ORIGINAL ARTICLE

PAI-1 and *tPA* gene polymorphisms and susceptibility to chronic obstructive pulmonary disease in a sample of Turkish population

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Abstract

Objective: The aim of this study was to assess the influence of plasminogen activator inhibitor-1 *(PAI-1) 4G/5G* or tissue plasminogen activator *(tPA) I/D* polymorphisms in chronic obstructive pulmonary disease (COPD) cases in a sample of Turkish population.

Methods: *PAI-1 4G/5G* and tPA Alu-repeat I/D genetic polymorphisms in 153 COPD subjects and 160 controls were investigated using PCR-RFLP and PCR methods, respectively.

Results: 4G allele frequency was 0.62 and 0.39 for COPD and control groups, respectively. 4G allele had an estimated 2.56-fold [95% CI = 1.85–3.53] increased risk of COPD. *tPA I* allele frequency was 0.55 and 0.50, for COPD and control groups, respectively. I allele had an estimated 1.19-fold [95% CI = 0.87–1.62] increased risk of COPD

Conclusions: *PAI-1 4G/4G* and 4G/5G genotypes seemed to play a key role in the pathophysiology of COPD in Turkish individuals.

Keywords: COPD; Genetic susceptibility; Polymorphisms; Tissue-type plasminogen activator *(tPA)*; Plasminogen activator inhibitor-1 *(PAI-1)*

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Introduction

Chronic obstructive pulmonary disease (COPD) is a preventable and treatable disease, characterized by progressive airflow limitation, which might be completely irreversible, with systemic effects, related to inflammatory response due to various harmful particles and gases. Although the exact mechanisms behind the development of COPD are not fully understood, potential mechanisms of the disease are thought to include protease/antiprotease imbalance, inhibition of antiproteases by oxidants, such as tobacco smoke, and oxidant/free radical mediated cellular and tissue damage. Risk factors for COPD include both genetic factors and environmental factors (e.g., cigarette smoking). The primary environmental risk factor is smoking, but other risk factors include history of respiratory infection, air pollution, second-hand smoke, and occupational exposures to certain industrial pollutants.[1]

Pathological studies have indicated that microthrombosis might occur in the pulmonary vessels of COPD patients and such changes might be a cause of disease exacerbation. Thrombosis could be due to platelet activation or the existence of prothrombotic condition in subjects with vascular and alveolar lesions but this possibility has never been throughly investigated in COPD subjects.[12]

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The degradation of fibrin is dependent on the fibrinolytic or plasminogen activator system. This system basically involves plasminogen activators likewise belonging to the class of serine proteases and inhibitors called serpins. Under physiological conditions, the major plasminogen activator contributing to fibrinolysis is tissue plasminogen activator *(tPA)*. The substrate plasminogen is cleaved at its lysine residues by *tPA* to form active plasmin. Finally plasmin acts on fibrin resulting in resorption of fibrin dots. Plasminogen activator and plasmin inhibitors regulate the conversion of plasminogen to active plasmin and thereby regulate fibrin clearance.[3] A 300 base pair Alu repeat insertion/deletion *(I/D)* polymorphism in intron of the tPA gene at chromosome 8p12-q11.2 was described by Ludwig et al.[4] Although *tPA* is the primary enzyme responsible for dissolving fibrous clots, few studies have evaluated the role of *tPA* polymorphisms and risk of poor fibrinolysis.

Plasminogen activator inhibitor-1 *(PAI-1)* is a potent and major inhibitor of *tPA*. The efficacy of fibrinolysis depends on the interactions of the plasminogen activators and inhibitory proteins of the plasminogen activator - plasmin system. Deficient expression of *PAI-1* can lead to relatively unrestricted expression of plasmin. This scenario promotes excessive degradation of fibrin and could result in an increased risk of bleeding. Conversely, excessive production of *PAI-1* would be expected to limit the generation of plasmin and facilitate persistence of fibrin clots. This is a central event that contributes to the pathogenesis of diverse processes including atherosclerosis, coronary artery disease, intravascular

thrombosis, extravascular fibrin deposition associated with tissue inflammation and airway remodeling associated with chronic obstructive pulmonary disease. The gene encoding for *PAI-1* is located at chromosome 7q22. A single guanine insertion/deletion (4G/4G) polymorphism in the promoter region of the *PAI-1* gene, 675 base pairs upstream from the transcriptional start, has been associated with plasma *PAI-1* levels. The deletion allele (4G) fails to bind repressor proteins, down-regulating fibrinolysis and up-regulating inflammatory activity.[5]

PAI-1 and *tPA* are expressed by numerous cells types in the lung including endothelial cells, epithelial cells, and alveolar macrophages. Then, *PAI-I* and *tPA* are involved in plasmin formation and plasmin contributes to extracellular matrix proteolysis.[6] For this reason, *PAI-1* and *tPA* are among the candidate genes that are thought to play a role in the pathogenesis of COPD. The aim of this study was to assess the influence of *PAI-1 4G/5G* or *tPA I/D* polymorphisms in COPD cases in a sample of Turkish population. To the best of our knowledge, this is the first study that investigated the effect of *PAI-1 4G/5G* or *tPA I/D* polymorphisms on COPD.

Methodology

The study was approved by the Clinical Research Ethics Committee of Yuzuncu Yıl University and all subjects gave written informed consent. Our patient group included 153 COPD subjects who have been treated in Yuzuncu Yıl University, Dursun Odabaş Medical Center, Department of Pulmonary Medicine, Van, Turkey. Diagnosis of COPD was based on symptoms, physical examination, and presence of risk factors. Diagnosis was confirmed by post-bronchodilator spirometry performed 15 min after administration of four doses of salbutamol sulfate. Pre-and post-bronchodilator spirometry was performed according to American Thoracic Society/European Respiratory Society recommendations using a spirometer in all subjects.[7] The diagnosis of COPD and its severity were determined according to GOLD criteria. Patients fulfilling the criteria for COPD were enrolled as cases and those who did not fulfill the standard diagnostic criteria were enrolled as controls. Clinical examination of respiratory system was carried out to document obstructive airway disease and to rule out other forms of pulmonary diseases.

The control group consisted of 160 healthy individuals who consulted to the laboratories of Yüzüncü Yıl University, Medical Faculty, Dursun Odabaş Medical Center and do not have any inherited, acquired or chronic illnesses, and airflow limitation. Healthy individuals were in the similar age and sex distribution to the subjects with COPD.

Forced expiration (FEV1), FEV1/ Forced vital capacity (FVC), mean platelet volume (MPV), platelet distribution width (PDW), platelet count (PLT) and plateletocrit (PCT) values, Prothrombin Time (PT), activated partial thromboplastin time (aPTT) and international normalized ratio (INR) levels were recorded from the patient folder among the COPD subjects.

DNA isolation: 5 mL peripheral blood was taken from COPD-diagnosed patients and control group individuals to K2-EDTA tubes and stored at + 4ºC until the study day. All studies were carried out in Yüzüncü Yıl University Pharmacy Faculty, Biochemistry Research Laboratory. In addition, the patient follow-up form was used for the detection of laboratory and clinical data of patients with COPD and these forms were filled in order to refer patients' polyclinic and service files. Genomic DNA isolation from whole blood samples was performed according to the Poncz method.[8] In this method firstly, 0.5 ml of human whole blood anticoagulated with EDTA-K_2 at 1 mg/mL blood was mixed with an equal volume of a lysis solution containing 1% Triton X-100 to lyse the cells and the nuclei were isolated as described. Isolated nuclei were suspended in an enzyme reaction solution containing 1% SDS and digested with 0.8 mg/ml proteinase K to liberate DNA from nuclear proteins. After 1h incubation, NaI solution was added to the nuclear lysate to the final concentrations of 4.5 M NaI and 0.4% SDS, and was followed by isopropanol addition. The content in the tube was mixed well by inversion until whitish precipitate appeared. The precipitate was collected by centrifugation and washed with the alcohol solutions. If required, contaminant RNA could be removed by pancreatic RNAse treatment prior to the proteolysis.[8]

Determination of Genotypes: Identification of *PAI-1* was assayed with PCR restriction fragment length polymorphism (PCR-RFLP) based methods, as described by Diamanti-Kandarakis et al,[5] and for tPA genotyping, PCR based method was performed as described by Ferrari et al.[9]

For genotyping of *PAI-1 4G/5G* polymorphism; Forward 5′-CACAGAGAGAGTCTGGCCACGT-3′ and Reverse 5′-CCAACAGAGGACTCTTGGTCT-3′ primers (Genbank accession codes: J03836.1) were used. After the PCR amplification, RFLP analysis was performed with the restriction enzyme BslI to detect the 4G>5G change. The samples carrying 4G genotype were identified as a single band; 99 bp, 5G genotype were identified as double bands; 77 bp and 22 bp, and 4G/5G genotype were identified as three bands; 99 bp, 77 bp and 22 bp. For genotyping of *tPA* Alu-repeat I/D polymorphism; Forward 5′-TCCGTAACAGGACAGCTCA-3′ and Reverse 5′-ACCGTGGCTTCAGTCATGGA-3′ primers (Genbank accession codes: X77531.1) were used.[9] Obtained fragment were 967 bp for II genotype, 655 bp for DD genotype, and

967 bp and 655 bp for ID genotype. The primers were provided by PRZ BioTECH (Bilkent, Ankara, Turkey). Sequenced reads were aligned against the human *PAI-1* and *tPA* genes using CLC Main Workbench Version 7.6.4 (www.clcbio.com) in order to assess for polymorphisms.

Statistical analysis: Assuming a probability of disease of 0.01, a risk genotype frequency in population of 0.6 and an odds ratio (OR) of 1.8 with a two-sided p value of 0.05, and a case–control design with a 1:3 ratio, by means of Power 3.9, we estimated that we would need at least 140 cases to reach a power of more than 95% under a recessive model of inheritance.[10] The distributions of the *PAI-1 4G/5G* or *tPA I/D* polymorphisms were compared by using the Hardy–Weinberg heredity equilibrium by χ2 tests. Odds ratios (ORs) with 95% confidence intervals (CIs) were also calculated to examine the association between the PAI-1 and tPA genotypes and the risk of COPD. Clinical features are presented as means ± standard deviation. All tests were performed using Statistical Package for the Social Sciences, version 14.0 (SPSS Inc., Chicago, IL, USA). p<0.05 was considered significant.

Results

The frequencies of *PAI-1 4G/4G* genotype in COPD and control group were 73(47.71%) and 51(31.87%), respectively. The frequency of the *PAI-1 4G/5G* genotype in COPD and control group were 45(29.41%) and 24(15.00%), respectively. The frequency of the PAI-1 5G/5G genotype in COPD and control group were 35(22.87%) and 85(53.13%), respectively. According to these data, we could suggest that *PAI-1 -675 4G/4G* genotype increase the COPD risk by 3.47-fold [95% CI = 2.04–5.92], and 4G/5G genotype increase the COPD risk by 4.55-fold [95% CI = 2.42–8.57] (p<0.05) (Table-1). 4G allele frequency was 191(62.42%) and 126(39.37%), 5G allele frequency was 115(37.58%) and 194(60.63%) for COPD and control groups, respectively. According to these data, we could suggest that 4G allele had an estimated 2.56-fold [95% CI = 1.85–3.53] increased risk of COPD (p<0.05) (Table-1).

Table-1: Genotype/allele frequencies and odds ratios of PAI-1 4G/5G and tPA I/D polymorphisms in control and COPD cases.

		COPD cases n (%)	Control group n (%)	Odds ratio [95% CI][a]	*p*-value [b]
PAI-1 4G/5G					
Genotype	4G/4G	73 (47.71)	51 (31.87)	3.47 [2.04-5.92]	<0.001
	4G/5G	45 (29.41)	24 (15.00)	4.55 [2.42-8.57]	<0.001
	5G/5G	35 (22.87)	85 (53.13)	1.00 (referance)	
Allele	4G	191 (62.42)	126 (39.37)	2.56 [1.85-3.53]	<0.001
	5G	115 (37.58)	194 (60.63)	1.00 (referance)	
tPA I/D					
Genotype	II	52 (33.99)	48 (30.00)	1.34 [0.75-2.39]	0.323
	ID	63 (41.18)	65 (40.63)	1.20 [0.69-2.08]	0.519
	DD	38 (24.83)	47 (29.37)	1.00 (referance)	
Allele	I	167 (54.58)	161 (50.31)	1.19 [0.87-1.62]	0.286
	D	139 (45.42)	159 (49.69)	1.00 (referance)	

a Crude odds ratio (OR), 95% CI = confidence interval at 95%; b Chi square

The frequency of *tPA II* genotype in COPD and control group were 52(33.99%) and 48(30.00%), respectively. The frequency of the *tPA ID* genotype in COPD and control group were 63(41.18%) and 65(40.63%), respectively. The frequency of the *tPA DD* genotype in COPD and control group were 38(24.83%) and 47(29.37%), respectively. According to these data, we could suggest that *tPA II*

Table-2: Clinical features of COPD subjects according to PAI-1 genotype and allele variants.

	Genotypes of *PAI-1* gene				Alleles of *PAI-1* gene		
	4G/4G (n=73)	*4G/5G (n=45)*	*5G/5G (n=35)*	*p values*	*4G (n=191)*	*5G (n=115)*	*p-values*
FEV1 (L)	**53.72±6.95**	66.75±9.63	65.17±7.62	*<0.001, **0.325	**55.18±6.14**	65.84±8.64	***<0.000
FEV1/FVC (%)	**42.13±5.63**	54.38±6.34	52.27±6.24	*<0.001, **0.079	**46.59±5.03**	53.97±7.13	***<0.000
MPV (μm³)	7.15±0.81	7.26±0.87	7.33±0.78	*0.233, **0.651	7.2±0.85	7.29±0.87	***0.375
PDW (%)	12.65±2.34	14.58±2.17	14.63±2.1	*0.916, **0.918	13.38±2.36	13.88±2.42	***0.076
PLT (x10³/mm³)	**262.6±31.57**	181.3±22.54	184.5±22.14	*<0.001, **0.527	**220.13±25.69**	183.64±22.69	***<0.000
PCT (%)	**0.18±0.02**	0.14±0.01	0.14±0.02	*<0.001, **1.000	**0.16±0.02**	0.13±0.02	***<0.000
PT (sec.)	**14.15±2.05**	11.37±2.36	11.59±2.06	*<0.001, **0.663	**13.69±1.67**	11.47±2.05	***<0.000
aPTT (sec.)	31.56±4.96	32.34±4.01	31.08±3.65	*0.611, **0.151	32.09±3.97	31.95±4.09	***0.768
INR	**0.92±0.14**	0.71±0.08	0.72±0.09	*<0.001, **0.601	**0.83±0.165**	0.72±0.08	***<0.000

*Shows p value between 5G/5G and 4G/4G genotypes; **Shows p value between 5G/5G and 4G/5G genotypes;
***Shows p value between 5G and 4G alleles; Bold data shows the significant differences as compared with 5G/5G genotype or 5G allele ($p<0.05$)

Table-3: Clinical features of COPD subjects according to tPA genotype and allele variants.

	Genotypes of *tPA* gene				Alleles of *tPA* gene		
	I/I (n=52)	*I/D (n=63)*	*D/D (n=38)*	*p-values*	*I (n=167)*	*D (n=139)*	*p-values*
FEV1 (L)	61.78±7.86	62.63±8.62	63.34±8.63	*0.375, **0.689	62.05±7.63	62.97±8.36	***0.315
FEV1/FVC (%)	52.39±6.95	54.67±6.27	53.37±6.51	*0.499, **0.322	53.34±6.54	53.97±6.58	***0.403
MPV (μm³)	7.22±0.86	7.18±0.86	7.21±0.83	*0.956, **0.864	7.22±0.86	7.19±0.85	***0.760
PDW (%)	13.67±2.34	13.78±2.61	13.64±2.36	*0.952, **0.787	13.71±1.52	13.69±1.46	***0.907
PLT (x10³/mm³)	221.64±25.96	219.67±25.94	214.38±24.65	*0.184, **0.314	221.08±24.96	216.94±28.96	***0.180
PCT (%)	160.02±22.64	159.48±18.04	154.56±22.94	*0.264, **0.234	159.62±18.63	155.97±22.34	***0.120
PT (sec.)	12.69±1.34	12.54±1.38	12.08±1.62	*0.054, **0.132	12.87±1.76	12.54±1.57	***0.087
aPTT (sec.)	31.24±3.64	31.98±4.06	31.74±3.85	*0.532, **0.770	31.62±4.52	31.85±3.95	***0.639
INR	0.856±0.95	0.835±0.09	0.863±0.09	*0.964, **0.133	0.845±0.09	0.853±0.15	***0.565

*Shows p values between D/D and I/I genotypes; **Shows p values between D/D and I/D genotypes; ***Shows p values between D and I alleles;
Bold data shows the significant differences as compared with D/D genotype or D allele ($p<0.05$).

genotype increase the COPD risk by 1.34-fold [95% CI = 0.75–2.39], and *tPA ID* genotype increase the COPD risk by 1.20-fold [95% CI = 0.69–2.08] (p>0.05) (Table-1). *tPA I* allele frequency was 167(54.58%) and 161(50.31%), D allele frequency was 139(45.42%) and 159(49.69%) for COPD and control groups, respectively. According to these data, we could suggest that I allele had an estimated 1.19-fold [95% CI = 0.87–1.62] increased risk of COPD (p>0.05) (Table-1).

Patients' FEV1, FVC and FEV1/FVC values, mean platelet volume (MPV), platelet distribution width (PDW), platelet count (PLT) and plateletocrit (PCT) values, PT, aPTT and INR levels were compared according to the genotype and allele distributions of *PAI-1* and *tPA* genes and showed in Table-2 and Table-3, respectively.

Discussion

Susceptibility to develop COPD results from a combination of environmental and genetic factors. Cigarette smoking is undoubtedly the main environmental risk factor for COPD in the developed world.[11] In addition, genetic factors are influential in the development of COPD. There are good reasons to assume that multiple genes, each with only a modest effect, contribute to the development of COPD. It could also be speculated that multiple predisposing gene variants are interacting with each other and with environmental risk factors.[11]

PAI-1 and *tPA* might play a vital role in the pathogenesis of COPD and are excellent candidate genes for a COPD association studies. First, both genes are expressed by numerous cells types in the lung including endothelial cells, epithelial cells, and alveolar macrophages. Second, *PAI-1* and *tPA* are involved in plasmin formation and plasmin contributes to extracellular matrix proteolysis.[2] In addition, plasmin regulates MMP-1 and MMP-9 activities and both proteinases have been implicated in the pathogenesis of COPD. Finally, *PAI-1* could be induced by inflammatory cytokines such as IL-1 and TNF-α, which are increased in patients with COPD.[11] As far as we know, *PAI-1* and *tPA* have not been previously investigated as candidate genes for COPD until now. This is the first time polymorphisms in these genes have been tested for an association with rate of decline in lung function.

In humans, elevated plasma levels of *PAI-1* have been associated with myocardial infarction and deep vein thrombosis.[12] Genetically modified mice have provided some insight into the function of *PAI-1*. Transgenic mice overexpressing *PAI-1* develop deep vein thrombosis and vascular fibrinolysis is accelerated in *PAI-1* deficient mice. In addition, *PAI-1* is believed to play an important role in a number of plasminogen dependent proteolytic events outside the vasculature. *PAI-1* knockout mice do not develop pulmonary fibrosis after lung injury.[13] Furthermore, evidence suggested that in a murine model of chronic asthma, PAI-1 deficient mice have increased ECM deposition in the airways because of decreased MMP-9 activity and increased fibrinolysis.[14] There is extensive and growing evidence that *PAI-1* is involved in ovarian follicular rupture, as well as angiogenesis and tumour invasion.[12]

Several polymorphisms have been characterized in *PAI-1* gene. A functional polymorphism in the promoter region of the *PAI-1* gene (-675/4G→5G) effects the binding of nuclear proteins regulating transcription and is significantly correlated with the plasma levels of *PAI-1*.[15] The 4G allele is associated with increased gene transcription and higher *PAI-1* plasma concentrations. The two alleles are almost equally distributed among the Caucasian population.[9,16] The 4G allele of this common -675/4G→5G promoter polymorphism is associated with myocardial infarction, coronary artery disease, abdominal aortic aneurysms, stroke, obesity, a poor survival rate after severe trauma, meningococcal disease, and asthma.[3,,5,9,15,16]

We have indicated that the prevalence of *PAI-1 4G* allele was higher in COPD patients than the control group. Heterozygous or homozygous carriage of 4G allele is thought to play a role in the development of COPD.

Up-regulated PAI-1 expression, because of the *PAI-1 4G* allele, indicate indirectly that COPD subjects may be in a hypercoagulative state.[17] Arboix[18] showed that the presence of COPD was a strong predictor of lacunar stroke. These studies suggested the presence of a hypercoagulative state in systemic circulation in COPD subjects.[11,12] These results showed that the effect of *PAI-1* 4G allele on COPD susceptibility was similar to other diseases.

Human *tPA* is an extracellular serine proteinase produced by numerous cells types in the lung including endothelial cells, epithelial cells, alveolar macrophages, and smooth muscle cells. Endothelial cells are considered the most important source of *tPA* in vivo. *tPA* is released from endothelial cells in a constitutive and regulated fashion. The *tPA*-mediated pathway is thought to be primarily involved in the resolution of blood clots.[12] Studies suggested that high plasma levels of *tPA* mark an increased risk of atherothrombotic ischaemic events such as myocardial infarction and stroke; elevated *tPA* levels may represent the activation of the endogenous fibrinolytic system in response to the existence of preclinical atherosclerosis. Genetic variation at the *tPA* locus has been characterized and extensively studied in association with plasma *tPA* levels.[4] Ladenvall et al, reported an association between SNPs at the *tPA* locus and vascular *tPA* release.[19]

The Alu-repeat *I/D* polymorphism is associated with vascular *tPA* release rates.[20]

These data showed that I allele increases the COPD risk by 1.19-fold and *tPA II* genotype increases the COPD risk by 1.34-fold, and ID genotype increases the COPD risk by 1.20-fold. Although the prevalence of the *tPA I* allele was higher in COPD subjects compared with those in control group, having the I allele, either in heterozygous or homozygous state, didn't have a significant risk factor for the COPD formation. No study has shown that the interaction between *tPA* Alu-repeat I/D polymorphism and COPD etiology in the literature, but some reseachers have indicated that *tPA I* allele has increased the risk of myocardial infarction, stroke and atherosclerosis.[20,21]

In our study, we also considered FEV1 value and FEV1/FVC ratio for verifying the pulmonary function, MPV, PDW, PLT and PCT values for the platelet indice analysis and PT, aPTT and INR levels for coagulation statue among the COPD subjects according to the genotype distribution of these two genes. We found that mean baseline FEV1 and FEV1/FVC was significantly lower in subjects carrying the *PAI-1 4G* allele than the 5G allele. Our study also revealed that mean baseline FEV1 and FEV1/FVC were significantly lower in subjects with PAI-1 4G/4G genotype than in those with either 4G/5G or 5G/5G genotype. We couldn't find a significant differance between PAI-1 4G/5G and 5G/5G genotypes for the mean baseline FEV1 and FEV1/FVC. According to our study, we could claim that PAI-1 4G allele and 4G/4G genotype could ruin the pulmonary functions and might be a risk factor for COPD formation.

We found that PLT, PCT, PT and INR values was significantly higher in subjects carrying the *PAI-1 4G* allele than the 5G allele, but there was no differences in PDW, MPV and aPTT values between PAI-1 4G and 5G carriers. Our study also showed that PLT, PCT, PT and INR values was significantly higher in subjects with PAI-1 4G/4G genotype than in those with either 4G/5G or 5G/5G genotype. There was no differences in PDW, MPV and aPTT values among three different genotypes. According to our study, we could express that *PAI-1 4G* allele and 4G/4G genotype could lead to hypercoagulation state and may be a risk factor for COPD formation. Our study also showed that *tPA I* or *D* alleles and *tPA II, ID* or *DD* genotypes did not effect FEV1 and FVC values, FEV1/FVC ratio and PLT, PCT, PDW, MPV, PT, aPTT and INR values in COPD subjects. Previous studies indirectly suggested that the presence of a prothrombotic condition in COPD subjects based on changes in the activities of platelets and clotting system.[22,23] Nenci et al, demonstrated platelet activation in COPD subjects by detection of high plasma levels of p-thromboglobulin, a substance released from activated platelets.[24]

Conclusion

PAI-1 4G/4G and 4G/5G genotypes seemed to play a critical role in the progression of COPD. We believe that this result might contribute to the development of new strategies in the treatment of COPD and other fibrinolytic system disorders.

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