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Investigation of autoantibodies against extracellular membrane antigens of pulmonary cells in patients with COPD

Dissertation

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Zusammenfassung

Hintergrund: Die chronisch obstruktive Lungenerkrankung (COPD) wird als eine chronisch-fortschreitende Krankheit definiert, die langfristige Atemwegssymptome und eine Einschränkung des Luftstroms verursacht. Zahlreiche Befunden weisen darauf hin, dass Patienten mit COPD Autoantikörpern gegen verschiedene Antigene exprimieren können. Angesichts der potenziellen Pathogenität von Autoantikörpern gegen extrazelluläre Antigene habe ich die Hypothese aufgestellt, dass Autoantikörper gegen extrazelluläre Membranantigene bei COPD vorhanden sind und diese mit der Pathogenese der Erkrankung in Verbindung stehen. Konsequenterweise war die Klärung des Zusammenhangs zwischen COPD und Autoantikörpern gegen extrazelluläre Membranantigene von primären Lungenzellen das Ziel dieser Studie

Methoden: In diese Studie wurde insgesamt wurden 147 klinisch gut charakterisierte Patienten mit COPD und 64 alters- und geschlechtsangepasste gesunde Kontrollen (HC) eingeschlossen. Die Serumspiegel von IgG- und IgM-Autoantikörpern gegen primäre menschliche bronchiale Epithelzellen (HBEC) und Lungenfibroblasten wurden mittels Durchflusszytometrie bestimmt. Die Anwesenheit von IgG- und IgM-Autoantikörpern gegen extrazelluläre Antigene von primären HBEC wurde durch indirekte Immunfluoreszenz und konfokale Mikroskopie bestätigt. Die Daten wurden unter Verwendung der Software GraphPad Prism 9.0 analysiert.

Ergebnisse: In den Seren der COPD-Patienten und gesunder Kontrollen ließen sich Autoantikörper gegen primäre HBEC, nicht jedoch gegen Lungenfibroblasten, nachweisen. Patienten mit COPD zeigten dabei signifikant erhöhte Serumspiegel von IgG- und IgM-Autoantikörpern gegen HBEC im Vergleich zu gesunden Kontrollen. Darüber hinaus wurde ein signifikanter Anstieg der Prävalenz von IgM-Autoantikörpern gegen HBEC mit zunehmender Schwere der COPD beobachtet. Bei der Klassifizierung von COPD-Patienten nach Raucherstatus zeigten Raucher höhere Spiegel von IgG-Autoantikörpern gegen primäre HBEC als Nichtraucher. Demgegenüber ließen sich keine signifikanten Zusammenhänge zwischen Autoantikörpern und dem Alter, dem Body-Mass-Index, Parametern der Lungenfunktionstests, kürzlich aufgetretenen Atemwegsinfektionen oder dem Vorhandensein von interstitiellen Anomalien bei Patienten mit COPD nachweisen.

Schlussfolgerungen: Zusammenfassend weist diese Studie eine Assoziation zwischen COPD und Autoantikörpern, die gegen extrazelluläre Membranantigene von primären HBEC gerichtet sind, nach. Darüber hinaus legen die Ergebnisse einen potenziellen Zusammenhang zwischen Zigarettenrauchen und der Induktion von Autoantikörpern gegen primäre HBEC nahe. Weitere Untersuchungen sind erforderlich, um die Pathogenität dieser Autoantikörper zu erforschen und ihren Beitrag zur Entwicklung von COPD zu verstehen.

Summary

Background: Chronic obstructive pulmonary disease (COPD) is defined as a chronic, progressive disease that causes long-term respiratory symptoms and airflow limitation. A growing body of evidence has accumulated regarding the presence of autoantibodies against various antigens in patients with COPD. Given the potential pathogenicity of autoantibodies to extracellular antigens, I hypothesized that autoantibodies against extracellular membrane antigens are present in and associated with COPD. This study aimed to determine the relationship between COPD and autoantibodies against extracellular membrane antigens of primary pulmonary cells.

Methods: A total of 147 clinically well-characterized patients with COPD and 64 age- and gender-matched healthy controls (HC) were enrolled. Serum levels of IgG and IgM autoantibodies against primary human bronchial epithelial cells (HBEC) and lung fibroblasts were determined using flow cytometry. The presence of IgG and IgM autoantibodies against extracellular antigens of primary HBEC was confirmed through indirect immunofluorescence and confocal microscopy imaging. Data were analyzed using GraphPad Prism 9.0 Software.

Results: Autoantibodies against primary HBEC, rather than lung fibroblasts, were detected in sera of both COPD patients and healthy subjects. Patients with COPD showed significantly increased serum levels of IgG and IgM autoantibodies against HBEC compared to healthy controls. Furthermore, the prevalence of IgM autoantibodies against HBEC tended to increase significantly with COPD severity. When COPD patients were classified according to smoking status, smokers showed higher levels of IgG autoantibodies against primary HBEC than never smokers. No significant associations were observed between autoantibodies against primary HBEC and age, body mass index, parameters of lung functional tests, recent respiratory infection, or the presence of interstitial abnormalities in patients with COPD.

Conclusions: Taken together, this study demonstrates an association between COPD and autoantibodies against extracellular membrane antigens of primary HBEC. Moreover, the results suggest a potential link between cigarette smoking and the induction of autoantibodies against

primary HBEC. Further investigations are warranted to explore the pathogenicity of these autoantibodies and their contribution to COPD development.

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Abbreviations

ACPA	anti-citrullinated protein antibody
ACTDA	antibodies to several connective tissue disease autoantigens
ANA	antinuclear antibody
APC	allophycocyanin
BAL	bronchoalveolar lavage
BB	bronchial brushings
BMI	body mass index
BOLD	Burden of Obstructive Lung Disease Study
BSA	bovine serum albumin
CCP	anti-cyclic citrullinated peptide antibodies
CFFCP	chimeric fibrin/filaggrin citrullinated synthetic peptide
cGVHD	chronic graft-versus-host disease
CMP	carbonyl-modified proteins
COPD	chronic obstructive pulmonary disease
DAPI	4′,6-diamidino-2-phenylindole
ECM	extracellular matrix
EDTA	ethylene-diamine-tetra acetic acid
FACS	fluorescence-activated cell sorting
FEV1	expiratory volume in 1 second
Fig.	figure
FVC	forced vital capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GWAS	genome-wide association studies
HBEC	primary human bronchial epithelial cells
HC	healthy controls
ILA	interstitial lung abnormality
MFI	median fluorescence intensity
PBS	phosphate-buffered saline

RA	rheumatoid arthritis
RT	room temperature
SAEC	primary human small airway epithelial cells
SD	standard deviation
SLE	systemic lupus erythematosus
WHO	World Health Organization
16HBE14o-	Human Bronchial Epithelial Cell Line

1. Introduction

1.1. Chronic obstructive pulmonary disease (COPD)

1.1.1. Definition and classification of COPD

COPD is defined as a chronic progressive disease that causes long-term respiratory symptoms and airflow limitation [1]. It is primarily contributed by two pathological conditions: chronic bronchitis and emphysema [2]. Dyspnea, cough, sputum production, and exacerbations are the most common respiratory symptoms in patients with COPD [1]. According to the World Health Organization (WHO), COPD is the third leading cause of death worldwide, causing 3.23 million deaths in 2019 [3]. In a recent systematic review and modelling analysis, Adeloye and colleagues reported that the global prevalence of COPD among people aged 30–79 years was 10.3% in 2019 [4]. Additionally, this systematic review has demonstrated that the estimated prevalence of COPD increase with age and is higher in men than in women [4].

The diagnosis of COPD is based on the presence of clinical symptoms and the fact that the ratio of post-bronchodilator forced expiratory volume in 1 second to forced expiratory vital capacity (FEV1/FVC) is less than 0.70 [5]. According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [6], the severity of airflow limitation in COPD is graded based on post-bronchodilator FEV1 expressed as percent of predicted values. Patients with FEV1/FVC<0.70 are classified into four categories in term of disease severity: “mild” (GOLD 1, FEV1 ≥80% predicted), “moderate” (GOLD 2, 50% ≦FEV1 <80% predicted), “severe” (GOLD 3, 30% ≦FEV1 <50% predicted), and “very severe” (GOLD 4, FEV1 <30% predicted).

1.1.2. Risk factors for COPD

COPD is a complex disease with multifactorial background. The risk of developing COPD is associated with cigarette smoke, biomass smoke, occupation exposures, respiratory infections, air pollutants, age, sex, and genetic factors [7].

1.1.2.1. Cigarette smoke

Cigarette smoke is the most important and common cause of COPD [8-11]. Among patients with COPD, only approximately 20% of people have never smoked [12]. Furthermore, the prevalence of COPD increases with the smoking burden [13]. By analyzing cross-sectional data from a large multicenter cohort, Bhatt and colleagues have shown that smoking duration is a stronger risk factor for COPD than the composite pack-years [14]. Regarding mortality, in the United States, the relative risk of death from COPD for current smokers aged 55 years or older is over 20 times than that for age-matched never smokers [15].

Second-hand smoke exposure is also an important risk factor for the development of COPD. It has been estimated that more than 30% of non-smokers worldwide were exposed to second-hand smoke [16]. Accordingly, second-hand smoke exposure is an independent risk factor for developing COPD in nonsmokers [17]. According to the estimation by Korsbæk et al., exposure to lifelong second-hand smoke may account for 2.9% of patients with COPD [18].

1.1.2.2 Exposure to biomass smoke

Exposure to biomass smoke has been identified as the primary risk factor for COPD development worldwide [19]. Several studies from different regions have shown that biomass fuel is a common risk factor for COPD, especially among women in rural areas [20-22]. Subsequently, by conducting a meta-analysis of 15 epidemiologic studies, Hu et al. [23] were able to show that individuals exposed to biomass smoke are 2.44 times more prone to developing COPD compared to those not exposed to biomass smoke. This factor has been clearly identified as a risk factor for the development of COPD in both women and men, and in both the Asian population and the non-Asian population.

1.1.2.3. Occupational exposures

The risk of developing COPD is also associated with occupational exposures, including exposure to

different dusts and fumes, cadmium, silica and so on [24]. In the USA, the fraction of COPD attributed to occupational exposures has been estimated as 19.2% overall and 31.1% among never smokers [25]. By performing a systematic review and meta-analysis of population-based studies, Alif and colleagues were able to show that low exposure to mineral dust, and high exposure to gases or fumes could increase the risk of COPD [26].

1.1.2.4. Respiratory infections

Previous studies consistently show that respiratory infections are environmental risk factors for COPD and play a key role in its onset and progression [6]. Children who have had pneumonia are at a higher risk of developing COPD in the future, and childhood pneumonia may play a key role in the early onset of COPD [27]. Additionally, previous studies have found a nearly 2.6 times increased risk of COPD with prior pulmonary tuberculosis (TB), and the association between TB and COPD is independent of smoking, income level, definition of COPD, definition of TB, and other confounding factors [28-30].

1.1.2.5. Air pollutants

Exposure to outdoor air pollutants is also a risk factor for the development of COPD. A recent meta-analysis demonstrates that outdoor air pollution, represented by 10 $\mu g/m^3$ increment of PM10, increases the incidence, prevalence and mortality of COPD in China, USA and European Union [31]. By conducting a cross-sectional study in four cities of southern China, Liu et al. [32] were able to show that COPD prevalence was significantly associated with elevated PM2.5. Subsequently, another large cross-sectional cohort study in the UK discovered significant associations between COPD prevalence and concentrations of PM2.5, PM10 and NO_2 [33]. Furthermore, Exposure to air pollutants not only heightens the risk of COPD in healthy individuals, but also elevates the risk of asthma-COPD overlap syndrome in asthmatics [34].

1.1.2.6. Age and sex

Due to the good nutrition and healthcare services, life expectancy rises worldwide, resulting in an increased prevalence of many chronic diseases, including COPD [35]. In a meta-analysis of 133 published studies, Rycroft et al. demonstrate that the prevalence of COPD increases with age, with highest prevalence in the population aged 75 years and older [36] .

Due to gender differences in tobacco smoking, COPD were usually more prevalent among males compared to females, especially in the developing countries [8, 37, 38]. However, the prevalence of COPD has increased substantially among females in developed countries [39-41], which could be largely explained by changes of smoking patterns [42]. In addition, results from a COPD surveillance in the United States from 1999 to 2011 show a higher age-adjusted prevalence in women compared with men (7.3% vs 5.7%) [43], suggesting that females are more susceptible to COPD than males.

1.1.2.7. Genetic factors

The development of COPD is also contributed by genetic predisposition. Alpha-1 antitrypsin deficiency is a well-established genetic risk factor for COPD [44]. In 1963, Laurell and Eriksson, for the first time, reported that α1 antitrypsin deficiency is associated with pulmonary emphysema [45], and this finding has been confirmed by numerous subsequent studies [46, 47]. α1-antitrypsin deficiency is an inherited disorder caused by mutations in serine proteinase inhibitor, clade A (*SERPINA1*) gene, leading to the development liver and lung disorders, including COPD [48]. It has been estimated that and 1-2% of patients with COPD have severe α1 antitrypsin deficiency [49]. For the treatment of patients with α1-antitrypsin deficiency-associated COPD, infusions of human plasma-derived α1-antitrypsin are used as an augmentation therapy in addition to standard therapies [48].

In addition to α1-antitrypsin deficiency, a number of genomic regions predisposing people to COPD have been identified from genome-wide association studies (GWAS). In 2009, Pillai and colleagues, for the first time, utilized GWAS to identify two loci associated with COPD susceptibility, including the α-nicotinic acetylcholine receptors 3–5 (*CHRNA* 3/5) and the hedgehog interacting protein (*HHIP)*

loci [50]. Subsequently, more genetic loci associated with COPD susceptibility have been identified through GWAS, including the family with sequence similarity member 13A (*FAM13A)* [51, 52], matrix metalloproteinase 12 (*MMP12)* [53] and transforming growth factor-β2 (*TGFB2)* [53]. Besides disease susceptibility, disease-related phenotypes and severity are associated with genetic loci. For example, it has been shown by Pillai et al. that the *CHRNA 3/5* locus is associated with smoking intensity and emphysema, the *HHIP* locus is associated with the frequency of COPD exacerbations, and the *FAM13A* locus is associated with lung function [54]. In addition, the *MMP12* locus has been shown to be associated the severity of COPD [55].

1.2. Autoimmunity and COPD

As mentioned above, cigarette smoking is the most common risk factor for the development of COPD, and studies from different countries demonstrate beneficial effects of smoking cessation in COPD patients [56-58]. Despite the positive effects of smoking cession, some harmful effects of cigarette smoking on pulmonary manifestations persist in former smoker. For example, it has been reported that airway inflammation persists even after quitting smoking in patients with established COPD [59]. In line with this notion, Ben-Shlomo et al. reported that ex-smokers may experience an elevated mortality even after 30 years of smoking cessation [60]. This intriguing phenomenon suggests that that there is some kind of self-perpetuating pathogenic process in COPD patients, which is triggered by cigarette smoke. Given that functionally well-organized lymphoid follicles are present around the small airways and lung parenchyma [61-63], it is conceivable that cigarette smoke triggers adaptive immune responses to certain non-cigarette antigens in COPD patients, such as autoantigens, lung microbiome, and infectious pathogens [64-66]. Among those candidates, autoantigens are of particular interest since cigarette smoke is capable of triggering autoimmunity [67]. In 2002, Cosio et al. proposed a new perspective that COPD could be regarded as an autoimmune disease triggered by cigarette smoke [68]. Subsequently, a growing body of evidence for the association of autoimmunity with COPD has been accumulated [69, 70], providing a new perspective for our understanding of the pathological mechanism of COPD.

1.2.1. Humoral autoimmunity in COPD

In 2007, Lee and colleagues, for the first time, reported that emphysema is characterized by the presence of autoimmunity to elastin, an essential component of pulmonary extracellular matrix [64]. According to this study, serum levels of anti-elastin antibody are elevated in patients with emphysema and positively correlated disease severity [64]. Subsequently, many efforts have been made and various autoantibodies have been suggested to be associated with COPD. These autoantibodies can be categorized into two groups according to their target antigens, namely autoantibodies against undefined antigens such as tissue, cell, or cell nucleus and autoantibodies against defined molecules (Table 1).

Table 1. Summary of serum autoantibodies in COPD

Autoantigens		Method	COPD vs control	Reference
Tissues	Human lung tissue	DIH	More prevalent	[71]
		ELISA	Significantly lower	[72]
	Rodent tissues	IIF	More prevalent	[73]
Cells	Primary human pulmonary airway epithelial cells	IIF	Significantly higher	[71]
	Human bronchial epithelial cells	IIF	Significantly higher	[74]
	Primary human pulmonary artery endothelial cells	IIF	Significantly higher	[71]
	HUVEC	ELISA	Significantly higher	[75]
		ELISA	Significantly higher	[76]
Cell nucleus	Nuclear	IIF	More prevalent	[73]
		IIF	Significantly higher	[77]
Molecules	Elastin	ELISA	Significantly higher	[64]
		PA	Significantly higher	[78]
	Rodent tissues	ELISA	No difference	[79]
	Elastin collagen I	ELISA	Significantly lower	[80]
		ELISA	Significantly lower	[81]
		ELISA	No difference	[64]
		ELISA	No difference	[79]
		PA	Significantly higher	[78]
	collogen II	PA	Significantly higher	[78]
	collagen IV	PA	Significantly higher	[78]
	Aggrecan	PA	Significantly higher	[78]
	cytokeratin 18	WB	Significantly higher	[82]
	collagen IV	ELISA	Significantly higher	[83]
	Aggrecan	ELISA	No difference	[72]
	cytokeratin 19	ELISA	Significantly higher	[83]
		IIF	More prevalent	[73]
		IIF	Significantly higher	[77]
	CCP	ELISA	Significantly higher	[84]
	CMP	ELISA	Significantly higher	[76]
	CFFCP	ELISA	Significantly higher	[85]

COPD, chronic obstructive pulmonary disease; IIF, indirect immunofluorescence; HUVEC, human umbilical vein endothelial cell; ELISA, enzyme-linked immunosorbent assay; DIH, direct immunohistochemistry; PA, protein array; WB, western blot; CCP, citrullinated peptides; CMP, carbonyl-modified proteins; CFFCP, chimeric citrullinated peptides of human fibrin and filaggrin.

1.2.1.1. Autoantibodies against tissue, cell, or cell nucleus

Given that autoantigens in COPD are largely unknown, determination of autoantibodies against lung tissue provides proof-of-principle evidence for the presence of autoantibodies in COPD. In 2008, Feghali-Bostwick and colleagues reported that IgG deposition within alveolar septa and small airway walls was detected by immunohistochemical staining in 6 out of 6 patients with COPD, but in none of the 6 controls [71], for the first time indicating the presence of anti-tissue autoantibodies in COPD patients. This notion was confirmed by another study using rodent tissues as antigen [73]. In this study, Núñez et al. could show that COPD patients have a significantly higher prevalence of anti-tissue IgG autoantibodies than healthy controls and that levels of the anti-tissue antibodies are associated with severity of airflow limitation and gas transfer impairment [73]. In addition, Cass et al. reported that increased serum levels of anti-tissue IgM autoantibodies are correlated with the airspace enlargement [86]. By contrast, Daffa et al. reported that serum levels of autoantibodies against lung tissue antigens detected by using enzyme-linked immunosorbent assay (ELISA) are significantly lower in COPD smokers than in healthy non-smokers [72].

Besides investigating anti-tissue autoantibodies, Feghali-Bostwick and colleagues determined anti-epithelial cell autoantibodies in patients with COPD [71]. Their results showed that the prevalence of autoantibodies against both the epithelial cell line and primary airway epithelial cells are significantly higher in COPD patients than both smoke and/or non-smoke controls, for the first time demonstrating the presence of anti-epithelial antibodies in COPD [71]. In line with this finding, Cheng et al. reported that prevalence of IgG and IgA autoantibodies against human bronchial epithelial cells is significantly higher in COPD patients than in healthy controls [74]. Despite the presence of autoantibodies against epithelial cells in patients with COPD, it is debatable whether the autoantibodies are associated with the severity of the disease [71, 74].

The presence of anti-endothelial cell autoantibodies in patients with COPD has been demonstrated in several studies. In the abovementioned study, Feghali-Bostwick et al. [71] reported that autoantibodies against primary pulmonary artery endothelial cells are present in 50% COPD patients, but not in healthy controls, for the first time suggesting an association of anti-endothelial cells

autoantibodies with COPD. This observation has been confirmed by two subsequent studies that determined anti-endothelial cell autoantibodies in COPD patients and controls using cell-based ELISA with human umbilical vein endothelial cells [75, 76]. Moreover, the association of anti-endothelial cell autoantibodies with COPD is further supported by experimental evidence. In 2005, Tarasevicient-Stewart and colleagues reported that rat injected intraperitoneally with xenogeneic endothelial cells produce anti-endothelial cell autoantibodies and develop emphysema [87].

In addition to tissues and cells, the cell nucleus is another type of undefined autoantigen. Due to its high prevalence in autoimmune disorders, particularly in autoimmune rheumatic diseases, antinuclear antibody (ANA) is a widely used serological marker of autoimmunity [88]. In 1976, Hodson et al. [89] for the first time determined ANA in patients with COPD. Their results demonstrates that ANA is present in low titre in 28% of patients with severe chronic bronchitis, but it is observed in only 4% of age- and sex-matched healthy controls, suggesting an increased prevalence of ANA in patients with severe COPD. The increased prevalence of ANA in COPD compared to healthy subjects has also been observed by subsequent studies [73, 77], confirming the association of COPD with ANA. Although the presence of ANA in serum is not associated with severity of COPD [73, 77, 89], Morissette et al. could show that there is a correlation between levels of ANA in the sputum and disease severity in COPD patients [90]. In line with this observation, Morissette et al. demonstrate that ANA is present in the lungs, but not the circulation of cigarette smoke-exposed mice [90].

1.2.1.2. Autoantibodies against defined molecules

To further explore the role of autoantibodies with COPD, some studies investigated autoantibodies against specific antigens which are suspected to be present in or functionally related to the disease, including extracellular matrix (ECM) protein, cytokeratin and neo-autoantigens.

ECM protein is a complex composition of molecules, such as elastin, collogen, aggrecan, fibronectin, and laminin. Given that ECM proteins play an essential role in maintaining the integrity and structure of the lung and that degradation of matrix proteins is a hallmark of emphysema, anti-ECM protein antibodies are considered potentially pathogenic in the development of COPD. This notion is

supported by the findings in a previous study where Lee et al. reported that levels of anti-elastin antibodies are significantly higher in emphysema patients compared with controls [64]. However, all subsequent studies failed to confirm this promising finding [79-81, 91], with only one exception [78], keeping this issue under the debate. In addition to elastin, collagens have been also extensively investigated as candidate autoantigens for COPD. In 2012, Packard et al. reported that serum levels of autoantibodies against collagen I, II and IV were significantly elevated in patients with COPD compared to healthy controls, suggesting an association of anti-collagen antibodies with the disease [78]. Similar to the finding of anti-elastin antibodies, this interesting observation has not been confirmed by other groups [64, 72, 79].

Beside ECM proteins, cellular proteins from pulmonary cells have also been considered as potential autoantigens in COPD. To identify novel autoantibodies in COPD, Kuo et al. performed an immunoblotting assays with lysates prepared from alveolar cells as targets [82]. Their results showed that autoantibodies against multiple antigens are more prevalent in sera of COPD patients than in healthy subjects. One of those autoantigens was identified as cytokeratin 18 (CK-18), a cytoskeletal protein expressed in epithelial cells [82]. The prevalence of anti-CK-18 antibodies is significantly higher in patients with COPD than in controls, and serum levels of anti-CK-18 are positively correlated with lung function in patients. This finding was confirmed by Xiong and colleagues who could show that levels of IgG, IgA and IgM autoantibodies against CK-18 are elevated in COPD patients and correlated with disease severity [83]. Moreover, Xiong et al. [83] reported that autoantibodies against another cytokeratin, CK-19, are also associated with development and severity of COPD. The association of autoantibodies against CK-18 and CK-19 with COPD is further supported by findings in animals. Mice exposed to cigarette smoke produce higher levels of anti-CK18 and anti-CK19 IgG than those exposed to room air [83].

Neo-autoantigens represent a groups of antigens expressed under specific conditions, rather than ubiquitously, such as citrullinated peptides and proteins [92]. Since cigarette smoke is capable of inducing abnormal post-translational modification proteins [93], some effort has been made to investigate antibodies against neo-autoantigens in COPD. Citrullination, a post-translational modification of proteins, is known to occur in smokers, and anti-cyclic citrullinated peptides (CCP) antibodies are a specific serologic marker for rheumatoid arthritis (RA) [94]. In 2014, Sigari et al.

[84] reported that the prevalence of anti-CCP antibodies in patients with wood smoke-induced COPD without RA is significantly higher than that in healthy controls, suggesting an association of COPD with anti-CCP antibodies. Beside anti-CCP antibodies, autoantibodies against two other types of neo-autoantigens, namely carbonyl-modified proteins (CMP) and chimeric fibrin/filaggrin citrullinated synthetic peptide (CFFCP), have been reported to be elevated in patients with COPD compared to healthy controls [76, 85].

1.2.2. Cellular autoimmunity and COPD

Like humoral autoimmunity, cellular autoimmunity is also associated with COPD. In a study by Lee and colleagues [64], the authors investigated both humoral and cellular autoimmunity to elastin in patients with emphysema. Besides showing that levels of anti-elastin antibodies are elevated in patients with emphysema compared to controls, their results revealed that peripheral blood CD 4^+ T cells isolated from individuals with emphysema, but not those from healthy subjects, proliferate and release interferon gamma (IFN-γ) in response to elastin peptides [64], suggesting the presence of T-helper type I (Th1) responses to elastin in COPD. In addition, there is a positive association between the severity of emphysema and the elastin peptides-induced IFN-γ from $CD4^+$ T cells. These findings link COPD to cellular autoimmunity against a specific antigen.

Similar to findings from Lee's study [64], Zhou and colleagues reported that anti-elastin antibodies and T cells specific for elastin peptides are increased in patients with COPD [95], confirming the association of COPD with cellular autoimmunity. Moreover, Zhou and colleagues provided more evidence for this notion using an animal model for COPD. In this animal model, mice exposed to cigarette smoke for two weeks generate elastin-specific T cell responses that leads to bronchitis-like airway inflammation and airspace enlargement following elastin recall challenge. Adoptive T cell transfer into *Rag1*$^{-/-}$mice demonstrates that T cells contribute to the development of experimental COPD, implicating an essential role of elastin-specific T cells in this process. Using matrix metalloproteinase-12 (*Mmp12*) deficient mice, Zhou et al. demonstrate that cigarette smoke exposure-induced elastin-specific T cell responses are MMP12 dependent, suggesting the

involvement of MMP12 in the generation of elastin fragments [95]. Notably, although both studies support association of autoreactive $CD4^+$ T cells to elastin with COPD, they differ from each other in the disease relevant-Th cell type. Lee et al. suggest that Th1 responses to elastin are associated with COPD [64], while Zhou demonstrates that elastin-specific Th17 responses are disease-relevant [95]. By contrast to findings from above-mentioned two studies, Rinaldi et al. reported that elastin-specific T cell responses are not present in COPD, making this issue controversial.

In addition to $CD4^+$ T cells, $CD8^+$ T cells has also been suggested to be associated with COPD. [95]. In above-mentioned study conducted by Zhou and colleagues, the authors determined peripheral blood $CD8^+$ T cell responses to elastin peptide using a synthesized tetramer. Their results showed that there were more $CD8^+$ T cells that bind to the tetramer in peripheral blood of patients with COPD than in healthy subjects [95], implicating the presence of elastin-specific $CD8^+$ T cell in COPD.

1.3. Hypothesis and aims

1.3.1. Hypothesis

Despite of a growing body evidence suggesting the presence of autoimmunity in COPD, the extent to which autoimmune responses contribute to the development of COPD remains largely unknown. For example, although elastin-specific Th17 cells have been shown to be capable of inducing bronchitis-like inflammation in mice [95], these cells have not been detected in patient with COPD [64, 95]. As far as humoral autoimmunity is concerned, COPD-associated autoantibodies targeting intracellular antigens such as ANA, CK-18 and CK-19 seem unlikely pathogenic due to the lack of access to their antigens. Autoantibodies against extracellular antigens, including elastin and collagens, are potentially pathogenic, but their presence in and association with COPD are still subjects of debate [64, 72, 78-81, 91].

Additionally, although several studies have provided compelling evidence supporting the association of COPD with autoantibodies against pulmonary cells [71, 74-76], these autoantibodies were detected using permeabilized cells, leaving uncertainty about whether they recognize intracellular or extracellular antigens. In a previous study, our laboratory employed a protein microarray comprising more than 19,000 human proteins to determine the autoantibody profiles of COPD [96]. Our results demonstrate that serum levels of autoantibodies against extracellular antigens increase in patients with COPD compared to non-COPD smokers, suggesting a shift toward potentially pathogenic autoantibodies in the disease. Given that autoantibodies against pulmonary cells are present in COPD and that autoantibodies against extracellular antigens are potentially pathogenic, I hypothesize that autoantibodies against extracellular membrane antigens of pulmonary cells are present in COPD and contribute to the development of the disease.

1.3.2. Aims of this study

To verify the hypothesis, this study recruited 147 patients with COPD and 64 age- and sex-matched

healthy subjects. This study aimed to achieve three objectives: i) determine whether autoantibodies against extracellular membrane antigens of human airway epithelial cells and lung fibroblasts are present in patients with COPD, ii) explore whether those autoantibodies, if present, are associated with risk of developing COPD, and iii) evaluate the association of these autoantibodies with demographic and clinical features of COPD.

2. Materials and Methods

2.1. Materials

2.1.1. Consumables and equipment

Products	**Manufacturer**
15ml, 50ml tubes	Sarstedt, Germany
6-channel µ-Slides	Ibidi, Germany
96-well plate	Costar, USA
Biosafety cabinet	Heraeus, Germany
Carbon dioxide cell incubator	Thermo Fisher, USA
Cell culture dishes	Corning, USA
Confocal laser scanning microscope Leica TCS SP5	Leica, Germany
Filters	Merck, Germany
Fluid aspiration system	Vacubrand, Germany
LSR II flow cytometer	BD, USA
Luer Syringe	Beckon Dickinson, USA
Pipette	Eppendorf, Germany
Pipette tips	Sarstedt, AG&CO, Germany
Refrigerator (-80 °C)	EWALD Innovationstechnik GmbH, Germany
Refrigerator (4°C, -20 °C)	LIEBHERR, Germany
Slides	R. Langenbrinck Gmbm, Germany
Super-centrifuge	Hettich, Germany
Syringe	Beckon Dickinson, UST
Water bath	Julabo, Germany

2.1.2. Chemicals and reagents

Products	**Manufacturer**	**Cat. No**
Bovine serum albumin (BSA)	PAN-Biotech, Germany	P06-139350
Roti®Histofix 4%	Roth, Germany	P087.1
Mounting medium	Thermo Fisher Scientific, USA	P36935
Fetal bovine serum	Biotech, Germany	10270-106
Penicillin/Streptomycin-solution	PAN-Biotech, Germany	P06-07100

2.1.3. Cells

Cells	Cat. No
16HBE14o-14o- Human Bronchial Epithelial Cell Line	P04-04515
Primary human lung fibroblasts	Kindly provided by Prof. Silke Meiners, Research Center Borstel, Germany
HBEC (primary human bronchial epithelial cells)	Prepared in house

2.1.4. Buffers

Buffer	**Recipe**
PBS-D	1.15g/L Sodium Phosphate Dibasic 0.2g/L Potassium Phosphate Monobasic 0.2g/L Potassium Chloride

	8g/L Sodium Chloride Water
FACS buffer	PBS-D with 0.1% BSA
Blocking buffer	PBS-D with 1.0% BSA
PneumaCult EX PLUSTM Complete Medium for cell culture (SAEC/HBEC)	PneumaCult EX PLUS Basal Medium 98ml PneumaCult EX PLUS 50* supplement 2ml PEST 1ml 96 µg/ml Hydrocortisone stock solution 0.1ml
Medium for cell culture (primary human lung fibroblasts)	MCDB 131 medium 10% FCS in MCDB 131 medium 1% Penicillin/ Streptomycin in MCDB 131 medium Glutamine 2.0ng/ml basic-FGF in MCDB 131 medium 0.5ng/EGF in MCDB 131 medium 5µg/ml insulin in MCDB 131 medium
Medium for cell culture (16HBE14o-)	DMEM 10% FCS in DMEM 1% Penicillin/ Streptomycin in DMEM

2.1.5. Antibodies

Antibodies	**Company**	**Cat. number**
APC anti-human IgG Fc	Biolegend	410712
APC rat IgG2a isotype control	Biolegend	400512
Alexa Fluor® 488 anti-human IgM Antibody	Biolegend	314534
Alexa Fluor® 488 mouse IgG1 isotype control	Biolegend	400129

2.2. Methods

2.2.1. Subjects

All patients with COPD and healthy controls were recruited from the First Affiliated Hospital of Xiamen University, Xiamen, China. COPD was diagnosed according to the guideline proposed by the Global Initiative for Chronic Obstructive Lung disease (GOLD) [1]. In brief, the diagnosis of COPD was based on the presence of clinical symptoms and a ratio of FEV1/FVC < 70%, while severity of COPD was graded based on post-bronchodilator FEV1% predicted values, including GOLD 1 (FEV1%pred ≥80%), GOLD 2 (50% ≦ FEV1%pred <80%), GOLD 3 (30% ≦FEV1%pred <50%) and GOLD 4 (FEV1%pred <30%). The presence of emphysema and interstitial lung abnormalities (ILA) were assessed by using high-resolution computed tomography (HRCT) of the chest. Emphysema was diagnosed when more than 6% of low attenuation below –950 Hounsfield units were found [97]. ILA was diagnosed when non-dependent abnormalities affect more than 5% of any lung region in HRCT, including ground-glass or reticular abnormalities, lung distortion, traction bronchiectasis, honeycombing, and non-emphysematous cysts [98]. The normal control group was composed of healthy subjects who received annual routine physical examination. This study was conducted according to the 1964 Declaration of Helsinki and its subsequent amendments or similar ethical standards and approved by the Ethics Committee of the University Lübeck (AZ 17-131, July, 2017), Germany, and the Clinical Research Ethics Committee of the First Affiliated Hospital of Xiamen University (No. 2021064, June, 2021), China.

2.2.2. Data collection

The demographic and clinical data of COPD patients were obtained from medical records. Demographic data included age, gender, body mass index (BMI), smoking status, and smoking history, while clinical features consisted of parameters of lung function test such as FEV1/FVC1 ratio, FEV1% predicted, GOLD stage, emphysema, ILA and respiratory infection in 6 weeks. Demographic data of healthy subject was also obtained for the evaluation. Before proceeding with any analysis, all data were fully anonymized by removing personally identifiable information.

2.2.3. Preparation of serum samples

Peripheral blood samples were collected from all subjects with BD Vacutainer Plus Plastic tubes which were coated with silicone and micronized silica particles to accelerate clotting. Samples were then centrifuged at 1500 x g for 15 min at room temperature, and supernatants were aliquoted and immediately stored at –80ºC for further evaluation.

2.2.4. Isolation of human bronchial epithelial cells (HBEC)

Primary human bronchial epithelial cells (HBEC) were isolated from surgical biopsies of the bronchus (Tumor-free tissues) of a patient with lung cancer as described [99]. Briefly, bronchial tissue separated from the lung was transferred to a tube containing 20 ml of RPMI1640, supplemented with 2 mM L-glutamin, 1.4 mg/ml pronase and 0.1 mg/ml DNase I, and placed at 4–8°C for overnight incubation. The next day, Fetal calf serum (FCS) was added to 10% to stop the action of the protease. Subsequently, bronchial tissue was discarded, and the cell-containing supernatant was transferred through EASY strainer Cell Sieves (100 and 40 µm) to new tubes and centrifuged 10 min at 600 × g. Epithelial cells were then collected into a new tube, counted and suspend in PneumaCult EX PLUS™ Complete Medium.

2.2.4. Cell culture

To culture primary HBEC, cells suspended in PneumaCult EX PLUS^{TM} Complete Medium were seeded on 100mm culture plates precoated with collagen I, and incubated at 37°C in a humidified 5% CO_2 incubator Primary human lung fibroblasts that were generously provided by Prof. Silke Meiners were cultured in MCDB 131 medium, containing 10% FCS, 1% Penicillin/ Streptomycin, glutamine, 2.0ng/ml basic-FGF, 0.5ng/ml EGF and 5µg/ml insulin. Cells were cultured at 37°C in a humidified 5% CO_2 incubator. The 16HBE14o- cell line was cultured in DMEM containing 10% FCS and 1% Penicillin/ Streptomycin in T-25 flask or T-75 flask at 37°C in in a humidified 5% CO_2 incubator. For all three types of cells, the medium was changed every 2–3 days until cells reached desired confluency.

2.2.5. Flow cytometry

Binding IgG and IgM autoantibodies from COPD and HC sera to pulmonary cells was determined using flow cytometry. Cultured cells were used for the determination when they reached 80-90% confluence. Cells were detached from the flask or dish with the Accutase™ Cell Detachment Solution and used for flow cytometry analysis. The detailed procedure of the analysis is summarized the table below.

1.1	suspend the cells in PBS-D +Life/Dead (1:1000 dilution) +Fc block (1:200 dilution)	20 min, 4°C
1.2	Cell suspension was incubated with sera (1:100 final dilution)	20 min, 4°C
1.3	Cells were washed three times with 200 µl FACS	5 min, 450g, 4°C
1.4	Cells were incubated with secondary antibodies and isotype controls (1:100 dilution)	20 min, 4°C
1.5	Cells were washed three times with 200 µl FACS	5 min, 450g, 4°C
1.6	Cells were fixed by 50 µl 4% PFA	30 min, 4°C
1.7	Cells were washed three times with 200 µl FACS	5 min, 450g, 4°C
1.8	Cells were filled up with 150µl FACS-Buffer in each well	-
1.9	Measurement	-

Briefly, after washing with cold FACS buffer, cells were suspended in 50 µl PBS with Fc block and live dead dye and incubated for 20 minutes at 4°C. Subsequently, 50 µl of COPD and HC sera (1:50 diluted with FACS buffer) were used for a further incubation at 4°C for 20 min. After that, cells were washed with FACS buffer three times, incubated with secondary antibody mixture of APC anti-

human IgG Fc and Alexa Fluor® 488 anti-human IgM or their isotype controls for 20 minutes at 4°C. Following the incubation, cells were washed three times with FACS buffer, resuspended in µ 100 µl FACS buffer, and fixed by adding 50 µl 4% PFA. Finally, samples were measured using a flow cytometer (BD FACSLSR II, USA), and the data were analyzed by FACS Express software (De Novo, USA, version 7.12.0020). Mean fluorescence intensity (MFI) values of the Alexa Fluor® 488 anti-human IgM and APC anti-human IgG Fc were regarded as levels of IgM and IgG autoantibodies, respectively.

2.2.6. Confocal microscopy

Immunofluorescence staining was employed to visualize the binding of IgG and IgM autoantibodies to the extracellular membrane of cells. Cells were seeded in 6-channel µ-Slides (µ-Slide VI 0.4; Ibidi, Martinsried, Germany), and incubated in the incubator at 37°C in a humidified 5% CO_2 incubator. After forming tight monolayers, the cells were washed with phosphate-buffered saline (PBS), and then blocked with 100 µl blocking buffer (1% BSA in PBS) for 45 minutes at room temperature (RT). Following washing with PBS, cells were incubated with COPD patient or healthy control sera (1:100 dilution in blocking buffer) for 1 hour at 4°C. Subsequently, cells were washed with PBS three times and incubated with 60 µl secondary antibody mixture (APC-anti-human IgG Fc / Alexa Fluor® 488 anti-human IgM or APC-mouse IgG2a isotype control / Alexa Fluor® 488-mouse IgG1 isotype control) in blocking buffer for 1 hour at 4°C. Finally, cells were washed with PBS three times and mounted with ProLong® Gold antifade reagent with DAPI (Thermo Fisher Scientific, USA). The Leica TCS SP5 confocal microscope (Leica, Germany) was used for visualizing the binding of IgG and IgM autoantibodies. The detailed procedure of the assay is summarized in the table below.

1.1	Each channel of µ-Slide VI 0.4 was coated with 5 µg/ cm² collagen I	1h, at 37 °C
1.2	30µl of cell suspension was seeded in each channel	Until forming tight monolayers, at 37 °C
1.3	Cells were washed with PBS and blocked with blocking buffer	45min, RT
1.4	Cells were washed with PBS and incubated with sera (1:100 dilution)	1h, 4°C
1.5	Cells were washed three times with PBS	-
1.6	Cells were incubated with 60 µl antibody combination	1h, 4°C
1.7	Cells were washed three times with PBS	-
1.8	Cells were mounted with ProLong® Gold antifade reagent with DAPI	-
1.9	Binding of autoantibodies were visualized by confocal microscopy	

2.2.7. Statistical analysis

Statistical analysis was conducted using GraphPad Prism Software (version 8.0.1). The D'Agostino Pearson test was performed to determine if variables follow a normal distribution. Quantitative variables with normal distribution were presented as mean ± standard deviation (SD), while variables not normally distributed were presented as median (Q1–Q3). For quantitative data that follow a normal distribution, the student t-test was used to determine statistical significance, otherwise, the Mann–Whitney U test was applied. For qualitative variables, statistical significance was determined using the chi-squared test, chi-squared test for trend, or Fisher's exact test. A P-value less than 0.05 was considered statistically significant.

3. Results

3.1. Demographic and clinical features of COPD patients and healthy controls

A total 147 patients with COPD patients and 64 healthy controls were recruited for the current study. Table 1 provides a summary of the demographic and clinical features of both patients and controls. The mean age of patients with COPD was 68.3±9.1 years (mean ± SD) which was comparable to the age of control subjects (65.6±10.7 years). Due to the male predominance in smokers in China [100], the COPD group consisted of 145 males and 2 females. To ensure sex-matched controls, 63 male and 1 female healthy subjects were recruited. Consistent with previous finding [101], the body mass index (BMI) of COPD patients was significantly lower than that of healthy subjects (20.8±3.5 vs 23.1±3.0, P=0.0003). As expected, majority of patients with COPD were smokers (93.9%), including ex-smokers and current smokers, while only 53.1% of healthy subjects were smokers. Additionally, the median pack-years was 40 in COPD patients, significantly higher than the value in controls.

Out of 147 COPD patients, 95 (64.6%) developed emphysema, 36 (24.7%) showed ILA, and 36 (24.7%) experienced a respiratory infection within 6 weeks prior to sample collection. In terms of spirometry parameters, the mean FEV1/FVC ratio in patients with COPD was 50%, and median of FEV1% predicted was 42%, both significantly lower than their corresponding values in control subjects. Following the GOLD guideline, 8 (5.4%), 44 (29.9%), 60 (40.8%) and 35 (23.8%) patients were classified into GOLD grades of 1, 2, 3 and 4, respectively.

Table 2. Demographic and clinical features of COPD patients and healthy controls (HC)

		COPD (n=147)	HC (n=64)	*P* value
Age		68±9	65.6±10.7	0.0608
Gender	Male	145 (98.6%)	63 (98.4%)	>0.9999
	Female	2 (1.4%)	1 (1.6%)	
BMI		20.8±3.5	23.1±3.0	0.0003
Current smokers		80 (54.4%)	26 (40.6%)	<0.0001
Ex-smokers		58 (39.5%)	8 (12.5%)	
Never smokers		9 (6.1%)	30 (46.9%)	
Pack-years		40（30-60）	30（16-47）	0.0097
Clinical features	Emphysema	95 (64.6%)	/	/
	FEV1/FVC	50.5±10.3	8.7	<0.0001
	FEV1%predicted	42（32-54）	99（90-103）	<0.0001
	GOLD 1	8 (5.4%)	/	/
	GOLD 2	44 (29.9%)	/	/
	GOLD 3	60 (40.8%)	/	/
	GOLD 4	35 (23.8%)	/	/
	Respiratory infection in 6 weeks §	36 (24.7%)	/	/
	ILA	36 (24.7%)	/	/

Quantitative data in a normal distribution are presented as mean ± SD, and the student t-test was used to determine statistical significance. Quantitative data that do not follow normal distribution are presented as median (Q1–Q3), and the Mann–Whitney U test was applied. For qualitative variables, they are presented as number (%), and statistical significance was determined using the chi-squared test or Fisher's exact test. § Lack of information of one patient. BMI: body mass index; FEV1: expiratory volume in 1 second; FVC: forced vital capacity; GOLD: global Initiative for chronic obstructive lung disease; ILA: interstitial lung abnormality.

3.2. Autoantibodies against lung cells in COPD patients and HC

3.2.1. Autoantibodies against primary human lung fibroblasts in COPD patients and HC

I initially investigated whether patients with COPD developed autoantibodies against cell surface antigens of primary lung fibroblasts. The cells were incubated with serum samples diluted at 1:100, followed by staining with secondary antibodies, APC anti-human IgG and Alexa Fluor® 488 anti-human IgM. The binding of IgG and IgM autoantibodies to the cells was determined by flow cytometry and expressed as the mean fluorescence intensity (MFI).

Figure 1A illustrates the gating strategy used to define single live cells for quantifying the binding MFI of IgG and IgM autoantibodies. As depicted by representative samples in Figure 1B and 1C, both fibroblasts incubated with COPD sera and those incubated with control sera exhibited comparable MFI of IgG and IgM autoantibodies to fibroblasts incubated with only secondary antibodies, suggesting little to no levels of IgG and IgM autoantibodies against extracellular membrane antigens of fibroblasts. Analysis of all COPD patient and healthy control samples revealed that the majority of samples exhibited a background level of MFI for IgG and IgM, with no significant difference observed between the two groups (Figure 1D, E).

3.2.2. Autoantibodies against primary HBEC in COPD patients and HC

Utilizing the same method, I subsequently investigated whether patients with COPD developed autoantibodies against cell surface antigens of primary HBEC. The gating strategy for the analysis is illustrated in Fig. 2A. In comparison to cells stained only with secondary antibodies, cells preincubated with human serum samples exhibited an increased MFI in both APC and Alexa Fluor 488 channels (Fig. 2B, C), indicating the presence of both IgG and IgM anti-HBEC autoantibodies. Further quantified analysis demonstrated that levels of anti-HBEC IgG antibodies in patients with COPD were significantly higher than those in control subjects (224.4 *vs* 186.2, P=0.0082) (Fig. 2D). Similarly, akin to IgG autoantibodies against HBEC, levels of anti-HBEC IgM antibodies were also

significantly elevated in COPD patients compared with healthy controls (88.1 *vs* 73.3, *P*=0.001) (Fig. 2E)

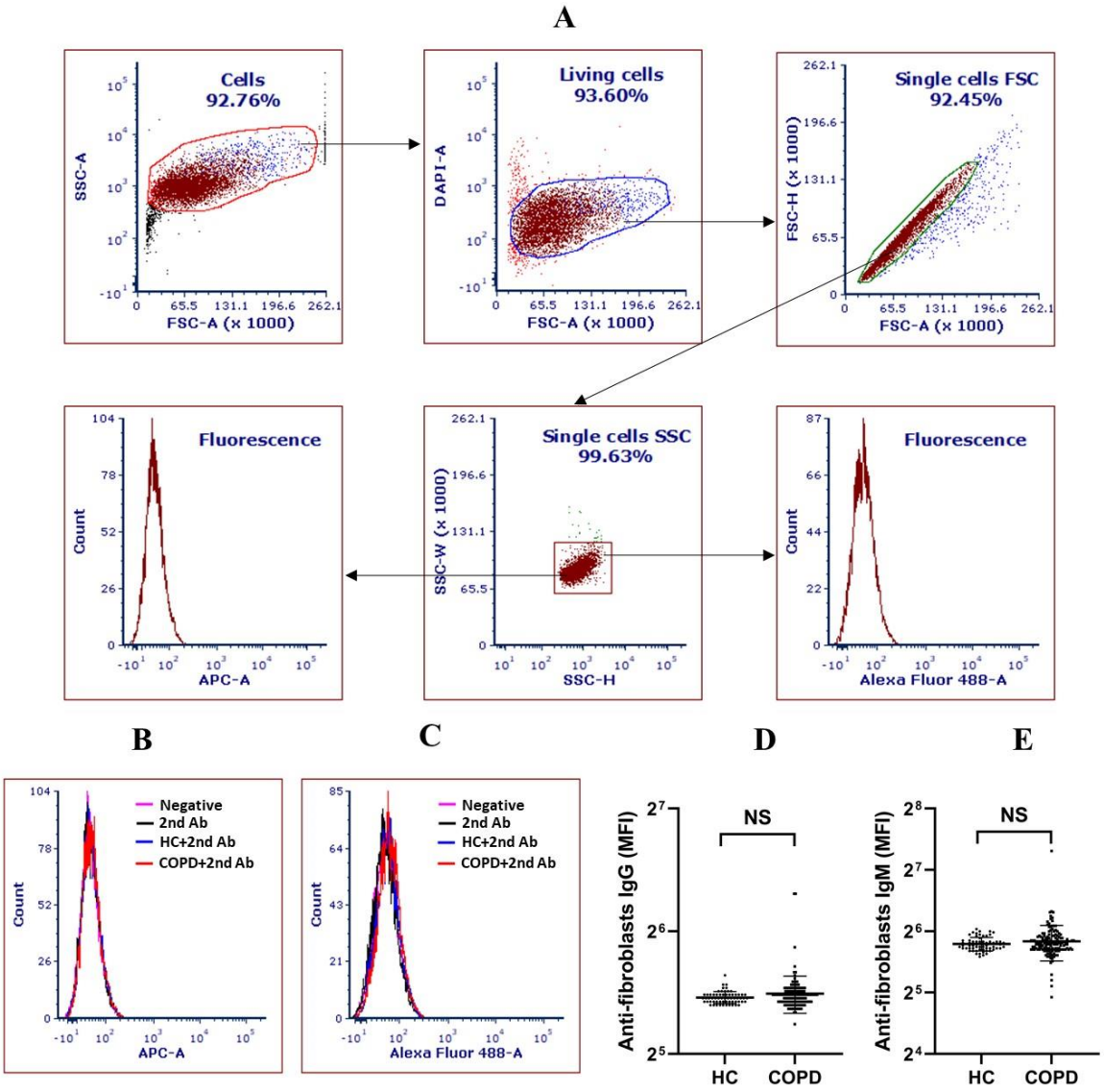

Figure 1. Autoantibodies against primary human lung fibroblasts in COPD patients and HC. A. Gating strategy for data analysis. Single live cells were gated for the analysis of binding of IgG (APC) and IgM (Alexa Fluor 488) autoantibodies against cell surface antigens of fibroblasts. Bindings of IgG and IgM autoantibodies were quantified as mean fluorescence intensity (MFI). Representative histogram of flow cytometry analysis of bindings of IgG (B) and IgM (C) autoantibodies. The violet, black, blue and red lines stand for negative control (cells without incubation with second antibodies), 2^{nd} Ab (cells incubated only with secondary antibodies), HC+2^{nd} Ab (cells incubated with control sera and secondary antibodies) and COPD + 2^{nd} Ab (cells incubated with COPD sera and secondary antibodies), respectively. Comparison of IgG (D) and IgM (E) autoantibodies between COPD patients and healthy controls (HC). Statistical analysis was performed using the Mann-Whitney U test.

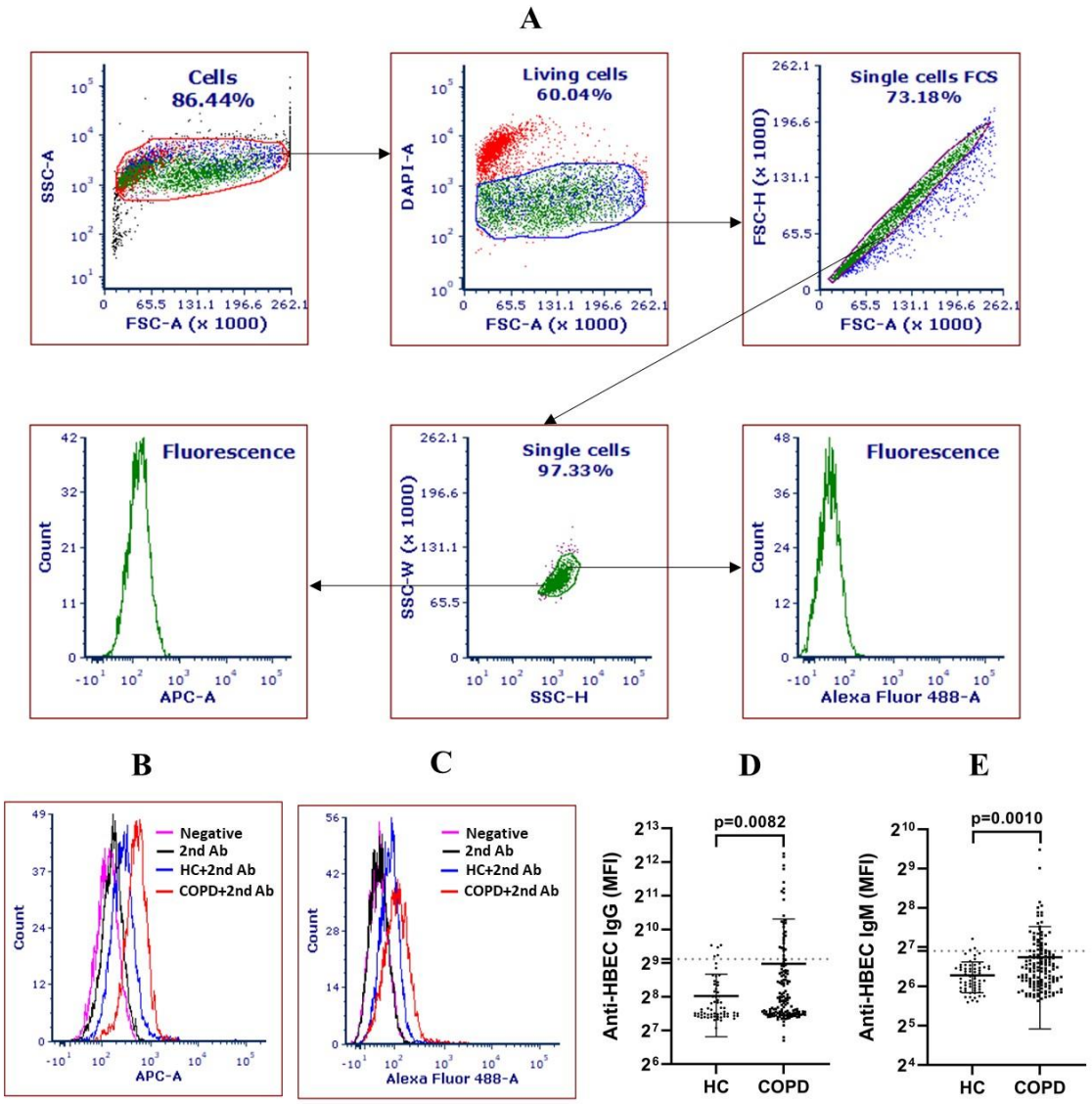

Figure 2. Autoantibodies against primary human bronchial epithelial cells (HBEC) in COPD patients and HC. Gating strategy for data analysis (A). Single live cells were gated for the analysis of binding of IgG (APC) and IgM (Alexa Fluor 488) autoantibodies against cell surface antigens of primary HBEC. Bindings of IgG and IgM autoantibodies were quantified as mean fluorescence intensity (MFI). Representative histogram of flow cytometry analysis of bindings of IgG (B) and IgM (C) autoantibodies against HBEC. The violet, black, blue and red lines stand for negative control (cells without incubation with second antibodies), 2^{nd} Ab (cells incubated with only secondary antibodies), HC+2^{nd} Ab (cells incubated with control sera and secondary antibodies) and COPD + 2^{nd} Ab (cells incubated with COPD sera and secondary antibodies), respectively. Comparison of IgG (D) and IgM (E) autoantibodies against HBEC between COPD patients and healthy controls (HC). Statistical analysis was performed by using the Mann-Whitney U test. Significant *P* values (<0.05) are shown.

3.2.3. Autoantibodies against primary HBEC do not react to 16HBE14o cell line

Given the association of autoantibodies against primary HBEC with COPD, in the next step I determined whether the autoantibodies reacted to 16HBE14o, a human bronchial epithelial cell line originally obtained from a 1-year old male and immortalized with the origin-of-replication defective SV40 plasmid [102]. Three COPD serum samples that were positive for both IgG and IgM autoantibodies against primary HBEC in the experiment described in 3.2.3 section were selected for the investigation.

As expected, primary HBEC incubation with the three serum samples led to a shift in the MFI of the APC channel compared to cells stained only with secondary antibodies (Fig. 3A), confirming the presence of IgG autoantibodies against primary HBEC. In contrast, such a shift was not observed in the 16HBE14o cell line (Fig. 3B), suggesting that IgG autoantibodies against primary HBEC do not bind to the 16HBE14o cell line. As for IgM autoantibodies against primary HBEC, similar results were obtained. As shown in Figure 3C and D, incubation with the three COPD serum samples led to the binding of IgM autoantibodies to primary HBEC but not to the 16HBE14o cell line. Therefore, these results demonstrate that autoantibodies against cell surface antigens of primary HBEC do not react to the 16HBE14o cell line.

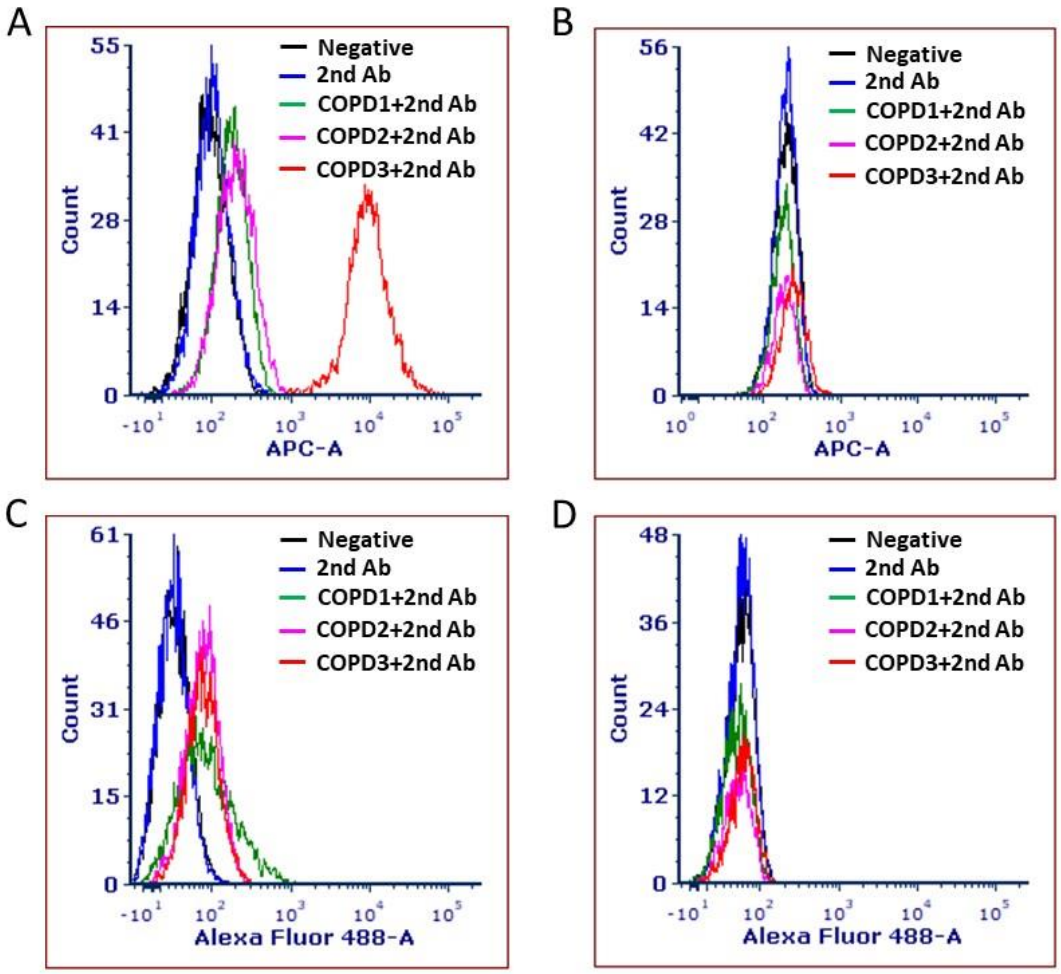

Figure 3. Binding of IgG and IgM autoantibodies to HBEC and 16HBE14o-. Three COPD serum samples that were positive for both IgG and IgM autoantibodies against primary HBEC were selected for the experiment. Serum samples were incubated with primary HBEC or 16HBE14o cell line, and the binding of IgG and IgM autoantibodies against cell surface antigens was detected using flow cytometry. Histogram of flow cytometry analysis of bindings of IgG (A) and IgM (C) autoantibodies against primary HBEC. Representative histogram of flow cytometry analysis of bindings of IgG (B) and IgM (D) autoantibodies against 16HBE14o.

3.2.4. Detection of the binding of autoantibodies against primary HBEC using confocal microscopy

To further confirm the presence of autoantibodies against primary HBEC in patients with COPD, I visualized the binding of IgG and IgM autoantibodies to the cells using confocal microscopy. For this purpose, intact primary HBEC cells were incubated with serum samples from COPD patients or healthy subjects, followed by incubation of secondary antibodies of APC anti-human IgG and Alex Fluor488 anti-human IgM. Using this indirect immunofluorescence staining, the binding of IgG and IgM autoantibodies was detected by confocal microscopy.

As shown by representative micrographs in Figure 4A, primary HBEC incubated with COPD serum showed strong binding of IgG antibodies, while cells incubated with control serum did not, confirming the presence of IgG autoantibodies against HBEC in COPD. Additionally, the binding of IgG antibodies to the cells showed a ring-like pattern, suggesting that IgG autoantibodies react to cell surface antigens. The binding of IgM autoantibodies to primary HBEC is shown in Figure 4B. Similar to findings in IgG autoantibodies, sera from COPD patients, but not that from healthy control, showed IgM reactivity to cell surface antigens of primary HBEC. In summary, the presence of IgG and IgM autoantibodies against cell surface antigens of primary HBEC was confirmed by confocal microscopy.

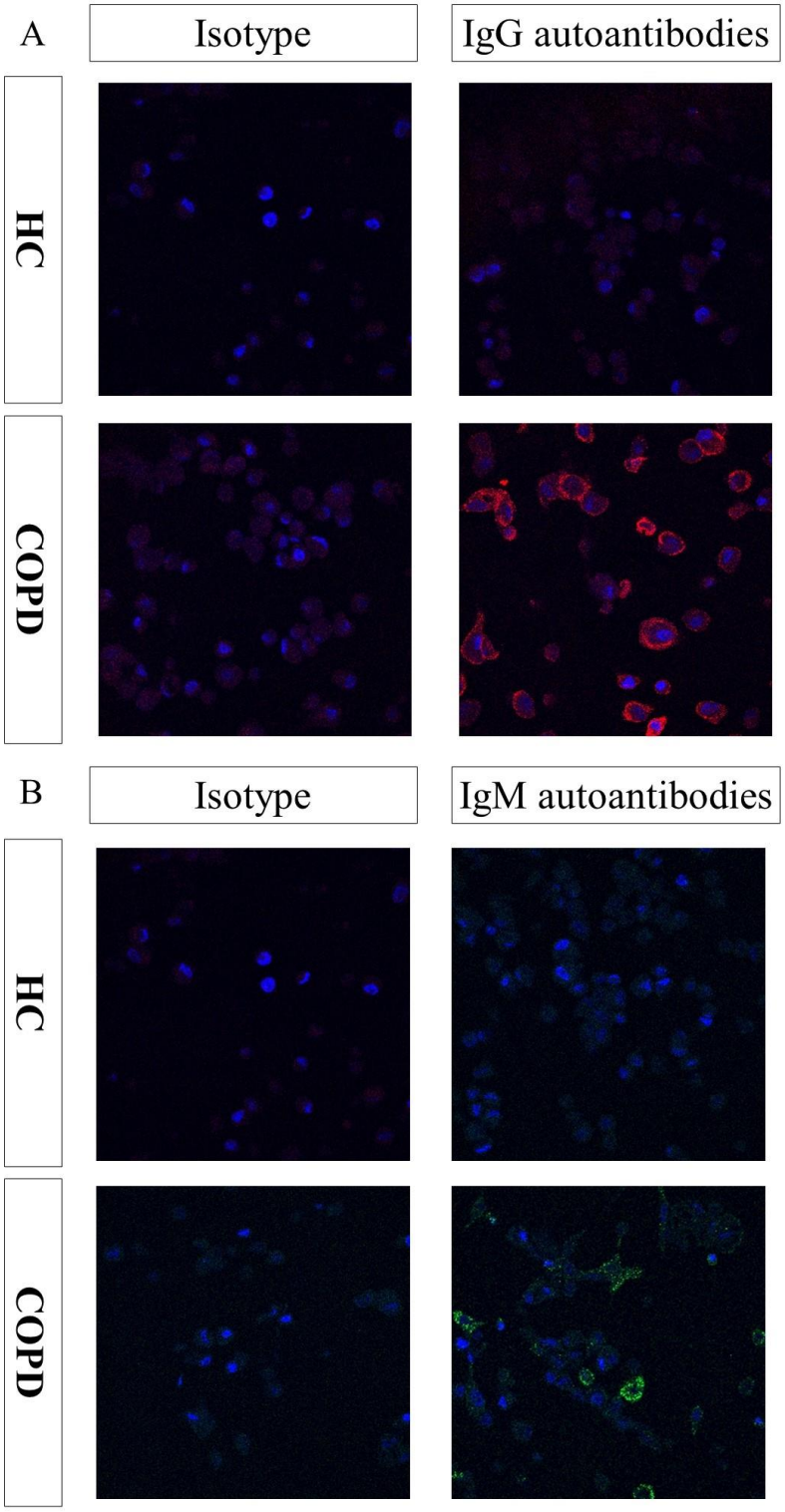

Figure 4. Binding of IgG and IgM autoantibodies to primary HBEC detected by confocal microscopy. Intact primary HBEC were incubated with serum samples from COPD patients or healthy subjects, and the binding of IgG and IgM autoantibodies was visualized using of APC anti-human IgG (red) and Alex Fluor488 anti-human IgM (green), respectively. DAPI (blue) was used to nuclear morphology. Representative confocal microscopy images at magnifications (630x) of the binding of IgG (A) and IgM (B) autoantibodies to primary HBEC are shown.

3.3. Association of autoantibodies against primary HBEC with demographic and clinical features of COPD patients

Given the association of autoantibodies against primary HBEC with COPD, the next investigation aimed to determine whether these autoantibodies were associated with the clinical and demographic characteristics of patients with COPD.

3.3.1. Association of autoantibodies against primary HBEC with emphysema

COPD is primarily attributed to two pathological conditions: chronic bronchiolitis and emphysema. In this study, all COPD patients exhibited chronic bronchiolitis, with 64.6% (95/147) of patients also developing emphysema. Considering the reported association between autoimmunity and emphysema [64], a stratified analysis was conducted by categorizing COPD patients into two subgroups: those without emphysema and those with emphysema.

As shown in Figure 5A, levels of IgG autoantibodies against primary HBEC in COPD patients without emphysema, but not in those without emphysema, were significantly higher than that in HC (P=0.0174). In contrast, levels of IgM autoantibodies against primary HBEC in COPD patients with emphysema, but not those without emphysema, were significantly elevated compared to HC (Fig. 5B, P=0.0010). However, no significant difference was observed in levels of IgG or IgM autoantibodies against primary HBEC between the two subgroups of COPD patients.

To calculate the prevalence of the autoantibodies, cut-off values were set as 2 standard deviations (SD) above the mean of MFI values in healthy controls. With these cut-off values, prevalence of IgG autoantibodies against primary HBEC in healthy subjects, COPD patients with emphysema, COPD patients without emphysema were calculated as 7.9%, 15.8% and 25.0%, respectively. Additionally, prevalence of IgM autoantibodies in the three groups were 4.8%, 26.3% and 19.2%, respectively (Fig. 5C, D).

Consistent with the results obtained from the analysis of autoantibody levels, the prevalence of anti-HBEC IgG autoantibodies in COPD patients without emphysema was significantly higher than that in HC (Fig. 5C, *P*=0.0189), and the prevalence of anti-HBEC IgM autoantibodies in both COPD patient without emphysema (*P*=0.0186) and COPD patients with emphysema (*P*=0.0005) were significantly higher than that in HC (Fig. 5D). Additionally, no difference in autoantibody prevalence was observed between the two groups of COPD patients.

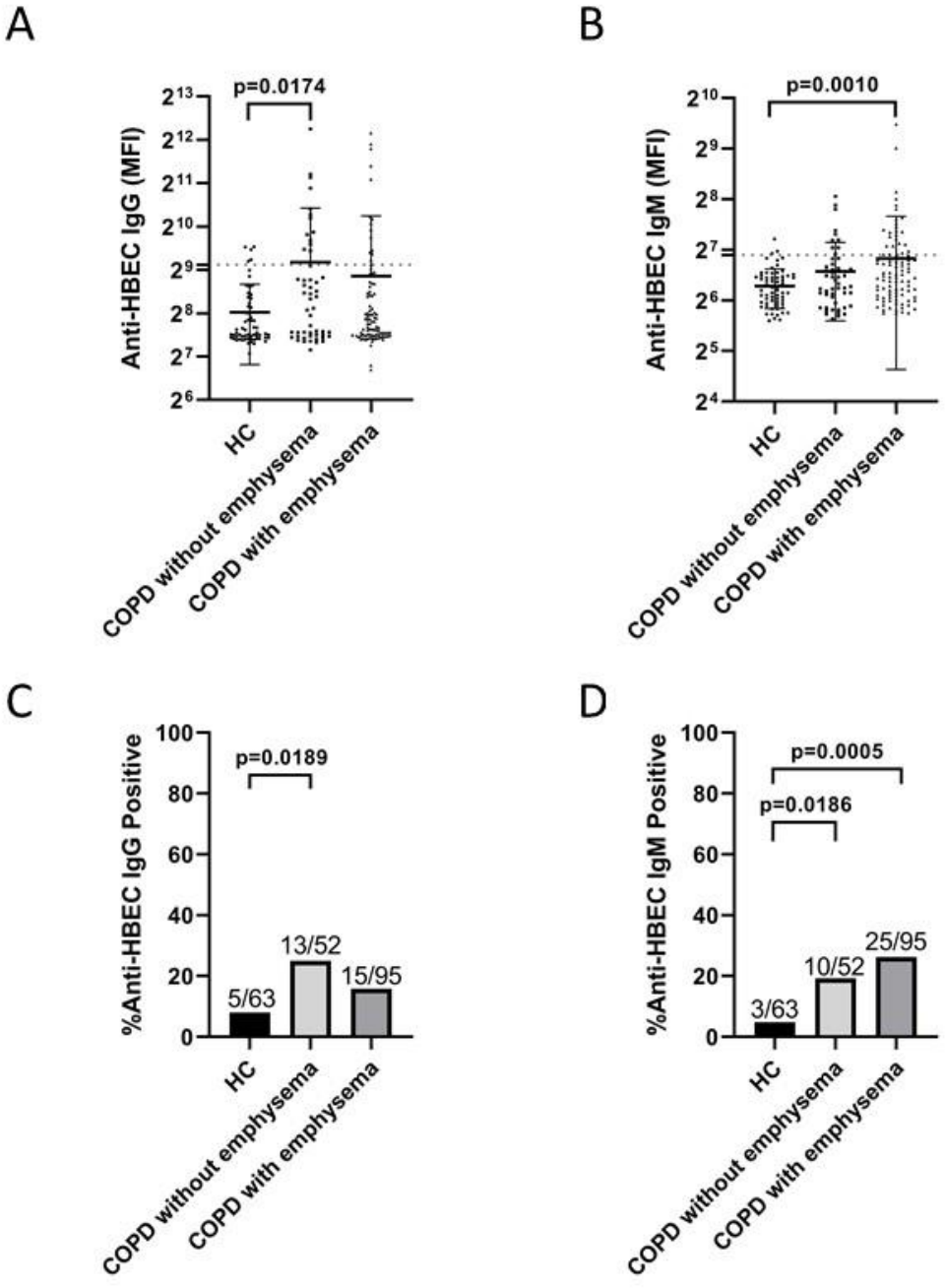

Figure 5. Analysis of association of emphysema with autoantibodies against primary HBEC. Comparison

of levels of IgG (A) and IgM (B) autoantibodies against HBEC among HC, COPD patients with and without emphysema. Cut-off values (dashed lines in A and B) used to define positivity of autoantibodies were set as two standard deviations above the mean of MFI values in HC. Prevalence of IgG (C) and IgM (D) autoantibodies against HBEC in HC and COPD patients with and without emphysema. The number of positive subjects and the number of total subjects in each group are depicted above corresponding bars. Statistical significance of the comparisons was determined using Kruskal-Wallis test (A, B) and Fisher exact test or Chi-square test (C, D). Significant *P* values (<0.05) are shown.

3.3.2. Comparison of autoantibodies against primary HBEC with GOLD stage in COPD patients

In 2016, Cheng and colleagues reported a positive correlation between the presence of IgG and IgA autoantibodies against HBEC and the GOLD stage of COPD [74], suggesting an association of autoantibodies against HBEC with disease severity. However, this interesting finding is not in line with the results from a previous study, making this issue controversial. To clarify the relationship between autoantibodies against HBEC and severity of COPD, I performed stratified analysis by dividing COPD patients into 4 subgroups according to GOLD criteria.

As depicted in Figure 6A, although there was a tendency for patients with moderate to very severe COPD (GOLD stage 2, 3, 4) to exhibit higher levels of IgG autoantibodies against HBEC compared to healthy controls and patients with mild COPD (GOLD stage 1), no significant difference was observed between HC and the four COPD subgroups. Similarly, there was no significant increase in the prevalence of IgG autoantibodies against HBEC with increasing GOLD stage (Fig. 6C). Regarding levels of IgM autoantibodies against HBEC, a significant difference was observed between patients with very severe COPD (GOLD stage 4) and HC (Fig. 6B, P=0.0023). Moreover, the prevalence of IgM autoantibodies against HBEC tended to increase significantly as the GOLD stage increased (Fig. 6D, P=0.0008).

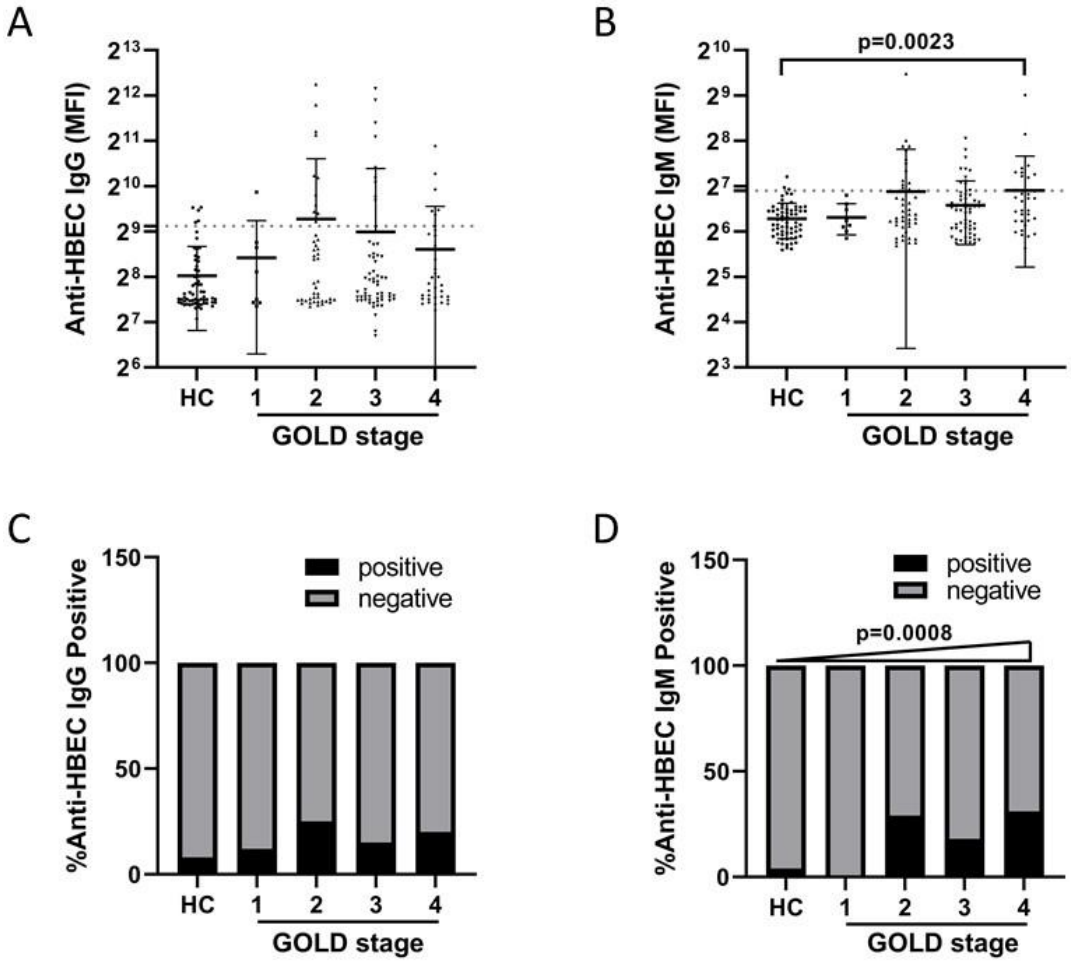

Figure 6. Analysis of the association of GOLD stages with autoantibodies against primary HBEC. Comparison of serum levels of IgG (A) and IgM (B) autoantibodies against HBEC between healthy controls (HC) and subgroups of COPD patients categorized according to GOLD criteria. Additionally, the comparison of the prevalence of IgG (C) and IgM (D) autoantibodies against HBEC in HC and subgroups of COPD patients categorized according to GOLD criteria. Statistical analysis was conducted using the Kruskal-Wallis test for (A) and (B), and the Chi-square test for trend for (C) and (D). Significant *P* values (<0.05) are indicated.

3.3.3. Analysis of correlation between autoantibodies against primary HBEC and lung function in COPD patients

Impaired lung function is a hallmark of COPD. There is controversy regarding the association between autoantibodies and lung function in COPD patients [77, 82]. To investigate this issue, I assessed the correlation between autoantibodies against HBEC and two parameters of lung function

tests, FEV1/FVC and FEV1% predicted, in COPD patients. As shown in Figures 7A and B, neither IgG nor IgM autoantibodies against HBEC were significantly correlated with the FEV1/FVC ratio in patients with COPD. Similarly, no significant correlation was observed between FEV1% predicted and the levels of IgG or IgM autoantibodies against HBEC (Figures 7C and D). Therefore, these results demonstrate that autoantibodies against HBEC are not correlated with parameters of lung function tests in COPD.

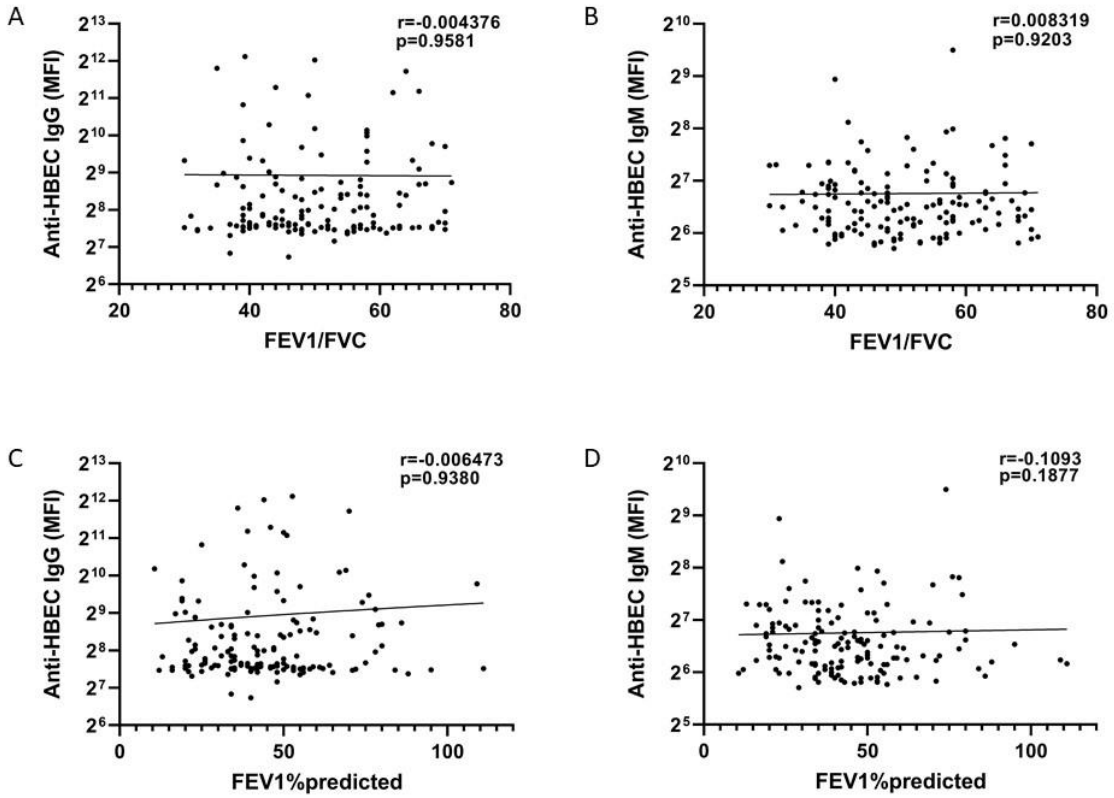

Figure 7. Analysis of correlation between lung functional test parameters and autoantibodies against primary HBEC in COPD patients. A. Correlation analysis of FEV1/FVC with IgG (A) and IgM (B) autoantibodies against HBEC. Correlation analysis of FEV1%predicted with IgG (C) and IgM (D) autoantibodies against HBEC. Spearman's rank correlation coefficient (r) and *P* values are shown.

3.3.4. Association of autoantibodies against primary HBEC with smoking status in COPD

It is widely believed that cigarette smoking promotes autoimmunity and is associated with increased risk of multiple autoimmune diseases [103]. However, it is unclear whether cigarette smoking is

associated with autoantibodies against HBEC. In the current study, 147 patients with COPD consisted of 138 smokers (including 80 current smokers and 58 ex-smokers) and 9 never smokers. The presence of autoantibodies against HBEC in COPD allows me to evaluate the association of the autoantibodies with cigarette smoking. For this purpose, stratified analysis was performed by dividing COPD patients into two subgroups: smokers and never smokers. As shown in Figure 8A, levels of IgG autoantibodies against HBEC in smokers were significantly higher than those in never smokers (P=0.0133). By contrast, no significant difference in anti-HBEC IgM autoantibodies was observed between never smokers and smokers in COPD patients (Fig. 8B). This finding was confirmed by the analysis of the prevalence of those autoantibodies in the two subgroups of patients with COPD. As shown in Figure 8C, the prevalence of IgG autoantibodies against HBEC in smokers was 20.3% (28/138), which was substantially higher, although not statistically significant, than the prevalence in never smokers (0%). By contrast, the prevalence of IgM autoantibodies against HBEC in smokers (23.8%) was comparable to that value in never smokers (22.2%) (Fig. 8D).

To further explore the association between autoantibodies against HBEC and cigarette smoking, a comparison of the autoantibodies between never smokers and smokers was also conducted in healthy subjects. As shown in Figure 8 E-H, there were no significant differences between smokers and never smokers in HC, regardless of the levels or prevalence of IgG or IgM autoantibodies against HBEC.

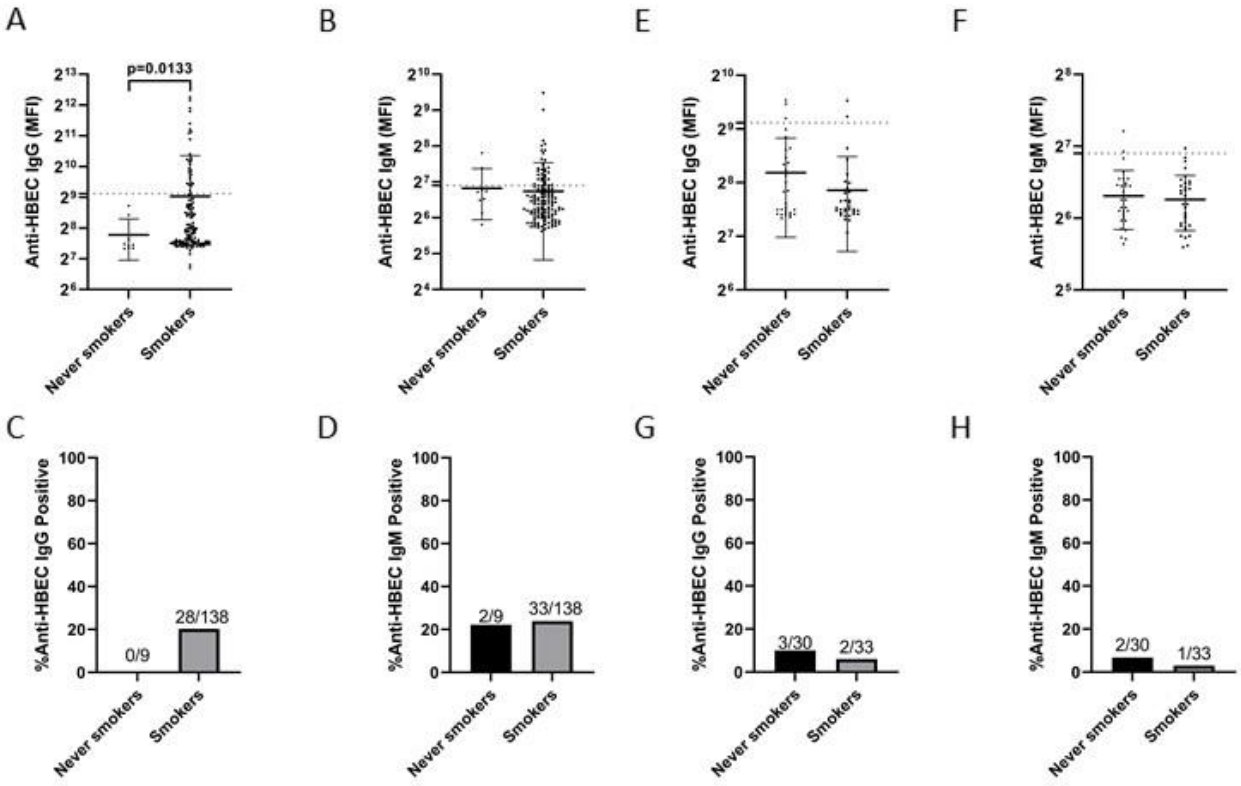

Figure 8. Analysis of the association between smoking status and autoantibodies against primary HBEC in COPD patients and healthy controls. Comparison of serum levels of IgG (A) and IgM (B) autoantibodies against HBEC between smokers and never smokers in COPD patients. Comparison of the prevalence of IgG (C) and IgM (D) autoantibodies against HBEC between smokers and never smokers in COPD patients. Comparison of serum levels of IgG (E) and IgM (F) autoantibodies against HBEC between smokers and never smokers in healthy controls. Comparison of the prevalence of IgG (G) and IgM (H) autoantibodies against HBEC between smokers and never smokers in healthy controls. Statistical analysis was performed using the Mann-Whitney U test (A, B, E, F) and Fisher exact test (C, D, G, H). Significant *P* values (<0.05) are indicated.

In the present study, 93.9% (138/147) of COPD patients and 53.1% (34/64) of healthy controls were smokers, and the difference was statistically significant. Given that autoantibodies against HBEC are associated with cigarette smoking status, I next determined whether the difference in autoantibodies against HBEC between COPD patients and healthy subjects was due to the difference in their smoking status. For this purpose, levels of autoantibodies against HBEC were compared between COPD smokers and healthy control smokers. As shown in Figure 9A, levels of anti-HBEC IgG autoantibodies were significantly higher in COPD smokers than in HC smokers (P=0.0020).

Although not significant, the prevalence of anti-HBEC IgG autoantibodies in COPD smokers (20.2%) was higher than that value in HC smokers (6.1%) (Fig. 9C). Similarly, both levels (*P*=0.0088) and prevalence (*P*=0.0062) of IgM autoantibodies against HBEC in COPD smokers were significantly higher than that in HC smokers (Fig. 9B, D). Therefore, these findings suggest that increased levels of autoantibodies against HBEC in patients with COPD compared with healthy subjects are not due to the difference in smoking status.

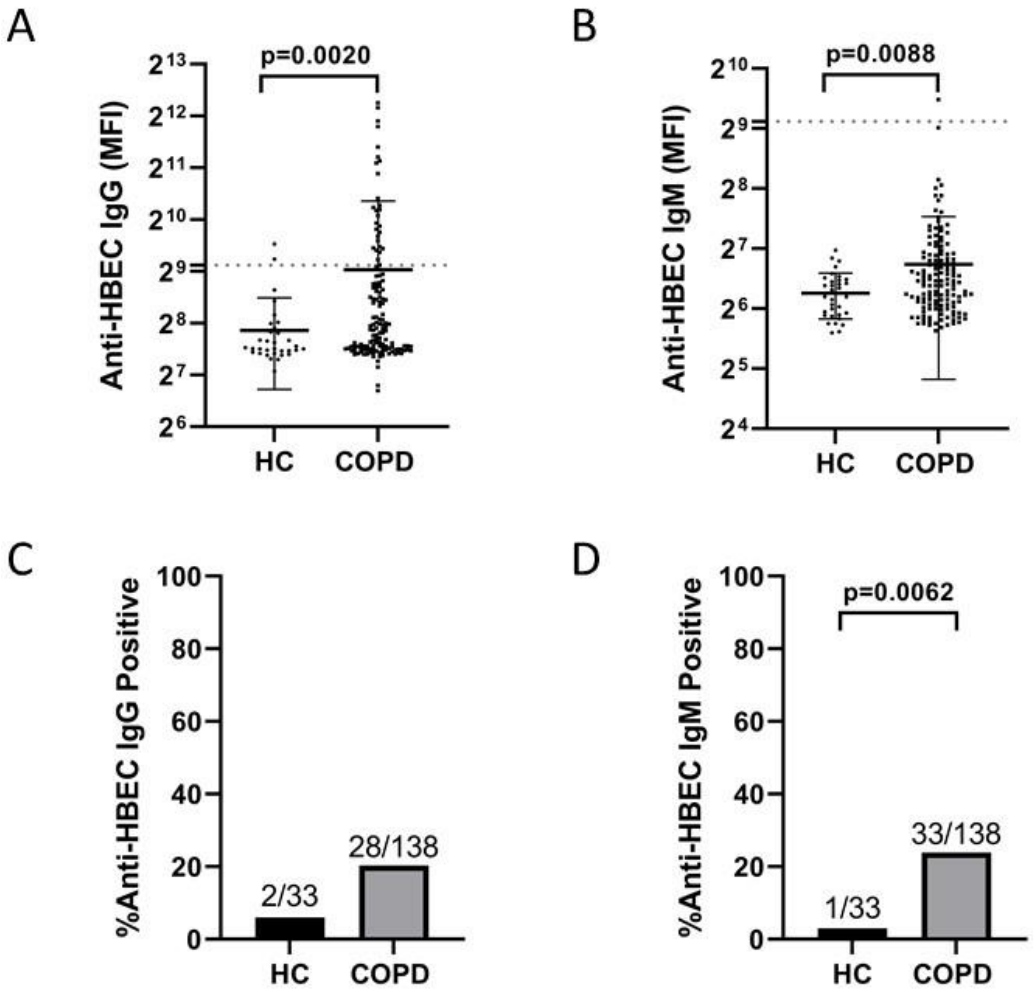

Figure 9. Comparison of autoantibodies against primary HBEC between healthy smokers and smoker with COPD. Serum levels of IgG (A) and IgM (B) autoantibodies against HBEC in healthy smokers and smokers with COPD. Prevalence of IgG (C) and IgM (D) autoantibodies against HBEC in healthy smokers and smokers with COPD. Statistical analysis was performed using the Mann-Whitney U test (A, B) and Fisher exact test (C, D). Significant *P* values (<0.05) are shown.

To further explore the association of cigarette smoking with autoantibodies against HBEC, I

evaluated the correlation between anti-HBEC antibodies and pack-year smoking history in smokers with COPD. As shown in Figure 10A, levels of IgG autoantibodies against HBEC were not correlated with pack-years (r=0.065, *P*=0.447). In addition, no significant correlation between levels of IgM autoantibodies against HBEC and pack-years was observed (r=0.163, *P*=0.056) (Fig. 10B).

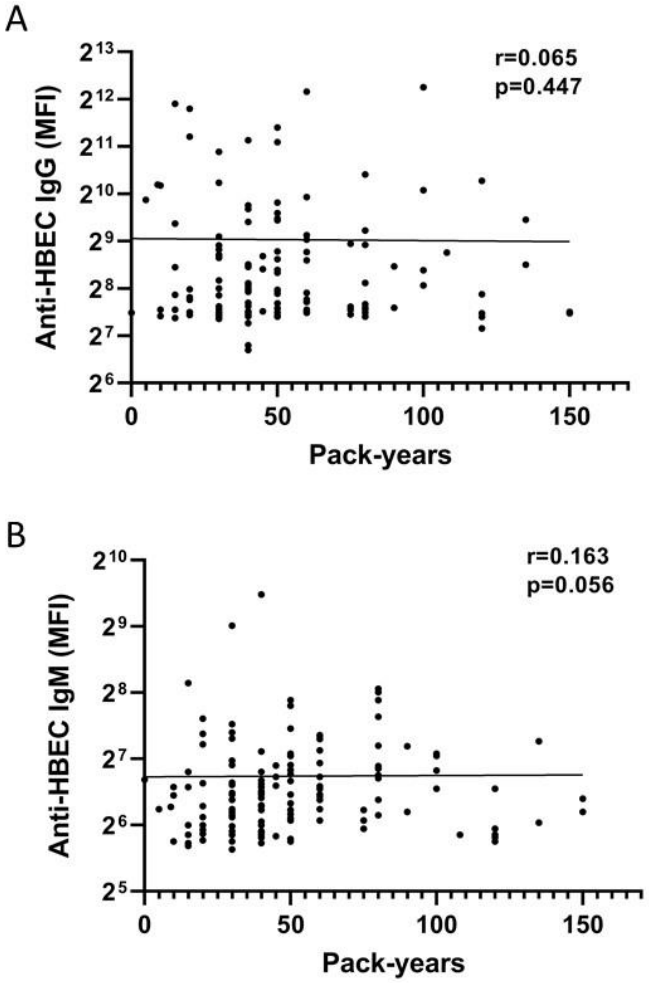

Figure 10. Relationship between autoantibodies against primary HBEC and pack-years in COPD smokers. Correlation analysis between pack-years and IgG (A) and IgM (B) autoantibodies against HBEC in COPD smokers. Spearman's rank correlation coefficient and *P* values are shown.

3.3.5. Correlation of autoantibodies against HBEC with age and BMI in patients with COPD

Given that age is a risk factor for the development COPD and associated with generation of autoantibodies [36, 73], I further determined the relationship between autoantibodies against HBEC and age in patients with COPD. As shown in Figure 11 A, no significant correlation was observed

between age of COPD patients and levels of IgG autoantibodies against HBEC (r=-0.025, p=0.768). Similarly, age of patients with COPD was not significantly correlated with IgM autoantibodies against HBEC (r=-0.039, *P*=0.637) (Fig. 11B).

It has been reported that low BMI is a hallmark of COPD patients and associated with severity of the disease [104]. To explore the relationship between BMI and autoantibodies against HBEC, Pearson's correlation analysis was performed. The results showed that BMI was not significantly correlated with levels IgG autoantibodies (r=-0.036, *P*=0.667) or IgM autoantibodies (r=-0.012, *P*=0.883) against HBEC in patients with COPD (Fig. 11C, D).

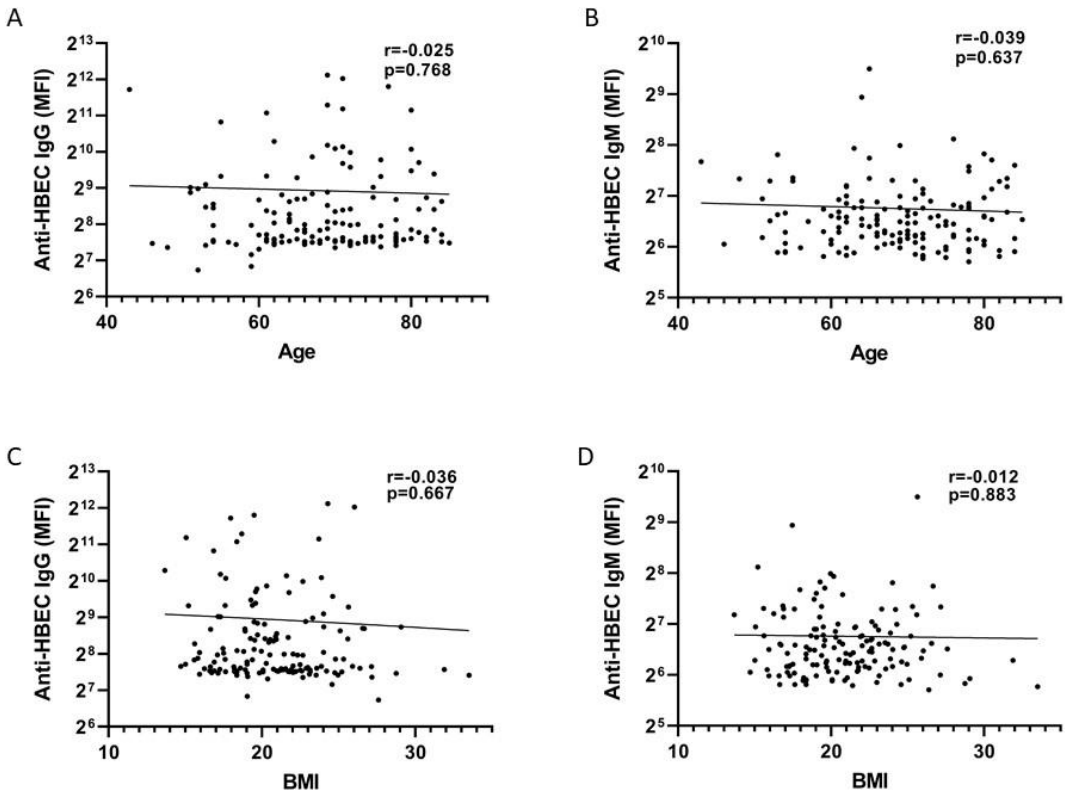

Figure 11. Relationship of autoantibodies against primary HBEC with age and BMI in COPD patients. Correlation analysis between age of COPD patients and IgG autoantibodies (A) and IgM autoantibodies (B) against HBEC. Correlation analysis between BMI and IgG (C) and IgM (D) autoantibodies against HBEC in COPD patients. Pearson's correlation coefficient and *P* values are shown.

3.3.6. Association of autoantibodies against HBEC with respiratory infection in COPD patients

In the present study, 24.7% (36/147) of patients with COPD had respiratory infections within 6 weeks prior to blood sampling. It has been reported that autoantibodies may be produced after respiratory infections [105, 106]. Therefore, I investigated whether recent respiratory infections were associated with the production of autoantibodies against HBEC in COPD patients. As shown in Figure 12A, levels of IgG autoantibodies in COPD patients with recent respiratory infections were comparable to those in COPD patients without recent respiratory infections. Furthermore, no significant difference was observed in levels of IgM autoantibodies against HBEC between the two subgroups of COPD patients (Fig. 12B). Consistent with this finding, the prevalence of both IgG and IgM autoantibodies against HBEC was comparable between COPD patients with and without recent respiratory infections (Fig. 12C, D).

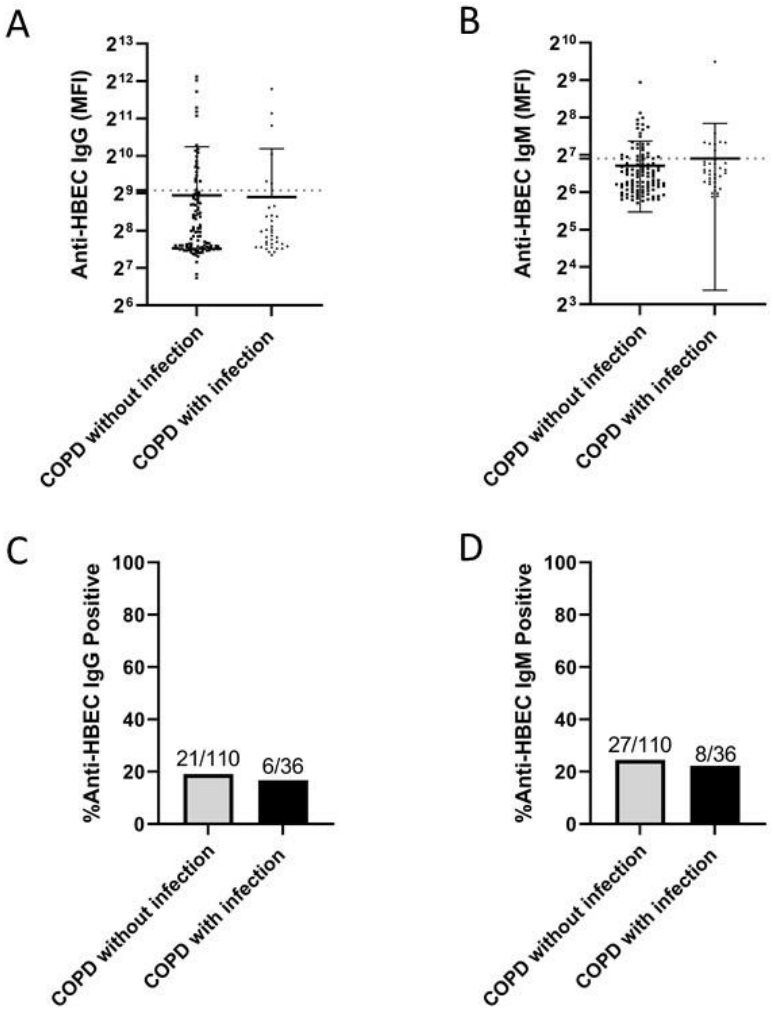

Figure 12. Analysis of the Association between Respiratory Infections and Autoantibodies against Primary HBEC in COPD Patients. Comparison of serum levels of IgG (A) and IgM (B) autoantibodies against HBEC between subgroups of COPD patients categorized according to the presence of respiratory

infections within 6 weeks prior to blood sampling. Prevalence of IgG (C) and IgM (D) autoantibodies against HBEC in subgroups of COPD patients categorized according to the presence of respiratory infections within 6 weeks prior to blood sampling. Statistical analysis was performed using the Kruskal-Wallis test (A, B) and Chi-square test (C, D).

3.3.7. Association of anti-HBEC autoantibodies with ILA in COPD patients

Since interstitial lung disease is associated with multiple autoimmune disorders [107-109], I next investigated whether autoantibodies against HBEC were associated with interstitial lung abnormalities (ILA) in patients with COPD. The results showed that levels of both IgG and IgM autoantibodies against HBEC were comparable between COPD patients with ILA and those without ILA (Fig. 13A, B). Additionally, no significant difference was observed between COPD patient with and without ILA in the prevalence of IgG or IgM autoantibodies against HBEC (Fig. 13C, D). Thus, the presence of ILA in patients with COPD was not associated with the production of autoantibodies against HBEC.

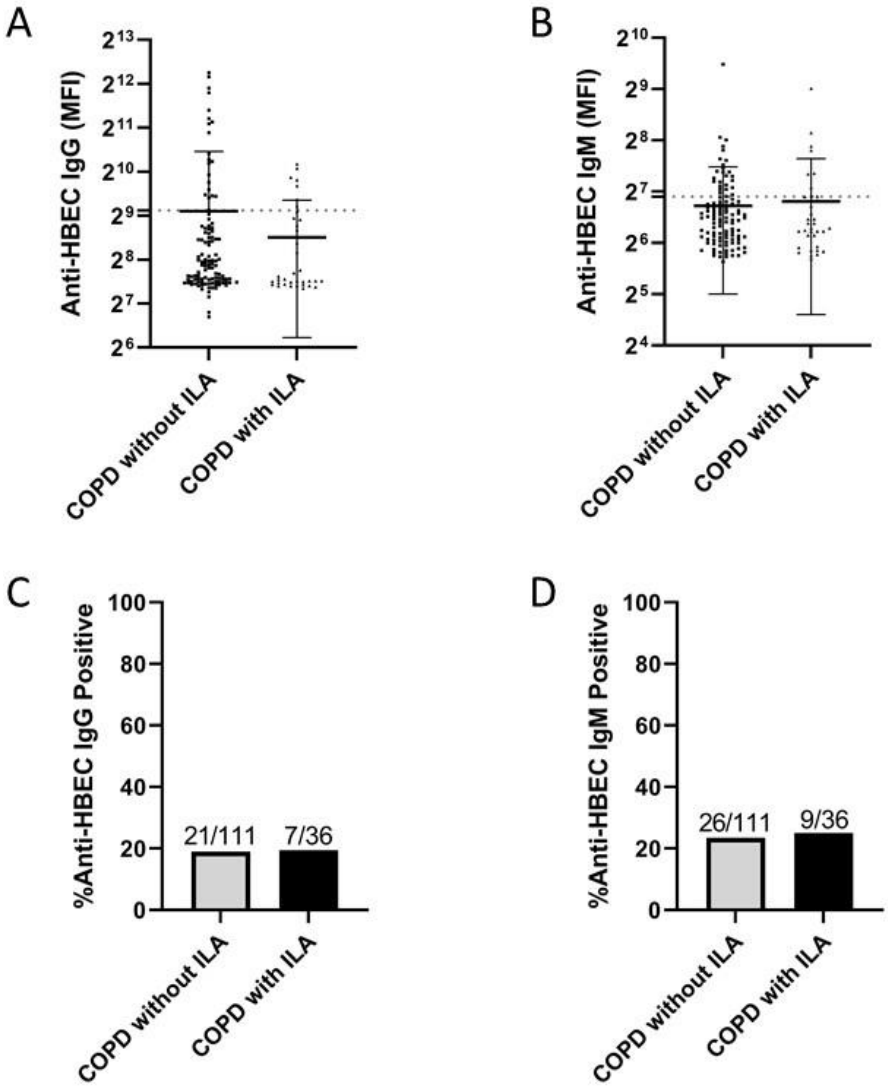

Figure 13. Analysis of the association between the presence of ILA and autoantibodies against primary HBEC. Comparison of serum levels of IgG (A) and IgM (B) autoantibodies against HBEC between COPD patients with and without ILA. Comparison of the prevalence of IgG (C) and IgM (D) autoantibodies against HBEC between COPD patients with and without ILA. Statistical analysis was performed using the Kruskal-Wallis test (A, B) and Chi-square test (C, D).

4. Discussion

In the current study, I investigated autoantibodies against extracellular membrane antigens of pulmonary cells, including primary human bronchial epithelial cells (HBEC) and lung fibroblasts, in COPD patients. The results revealed the presence of both IgG and IgM autoantibodies against primary HBEC in patients with COPD. Serum levels of these autoantibodies were found to be significantly higher in COPD patients compared to age- and gender-matched healthy subjects. Conversely, no or negligible levels of autoantibodies against primary lung fibroblasts were detected in both COPD patients and controls.

Upon classifying COPD patients according to disease severity as defined by GOLD criteria, individuals with very severe COPD (GOLD stage 4) exhibited a significant increase in the level of IgM autoantibodies against HBEC compared to healthy subjects. Additionally, there was a trend of increased prevalence of IgM autoantibodies against HBEC with higher COPD severity. Moreover, a significant association was observed between autoantibodies against primary HBEC and smoking status. Smokers with COPD had significantly higher levels of IgG autoantibodies against primary HBEC compared to COPD patients who never smoked. However, no significant associations were found between autoantibodies against primary HBEC and other demographic and clinical features of COPD patients, such as age, BMI, lung function parameters, recent respiratory infections, and the presence of interstitial lung abnormalities (ILA). To the best of my knowledge, this study represents the first investigation into the association of COPD with autoantibodies against extracellular membrane antigens of pulmonary cells.

4.1. Autoantibodies against the extracellular membrane antigens of pulmonary cells

Over the past two decades, a growing body of evidence has been accumulated on the association of COPD with autoantibodies [110, 111]. However, it remains unclear whether those autoantibodies contribute to the development of COPD. As a major component of humoral autoimmunity, autoantibodies play an essential role in the pathogenesis of autoimmune disorders. Autoantibodies are capable of mediating pathogenic changes via two major mechanisms [112]. Classically,

autoantibodies bind to antigens via the Fab domain and mediate subsequent immune responses via the Fc domain, leading to inflammation in and destruction of target tissues. Alternatively, autoantibodies of a second type are able to mediate pathogenic conditions solely via binding to and interacting with target antigens using Fab domains. Regardless of the mechanism utilized, access to the autoantigen is an indispensable step for autoantibody-induced pathology. Therefore, autoantibodies targeting extracellular antigens such as extracellular membrane antigens, extracellular matrix proteins and secreted molecule are potentially pathogenic.

Autoantibodies against extracellular membrane antigens have been found in patients with various autoimmune diseases. For example, IgG autoantibodies against thyroid stimulating hormone receptor (TSHR) act as a long-acting thyroid stimulator and lead to the development of Grave's disease [113]. In addition, autoantibodies against extracellular antigens of human endothelial cells, melanocyte and neurons have been reported to be present in systemic sclerosis (SSc), vitiligo and autoimmune encephalitis [114-116], respectively.

In the first part of the current study, the relationship between COPD and autoantibodies against extracellular membrane antigens of pulmonary cells was examined. The findings indicate the absence or negligible presence of autoantibodies against extracellular membrane antigens of primary fibroblasts in both COPD patients and healthy subjects. Previous research by Chizzolini and colleagues has demonstrated the existence of autoantibodies against cell membrane antigens of dermal and lung fibroblasts in a significant proportion of sera from patients with systemic sclerosis (SSc) [117]. Furthermore, these autoantibodies have the capacity to induce a proinflammatory function in fibroblasts [117]. Notably, the occurrence of autoantibodies against extracellular membrane antigens of fibroblasts has been specifically observed in patients with SSc, indicating disease specificity [118, 119]. Consistent with this perspective, the current study establishes that neither COPD patients nor healthy subjects exhibit autoantibodies against extracellular membrane antigens of fibroblasts.

In contrast to anti-fibroblasts antibodies, autoantibodies against primary HBEC were detectable in

both patients with COPD and healthy subjects. Moreover, levels of both IgG and IgM autoantibodies against primary HBEC were significantly higher in patients with COPD than in healthy controls. This finding establishes a connection between COPD and autoantibodies against primary HBEC. Two previous studies have reported elevated levels of autoantibodies against airway epithelial cells in patients with COPD compared to healthy controls [71, 74], aligning with our findings. However, in these studies, autoantibodies against epithelial cells were detected using permeabilized cells [71, 74], making it not possible to determine whether these autoantibodies recognize intracellular or extracellular antigens. Therefore, this study, for the first time, demonstrates the association of COPD with autoantibodies against membrane antigens of primary airway epithelial cells.

Importantly, autoantibodies against primary HBEC exhibit no reactivity towards the 16HBE14o cell line, indicating the specificity of these autoantibodies for primary airway epithelial cells. The 16HBE14o- cell line, derived from bronchial epithelial cells of a 1-year-old male cardiopulmonary patient and immortalized with a replication origin-deficient SV40 plasmid, maintains key functions of primary bronchial epithelial cells, including tight junctions and cytokine expression [102, 120]. Nevertheless, studies have reported differences between primary HBEC and the 16HBE14o cell line in cytokine-induced barrier function, paracellular permeability, and in vitro differentiation [121]. The current study's findings further emphasize distinctions in the expression of cell surface molecules between primary HBEC and the 16HBE14o- cell line.

4.2. Association of autoantibodies against primary HBEC with clinical features of COPD

In the second part of the current study, the association of autoantibodies against primary HBEC and clinical features of COPD, including emphysema, GOLD stage and parameters of lung function tests, was investigated

Chronic bronchitis and emphysema are the two most common conditions that contribute to COPD, and the majority of COPD patients exhibit both conditions [122, 123]. While previous studies have reported associations between COPD and various autoantibodies [64, 72, 79-81, 91, 96], only one

study has investigated the differences in autoantibodies between COPD patients with and without emphysema [78]. Utilizing an autoantigen array, Packard et al. examined autoantibodies against a broad spectrum of autoantigens in patients with emphysema, patients with chronic bronchitis, and healthy controls [78] . The authors have demonstrated that levels of autoantibodies to thyroid peroxidase are significantly higher in patients with chronic bronchitis compared to patients with emphysema and healthy controls. Patients with emphysema show increased levels of autoantibodies against collagen II, aggrecan, and intactin compared with patients with chronic bronchitis and healthy controls, suggesting that autoantibodies in COPD are associated with specific pathological conditions [78]. However, since only 5 healthy controls, 7 patients with chronic bronchitis and 9 patients with emphysema were utilized in Packard's study, the finding is compromised and needs validation.

In the present study, stratified analysis was performed by classifying COPD patients into two subgroups based on the presence or absence of emphysema. The results showed that levels of IgG autoantibodies against HBEC were significantly higher in COPD patients without emphysema than in healthy controls, while levels of IgM autoantibodies against HBEC were significantly higher in COPD patients with emphysema than in healthy controls. However, no significant difference in IgG or IgM autoantibodies against HBEC was observed between COPD patients with and without emphysema. Therefore, the results from the current study do not support an association of autoantibodies against primary HBEC with specific COPD conditions.

Classically, the disease severity of COPD is defined by GOLD stage which is classified based on parameters of lung function tests, including post-bronchodilator FEV1/FVC and FEV1% predicted [6]. In a large clinical study involving 3257 Japanese residents, Nakano and colleagues demonstrated a significant inverse correlation between serum levels of autoantibodies against several connective tissue disease autoantigens and lung function test parameters, including FEV1/FVC and FEV1% predicted [124], This suggests an association between autoimmunity and lung function in the general population. It is conceivable that autoantibodies correlate with severity of COPD, which can be verified by evaluating the relationship of autoantibodies with GOLD stage, FEV1/FVC and FEV1% predicted. For example, Kuo and colleagues reported that anti-CK-18 antibodies are inversely correlated with FEV1% predicted, suggesting an association of the autoantibody with severity of

COPD [82]. This finding is supported by subsequent evidence. Also, it has been shown by Xiong and colleagues that serum levels of IgG and IgM autoantibodies against CK18 as well as IgA and IgM autoantibodies against CK19 are gradually elevated with the increase of the severity of COPD [83]. Besides anti-CK-18 and anti-CK-19, autoantibodies against CD80 have been suggested to be associated with the severity of COPD. In 2016, Luo et al. [125] demonstrated that levels of autoantibodies against CD80 are significantly higher in patients with very severe COPD (GOLD stage 4) than in patients with moderate or severe COPD (GOLD stage 2 or 3).

Interestingly, the results of the current study indicate that levels of IgM autoantibodies against HBEC were significantly elevated in COPD patients with very severe disease (GOLD stage 4) compared to healthy subjects, suggesting an association between IgM autoantibodies against HBEC and the severity of COPD. Consistent with this finding, the prevalence of IgM autoantibodies against HBEC tended to increase significantly as the GOLD stage increased. Therefore, these results suggest that autoantibodies against HBEC, particularly of the IgM class, are linked to the severity of COPD. However, it is important to note that the association needs further validation. Despite the observed association with disease severity, the analysis of the relationship between autoantibodies against HBEC and parameters of lung function tests did not reveal any significant correlation. Neither IgG nor IgM autoantibodies against HBEC were significantly correlated with FEV1/FVC and FEV1% predicted. Taken together, while autoantibodies against HBEC, especially of the IgM class, appear to be associated with the severity of COPD, additional validation studies are warranted to confirm and further elucidate this relationship.

In the context of the association between autoantibodies and airway epithelial cells, a study reported a positive correlation between the presence of IgG and IgA autoantibodies against HBEC and the GOLD stage of COPD [74]. However, this finding is in contrast with results from a previous study. In 2008, Feghali-Bostwick et al. [71] reported no significant differences in the prevalence of anti-epithelial cell antibodies between subgroups of COPD patients classified according to GOLD criteria.

The divergence in results between the present study and the aforementioned two previous studies could be attributed to differences in methods and cells employed for antibody detection. Feghali-

Bostwick's study utilized the HEp-2 cell line [71], whereas our study and Cheng's study used primary HBEC [74]. Additionally, unlike Cheng et al. and Feghali-Bostwick et al., we did not permeabilize cells before incubating serum samples. Consequently, the autoantibodies detected in the current study target extracellular membrane antigens, while those identified in the two previous studies recognize both intercellular and extracellular molecules.

4.3. Association of autoantibodies against primary HBEC with cigarette smoking

In addition to clinical features, demographic characteristics, BMI, and smoking status were investigated for their association with autoantibodies against primary HBEC in patients with COPD. Due to the limited inclusion of only 2 female patients with COPD in the study, it was not possible to assess the association between sex and the autoantibodies. Results indicated that neither IgG nor IgM autoantibodies against primary HBEC correlated with age in patients with COPD. Similarly, no significant correlation of BMI with IgG or IgM autoantibodies against primary HBEC was observed. Notably, the current study revealed a significant association between IgG autoantibodies against primary HBEC and cigarette smoking. Among COPD patients, levels of IgG autoantibodies against primary HBEC were significantly higher in smokers than in never smokers, suggesting that cigarette smoking promote the production of autoantibodies against airway epithelial cells.

The association between cigarette smoking and autoimmunity has been extensively investigated in patients with autoimmune diseases. It is well-known that cigarette smoking is linked to an increased risk for multiple autoimmune disorders, such as systemic lupus erythematosus (SLE) [126], rheumatoid arthritis (RA) [127], and multiple sclerosis [128]. Although the underlying mechanisms remain largely unknown, it has been reported that cigarette smoking is associated with the production of autoantibodies in these diseases. For example, Freemer and colleagues demonstrated that cigarette smoking is associated with a significantly higher risk of producing autoantibodies against dsDNA in patients with SLE [129]. In line with this observation, it has been shown that cigarette smoking increases the risk for anti-CCP antibodies and rheumatoid factor in patients with RA, particularly in RA patients carrying HLA-DRB1 shared epitope [94] [130]. According to Karleskog et al., cigarette smoking may trigger RA-specific immune reactions to citrullinated proteins, leading to the

production of anti-CCP antibodies [131].

In addition to clinical observations, results obtained from animal studies support the notion that cigarette smoking triggers the production of autoantibodies. For instance, in 2010, Brandsma et al. demonstrated a significant increase in anti-decorin IgM, anti-collagen IgM and anti-elastin IgM antibodies in C57BL/6 mice after chronic exposure to cigarette smoke for 3 to 6 months compared with mice not exposed to smoke [132]. In 2012, Netkirk and colleagues reported that chronic smoke exposure induced rheumatoid factor and IgG autoantibodies to heat shock protein 70 in AKR/J mice [133]. Additionally, Xiong et al. reported that serum levels of IgG autoantibodies against CK18 and CK19 were significantly higher in C57BL/6J mice exposed to cigarette smoke for 6 months than in control mice exposed to room air [83]. Taken together, animal studies have demonstrated that chronic exposure to cigarette smoke is sufficient to trigger the production of both IgG and IgM autoantibodies against various antigens.

Given that cigarette smoking increases the risk for autoantibodies in both human patients and experimental animals, it is not surprising that it increases the risk of autoantibody production in COPD. However, due to the fact that only a small proportion of COPD patients are never smokers, the association between cigarette smoking and autoantibodies in COPD has been explored in only a few studies. In 2011, Bonarius and colleagues reported no significant difference in the prevalence of ANA between smokers and never-smokers in patients with COPD [77], suggesting that ANA is not associated with cigarette smoking. By comparing subgroups of smokers in patients with COPD, Brandsma et al. could show that levels of IgG autoantibodies against decorin are significantly higher in former smokers than in current smokers [79]. The two previous studies that investigated autoantibodies against epithelial cells did not determine the association of the autoantibodies with cigarette smoking [71, 74]. Therefore, for the first time, the current study demonstrates that IgG autoantibodies against primary HBEC are associated with cigarette smoking.

Interestingly, no significant difference in levels and prevalence of autoantibodies against primary HBEC was observed between never-smokers and smokers in healthy controls. This finding suggests that the association of IgG autoantibodies against primary HBEC with cigarette smoking is specific

to COPD. One possible explanation for this COPD-specific association is that cigarette smoke increases the production of IgG autoantibodies against primary HBEC in a condition-dependent manner. This condition-dependent effect of cigarette smoke on autoantibody production has been observed in patients with RA. In 2006, two studies demonstrated that the increased production of anti-CCP antibodies in smokers of RA patients is dependent on HLA-DRB1 shared epitope [94, 131]. Finally, despite the association between levels of IgG autoantibodies against primary HBEC and cigarette smoking in COPD patients, no significant correlation was observed between pack-years and levels of the autoantibodies. This result suggests that it is the presence or absence of smoking, rather than the amount of smoking, that affects the production of IgG autoantibodies against primary HBEC.

4.4. Limitations of the study

This study has three major limitations.

Firstly, the recruitment of predominantly male COPD patients, with only 2 females out of a total of 147 patients, stems from the significantly higher smoking rate among males than females in China[134, 135]. Consequently, the outcomes of this study may only be applicable to male patients with COPD.

Secondly, there is a notable disparity in the proportion of never-smokers between the healthy control group and patients with COPD. This mismatch in smoking status may compromise the adequacy of the control group in this study. Given our data indicating an association between cigarette smoking and autoantibodies against primary HBEC, the divergence in smoking status between patients and controls may potentially impact the results. However, it is important to note that this potential compromise has been addressed, at least partially, by demonstrating that levels of both IgG and IgM autoantibodies against primary HBEC were higher in smokers with COPD than in healthy control smokers.

Finally, all COPD patients included in this study were hospitalized cases. Approximately 80% of these hospitalizations were due to acute exacerbations, while the remaining cases were primarily

related to co-morbidities requiring treatment. Consequently, the COPD patients studied may not be fully representative of the entire spectrum of COPD cases.

5. Conclusion and outlook

5.1. Conclusion

The present study revealed a significant increase in serum levels of autoantibodies, including both IgG and IgM classes, against primary HBEC in patients with COPD compared to age- and gender-matched healthy control subjects. Furthermore, levels of IgG autoantibodies against primary HBEC were higher in smokers compared to never-smokers among COPD patients. Additionally, the prevalence of IgM autoantibodies against HBEC tended to increase significantly with COPD severity. In conclusion, these findings suggest an association between autoimmunity to primary HBEC and the development of COPD, with this autoimmunity potentially being triggered by cigarette smoking.

5.2. Outlook

Different from previous studies that examined autoantibodies against epithelial cells in patients with COPD [71, 74], the current study specifically investigated autoantibodies against extracellular membrane antigens. Considering the potential pathogenicity of autoantibodies targeting extracellular antigens, the association of autoantibodies against primary HBEC with COPD suggests their possible contribution to the development of the disease, warranting further investigation.

Autoantibodies against primary HBEC might affect target cells in two ways. On the one hand, the binding of these autoantibodies to epithelial cells might initiate subsequent immune responses, such as complement activation, recruitment of immune cells, and antibody-dependent cell cytotoxicity via its Fc domain [136]. On the other hand, these autoantibodies might act as functional autoantibodies and interfere with the homeostasis of target cells solely by binding to and interacting with target antigens using their Fab domains. Both possibilities need to be explored in future studies [112].

Given the numerous antigens present on the extracellular membrane of airway epithelial cells, it is essential to identify the target antigen(s) of COPD-associated autoantibodies. This will be achieved through immunoprecipitation followed by liquid chromatography-tandem mass spectrometry [137, 138]. Once the autoantigen is identified, an animal model can be generated by immunizing mice with

the antigen to induce specific antibody responses. Subsequently, the function and pathogenicity of the induced antibodies will be evaluated *in vitro* and *in vivo*.

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8. Declaration

I declare that this thesis has been written without the help of others and that no other than the stated auxiliaries have been use. Furthermore, the applicant has not participated in another doctoral procedure. Lastly, no other application for admission has been placed, and that the thesis has not been presented anywhere else.

9. Curriculum Viate

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