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Association of *SFTPB* Gene Polymorphisms with Chronic Obstructive Pulmonary Disease (COPD) Susceptibility in the Population of Southern Punjab, Pakistan

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SS, AB and TS developed the idea and supervised and co-supervised the complete research, respectively and critically analyzed the manuscript. SM helped in the statistical analysis of the results and critically analyzed the manuscript.

Key words
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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality globally and its prevalence is increasing in Pakistan. COPD has heterogeneous etiologies ranging from environmental causes to genetic factors. Single nucleotide polymorphisms (SNPs) in *SFTPB* gene have been shown to be associated with pathogenicity of COPD. This study was aimed to examine the SNPs in *SFTPB* gene along with some demographic and clinical parameters in a cohort of COPD ascertained from Southern Punjab, Pakistan, in a case-control study. Three hundred subjects (150 cases and 150 controls) were recruited and four SNPs rs3024791, rs1130866, rs2118177 and rs2304566 were genotyped through ARMS-PCR. Results showed that cigarette smoking and pulmonary function parameters were significant risk factors of COPD in Southern Punjab, Pakistan. Two SNPs, rs1130866 and rs2118177, were significantly associated with pulmonary function. Two of the four studied SNPs, rs1130866 and rs2304566, were found to be significantly associated with COPD in different inheritance models. Haplotype analysis showed that three haplotypes "CCCC", "CATT" and "TCTT" at the four studied SNPs were significantly associated with reduced risk of COPD and statistically significant linkage disequilibrium was found between two *SFTPB* gene SNPs rs2118177 and rs2304566. Collectively, our findings show that SNPs in *SFTPB* may be utilized as predictor of COPD in the study population and may help guide in the prospective personalized medicine.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) belongs to the group of obstructive lung diseases which is characterized by chronically poor airflow (Yu *et al*., 2021). It becomes extremely hard to remove air completely from the lungs in this disease which is called as airflow obstruction. This airflow obstruction can further cause problems such as shortness of breath or feeling of tiredness because the affected person is working harder to breathe (Vestbo *et al*., 2013). The term COPD is comprised of conditions like chronic bronchitis, emphysema, or a combination of both (American Thoracic Society, 2005).

Worldwide, 329 million people are affected by COPD and this makes up almost 5% of the population (Vos *et al*., 2012). In Pakistan, an estimated 15 million children and 7.5 million adults are suffering from asthma and related conditions (Khan, 2022).

COPD is a complex disease and its common triggers are environmental factors such as cigarette, occupational and environmental exposure to dust and gases, as well as genetic predisposition (Seifart *et al*., 2002; Shahid *et al*., 2019). Among the genetic factors involved in COPD susceptibility, the gene for surfactant protein B (*SFTPB*) is one of the most important and has been investigated in diverse ethnic groups (Yang *et al*., 2014). The *SFTPB* protein is a member of the surfactant protein family which plays an important role in bronchiolar stability, immune defense and the regulation of inflammatory processes in the lung (Bækvad-Hansen *et al*., 2011).

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There is paucity of knowledge on the association of *SFTPB* gene polymorphisms with COPD in Pakistan. The present study was aimed to investigate the association of *SFTPB* gene polymorphisms, (rs3024791, rs1130866, rs2118177 and rs2304566), with COPD susceptibility in a case-control cohort ascertained from Southern Punjab.

MATERIALS AND METHODS

Ethical approval for current research study was obtained from the University Research Ethics Committee (UREC) of The Women University Multan via letter No: WUM/UREC/00010. Written informed consent was obtained from all subjects. Cases (n=150) were selected from the diagnosed COPD patients visiting Nishter Hospital, Multan and Al-Khaliq Hospital, Multan belonging to different parts of Southern Punjab, Pakistan. Healthy individuals without any history of lung disease were chosen as controls (n=150) from the general population of Multan, Pakistan. History of smoking was recorded in pack-years where one pack-year is defined as, smoking 20 cigarettes every day for one year. Subject's height was measured in meters, weight was noted in kilograms and body mass index (BMI) was calculated in kg/m^2. Only those patients were included in this study that showed airflow obstruction in spirometry tests. The criterion for airflow obstruction was taken as a ratio of FEV1/FVC of < 70% after administration of 200 µg of inhaled salbutamol using a spacer device. The COPD was staged based on FEV1 as a percentage of predicted. The electronic spirometer (Spirolab III, MIR, Italy) was used to record lung function of subjects.

DNA was isolated from 3 ml blood of each study subject and DNA extraction was carried out manually by following the salt precipitation method. Tetra ARMS-PCR was used to genotype the SNPs of *SFTPB* gene. For this purpose, primers were designed by using the Primer1 software (http://cedar.genetics.soton.ac.uk). PCR was performed in a final reaction volume of 20 µl for each primer set. For primer SPB1, each reaction consisted of 1X $(NH_4)_2SO_4$ buffer, $MgCl_2$ (2.5 mM), dNTPs (2.5 mM), SPB1 primers (15 pM each), 1.25 units of *Taq* DNA polymerase (Thermo Fisher Scientific, USA, Catalog No. EP0405), human genomic DNA (100 ng) and sterile water. For primers SPB2, SPB3 and SPB4, every reaction consisted of 1X buffer ($(NH_4)_2SO_4$ buffer for SPB2 and KCl buffer for SPB3 and SPB4), 2.5 mM $MgCl_2$, dNTPs (2.5 mM), 15 pM of each outer primer and 17.5 pM of each inner primer, 1.25 units of *Taq* DNA polymerase (Thermo Fisher Scientific, USA, Catalog No. EP0405), 100 ng of human genomic DNA and sterile water. Annealing temperature for each of the primer set used was different but the rest of the thermal profile was same for all of them. An initial denaturation step of 7 minutes was carried out at a temperature of 95°C. The initial denaturation step was followed by 35 cycles comprising of 3 steps: Denaturation of 30 sec at 95°C, annealing of 30 sec (at 57°C for SPB1, 60°C for SPB2, 56°C for SPB3 and 58°C for SPB4) and extension of 45 sec at 72°C. The thermal profile was concluded with a final step of extension of five minutes at a temperature of 72°C. The PCR-products were analyzed by 2% agarose gel electrophoresis.

Statistical analysis

Data were presented as mean ± standard deviation. Chi-square test was applied for categorical data and to compare two means Student's t-test was applied using SPSS software (version 20.0). $P \leq 0.05$ was considered statistically significant for all tests. Associations between the four SNPs and pulmonary function were analyzed by linear regression analysis. For the estimation of haplotype frequencies, a frequency threshold of 0.01 for rare haplotypes was taken. Haplotype association with COPD (disease status) was calculated. The relationship of genotypes with the risk of developing COPD was checked under multiple gene models. The respective odd ratios (OR) and 95%CI were calculated through STATA (version 11). Minor allele frequencies (MAF) were obtained from the online databases like ExAC, 1000 Genome, and SNPedia. Positions and maps of the SNPs included in this study were obtained by using the UCSC genome browser (GRCh38).

RESULTS

The demographic and clinical characteristics of the study subjects are presented in Table I. The difference in these parameters between COPD patients and controls was highly significant. Out of the total subjects (n=300), in the current study, majority were men (n=295) with the exception of 5 women. Statistical analysis showed significantly high difference among the groups ($p<0.001$). The smoking history of COPD patients was 23.85 ± 7.75 pack years which was more than controls 10.74 ± 4.74 pack years. Statistical analysis showed significant difference between groups ($p<0.001$). The BMI of COPD patients was high 24.05 ± 7.43 kg/m^2 than that of controls 21.77 ± 1.77 kg/m^2. Statistical analysis showed significant difference between the groups ($p<0.001$). Various pulmonary function test parameters, that are important for the screening of COPD patients, were measured and compared between cases and controls. Significantly low values were found of FVC % Predicted and FEV1 % Predicted in COPD patients compared to controls

(p=<0.001). The ratio of FEV1/FVC (forced expiratory ratio) in COPD patients was also significantly low than that of the controls (p=0.002).

Table I. Clinical and demographic parameters of COPD patients and controls. The values are Mean±SD.

Parameter	COPD patients (n = 150)	Controls (n = 150)	p-value
Age (in years)	61.22 ± 11.76	51.36 ± 11.24	<0.001***
Smoking history			
Smokers	85	128	
Ex-smokers	56	---	---
Non smokers	9	22	
Smoking (pack-years)	22.42 ± 9.42	10.74 ± 4.74	<0.001***
BMI (kg/m^2)	24.05 ± 7.43	21.77 ± 1.77	<0.001***
FVC % predicted	38.59 ± 22.42	93.74 ± 11.23	<0.001***
FEV1% predicted	43.16 ± 27.92	101.07±10.80	<0.001***
FEV1/FVC %	104.35 ± 19.59	109.65 ± 7.12	0.002*

*p≤0.05= significant; **p≤0.01=highly significant; ***p≤0.001= very highly significant. FVC or 'forced vital capacity' is a lung volume parameter that determines the maximum amount of air that can be forced out after full inhalation. FEV1 or 'forced expiratory volume at the first second of the expiration' is a lung volume parameter that determines the maximum amount of air that can be blown out during the first second of the FVC process. FEV1/FVC is the ratio of the two measurements: FVC and FEV1.

Table II. Allelic frequencies of *SFTPB* gene SNPs and Chi-square *p*-values to test deviations from Hardy-Weinberg equilibrium.

SNP ID	p-value for HWE in cases/ Controls	Alleles	Cases (%)	Controls (%)	p value
rs3024791	0.877/0.283	C	48.7	46	0.513
		T	51.3	54	
rs1130866	<0.001/<0.001	A	45.7	57.0	0.005*
		C	54.3	43.0	
rs2118177	0.003/0.105	C	61.3	68.7	0.060
		T	38.7	31.3	
rs2304566	<0.001/0.267	C	45.3	47.3	0.623
		T	54.7	52.7	

For statistical details see Table I.

As shown in Table II, in SNP rs3024791 the distribution of alleles among the cases and controls was statistically not significant. For the second SNP rs1130866, the frequency of allele A was high in controls while the allele C was high in cases (p=0.005). In the SNP rs2118177, the frequency of C allele was high in controls while the allele T was high in cases. For the last SNP rs2304566 the frequency of C allele was slightly high in controls while the allele T was slightly high in cases.

The relationship between *SFTPB* SNPs and risk of COPD was analyzed by using four inheritance models: Codominant, Dominant, Recessive and Overdominant (Table III). The genotypes of SNPs rs3024791 and rs2118177 were not associated with the risk of COPD in any of these models. The genotypes of SNP rs1130866 were significantly associated with reduced risk of COPD in codominant model, genotype A/C (OR = 0.68, 95%CI = 0.39-1.18, p = 0.054) and C/C (OR = 0.50, 95%CI = 0.29-0.88, p = 0.054); in dominant model, genotype C/C and A/C (OR = 0.59, 95%CI = 0.36-0.95, p = 0.029); and in recessive model, genotype C/C (OR = 0.61, 95%CI = 0.73-1.00, p = 0.047). Genotypes of SNP rs2304566 were significantly associated with increased COPD risk in the codominant model, genotype C/T (OR = 2.19, 95%CI = 1.27-3.78, p = 0.0074) and C/C (OR = 1.07, 95%CI = 0.60-1.90, p = 0.0074); in dominant model, genotype C/C and C/T (OR = 1.60, 95%CI = 0.99-2.58, p = 0.053); and in overdominant model, genotype T/C (OR = 2.13, 95%CI = 1.32-3.45, p = 0.0018).

The linear regression model was used to find out whether there was an association between the *SFTPB* SNPs and pulmonary function in all study subjects. According to results the SNP rs1130866 was significantly related with FEV1 % predicted (p=0.002) and FVC % predicted (p=0.003) whereas FEV1/FVC % had no significant association with the SNP (p=0.445). Another statistically significant relationship was observed between the SNP rs2118177 and the pulmonary function, FVC % predicted (p=0.041). However, the association of this SNP was not significant with the other two pulmonary functions, FEV1 % predicted (p=0.095) and FEV1/FVC % (p=0.767). The SNPs, rs3024791 and rs2304566 had no statistically significant association with any of the pulmonary functions included in the study (Table IV).

Haplotype analysis showed that the haplotype CCCC (OR=0.270, 95%CI=0.09-0.84, p=0.024), CATT (OR=0.260, 95%CI=0.09-0.74, p= 0.012) and TCTT (OR=0.190, 95%CI= 0.04-0.80, p=0.025) in the four SNPs rs3024791, rs1130866, rs2118177 and rs2304566 were significantly related to reduced risk of developing COPD (Table V).

Further haplotype analysis detected statistically significant linkage disequilibrium between SNPs rs2118177 and rs2304566 (p=0.0382; Fig. 1).

Table III. Genotype frequencies of the study SNPs and their relation with risk of COPD.

SNP ID	Model	Genotype	Cases		Controls		Chi-test p-value	OR	95%CI	p value
			No.	%	No.	%				
rs3024791	Codominant	T/T	40	26.7	47	31.3	0.831	1.00	---	0.66
		C/T	74	49.3	68	45.3	---	0.78	0.46-1.34	---
		C/C	36	24.0	35	23.3	---	0.83	0.44-1.55	---
	Dominant	T/T	40	26.7	47	31.3	0.445	1.00	---	0.37
		C/C-C/T	110	73.3	103	68.7	---	0.8	0.48-1.31	---
	Recessive	T/T-C/T	114	76.0	115	76.7	1.00	1.00	---	0.89
		C/C	36	24.0	35	23.3	---	0.96	0.57-1.64	---
	Overdominant	T/T-C/C	76	50.7	82	54.7	0.488	1.00	---	0.49
		C/T	74	49.3	68	45.3	---	0.85	0.54-1.34	---
	Log-additive	---	---	---	---	---	---	0.90	0.66-1.24	0.52
rs1130866	Codominant	A/A	43	28.7	61	40.7	0.054	1.00	---	0.054*
		A/C	51	34.0	49	32.7	---	0.68	0.39-1.18	---
		C/C	56	37.3	40	26.7	---	0.50	0.29-0.88	---
	Dominant	A/A	43	28.7	61	40.7	0.039	1.00	---	0.029*
		C/C-A/C	107	71.3	89	59.3	---	0.59	0.36-0.95	---
	Recessive	A/A-A/C	94	62.7	110	73.3	0.063	1.00	---	0.047*
		C/C	56	37.3	40	26.7	---	0.61	0.37-1.00	---
	Overdominant	A/A-C/C	99	66	101	67	0.807	1.00	---	0.81
		A/C	51	34	49	33	---	0.94	0.58-1.52	---
	Log-additive	---	---	---	---	---	---	0.71	0.54-0.94	0.016**
rs2118177	Codominant	C/C	65	43.3	75	50.0	0.163	1.00	---	0.16
		C/T	54	36.0	56	37.3	---	0.90	0.55-1.48	---
		T/T	31	20.7	19	12.7	---	0.53	0.27-1.03	---
	Dominant	C/C	65	43.3	75	50.0	0.30	1.00	---	0.25
		T/T-C/T	85	56.7	75	50.0	---	0.76	0.49-1.20	---
	Recessive	C/C-C/T	119	79.3	131	87.3	0.088	1.00	---	0.062
		T/T	31	20.7	19	12.7	---	0.56	0.30-1.04	---
	Overdominant	C/C-T/T	96	64	94	62.7	0.811	1.00	---	0.81
		T/C	54	36	56	37.3	---	1.06	.66-1.69	---
	Log-additive	---	---	---	---	---	---	0.76	.56-1.04	0.084
rs2304566	Codominant	T/T	61	40.7	45	30.0	0.0076	1.00	---	0.0074*
		C/T	42	28.0	68	45.3	---	2.19	1.27-3.78	---
		C/C	47	31.3	37	24.7	---	1.07	0.60-1.90	---
	Dominant	T/T	61	40.7	45	30.0	0.0698	1.00	---	0.053*
		C/C-C/T	89	59.3	105	70.0	---	1.60	0.99-2.58	---
	Recessive	T/T-C/T	103	68.7	113	75.3	0.247	1.00	---	---
		C/C	47	31.3	37	24.7	---	0.72	0.43-1.19	0.20
	Overdominant	T/T-C/C	108	72.0	82	54.7	0.0018	1.00	---	0.0018***
		T/C	42	28.0	68	45.3	---	2.13	1.32-3.45	---
	Log-additive	---	---	---	---	---	---	1.07	0.80-1.42	0.66

Table IV. Association of *SFTPB* SNPs with pulmonary function in all study subjects (n=300).

SNP ID	FEV1 % predicted		FVC % predicted		FEV1/FVC %	
	β-value	p-value	β-value	p-value	β-value	p-value
rs3024791	-0.023	0.685	-0.020	0.728	-0.005	0.936
rs1130866	-0.176	0.002*	-0.173	0.003*	-0.044	0.445
rs2118177	-0.096	0.095	-0.117	0.041*	-0.017	0.767
rs2304566	-0.061	0.289	-0.053	0.350	-0.076	0.193

For statistical details see Table I.

Table V. Haplotype frequencies of studied *SFTPB* polymorphisms and their relationship with risk of COPD.

rs3024791	rs1130866	rs2118177	rs2304566	Frequency	OR	95%CI	p-value
T	A	C	T	0.100	1.000	---	---
T	C	C	C	0.094	0.470	0.17 - 1.32	0.150
C	C	C	T	0.093	0.930	0.36 - 2.36	0.880
T	A	C	C	0.086	0.730	0.25 - 2.17	0.570
C	A	C	C	0.077	1.010	0.36 - 2.82	0.990
T	C	C	T	0.068	0.410	0.12 - 1.41	0.160
C	C	C	C	0.068	0.270	0.09 - 0.84	0.024*
C	A	C	T	0.065	0.300	0.09 - 1.08	0.067
C	A	T	T	0.061	0.260	0.09 - 0.74	0.012*
T	C	T	T	0.054	0.190	0.04 - 0.80	0.025*
T	A	T	T	0.054	0.850	0.22 - 3.26	0.810
C	C	T	T	0.041	0.510	0.13 - 1.97	0.330
T	A	T	C	0.041	0.800	0.22 - 2.90	0.730
C	C	T	C	0.040	0.860	0.24 - 3.09	0.810
C	A	T	C	0.029	0.510	0.10 - 2.47	0.400
T	C	T	C	0.029	0.190	0.01 - 3.12	0.250

Global haplotype association *p*-value: 0.024 (*=statistically significant).

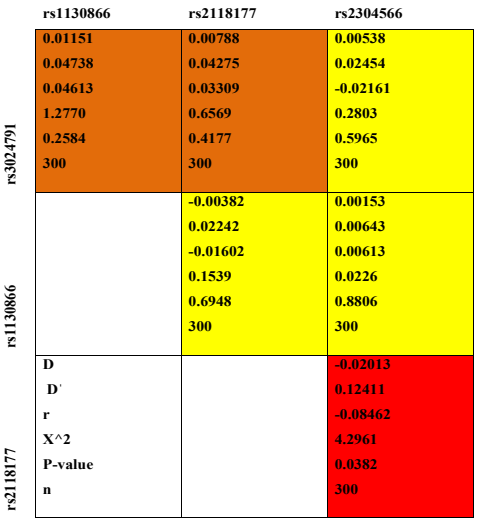

Fig. 1. Linkage disequilibrium map for the studied polymorphisms in *SFTPB* gene.

DISCUSSION

SNPs in *SFTPB* have been associated to COPD in different studies and, therefore, could be responsible for risk of COPD in the different populations of the world. The present study aimed to find out whether the four SNPs in *SFTPB* were associated with COPD susceptibility in Southern Punjab population.

In the present study, statistically significant differences were observed in the clinical features between COPD patients and controls. Increase in age combined with smoking and pack-years of smoking were the most important risk factors for COPD where the cases had been exposed to more pack-years of smoking as compared to the controls. Various studies focusing on the relationship between COPD and smoking have been conducted in the past. Blasi *et al*. (2017) carried out a study on the epidemiology of COPD in Italy and concluded that COPD was a major cause of morbidity and mortality worldwide,

especially in smokers over 40 years of age. Similar results were reported in a study by Arsalan *et al.* (2013) on the prevalence of COPD in Karachi, Pakistan, where majority of the COPD patients were found to be smokers as compared to non-smokers. In another study on COPD carried out by Afonso *et al.* (2011), it was concluded that smoking or exposure to tobacco was a primary cause of COPD with the mortality ten times higher in smokers as compared to non-smokers. In a study carried out by Bækvad-Hansen *et al.* (2010) in a general Danish population it was concluded that even though not all smokers develop COPD yet the risk of developing the disease increases with the increased rate of smoking.

In the present study, statistically significant differences were calculated in the values of different pulmonary function parameters between COPD patients and controls. FEV1 values were significantly low in the COPD patients as compared to the controls. Furthermore, COPD patients belonging to older age groups and having a smoking history had reduced FEV1 values compared to the patients who were ex-smokers or non-smokers and did not belong to older age groups. In two different studies conducted by Sherman *et al.* (1992) in America and Kerstjens *et al.* (1997) in Netherlands it was observed that FEV1 decreased with increasing age and the rate of reduction served as a vital spirometric indicator of how the disease advanced in COPD. Furthermore, our results showed that the values of FVC (forced vital capacity in liters) % predicted, FEV1 (forced expiratory volume in one second) % predicted and FEV1/FVC % predicted were significantly lower in COPD patients as compared to controls. Similar to the results of the present study, previous research also shows that various pulmonary function parameters are found to be reduced in COPD patients as compared to normal individuals (Blasi *et al*., 2017; Camiciottoli *et al*., 2013; Hillas *et al*., 2015; Kakavas *et al*., 2021; Lopes and de Melo, 2016).

The association of *SFTPB* gene SNPs and risk of COPD has been assessed in various populations. Guo *et al.* (2001) observed a significant relationship between rs1130866 and COPD development in Mexican population (p=0.02). In another study Hersh *et al.* (2005) witnessed an association between the SNP rs1130866 and moderate-to-severe airflow obstruction in American population (p=0.03). In another research by Bækvad-Hansen *et al.* (2011) there was no significant relation between the *SFTPB* SNP rs3024791 and lung function and COPD in a population of Denmark. In a study by Yang *et al.* (2014) no association was found between the SNPs, rs3024791, rs2118177 and rs2304566 with either COPD or lung function in a population of China.

The present study revealed a statistically significant relationship between rs2118177 and only one pulmonary function, FVC % predicted (p=0.041). This SNP was associated with a decreased FVC % predicted. Another SNP, rs1130866 was significantly related with two parameters; FEV1 % predicted (p=0.002) and FVC % predicted (p=0.003) where this SNP was significantly related with a decreased FEV1 % predicted and FVC % predicted. In the present study, the reduced values of the three pulmonary function parameters may be caused due to the prolonged exposure of the study subjects with cigarette smoking which results in constriction of the airways and makes it difficult to breathe, therefore resulting in decreased pulmonary function. However, in contradiction to the current study, in another study conducted by Yang *et al.* (2014), it was verified that the T allele of rs1130866 protected Chinese Han subjects from COPD and the SNP was associated with an increased FEV1% predicted. The remaining SNPs, rs2304566 and rs3024791 had no statistically significant association with any of the three pulmonary functions.

Haplotype analysis for all the study subjects, of the current study, using the four *SFTPB* gene SNPs showed that the following haplotypes were significantly linked with reduced risk of COPD: CCCC (OR=0.270, 95%CI=0.09-0.84, p=0.024), CATT (OR=0.260, 95%CI=0.09-0.74, p=0.012) and TCTT (OR=0.190, 95%CI=0.04-0.80, p=0.025). Since no studies related to haplotype analysis of *SFTPB* gene SNPs are present in Pakistan, it can be said that this association is being reported for the first time.

Further haplotype analysis detected statistically significant linkage disequilibrium between two *SFTPB* gene SNPs rs2118177 and rs2304566 (p=0.0382). In accordance with the present study, a study by Yang *et al.* (2014) in a Chinese population showed significant linkage disequilibrium between the two *SFTPB* gene SNPs rs2118177 and rs2304566. Similarly, another study conducted by Foreman *et al.* (2008) in America also showed linkage disequilibrium between the two *SFTPB* gene SNPs rs2118177 and rs2304566.

CONCLUSION

Cigarette smoking along with increased pack-years of smoking was found to be important risk factor for COPD in the present study. Furthermore, pulmonary function parameters were also found to be significant risk factors of COPD in Southern Punjab, Pakistan. It was observed that two of the four studied SNPs (rs1130866 and rs2304566) were found to be significantly associated with COPD. No studies have been carried out in Pakistan previously to determine the role of *SFTPB* polymorphisms in causing COPD, therefore, the present study provides initial data which can be useful in paving a way for future researches

in this regard. The current study showed that three haplotypes were significantly linked with reduced risk of COPD. Further haplotype analysis detected statistically significant linkage disequilibrium between two *SFTPB* gene SNPs rs2118177 and rs2304566. This finding, which is being reported for the first time in Pakistan, can be helpful in carrying out future projects related to haplotype analysis of *SFTPB* polymorphisms in Pakistan.

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Ethical approval

The present study was approved by the University Research Ethics Committee (UREC) via letter No: WUM/UREC/00010.

Statement of conflict of interest

The authors have declared no conflict of interest.

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