolkit (GATK) for zygosity analysis and the command “HaplotypeCaller” to access the variants. To facilitate the interpretation and mapping to the latest human genome version, we translated the GRCh37/hg19 genome coordinates into the corresponding coordinates of the latest human genome version, namely GRCh38/hg38.

### Statistical Analysis

We used the commercial statistical package SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) to perform all the statistical analyses. Descriptive data are presented as mean with standard deviation (SD) or 95% confidence intervals (CI) or numbers with percentage. We used Fisher's exact test to study the differences between categories, while we used Mann–Whitney U (or Kruskal–Wallis)

test to evaluate differences between numerical variables. A  $p$ -value  $< 0.05$  was considered significant.

## Results

We recruited 38 subjects with a mean ( $\pm$  SD) age of 34.1 ( $\pm$  10.3) years. The baseline demographics, spirometry, and immunological findings of the study population are summarized in Table 1. There were 18, 12, and eight participants of ABPA, asthma, and healthy controls, respectively (Fig. 1). The asthmatic subjects were significantly older than healthy controls or ABPA subjects. The sex distribution was similar in the three groups. Of the 12 asthmatics, eight had aspergillus sensitization. The sweat chloride values were higher in ABPA and asthma than the healthy controls; however, the absolute values were  $< 30$  mmol/L in all the study participants.

The SNP frequency was higher in asthmatic controls than ABPA or healthy controls (Table 2). However, this was not statistically significant (9/12 [75%] vs. 11/18 [61.1%] vs. 3/8 [37.5%],  $p = 0.24$ ). Compared to healthy controls, subjects with asthma and ABPA had a higher frequency of CFTR mutations (3/8 vs. 20/30;  $p = 0.03$ ). The frequency of detecting two or more SNPs was 25% (2/8), 50% (9/18), and 75% (8/12) in healthy controls, ABPA, and asthmatic subjects, respectively. We also found the frequency of

SNPs higher, albeit not significant ( $p = 0.18$ ), in subjects with ABPA-HAM (5/6, 83.3%) and ABPA-B (5/8, 62.5%) than ABPA-S (1/4, 25%). The subjects with SA had a similar frequency (6/8, 75%) of SNPs than UA (3/4, 75%).

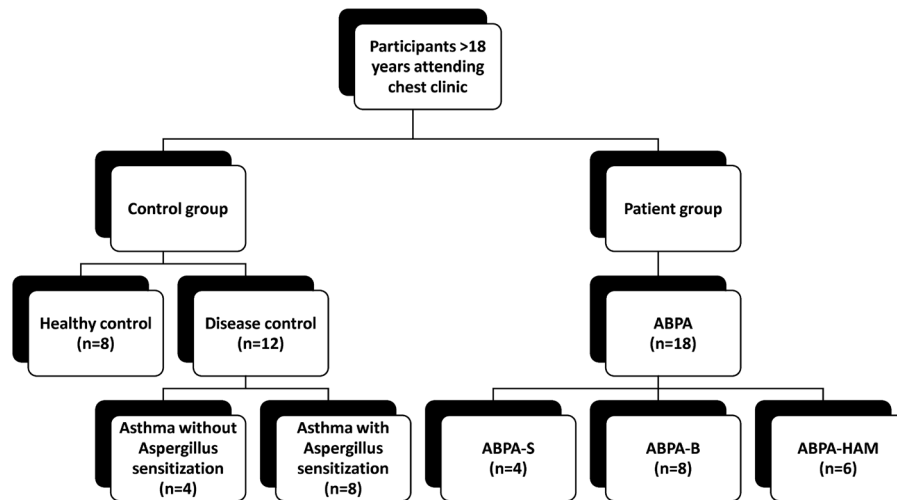
We identified 50 variants from the vcf files of 38 subjects. The summary of the SNPs is provided in Table S1. These 50 SNPs were found among 23 subjects (healthy controls [ $n = 5$ ], ABPA [ $n = 22$ ], asthma [ $n = 23$ ], Table S2). We found none of the 21 common mutations in the CFTR gene of ABPA patients (del 508, G542X, G551D, R553X, W1282X, N1303K, R117H, R347P, R334W, A455E, G551S, 2789 + 5 G > A, D1152H, 3849 + 10 kb C > T, G427X, I507del, 621 + 1 G > T, 1717 -1 G > A, R1162X, R560T, S549N, G542X) [26–29]. We found six unique SNPs not previously reported in ABPA or asthmatic subjects. Four have been cataloged previously and assigned reference SNP cluster ID (rs\_ID) in the NCBI dbSNP database (Table 3). We identified two novel SNPs (chr7:117250703, chr7:117282655) in four (ABPA [ $n = 1$ ], asthma [ $n = 3$ ]) samples without corresponding reference SNP. The zygosity analysis performed by GATK HaplotypeCaller classified the majority (85.5%) of the SNPs as heterozygous. Seven SNPs in four subjects (healthy control [ $n = 1$ ], ABPA [ $n = 2$ ], and asthma [ $n = 1$ ]) were marked as a homozygous alternate.

**Table 1** Baseline characteristics of the study population

Variable	ABPA (n = 18)	Asthma (n = 12)	Healthy controls (n = 8)	P-value
Age, years	31.4 (26.3–36.6)	41.2 (34.7–47.6)	29.4 (26.4–32.3)	0.01
Sex (M/F)	14/4	6/6	3/5	0.10
Expectoration of brownish mucus plugs	6 (33.3%)	1 (8.3%)	–	0.20
Hemoptysis	2 (11.12%)	–	–	0.51
Family history of asthma	9 (50%)	3 (25%)	2 (25%)	0.27
Forced expiratory volume in the first second, liters	2.43 (1.98–2.88)	2.28 (1.73–2.85)	–	0.83
Forced vital capacity, liters	2.56 (2.08–3.03)	2.34 (1.69–3.01)	–	0.45
Serum total IgE, IU/mL	3330 (2228–4431)	922 (428–1415)	–	0.001
Serum <i>A. fumigatus</i> -specific IgE, kUA/L	26.8 (15.1–38.5)	2.6 (-0.67–5.8)	–	0.0001
Serum <i>A. fumigatus</i> -specific IgG, mgA/L	117.9 (84.8–151.2)	20.5 (10.2–30.8)	–	0.0001
Peripheral blood eosinophil count, cells/ $\mu$ K	893 (672–1114)	293 (142–444)	–	0.0001
Sweat chloride, mmol/L, median (range)	11 (3–27)	15 (3–24)	5.5 (3–13)	0.045

ABPA Allergic bronchopulmonary aspergillosis

All the values are represented as number (percentage) or mean (95% confidence intervals)



**Fig. 1** Schematic representation of the study population

## Discussion

We found a high frequency of CFTR mutations in subjects with ABPA (61%) and asthma (75%) than healthy controls (37.5%). We also observed a higher proportion of mutations in the severe forms of ABPA (ABPA-HAM and ABPA-B than ABPA-S), similar to a previous study [27].

Only a few studies have explored the occurrence of CFTR mutations in ABPA complicating asthma [26–31]. The prevalence of CFTR mutation in these studies ranges from 11.5–66.6%, with the highest frequency seen in the study that used whole-exome sequencing [31]. In a study of 18 patients with ABPA, Lebecque et al. found the prevalence of CFTR mutation and multiple SNP frequency of 67 and 22%, respectively [31]. In the present study, we screened for 21 documented mutations in the CFTR gene and did not find them in our ABPA subjects. Even in previous studies from India, it has been shown that these common mutations, including  $\Delta F508$ , are uncommon [32, 33]. Instead, we found six SNPs, hitherto unreported, in patients with ABPA or asthma. Four SNPs are listed in the NCBI database, while two were novel. In CF, the former four SNPs have been considered either benign or of uncertain significance (Table 3). Of the two novel SNPs, one was recently reported in CF, [34] while one not previously reported was present in an asthma subject in the current study.

We found a higher CFTR-SNP frequency in asthmatics than in previous studies, where the

frequency of CFTR mutations was higher in ABPA [26, 27]. However, a high prevalence of CFTR mutations has been described in asthma patients, especially those with excess mucus production [35]. On the contrary, some studies have demonstrated that the heterozygous CFTR state is either protective or not associated with asthma [36, 37]. Asthma and ABPA have also been considered a continuum of a single illness, where patients with asthma develop aspergillus sensitization and eventually ABPA [38]. Likely, the patient's environmental and epigenetic factors and the heterozygous CFTR mutations influence the development of ABPA in asthmatic patients [39–42]. It is possible that the asthmatic patients in our study may develop ABPA in the future.

What is the clinical relevance of our study? We found a high prevalence of SNPs in ABPA and multiple SNPs in individual subjects, like in a previous study [31]. Majority of the SNPs in the current study and the study by Lebecque et al. [31], had a heterozygous alternate state. Whether ABPA complicating asthma should be considered a CFTR-related disorder remains unclear [14, 43]. In the current study, two patients with ABPA and one with asthma had a homozygous state of CFTR. However, none of these patients had a history of malabsorption, and the sweat chloride values were below 30 mmol/L on two occasions. Should these patients be labeled as classic CF solely based on the presence of ABPA, and the novel mutation also remains uncertain? Overall, our study results suggest that CFTR mutations may

**Table 2** Status of single nucleotide polymorphisms (SNPs). The positions of the SNPs and the base change are shown in the brackets for easy identification from table S1

	Group	SNPs
	Control—1	Positive (1408G > A, 2502 T > G)
	Control—2	Negative
	Control—3	Positive (1408G > A)
	Control—4	Positive (1408G > A, 2502 T > G)
	Control—5	Negative
	Control—6	Negative
	Control—7	Negative
	Control—8	Negative
	ABPA-S—1	Negative
	ABPA-S—2	Positive (2502 T > G, 4389G > A)
	ABPA-S—3	Negative
	ABPA-S—4	Negative
	ABPA-B—1	Negative
	ABPA-B—2	Positive (2502 T > G)
	ABPA-B—3	Positive (1408G > A)
	ABPA-B—4	Positive (1408G > A, 2502 T > G, 4389G > A)
	ABPA-B—5	Positive (2502 T > G, 4389G > A)
	ABPA-B—6	Positive (2502 T > G, 3177A > G)
	ABPA-B—7	Negative
	ABPA-B—8	Negative
	ABPA-HAM—1	Positive (1408G > A)
	ABPA-HAM—2	Negative
	ABPA-HAM—3	Positive (1408G > A, 2502 T > G, 4389G > A, c.3119 T > A)
	ABPA-HAM—4	Positive (1408G > A, 4389G > A)
	ABPA-HAM—5	Positive (2502 T > G, 4389G > A)
	ABPA-HAM—6	Positive (1408G > A, 2502 T > G, 4389G > A)
	UA—1	Positive (1408G > A, 2502 T > G, 4389G > A)
	UA—2	Positive (1408G > A, 2502 T > G, 4389G > A)
	UA—3	Positive (c.3119 T > A)
	UA—4	Negative
	SA—1	Positive (1408G > A, 2502 T > G)
	SA—2	Negative
	SA—3	Positive (1408G > A, 2502 T > G)
	SA—4	Positive (1408G > A, 2502 T > G, c.3119 T > A)
	SA—5	Positive (1408G > A, 2502 T > G, 4389G > A)
	SA—6	Positive (2502 T > G, 4389G > A)
	SA—7	Positive (1408G > A, 2502 T > G)
	SA—8	Negative
<i>ABPA-B</i> ABPA with bronchiectasis; <i>ABPA-HAM</i> ABPA with high-attenuation mucus; <i>ABPA-S</i> Serological ABPA; <i>UA</i> <i>Aspergillus fumigatus</i> unsensitized bronchial asthma; <i>SA</i> <i>Aspergillus fumigatus</i> sensitized asthma		

contribute to the high prevalence of ABPA in the North Indian population.

Finally, our study is not without limitations. A significant limitation is the small sample size. We could include only 38 subjects due to financial constraints. Other drawbacks include mapping our

sequences with the human GRCh37/hg19 reference genome only, which constitutes Caucasian and Chinese populations. Hence, any SNP or variations specific to the Indian populations might be missed. We could not follow up the asthma cohort with CFTR mutations to evaluate whether they developed ABPA.

**Table 3** Summary of SNP with a review of clinical significance (genome coordinates translated to GRCh38)

SNP ID	rs213950	rs200735475	rs1800113	rs1800136	–
Position	chr7:117559479 (GRCh38.p12)	chr7:117594941 (GRCh38.p12)	chr7:117611618 (GRCh38.p12)	chr7:117667054 (GRCh38.p12)	chr7:117250703 (GRCh38.p12)
Alleles	G > A	T > G	A > G	G > A	c.3119 T > A
Variant type	SNV	SNV	SNV	SNV	SNV
Allele Frequency (%)	A = 0.47474 (119,239/251166, GnomAD_exome)	G = 0.00029 (74/ 251324, GnomAD_exome)	G = 0.00004 (11/ 250820, GnomAD_exome)	A = 0.21645 (54,312/250926, GnomAD_exome)	1 (0.07)
	G = 0.44781 (56,231/125568, TOPMED)	G = 0.00007 (9/ 125568, TOPMED)	G = 0.00007 (9/ 125568, TOPMED)	A = 0.20936 (26,289/125568, TOPMED)	
Gene: consequence	CFTR: Missense Variant CFTR-AS1: Intron Variant	CFTR: Missense Variant	CFTR: Synonymous Variant	CFTR: Synonymous Variant	CFTR: Missense Variant
Clinical significance	Allele: G ClinVar Accession: RCV000119035.2 Hereditary pancreatitis Benign Allele: A ClinVar Accession: RCV000007550.5 Cystic fibrosis, Likely-Benign	Cystic fibrosis Uncertain-Significance ClinVar Accession: RCV000542704.2	Not Reported in ClinVar	Cystic fibrosis Likely-Benign ClinVar Accession: RCV000353586.1	Unknown
Publication	Iso et al. Hum Genome Var 2019; 6: 17 Li et al. Lung Cancer 2010; 70(1): 14–21	None	None	Vecchio-Pagán et al Hum Genome Var 2016; 3: 16,038 Elahi et al. J Mol Diagn 2006; 8(1): 119–27	Alibakshi et al., Pediatr Pulmonol 2021; 56(12): 3644–3656

SNP Single nucleotide polymorphisms; SNV Single nucleotide variation

The merit of our study is the robust phenotyping followed while enrolling the ABPA patients and the use of next-generation sequencing to detect and analyze all known and unknown mutations in the CFTR gene. The current study is also the first to evaluate the significance of CFTR mutations in an Asian ABPA population, including subjects of Indian origin. All previous studies on the association of the CFTR in ABPA were conducted on Caucasian populations.

In conclusion, our preliminary data found novel mutations in Indian subjects with asthma and ABPA. The high frequency of mutations in ABPA might point to a plausible role of CFTR mutations in the pathogenesis of ABPA. Whether asthmatic patients with

CFTR mutations are destined to develop ABPA in the future remains speculative. Further studies with a larger sample size are needed to confirm our findings.

**Author Contributions** RK: data collection, preparation of the first draft, and revision of the manuscript, AA: data collection, data analysis, preparation of the first draft, and revision of the manuscript, AC: data collection and revision of the manuscript, SMR: data collection and revision of the manuscript, RA: conceptualization of the project, data collection, preparation of the first draft, and revision of the manuscript.

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**Data availability statement** Data will be made available on request with the corresponding author.

## Declarations

**Conflict of interest** None.

**Ethical approval** This is an observational study. The Institute Ethics Committee approved the study protocol.

**Informed consent** We obtained written informed consent from all the study subjects. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

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