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Women in science - pulmonary medicine 2023

Edited by
Suzana Erico Tanni and Zhihong Chen

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Women in science - pulmonary medicine 2023

Topic editors

Suzana Erico Tanni — Sao Paulo State University, Brazil

Zhihong Chen — Fudan University, China

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Michel Goldman,
Université Libre de Bruxelles, Belgium

*CORRESPONDENCE
Zhihong Chen
✉ chen.zhihong@zs-hospital.sh.cn

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Editorial: Women in science - pulmonary medicine 2023

Tao Zhu [1] and Zhihong Chen [2*]

[1]Department of Respiratory Medicine and Critical Care Medicine, Suining Central Hospital, Suining, Sichuan, China, [2]Department of Respiratory Medicine and Critical Care Medicine, Zhongshan Hospital of Fudan University, Shanghai Institute of Respiratory Disease, Shanghai, China

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Editorial on the Research Topic
Women in science - pulmonary medicine 2023

Although the proportion of women in science, technology, engineering, and mathematics (STEM) has been gradually rising in recent decades, women are still underrepresented both as scientists and as participants in medicine research. Globally, it is estimated that only 30% of researchers are women (1, 2). Meanwhile, evidence indicates that a sex bias still exists throughout many academic fields. In spite of this, women contribute substantially to advancing medicine knowledge (1, 2). Pulmonary diseases are one of leading causes of morbidity and mortality worldwide. The Research Topic "*Women in science - pulmonary medicine 2023*" highlights work led by women in the field of pulmonary medicine.

All manuscripts in this Research Topic have been undergone a rigorous peer review process. Ultimately, 10 publications (eight research studies, one clinical study protocol, and one review paper), which were all led by senior and early career women from across the world, focusing on lung health, were included.

COPD is one of leading cause of death. In this Research Topic, three research studies and one study protocol focused on different aspects of COPD management. Mou et al., investigated the anxiety-associated clinical profile in older patients with COPD. In this study, a total of 424 older COPD patients were enrolled. Among them, 84 had anxiety, and 340 were without anxiety. There data showed that the BODE index, mMRC, CAT score, comorbidities, and acute exacerbations were independently associated with anxiety in older COPD patients. Then, these results imply that anxiety accounts for worsening symptoms, more comorbidities, and frequent acute exacerbation in older patients with COPD. Thus, more attention should be provided to anxiety in COPD management. In a cross-sectional study, Shen et al. explored the values of five methods, COPD-PS scale, COPD-SQ scale, peak expiratory flow (PEF), COPD-PS scale combined with PEF, and COPD-SQ scale combined with PEF, in diagnosing COPD. They revealed that the sensitivity and specificity of both COPD-SQ questionnaire and COPD-PS questionnaire in prediction COPD was markedly promoted by combing with PEF. Furthermore, among them, the performance of COPD-SQ questionnaire combined with PEF was better. Meanwhile, Shi et al. designed a supervised, single-blinded, randomized controlled clinical trial to investigate the efficacy and safety of music-therapy facilitated pulmonary telerehabilitation program in COPD management. Then, shuttle walking test was used as primary outcome. Additionally, in a pre-clinical study, Brito et al. reported the role of photobiomodulation (PBM) in mediating cytokines, chemokines, and transcription factors expressions in variety of effector and

regulatory T cells, such as CD4+STAT4 cells, CD4+CD25+Foxp3+ cells, etc, in the lung in a murine model of COPD. The results showed that lung pathological alterations, airway inflammation, and inflammatory mediators were attenuated by PBM, possibly through promoting regulatory T cells (CD4+CD25+Foxp3+) population and enhancing their IL-10 releasing in COPD mice.

Asthma is a common non-communicable airway disorder, affecting more than 300 million people worldwide (3). Severity evaluation is essential for asthma treatment. Mao et al. explored the values of neutrophils and related oxidative stress-associated molecules in peripheral blood and induced sputum in the prediction of asthma severity. Compared to non-severe asthma, severe asthma had higher levels of neutrophils, neutrophils%, and 8-iso-PGF2a in the peripheral blood, and increased ROS concentration of neutrophils in the induced sputum. Additionally, they also found that neutrophils and 8-iso-PGF2a in peripheral blood were the promising biomarkers for asthma severity prediction.

It is well-known that smoking is a common risk factor for a variety of diseases, particularly respiratory and cardiovascular system. Batista A. N. R. et al. explored the association between cardiac morphometric features and myocardial fat deposition in young adults. Meanwhile, the role of smoking cessation on the lipid metabolism were also investigated. In this cross-sectional study, it is found that smoking history was positively correlated with myocardial triglyceride (TG) deposition. Then, compared to non-smoking group, high-density lipoprotein cholesterol was lower, and TG and very-low-density lipoprotein cholesterol were higher in smoking group. Their findings indicate smoking can lead to cardiac morphometric abnormal and promote myocardial fat deposition. Then, smoking cessation improves cardiac function and lipid profile disorder. Otherwise, an exciting review was presented by Batista D. R. et al. about the relation of electronic nicotine delivery systems (ENDS) exposure and metals in biological samples. It was showed that both primary and second-hand electronic nicotine delivery systems (ENDS) exposure resulted in increased levels of a variety of metals, including lead and cadmium, in biological samples. Meanwhile, they also revealed that conventional combustible cigarette users have similar or higher metal levels than ENDS users.

Interstitial lung disease (ILD) is a group of pulmonary disorders characterized by inflammation and/or fibrosis (4). Idiopathic pulmonary fibrosis (IPF) accounts for approximately more than 30% of all cases of ILD. However, its etiology and pathogenesis are still not clear. Liu et al. explored the potential key genes in IPF and their roles in immune cells using integrated bioinformatics analysis. Subsequently, these findings were verified both *in vivo* and *in vitro*. They identified that CFH and FHL2 were essential for IPF, which also played the hub roles in different immune cells. Collectively, these results indicate that CFH and FHL2 were promising biomarkers for IPF diagnosis. Silicosis is another pulmonary fibrosis disease with poor prognosis. In a retrospective study, 246 adult patients with silicosis were included from China. Kang et al. used a novel inflammatory biomarker, the systemic immune-inflammation Index (SII), to assess the severity of silicosis. Then, they found that SII level was independently associated with advanced stage of silicosis. Additionally, 444.1 could be used as the cut-off value of SII index to predict advanced stage of silicosis.

Lastly, Fischer et al. developed a new questionnaire, the HeLP (Health Literacy in Pulmonary Embolism)-Questionnaire, which was composed of 23 items in four domains, to assess pulmonary embolism-specific issues of health literacy and to evaluate its psychometric properties.

Collectively, this Research Topic highlighted that current women-led investigations contribute substantially to pulmonary medicine. Then, we hope that it can further promote and inspire female medical researchers and clinicians to continue their explorations into novel advances in academic fields.

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EDITED BY
Zhihong Chen,
Fudan University, China

REVIEWED BY
Zhilong Jiang,
Fudan University, China
Assis Kamu,
Universiti Malaysia Sabah, Malaysia
Li Li,
National Jewish Health, United States
Shujing Chen,
Fudan University, China

*CORRESPONDENCE
Simone Fischer
✉ simone.fischer@med.uni-augsburg.de

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Health literacy in patients with pulmonary embolism: development and validation of the HeLP (Health Literacy in Pulmonary Embolism)-Questionnaire

Simone Fischer[1,2]*, Anja Kalch[3], Constanze Küchler[3], Aliscia Rebecca Albani[3], Helena Bilandzic[3], Dirk Horenkamp-Sonntag[4], Thomas M. Berghaus[5], Christine Meisinger[1] and Inge Kirchberger[1,2]

[1]Epidemiology, Faculty of Medicine, University of Augsburg, Augsburg, Germany, [2]Institute for Medical Information Processing, Biometry and Epidemiology (IBE), LMU München, Munich, Germany, [3]Department of Media, Knowledge and Communication, University of Augsburg, Augsburg, Germany, [4]Techniker Krankenkasse, Healthcare Management, Hamburg, Germany, [5]Department of Cardiology, Respiratory Medicine and Intensive Care, University Hospital Augsburg, Augsburg, Germany

Background: Pulmonary embolism (PE) is a common cardiovascular disease and health literacy is necessary to deal with its consequences after the acute event. The aim of this study was to develop and validate a new questionnaire to measure PE-specific health literacy.

Methods: A mixed-methods design with qualitative and quantitative elements was used in the development process. A literature review about health literacy concepts and instruments and interviews with patients with PE and clinicians were conducted. Quantitative analyses included factor analyses, item response theory with a graded partial credit model, and reliability analyses in different test and validation samples. Furthermore, convergent and known-groups validity and responsiveness were assessed.

Results: The qualitative results supported a concept of PE-related health literacy with four main topics: dealing with PE-related health information, disease management, health-related selfcare, and social support. An initial item pool of 91 items was developed. Further interviews and an online survey with patients with PE (n = 1,013) were used to reduce the number of items and to confirm structural validity. Confirmatory factor analyses in the final evaluation study with patients with PE (n = 238) indicated a good model fit of the four-factor structure. The Health Literacy in Pulmonary Embolism (HeLP)-Questionnaire showed good reliability (Cronbach's alpha: 0.82 to 0.90). All four subscales were responsive toward receiving a brochure with PE-related health information.

Conclusion: The newly developed German HeLP Questionnaire comprises 23 items in four domains and showed good psychometric properties. Further evaluation of the questionnaire in different samples of patients with PE is needed.

KEYWORDS

health literacy, pulmonary embolism, disease-specific questionnaire, psychometric evaluation, factor analyses, item response theory

1. Introduction

Pulmonary embolism (PE) is globally the third most common cardiovascular disease, after myocardial infarction and stroke, and the incidence rates are rising (1). PE is potentially fatal and patients surviving a PE may suffer from long-term consequences including persistent dyspnoea, impaired physical functioning, right heart failure, and chronic thromboembolic pulmonary hypertension (2). Bleeding risk due to anticoagulant medication intake and fears regarding a potential recurrent event may also affect mental health (3). Studies revealed that a considerable amount of patients suffer from symptoms of depression or anxiety after PE (4–6). The acute event is usually followed by an outpatient treatment with anticoagulant medication for at least 3 to 6 months or even longer. Patients are faced with finding a way to cope with their disease, adhering to the treatment, recovering, and preventing further events. For these processes patients' health literacy plays an important role in the active and responsible management of their disease. Health literacy comprises the skills to manage a disease and promote one's own health. Among many existing definitions of health literacy, a comprehensive definition was developed in the European Health Literacy Survey (HLS-EU) and has meanwhile been spread widely: "Health literacy is linked to literacy and entails people's knowledge, motivation and competences to access, understand, appraise and apply health information in order to make judgments and take decisions in everyday life concerning health care, disease prevention and health promotion to maintain or improve quality of life during the life course" (7). The World Health Organization identified health literacy as a key determinant of health. Limited health literacy results in a variety of negative health related outcomes, such as "less healthy choices, riskier behavior, poorer health, less self-management and more hospitalization" (8). Among older patients, poor health literacy is associated with higher mortality rates (9). In a study about patients with chronic obstructive pulmonary disease (COPD) poor health literacy was associated with greater disease severity, greater helplessness, worse respiratory-specific quality of life, and more frequent utilization of COPD-related emergency health-care (10). Since health literacy has been shown to have a crucial impact on several general and disease-specific health outcomes, sound health literacy measures are needed to identify potential deficits. A number of instruments for measuring general health literacy already exist. Some focus on functional health literacy and are linked to reading and numerical literacy. Others, e.g., the HLS-EU Questionnaire (11) or the Health Literacy Questionnaire (HLQ) (12), involve a more comprehensive definition of health literacy and comprise dimensions about engagement with health care providers, the appraisal of health information or the existence of sufficient social support. Health literacy is important to promote general health, but different diseases may require different competencies. For some diseases, such as diabetes, multiple sclerosis, or heart failure, specific health literacy tools have already been developed (13–16). Similarly, in addition to generic health literacy (e.g., understanding information about the disease), patients with PE also face specific challenges (e.g., dealing with anticoagulant medications) that need to be mastered. To our knowledge, no instrument for measuring PE-related health literacy exists. The aim of this study was to develop a questionnaire that addresses PE-specific issues of health literacy and to evaluate its psychometric properties.

2. Methods

2.1. Study aim and design

The Health Literacy in Pulmonary Embolism (HeLP)-Questionnaire was developed and validated between May 2020 and November 2022 as a part of the research project INFO-LE (Evidence-based health information for patients with PE) which is a collaboration between epidemiologists and communication scientists of the Ludwig-Maximilian-University Munich and the University of Augsburg. The main goal of the project was to develop evidence-based health information for patients with PE which was accompanied by the development of a PE-specific health literacy questionnaire. For the development and validation process, we used a mixed-methods design with qualitative and quantitative approaches (Figure 1). We planned all steps and analyses in accordance with the Consensus Based Standards for the Selection of Health Measurement Instruments (COSMIN) checklist to assure high methodological quality (17). The study was approved by the Ethics Committee of the Ludwig-Maximilians-Universität München (Date of approval: 6 July 2020. Reference number: 20–452). The study was performed according to the Declaration of Helsinki.

2.2. Phase I: literature search and qualitative interviews

The development process started with a literature review about concepts and measures of health literacy. To develop a comprehensive concept of PE-specific health literacy we explored experiences of patients and physicians by conducting interviews. Patients were recruited from the LEA cohort study and via Social Media channels. The LEA study is a long-term observational cohort study including patients 18 years and older with PE who were treated at the University Hospital Augsburg. Patients with incident or recurrent confirmed PE diagnosis based on multidetector CT pulmonary angiography or ventilation–perfusion lung scanning are included in the cohort (18). For our interviews, we tried to include a heterogenous sample of patients with PE to cover different perspectives. Patients received postal information about the study and an invitation for a face-to-face interview. Due to restrictions regarding the COVID-19 pandemic, some interviews were carried out by phone or via an online video call. The topics comprised the definition of health literacy, specific PE-related aspects, and how high and low health literacy related to PE could be defined. The interviews were analyzed using qualitative content analysis. As a result, a concept of PE-specific health literacy and a list of questionnaire items were developed. A second round of patient interviews including cognitive debriefing was carried out to investigate the face validity of the first item pool and to eliminate repetitive or inadequate items (19).

2.3. Phase II: quantitative analyses of online and postal survey

The first part of the quantitative phase consisted of an online survey of members of a German health insurance company, who were

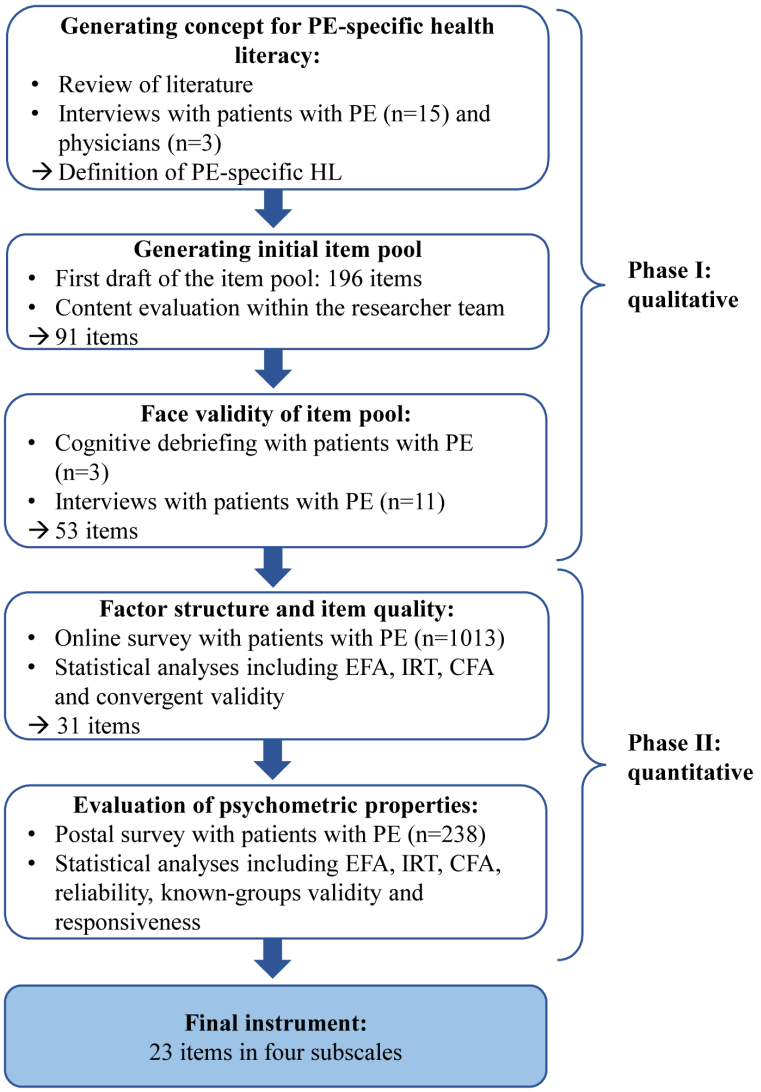

FIGURE 1
Development and validation process of the HeLP questionnaire.

at least 18 years old, and had at least one PE event in the past 2 years. The patients were contacted by the health insurance company and received an access link to the online questionnaire. The survey data were used to investigate the factorial structure using explanatory factor analysis (EFA) and confirmatory factor analysis (CFA). The quality of each item was additionally investigated using item response theory (IRT). In this step, the sample was divided into a test and a validation sample. The test sample was used for EFA and IRT. In the EFA, we used weighted least squares (WLS) estimation and different rotation methods to decide which fits the theoretical concept best (20). Bartlett's Test of Sphericity and Kaiser–Meyer–Olkin (KMO) index were used to determine the appropriateness of the sample for factor analysis (21). Parallel analyses and Empirical Kaiser Criterion (EKC) were used to examine the number of factors to be extracted. Items with factor loadings < 0.3 or cross loadings > 0.3 and with a difference between loadings < 0.2 were excluded. EFA was repeated with the remaining items until an interpretable solution was obtained. After EFA, the items were analyzed using IRT. Since unidimensionality is an important assumption of IRT, a graded partial credit model (GPCM) was calculated separately for each previously extracted factor. We inspected slope (a) parameters for discrimination and location (b_i) parameters for item difficulty, item trace lines and test information curves. Items with unordered b_i parameters or $a < 1$ were eliminated unless they were seen as indispensable for the measured construct.

After this item reduction process, a second EFA was conducted to identify possible changes to the first factor solution. In the next step, a CFA using data of the validation sample was conducted. For assessing model fit we used different global goodness of fit indices: Chi-square test statistics (χ^2), Tucker-Lewis Index (TLI), Comparative Fit Index (CFI), Root Mean Square Error of Approximation (RMSEA) and Standardized Root Mean Square Residual (SRMR). For good model fit, the chi-square test statistics should be non-significant and the ratio $\chi^2/df < 2$ (or at least < 3), TLI and CFI ≥ 0.95 (or at least $\geq$ 0.90) (22), RMSEA ≤ 0.05 (or at least $\leq$0.08) and SRMR $\leq$0.05 (or at least ≤ 0.10) (23). For handling not normally distributed data, we used robust maximum likelihood (MLR) and full information maximum

likelihood method to account for missing data. Modification indices were used to identify local dependency which was modeled where necessary. Spearman correlation coefficients with the validated German version of the Health Literacy Questionnaire (HLQ) were calculated. The HLQ is an instrument that measures generic health literacy and comprises nine different domains: "feeling understood and supported by healthcare providers," "having sufficient information to manage my health," "actively managing my health," "social support for health," "appraisal of health information," "ability to actively engage with healthcare providers," "navigating the healthcare system," "ability to find good health information," and "understanding health information well enough to know what to do" (12). For convergent validity, we assumed at least moderate correlations with some of the HLQ scales.

The last part was an evaluation study based on a postal survey of patients with PE who were treated at the University Hospital Augsburg. We examined completeness of the data and floor and ceiling effects which are presented as proportions of participants with minimal and maximal possible scores. We considered them acceptable if they accounted for <15% (24). Another EFA and CFA was conducted to confirm the factor structure in this sample. We tested psychometric properties including internal consistency, known-groups validity and responsiveness of the HeLP questionnaire with all finally selected items. Internal consistency was measured by Cronbach's alpha, McDonald's omega, and average inter-item correlation. Cronbach's alpha and McDonald's omega coefficients were considered appropriate if they ranged between 0.70 and 0.95 (24). Known-groups validity requires the questionnaire to discriminate between groups that are known to differ on the construct of interest (25) and was tested with Mann–Whitney *U*-tests and rank-biserial correlation as effect size ($r < 0.3$: small, $r = 0.3$–0.5: moderate, $r > 0.5$: large) (26). The variables education, job situation and age were selected for building the groups.

Some of the participants completed the questionnaire twice to test responsiveness which was considered as the ability of the measure to detect change. After the first assessment, they received a brochure with evidence-based health information about PE which was newly developed as a part of the INFO-LE study. We hypothesized that PE-related health literacy may be improved by this intervention and conducted Wilcoxon signed-rank tests with rank-biserial correlation (r) as effect size for all four subscales before and after the participants have received the brochure. Additionally, we report the standardized response mean (SRM) as a second effect size which is calculated by dividing the mean change between the measurements by the standard deviation of the change score (27). All analyses were performed using the statistic software R version 4.2.1 (28) mainly with the packages "lavaan," "mirt," and "psych."

3. Results

3.1. Phase I

3.1.1. Developing a concept of PE-specific health literacy and an initial item pool

On the basis of a comprehensive literature review about general and disease-specific health literacy and existing instruments, three interviews with physicians (pulmonologists and cardiologists) and 15 interviews with patients with PE, a concept of *PE-specific health literacy* was developed. In accordance with Begoray and Khan (29) and Sorensen (7) we defined the concept as follows:

> "*Pulmonary embolism (PE)-specific health literacy covers people's motivation, knowledge and competences to access, understand, appraise, and apply information on PE to engage with the demands of PE health care, prevention and health promotion to maintain and promote post PE health and health-related quality of life.*"

The interviews revealed that four topics related to health literacy seem to be important for patients with PE, namely: dealing with PE-related health information, actively managing the disease, selfcare, and seeking and accepting social support. An item pool with initially 196 items was created, covering these four domains. A 5-point Likert scale was chosen as response format ranging from "very difficult," "rather difficult," "little difficult," "rather easy," to "very easy." After repeated evaluation in terms of item wording and content within the project team, we rephrased or eliminated several items, leaving 91 items to be tested with patients for the first time.

3.1.2. Face validity of first item pool

The first three interviews included cognitive debriefing. In this process, detailed questions about the meaning, relevance and difficulty of each item as well as the appropriateness of the response categories were examined. The following 11 interviews included more general questions about the item pool. All participants of the interviews completed the entire pool of 91 items. Six patients were involved in more than one interview, e.g., shared their experience with PE in the first round and were also part of the cognitive debriefing of the first item pool. Sample characteristics of all 29 interviewed participants are shown in Supplementary Table S1. Participants were 19 to 79 years old, 14 were male, 22 of them were still taking anticoagulant medication, and the time since their last PE event varied from 1 month to 8 years. The direct feedback from the target group provided us with valuable insights into the appropriateness of the items. Misleading or repetitive items were eliminated and we were able to shorten the questionnaire to 53 items which were then tested in the quantitative analyses.

3.2. Phase II

3.2.1. Factor structure and item quality

For the online survey, 3,200 patients were contacted and 1,154 participated, of whom 1,118 reported that they actually had at least one PE. In this step, we examined the distribution and frequencies of the response categories. One response category named 'not applicable for me' was included at this stage to identify items that do not apply to a large proportion of patients with PE. However, none of the items was excluded for this reason. For further analyses, we excluded cases who did not complete all questions about PE-specific health literacy. Two more participants were excluded because they solely used the category 'not applicable for me'. The final sample included 1,013 participants and was randomly divided in a test sample ($n = 505$) and a validation sample ($n = 508$). Sample characteristics of the online survey are shown in Supplementary Table S2. With 29% most of the patients were in the age group between 61 to 70 years. Thirty-four percent of the

TABLE 1 Convergent validity with the HLQ.

HLQ scales	F1	F2	F3	F4
Feeling understood and supported by healthcare providers	0.28	0.31	0.28	0.45
Having sufficient information to manage my health	0.51	0.40	0.44	0.52
Actively managing my health	0.16	0.27	0.35	0.21
Social support for health	0.27	0.34	0.37	0.45
Appraisal of health information	0.29	0.08	0.10	0.20
Ability to actively engage with healthcare providers	0.55	0.50	0.49	0.67
Navigating the healthcare system	0.56	0.50	0.56	0.69
Ability to find good health information	0.68	0.38	0.37	0.50
Understand health information well enough to know what to do	0.53	0.39	0.32	0.34

Spearman correlation coefficients; all statistically significant with $p < 0.05$. F1, Dealing with PE-related health information; F2, Disease management; F3, Health-related selfcare; F4, Social support.

patients were female and about 18% had two or more PE events. The median of the time interval since their last PE was 18 months.

Based on the investigation of item distribution, three items were excluded due to very high ceiling effects of 40%, 31%, and 60%, respectively. One item about medication intake showed a similar high ceiling effect (54%), but remained in the item pool because of its relevance in terms of content. We examined the correlation matrix and excluded five items with very high inter-item correlation (r = 0.79 to r = 0.87). We then conducted an EFA with the test sample to examine the underlying factor structure of our item pool. Bartlett's test and KMO confirmed the adequacy of our sample (KMO = 0.95 and $\chi^2 = 14920.55$, $p < 0.001$). Both, EKC and parallel analysis, suggested four factors to be extracted from the data. We also tried three and five factor solutions and different oblique rotation methods, but four factors with bentler rotation resulted in the best interpretable solution. During EFA we removed eight items that did not load on any factor above 0.3 or showed cross loadings on two or more factors.

We conducted EFA before using IRT models to assure that the assumption of unidimensionality is met. Therefore, one IRT model (GPCM) was fitted separately for each factor. Six items with disordered thresholds or very low slopes were excluded. Four items remained in the questionnaire despite low slopes or slightly disordered thresholds because of their theoretical relevance. They addressed medication intake and dealing with symptoms of PE such as dyspnoea, which were considered important by the interviewees. The test information of all factors showed a peak in the area slightly below average.

After reducing the number of items, we conducted EFA again to confirm that the factor solution has not changed. The factor loadings at this stage ranged from 0.39 to 0.88 in factor 1, from 0.32 to 0.90 in factor 2, from 0.32 to 0.89 in factor 3 and from 0.53 to 0.84 in factor 4. Correlation between the four factors ranged from 0.45 to 0.66 and cumulative variance explained by the four factors was 54%.

To confirm this four-factor structure in the validation sample we conducted a CFA. After inspection of modification indices, we correlated four error terms that were supported by theoretical rationale and each within one factor. Two questions cover finding information, two address understanding information, two relate to checking the quality and sources of information, and two were about following physicians' recommendations. Therefore, they all had a high overlap in content. After this step, the model yielded acceptable fit statistics: $\chi^2 = 1028.371$, df = 424, $p < 0.001$, $\chi^2/df = 2.4$, TLI = 0.896, CFI = 0.905, RMSEA = 0.058 (0.054; 0.063), SRMR = 0.066.

We built mean scores for each subscale with higher scores indicating better PE-specific health literacy. To examine convergent validity, we correlated our new factor scores with the scales of the HLQ. All correlation coefficients were statistically significant ($p < 0.05$) and are shown in Table 1. Highest correlation coefficients for each factor varied between 0.50 for factor 2 and 0.69 for factor 4.

The analysis of the online survey resulted in a pre-final version of 31 items in four subscales.

3.2.2. Evaluation study with final psychometric properties

For the evaluation study, we recruited 300 patients who received an information brochure and the pre-final version of the health literacy questionnaire. Finally, the response rate was 80% with 240 participants. Some of them were asked to complete the questionnaire twice, before and after receiving the brochure. The mean time interval between the first and the second questionnaire after the brochure was 32 ± 14 days. Since two of them did not return the second questionnaire, our final sample included 238 patients. Patients' characteristics are presented in Table 2. Forty-five percent were women and the age ranged from 21 to 91 years with a mean age of 63 ± 15 years. Seventeen percent had already two or more PE events. The mean time between the PE event and study participation was 32 ± 21 months.

For all items, missing values were below 5%, indicating good acceptability. Two items were eliminated due to high mean inter-item correlation > 0.75 and redundancy of content. Items related to medication intake (final items 9 and 10) showed ceiling effects but again remained in the questionnaire because of their theoretical relevance. Another exploratory factor analyses (following the same procedure as for the data of the online survey) resulted in the deletion of three items with insufficient factor loadings. GPCMs were again fitted separately for each factor to identify unordered thresholds. The final questionnaire included only three items without ordered thresholds (Table 3). Thresholds ranged between −2.77 and 2.28 for "dealing with health information," −2.74 and 1.07 for "disease management," −2.91 and 1.71 for "health-related selfcare," and between −1.89 and 1.43 for

TABLE 2 Sample characteristics of the evaluation study.

Variable	*N*	
Mean (SD)		
Age	238	63.2 (14.6)
Time since last PE event (months)	220	32.1 (21.1)
***n* (%)**		
Sex	238	
Men		129 (54.2)
Women		109 (45.8)
Marital status	237	
Married		151 (63.7)
Single		35 (14.8)
Divorced		25 (10.5)
Widowed		26 (11.0)
Living alone	236	
Yes		66 (28.0)
No		170 (72.0)
Education level	237	
≤9 years school education		84 (35.4)
10 years school education		56 (23.6)
≥12 years school education		32 (13.5)
University degree		65 (27.4)
Occupation	235	
Employed (part or full time)		90 (38.3)
Not employed		13 (5.5)
Retirement		130 (55.3)
In education		2 (0.9)
Number of PE events	234	
1		194 (82.9)
>1		40 (17.1)
Prior diseases		
Thrombophila	223	67 (30.0)
Diabetes	221	23 (10.4)
Hypertension	226	108 (47.8)
Chronic heart failure	216	25 (11.6)
Myocardial infarction	218	11 (5.0)
Stroke	219	11 (5.0)
Mental disorder	220	25 (11.4)
Pulmonary hypertension	217	6 (2.8)
Cancer	224	43 (19.2)

PE, pulmonary embolism.

"social support" (Supplementary Table S3). Results of the CFA with MLR estimation approved a four-factor model. The inspection of modification indices led to the exclusion of three items due to high correlations and cross loadings and an inclusion of covaried error-terms between the items regarding medication intake (item 9 and item 10). The final model yielded good fit statistics: $\chi^2 = 381.353$, df = 223, $p < 0.001$, $\chi^2/df = 1.7$, CFI = 0.931, TLI = 0.921, RMSEA = 0.060 (0.050, 0.070) and SRMR = 0.061. All factor loadings were significant and above 0.5 (Table 3; Supplementary Figure S1). The correlations between the four latent factors ranged from 0.33 to 0.66. Cronbach's alpha and McDonald's omega indicated very good reliability ranging from 0.82 to 0.90 and 0.78 to 0.91, respectively. Average inter-item correlation ranged between 0.49 and 0.61. The final subscales showed no relevant floor or ceiling effects (Table 4).

Known-groups-validity was tested for age, years of education and job situation. Younger patients and patients with a higher education level showed significantly higher scores on the scale for "dealing with health information." Scores on "health-related selfcare" were higher for older patients, patients who are retired or not employed, and patients who live alone. Effect sizes were small, ranging from 0.16 to 0.27 (Table 5). After receiving the brochure with PE-related health information, patients showed significantly higher scores on all four subscales with moderate (r = 0.31) to large (r = 0.78) effect sizes (Figure 2), indicating good responsiveness of the questionnaire. The calculation of the SRM revealed small to moderate effects with SRM = 0.54 for the scale "dealing with health information," SRM = 0.30 for "disease management," SRM = 0.20 for "health-related selfcare," and SRM = 0.31 for "social support."

4. Discussion

In this study, we developed the first questionnaire about PE-related health literacy using a mixed-methods approach. A literature review on concepts and measures of health literacy as well as 29 interviews with patients and three interviews with physicians were conducted and qualitatively analyzed. Quantitative analyses included factor analyses, methods of IRT, reliability analyses, convergent validity, known-groups validity and responsiveness. Data basis was an online survey with 1,013 participants and a postal survey with 238 patients with PE. We found four domains that are relevant for PE-related health literacy: "dealing with health information," "disease management," "health-related selfcare," and "social support." The scale "dealing with health information" comprises items about accessing, understanding, appraising, and applying health information. These four competencies are in accordance with the definition of health literacy by Sørensen et al. (7) and are also captured in the related questionnaire HLS-EU-Q47 (11). The items of the scale 'disease management' ask whether patients have difficulty regarding PE-specific recommendations such as reducing their thrombosis risk or medication intake. The scale "health-related selfcare" contains aspects about physical and mental well-being including resting and setting boundaries in favor of their own health. The fourth scale about "social support" includes items about seeking support from various contact persons if help or advice in dealing with the PE is needed. Many health literacy questionnaires focus on health information. Disease-specific questionnaires can additionally address competencies that are directly related to the reality of patients' disease experiences.

TABLE 3 Item content and properties.

		Mean	SD	Ordered thresholds (GPCM)	Factor loadings (CFA)	R^2
Dealing with health information						
Item 1	Find information about pulmonary embolism that I can understand well.	3.35	0.97	Yes	0.67	0.45
Item 2	Understand advice or instructions from physicians and therapists.	3.86	0.84	Yes	0.75	0.56
Item 3	Understand written information about pulmonary embolism.	3.69	0.88	Yes	0.78	0.60
Item 4	Understand how I can reduce the risk of pulmonary embolism.	3.89	0.90	Yes	0.77	0.59
Item 5	Evaluate the quality of information about pulmonary embolism.	3.21	1.03	Yes	0.67	0.45
Item 6	Make decisions for my health after pulmonary embolism using health information.	3.49	0.89	Yes	0.73	0.53
Item 7	Recognize symptoms of an occurring pulmonary embolism.	3.22	0.98	Yes	0.54	0.29
Disease management						
Item 8	Follow recommendations on how to behave in order to avoid another pulmonary embolism (e.g., wear compression stockings, stop smoking, etc.)	3.86	0.96	Yes	0.72	0.52
Item 9	Take the prescribed medication (anticoagulants) regularly and on time.	4.48	0.81	No	0.50	0.25
Item 10	Deal well with the side effects of the medication (anticoagulants).	4.17	0.87	No	0.57	0.33
Item 11	Actively reduce my risk of thrombosis.	3.74	0.87	Yes	0.80	0.65
Item 12	Follow the advice of my physicians and therapists.	4.08	0.81	Yes	0.76	0.58
Health-related selfcare						
Item 13	Find the right balance of activity and rest.	3.62	0.88	Yes	0.75	0.56
Item 14	Take care of what is good for my health.	3.78	0.83	Yes	0.83	0.69
Item 15	Let my body rest when it is necessary.	3.83	0.94	Yes	0.84	0.71
Item 16	Take care of myself and my health needs on a regular basis.	3.63	0.90	Yes	0.86	0.75
Item 17	Recognize changes in my physical well-being.	3.70	0.92	Yes	0.73	0.54
Item 18	Set boundaries to not overload myself.	3.42	0.95	Yes	0.68	0.46
Social support						
Item 19	Find the right health care provider for my needs.	3.32	1.10	Yes	0.74	0.55
Item 20	Call upon someone when I have anxiety or depression about the pulmonary embolism.	3.30	1.18	Yes	0.69	0.47
Item 21	Ask my physicians and therapists if I am unsure about anything.	3.87	0.95	No	0.67	0.45
Item 22	Talk to other people about pulmonary embolism.	3.20	1.15	Yes	0.69	0.48
Item 23	Seek support from others when I need help dealing with pulmonary embolism.	3.27	1.11	Yes	0.84	0.70

CFA Model fit: $\chi^2/df = 1.7$, robust CFI: 0.93, robust TLI: 0.92, robust RMSEA and CI: 0.060 (0.050, 0.070), SRMR: 0.061.

TABLE 4 Acceptability and internal consistency.

Scale	Number of items	Missings % (*n*)	Floor effects %	Ceiling effects %	Cronbach's alpha	McDonald's omega	Average inter-item correlation
Dealing with health information (HI)	7	1.3 (3)	0	1.3	0.87	0.87	0.49
Disease management (DM)	5	0.4 (1)	0	11.3	0.82	0.78	0.49
Health-related selfcare (SC)	6	0 (0)	0	2.9	0.90	0.91	0.61
Social support (SU)	5	2.5 (6)	0	6.7	0.85	0.85	0.52

We included the scale "disease management" to reveal specific problems with handling the disease and its consequences such as medication intake and other thrombosis prophylaxes. Items regarding disease management are also part of health literacy questionnaires for other diseases such as multiple sclerosis (13). Since challenges with setting boundaries and dealing with limited physical performance were frequently mentioned in the interviews with patients, it seemed important to additionally include items

TABLE 5 Known-groups validity.

	Groups		*p*-value[b]	|r|[c]
Age	**<65 years**	**≥65 years**		
GI	3.6 (3.3, 4.1)[a]	3.3 (3.0, 3.7)	0.013*	0.19
KM	4.0 (3.6, 4.4)	4.2 (3.8, 4.6)	0.009*	0.19
SF	3.5 (3.0, 4.0)	3.8 (3.3, 4.3)	<0.001*	0.27
SU	3.4 (2.8, 4.0)	3.4 (3.0, 4.0)	0.627	-
Education	**<10 years**	**≥10 years**		
GI	3.3 (3.0, 3.9)	3.6 (3.1, 4.1)	0.028*	0.17
KM	4.2 (3.6, 4.7)	4.1 (3.6, 4.4)	0.123	-
SF	3.8 (3.2, 4.3)	3.7 (3.2, 4.0)	0.046*	0.16
SU	3.6 (3.0, 4.0)	3.4 (3.0, 4.0)	0.493	-
Job situation	**Retired/not employed**	**Employed/in education**		
GI	3.3 (3.0, 3.9)	3.7 (3.3, 4.1)	<0.001*	0.26
KM	4.2 (3.6, 4.6)	4.0 (3.6, 4.4)	0.178	-
SF	3.8 (3.2, 4.3)	3.5 (3.0, 3.9)	0.001*	0.25
SU	3.4 (2.8, 4.0)	3.6 (3.0, 4.0)	0.399	-
Living situation	**Living alone**	**Living with other persons**		
GI	3.4 (3.0, 4.0)	3.5 (3.1, 4.0)	0.486	-
KM	4.1 (3.6, 4.6)	4.2 (3.6, 4.6)	0.855	-
SF	3.8 (3.2, 4.5)	3.7 (3.2, 4.0)	0.025*	0.19
SU	3.4 (2.7, 4.1)	3.4 (3.0, 4.0)	0.818	-

[a]Median (Q_{25}, Q_{75}).
[b]Mann–Whitney *U*-test.
[c]Rank-biserial correlation.
*Significant with alpha = 0.05.

about "health-related selfcare." This demonstrates the benefits of developing a disease-specific instrument and of the involvement of patients in the development process in order to consider PE-specific challenges reported by the patients.

Overall, the final questionnaire obtained good psychometric properties. A four-factor structure was confirmed by CFA with adequate fit statistics. The final model showed significant chi-square test statistics with $p < 0.001$, which would indicate bad fit, but the ratio χ^2/df was 1.7, which is considered as good fit. It has previously been discussed that robust estimation can lead to over-rejection by corrected chi-square test statistics (30). Covaried error-terms between the two items about medication intake helped to improve the model fit but the respecification is supported by theoretical rationale and within the same scale. No overall score was built because the four subscales are considered to be interpreted separately. The four domains represent different aspects and we assume that an overall score would mask patients' individual needs in specific areas of health literacy. The HLQ for general health literacy has a similar structure and even distinguishes nine scales (31). Moderate to large correlations with HLQ scales indicated good convergent validity. Similar to the HLQ and the HLS-EU (11), we also used a Likert scale for difficulty to represent the different competencies levels. The test information functions calculated by the GPCMs were peaked in the area below average for each factor, indicating that the subscales provide more information on the lower spectrum of PE-related health literacy. Consequently, the subscales may not be sound to differentiate between patients with high or very high PE-related health literacy. However, since distinguishing the levels in terms of limited health literacy seems more important to identify needs, we do not consider this aspect as a real limitation of our questionnaire.

Known-groups validity of the questionnaire was confirmed, as it was able to distinguish between different levels of education, job situations, and age groups. Our findings for "dealing with health information" are consistent with previous studies which revealed higher proportions of limited general health literacy in older patients and patients with low education and low social status (32, 33). Remarkably, the scores on the scale regarding "health-related selfcare" were higher in older patients, in unemployed patients and patients who were living alone. This may be explained by the fact that the time available for selfcare activities may be higher without a job or other family responsibilities that younger patients often face.

The investigation of the questionnaire's responsiveness after receiving PE-related health information revealed small to large effects. Considering the fact that the brochure was a rather mild intervention and it could not be guaranteed whether the entire brochure has been read or how often the brochure has been used, the results are particularly good. Consequently, the questionnaire seems to be an appropriate tool to assess effects of intervention studies on PE-related health literacy.

We had only few missing values in our surveys and we excluded them for the validation process after inspection of their frequencies. For future studies, we recommend that one missing item per scale may be imputed, e.g., by the mean of the other items, otherwise the scale should not be calculated.

A strength of our study is the mixed-methods design with comprehensive involvement of patients who were directly affected by PE and could contribute by sharing their personal experiences. Since patients differ in age, history of cancer or other diseases, presence of symptoms, and medication intake, we tried to cover a broad spectrum of patients with PE in the interviews. The sample of the online survey was large enough to divide it into a test and validation sample. Our final questionnaire covers four domains, but with 23 self-administered items it is still short enough to be applied in a clinical setting. However, the study has some limitations. The sample of the evaluation study included many patients who were already part of a larger PE study cohort. Therefore, participants may be more sensitive toward study participation, have less long-term consequences after PE and may not represent the whole range of patients with PE. Cognitive debriefing was only conducted once for each item due to the large number of items included in the first draft of the item pool. The ability to detect change was also only tested for a small part of the sample in the evaluation study. Due to the postal survey, we were dependent on when patients returned their questionnaires to us and the time gap between the first and the second assessment were not the same for all participants. We were able to investigate many indicators of psychometric quality but we did not examine test–retest reliability to test the stability of the measure, which should be part of future studies.

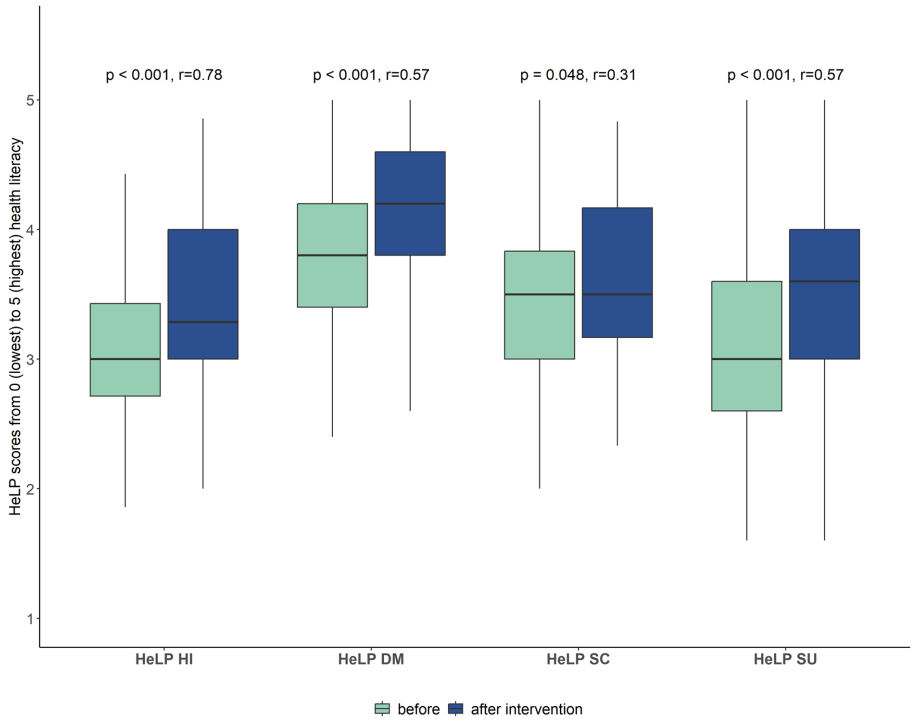

FIGURE 2
Responsiveness of the HeLP-questionnaire. Scores on the four subscales before and after receiving the brochure with evidence-based health information (Wilcoxon signed rank test, $n = 58$). Higher scores indicate higher health literacy.

5. Conclusion

The newly developed German HeLP questionnaire comprises 23 items in four domains and is the first instrument to measure health literacy in patients with PE. Overall, the questionnaire shows good validity and reliability. Further evaluation of the applicability of the questionnaire in different samples with patients with PE is required. The questionnaire can be used to identify patients with low health literacy who may require additional support from the healthcare system and to evaluate the impact of disease-specific interventions to improve PE-related health literacy.

Data availability statement

The datasets presented in this article are not readily available because of ethical restrictions. The study participants did not agree that their data will be used by other researchers. Requests to access the datasets should be directed to SF, simone.fischer@med.uni-augsburg.de.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Ludwig-Maximilians-Universität München, Pettenkoferstr. 8a, 80336 Munich (Date of approval: 6 July 2020. Reference number: 20–452). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SF organized and conducted the interviews, performed the statistical analyses, and drafted the first version of the manuscript. CK and AA developed the interview guide, conducted the interviews, and helped to revise the manuscript. AK conducted the interviews, was involved in designing and supervising the study, and provided critical feedback on the manuscript. DH-S organized the data collection of the online study and provided critical feedback as an advisor. TB was involved in the organization of the evaluation study and supported the interpretation of the results from a medical perspective. HB and CM were involved in planning and supervising the research and provided critical feedback on the manuscript. IK designed and supervised the study, supported the interpretation of the results, and participated in the writing and critical revision of the manuscript. The brochure with evidence-based information for patients with PE was developed by AA, CK, AK, and HB. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2023.1167499/full#supplementary-material

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EDITED BY
Giuseppe Murdaca,
University of Genoa, Italy

REVIEWED BY
Lilia M. M. Sierra-Galan,
The American British Cowdray Medical Center, Mexico
Nazareno Paolocci,
Johns Hopkins University, United States

*CORRESPONDENCE
Robson Prudente
✉ robsonapp@gmail.com

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Cardiac function, myocardial fat deposition, and lipid profile in young smokers: a cross-sectional study

Ana Natália Ribeiro Batista[1], Thaís Garcia[1], Robson Prudente[2]*, Maurício F. Barbosa[3], Pamela Modesto[1], Estefânia Franco[2], Irma de Godoy[1], Sergio Paiva[1], Paula Azevedo[1] and Suzana Erico Tanni[1]

[1]Pneumology Area, Department of Internal Medicine, Botucatu School of Medicine, São Paulo State University (UNESP), Botucatu, Brazil, [2]Pulmonary Function Laboratory, Clinical Hospital of Botucatu Medical School, São Paulo State University (UNESP), Botucatu, Brazil, [3]Department of Tropical Diseases and Diagnostic Imaging, Botucatu School of Medicine, São Paulo State University (UNESP), Botucatu, Brazil

Background: There is a possibility that cardiac morphometric characteristics are associated with the lipid profile, that is, the composition and concentration of triglycerides, total cholesterol, HDL, LDL, and others lipoproteins in young smokers without comorbidities. Thus, this study aimed to evaluate the association of cardiac morphometric characteristics, myocardial fat deposition, and smoking cessation with the lipid profile of young smokers.
Methods: A clinical and laboratory evaluation of lipids and the smoking status was performed on 57 individuals, including both a smoker group and a control group. Cardiac magnetic resonance imaging (MRI) with proton spectroscopy was performed to identify cardiac changes and triglyceride (TG) deposition in myocardial tissue.
Results: No differences were observed between the groups (control vs. smokers) in relation to the amount of myocardial TG deposition ($p = 0.47$); however, when TG deposition was correlated with cardiac MRI variables, a positive correlation was identified between smoking history and myocardial TG deposition [hazard ratio (95% CI), 0.07 (0.03–0.12); $p = 0.002$]. Furthermore, it was observed that the smoking group had lower high-density lipoprotein cholesterol [51 (45.5–59.5) mg/dl vs. 43 (36–49.5) mg/dl, $p = 0.003$] and higher TG [73 (58–110) mg/dl vs. 122 (73.5–133) mg/dl, $p = 0.01$] and very-low-density lipoprotein cholesterol [14.6 (11.6–22.2) mg/dl vs. 24.4 (14.7–26.6) mg/dl, $p = 0.01$] values. In the control and smoking groups, a negative correlation between TGs and the diameter of the aortic root lumen and positive correlation with the thickness of the interventricular septum and end-diastolic volume (EDV) of both the right ventricle (RV) and left ventricle (LV) were noted. Moreover, in the RV, positive correlations with the end-systolic volume (ESV) index (ESVI), stroke volume (SV), ESV, and EDV were observed. Regarding serum free fatty acids, we found a negative correlation between their values and the diameter of the lumen of the ascending aortic vessel. Lipoprotein lipase showed a positive correlation with the SV index of the RV and negative correlation with the diameter of the lumen of the ascending aortic vessel.
Conclusion: Several associations were observed regarding cardiac morphometric characteristics, myocardial fat deposition, and smoking cessation with the lipid profile of young smokers.

KEYWORDS

smoking, lipids, heart function tests, proton magnetic resonance spectroscopy, myocardial fat deposition

1. Introduction

Smoking is the leading cause of preventable death worldwide, which is the main risk factor for the development of several comorbidities. It corresponds to one of the most important health problems globally, causing dependence and reaching different ages and social classes (1, 2).

It is estimated that every year, more than eight million people die due to tobacco use and approximately 1.2 million of these deaths result from passive exposure to smoking.(1) Thus, passive exposure, occasional smoking, and/or consumption of a few cigarettes a day is considered sufficient to be related to the risk of heart disease (3). On the other hand, withdrawal from tobacco exposure reduces the risk of cardiovascular events by 50% after 1 year of abstinence.(2).

In this context, tobacco is recognized as the primary isolated risk factor for acute myocardial infarction (AMI) and plays a significant role in the onset and progression of coronary artery disease (1, 4). This is due, in part, to chronic exposure to nicotine, which leads to vascular endothelial dysfunction. This dysfunction is characterized by diminished nitric oxide synthesis, vasoconstriction, and increased adhesion of leukocytes to the endothelium, contributing to the development of atherosclerosis. This process is further exacerbated by the composition and concentration of lipoproteins, including triglycerides, total cholesterol, HDL, LDL, and other lipoproteins (lipid profile), as well as abnormal plasma lipoproteins. Notably, there is a decrease in high-density lipoprotein (HDL) levels, while levels of very-low-density lipoprotein (VLDL) and triglycerides (TG) increase (5–8).

In addition, pathophysiological mechanisms are involved in the increase in cardiovascular risk. There is evidence for a direct toxic effect of cigarette smoke on the myocardium, culminating in cardiac remodeling.(9, 10) A previous experimental study showed that cigarette smoke was associated with eccentric cardiac hypertrophy, regardless of its hemodynamic effects. In addition, the activity of enzymes responsible for the oxidation of fatty acids (FA) decreased, and consequently, the cardiac TG levels increased; other findings included cell death, hypertrophy, and myocardial dysfunction (11–14).

Although smoking is directly related to the increase in the circulating free FA (FFA) levels, it is not known exactly whether there are changes in glucose and FA metabolism in the myocardial tissue of smokers or there is an association between the accumulation of TG in the heart and hypertrophy and myocardial dysfunction in these individuals (15–17). We previously observed a strong association between smoking in young adults and decline in heart function, confirming that smoking can directly influence cardiac function, even without atherosclerosis or other chronic comorbidities, suggesting that other mechanisms are involved in the cardiac remodeling process, such as insulin resistance (IR) and changes in glucose metabolism (18, 19). Therefore, cardiac magnetic resonance imaging (MRI) is an important tool for identifying cardiac alterations, and this together with proton spectroscopy can evaluate TG deposition in myocardial tissue, thus favoring a more accurate assessment of cardiac function in this population (18, 20–24).

In this sense, our hypothesis is that cardiac morphometric characteristics are associated with the lipid profile and TG deposition in the myocardium of young smokers without comorbidities. This study is justified by expanding our knowledge about smoking and alterations in heart disease, aiming to enhance the understanding of the mechanisms of action of cigarettes on the cardiovascular system. Given the aforementioned, our aim was to evaluate the association between cardiac morphometric characteristics and myocardial fat deposition with lipid profiles in young smokers.

2. Methods

This is a cross-sectional study in which patients from the "smoking cessation outpatient clinic" at the Clinical Hospital of Botucatu Medical School were invited to participate in the study. For the control group, posters were distributed locally, and those who also showed an interest in the study were selected (approval number, 2.076.232).

The inclusion criteria for the smoking group were individuals aged >18 years with a minimum smoking history of 10 pack-years and smoking consumption of at least one cigarette per day in the month prior to the evaluation. In the control group, individuals without previous smoking and without any comorbidity were considered. The exclusion criteria for both groups were the presence of coronary or heart failure, systemic atherosclerosis, AMI, and chronic diseases such as systemic arterial hypertension; diabetes mellitus; dyslipidemia; respiratory, hepatic, renal, psychiatric diseases; and cancer of any kind. In addition, patients who did not undergo a previous echocardiographic examination (to exclude any ischemic heart disease or cardiac remodeling), had restrictions on performing cardiac MRI, and presented with alterations in pulmonary function examination were excluded.

All study participants were evaluated between 2017 and 2018 based on their clinical history and complete physical examination. Smoking history (pack-years) and current smoking status were investigated and complemented by assessing the intensity of nicotine dependence (Fagerström test) (25). Smoking status was confirmed by measuring carbon monoxide (CO) in expired air using a standardized technique with specific equipment (Micro + Smokerlyzer; Bedfont, England, UK). Values above 6.0 ppm of expired CO were considered significant for active smoking (26). For the treatment of smoking cessation, first-line drugs were administered in this study, dispensed according to medical prescriptions. Both the follow-up of these individuals and the verification of smoking cessation occurred qualitatively (direct questions), quantitatively (Fagerström test) and biological methods, especially through the analysis of carbon monoxide in exhaled breath (27, 28).

Laboratory evaluation included the analysis of complete blood count, fasting blood glucose, fasting insulin, C-reactive protein

(CRP), and serum lipid profile—total (serum) cholesterol, HDL, low-density protein (LDL), VLDL, and TG. The homeostatic model assessment (HOMA) index was used to assess IR (24, 29). FAs were analyzed through conversion to their coenzyme A derivatives, and for lipoprotein lipase (LPL) analysis, assays were performed in duplicate using commercially available enzyme-linked immunosorbent assay kits.

All participants of the research underwent cardiac MRI examination, which were carried out in the 3-T MR device (Magnetom Verio, Siemens AG, Health care Sector, Erlangen, Germany) according to the study protocol. The localizers were obtained through image cut sequences of the heart to the programming of sequences of posterior images. Images on cine-MRI in short and long axes of the left ventricle (LV) using the Steady-State Free Precession sequence were used for calculations of ventricular volumes and functions. Proton spectroscopy was performed with voxel placement in the interventricular septum (IVS) to quantify the myocardial fat deposits (**Figure 1**) (30, 31). After spectroscopy, gadolinium contrast was injected (Gadolinium DTPA – 0.15 mmol/kg) and new images of late enhancement were obtained after 15 min using the phase-sensitive inversion-recovery – PSIR sequence in the short and long axes of the LV and also the T1 maps at the same anatomical plans (32–34).

Regarding the reference values of ventricular volumes for cardiac MRI, the data provided by Kawel-Boehm et al., for healthy adults of both sexes, were used.(32).

For image analysis, ventricular function, volumes and mass of the LV were calculated through the Ventricular Function Argus software (Siemens AG, Healthcare Sector). All volumes and ventricular mass were indexed to the body surface area (21). Using standardized LV segmentation, we divided the T1 maps into 16 myocardial segments for T1 time measurements independently (35). The apex (segment 17) was not analyzed because it was impossible to avoid the partial volume effect in this segment. Regions of interest (ROIs) were drawn on the pre-contrast image and copied to the postcontrast images. The extracellular volume (ECV) was calculated manually using T1 measurements before and 15 min after the administration of intravenous contrast (36). T2 measurement was performed with the ROIs positioned in the IVS to exclude the possibility that eventual increases in native T1 were due to edema.

The following formula was used to calculate the ECV: ECV = (1-Hct) × λ, where Hct is the hematocrit and λ is the gadolinium partition coefficient. To calculate spectroscopy, two simultaneous cardiac images were used (four chambers and a short axis) with placement of the voxel in the IVS for quantification. The first image was taken without water suppression for peak-water

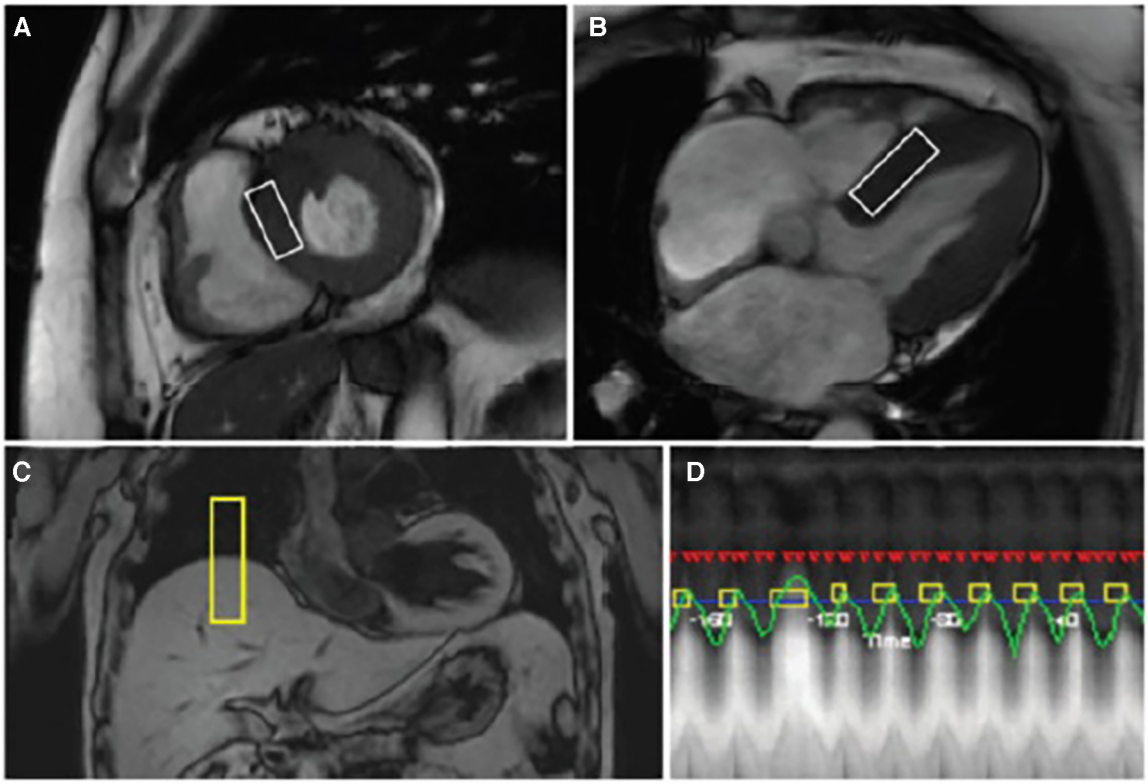

FIGURE 1
Cardiac magnetic resonance images with voxel location in the interventricular septum of the myocardium for image acquisition of a non-smoker subject. Options are: (A) left ventricular short axis; (B) four chambers; (C) capturing respiratory movement through the trigger located between the liver and the thorax; and (D) acquiring proton spectroscopy data.

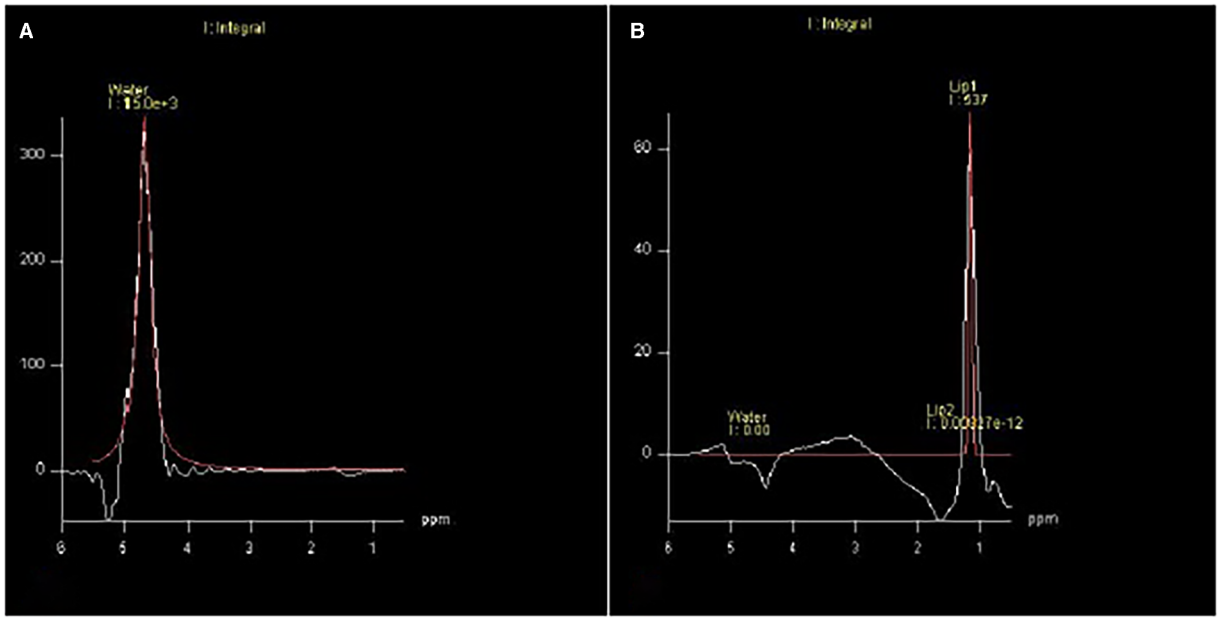

FIGURE 2
Water suppression to determine TG peaks, which were summed to obtain the TG peak value. (A) Without water suppression and (B) With water suppression.

determination. The second image was obtained with water suppression to determine TG peaks, which were summed to obtain the TG peak value (Figure 2). For the final quantification of myocardial TGs, the following formula was used: Lipd1 + Lipd2/water × 100 (9). Data were analyzed using Spectroscopy Evaluation software (Siemens AG, Healthcare Sector, Erlangen, Germany).

Descriptive statistics were used to describe the characteristics of all participants. Means ± standard deviation or medians and interquartile range (25%–75%) were used depending on the data distribution. Categorical variables are expressed as percentages. The χ^2 test was used to compare categorical variables.

The comparison between two independent groups was performed using Student's *t*-test or Mann–Whitney *U* test. Paired Student's *t*-test or Wilcoxon test were used to compare two dependent groups, especially before and after smoking cessation. To study the associations between the functional and morphometric variables of MRI with fat deposition, correlation coefficient analysis was performed using the Pearson or Spearman correlation test. Linear regression was used to evaluate the association of pack years, gender or age, and TG cardiac deposition.

The significance level was set at $p < 0.05$. All data were analyzed using SPSS version 17.0 (IBM Software, Dallas, TX, USA) and SAS (USA).

3. Results

Initially, 97 individuals of both sexes were invited to participate in this study: 47 in the control group (nonsmokers) and 50 in the active smoker group. After applying the inclusion and exclusion criteria, 28 and 29 participants remained in the control and smoking groups, respectively. Of the 29 individuals included in the smoking group, only 10 had ceased smoking at the end of the study. Table 1 shows the main characteristics of the 57 participants included in this study.

Regarding the lipid profile, the comparison between the two groups showed a significant reduction in the HDL cholesterol levels [51 (45.5–59.5) vs. 43 (36–49.5), $p = 0.003$] and increase in the TG [73 (58–110) vs. 122 (73.5–133), $p = 0.01$] and VLDL [14.6 (11.6–22.2) vs. 24.4 (14.7–26.6), $p = 0.01$] levels in the smoking group (Table 2).

When the TG deposition in the myocardium was quantified by proton spectroscopy, there was no statistically significant difference between them [0.311 (0.156–0.610) vs. 0.197 (0.116–0.572), $p = 0.47$]. As for the analysis of TG deposition in the myocardium by proton spectroscopy before and after smoking cessation, we did not observe significant differences between the initial and final moments (0.56 ± 1.08% vs. 0.16 ± 0.22%, $p = 0.28$).

TABLE 1 Characteristics of participants.

Variables	Control ($n = 28$)	Smokers ($n = 29$)	*p*-value
Man, %	60.7	41.4	0.14[a]
Age, years	34.7 ± 4.5	36.7 ± 5.7	0.14[a]
Weight, kg	75.8 ± 12.4	71.5 ± 15.7	0.26[a]
Height, m	1.73 ± 0.11	1.65 ± 0.09	0.003[a]
BMI, kg/m^2	25.1 ± 3.3	26.0 ± 4.0	0.39[a]
CO, ppm	2.0 (1.0–3.0)	9.5 (5.7–15.5)	<0.001[b]

Kg, kilograms; M, meters; BMI, body mass index; CO, carbon monoxide; ppm, parts per million.
Data expressed as mean ± standard deviation or median (25–75%) or percentage ($p < 0.05$).
[a]Student's *t*-test or
[b]Mann–Whitney.

TABLE 2 Laboratory assessment.

Variables	Control ($n = 28$)	Smokers ($n = 29$)	*p*-value
Free fatty acids, mmol/L	0.42 ± 0.18	0.45 ± 0.21	0.56[a]
CRP, mg/L	0.6 (0.5–0.7)	0.6 (0.5–1.1)	0.77[b]
HOMA index, *n*	0.99 (0.50–1.44)	1.42 (0.87–2.38)	0.05[b]
Blood glucose, mg/dl	88.4 ± 20.7	85.6 ± 12.2	0.70[a]
TG, mg/dl	73 (58–110)	122 (73.5–133)	0.01[b]
Total cholesterol, mg/dl	182.5 ± 22.8	187.9 ± 31.4	0.61[a]
HDL, mg/dl	51 (45.8–59.5)	43 (36–49.5)	0.003[b]
LDL, mg/dl	114.7 ± 22.7	119.8 ± 34.7	0.71[a]
VLDL, mg/dl	14.6 (11.6–22.2)	24.4 (14.7–26.6)	0.01[b]

CRP, c-reactive protein; HOMA, homeostasis model assessment; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein.
Data expressed as mean ± standard deviation or median (25–75%) or percentage ($p < 0.05$).
[a]Student's *t*-test or.
[b]Mann–Whitney.

Myocardial TG deposition was evaluated with cardiac MRI variables in the control and smoker groups, and there was a positive correlation between smoking history and myocardial fat deposition [coefficient (95% CI), 0.07 (0.03–0.12); $p = 0.002$]. We did not identify male influence (coefficient: 4.04; 95% CI: −6.27–14.4; $p = 0.43$; R^2: 3%) or age (coefficient: 0.79; 95% CI: −0.33–1.92; $p = 0.16$; R^2: 4%) in the deposition of triglycerides. No significant association was found between myocardial TG deposition and serum lipid profile variables in the control group; however, the smoking group showed a negative correlation with the LPL (Table 3). When the controls and smokers were grouped together, we did not identify a significant correlation between TG deposition and serum markers.

When analyzing the serum markers of all patients with cardiac MRI variables in relation to the LV, we identified a positive correlation between TG, end-diastolic volume (EDV), and IVS size. In relation to the right ventricle (RV), there was a positive correlation between the LPL and stroke volume (SV) index (SVI) and TG with EDV, end-systolic volume (ESV), SV, EDV index (EDVI), and ESV index (ESVI). Furthermore, we found a negative correlation between the ascending aorta diameter and LPL, FFAs, and TG in the aortic root (Table 4).

TABLE 3 Correlation between myocardial fat deposition and lipid profile variables for the smokers group.

	Variables	*R*	*p*-value
Triglyceride deposition	LPL, U/L	−0.38	0.04
	FFA, mmol/L	−0.07	0.72
	TC, mg/dl	0.10	0.61
	LDL, mg/dl	0.11	0.62
	HDL, mg/dl	−0.06	0.75
	VLDL, mg/dl	0.15	0.46
	TG, mg/dl	0.15	0.46
	HOMA, *n*	0.14	0.56

Correlation data between fat deposition and lipid profile variables were performed using Pearson's correlation for data with normal distribution or Spearman's correlation for non-parametric data.
LPL, lipoprotein lipase; FFA, free fatty acids; TC, total cholesterol; LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very-low-density lipoprotein; TG, triglycerides; HOMA, homeostasis model assessment.

TABLE 4 Correlation between myocardial fat deposition and lipid profile variables for the control and smoker groups.

	Variables		*r*	*p*-value
Left ventricle	TG, mg/dl	EDV, ml	0.27	0.04
		IVS, mm	0.30	0.03
Right ventricle	LPL U/L	SVI, ml/m^2	0.34	0.01
	TG, mG/dl	EDV, ml	0.30	0.03
		ESV, ml	0.29	0.03
		SV, ml/m^2	0.28	0.04
		EDVI, ml/m^2	0.28	0.04
		ESVI, ml/m^2	0.29	0.03
		Aortic root, mm	−0.35	0.008
	AAo, mm	LPL, U/L	−0.29	0.04
		FFA, mmol/L	−0.29	0.04

Correlation data between fat deposition and lipid profile variables were performed using Pearson's correlation for data with normal distribution or Spearman's correlation for non-parametric data.
TG, triglycerides; LPL, lipoprotein lipase; AAo, ascending aorta; EDV, end-diastolic volume; IVS, interventricular septum; SVI, stroke volume index; ESV, end-systolic volume; SV, stroke volume; EDVI, end-diastolic volume index; ESVI, end-systolic volume index; FFA, fat free acids.

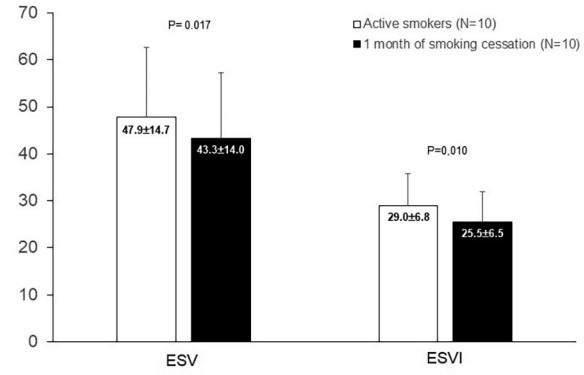

FIGURE 3
Comparison of functional variables of right ventricular of smokers at baseline and after one month of smoking cessation.

When comparing before and after 1 month of smoking cessation, in relation to the analysis of RV morphometric and functional variables, we identified a statistically significant difference only in the ESV (47.9 ± 14.7 vs. 43.3 ± 14.0, $p = 0.017$) and ESVI (29.0 ± 6.8 vs. 25.5 ± 6.5, $p = 0.010$) (Figure 3). In the LV, we identified a statistically significant difference only in the end-systolic diameter (ESD) (32.2 ± 4.6 vs. 33.8 ± 4.9, $p = 0.03$).

4. Discussion

In the present study we did not find strong associations between the variables analyzed and myocardial fat deposition, however, the smoking group had lower HDL and TG levels. These findings are well established in the literature, likewise the TG values and their influence on smokers have also been discussed (37–40). By itself, abdominal visceral fat accumulation is strongly correlated with higher TG levels, lower HDL values, and IR (41–43). In this sense, Freeman et al. (37) reported that

smoking had a small impact on TGs of active smokers, and more recently, Koda et al. (41) demonstrated that in smokers with a greater area of abdominal visceral fat, the TG values were also higher. However, in those with less visceral fat deposition, their numbers did not differ statistically between smokers and nonsmokers.

In the cardiac MRI analysis, we found a negative correlation between the TG values and diameter of the aortic root lumen and positive correlation between the thickness of the IVS and EDV of both the RV and LV; in the RV, positive correlations with the following volumes were also noted: ESV, SV, EDVI, and ESVI. Regarding FFA, we observed a negative correlation between its values and the diameter of the vessel lumen of the ascending aorta.

Fat deposition in the cardiac tissue and adjacent structures can generate mechanical overload with consequent remodeling of the cardiac mass, alteration of vascular resistance, and ejection fraction, causing a decrease in LV performance due to eccentric changes in the ventricular chamber, systolic changes, and increased ventricular wall tension (33, 35, 40–42, 44, 45).

Notably, the greater the fat deposition in certain anatomical structures, the greater the predisposition to the emergence and development of associated dysfunctions (46). Thus, we observed several cardiovascular structural and functional alterations correlated with both TG and FFA values, and presumably, such findings may be useful in the early identification of the risk of systemic arterial hypertension, atherosclerosis, and heart failure, among others directly or indirectly related to the cardiovascular system.

Regarding IR, although below the reference value, the HOMA index was also higher in smokers, but the difference was not statistically significant. We speculate that the sample size may have hampered the analysis; however, the highest absolute values in smokers were in agreement with other studies on the subject (42, 47). IR can be a predictor of arterial hypertension and dyslipidemia, which, in turn, favor fat deposition in the cardiovascular system; however, in our sample, in the MRI analysis, we did not find correlations between the HOMA index and cardiac structures and functions (19).

Few studies have evaluated myocardial fat deposition and its clinical associations, especially with IR (48, 49). Iacobellis and Leonetti (49), using transthoracic echocardiography, demonstrated a good correlation between IR and pericardial lipid droplet accumulation. Silva et al. (45), on the other hand, found through necropsies that fat deposition in the LV is associated with risk factors for cardiovascular diseases, such as smoking and atherosclerotic disease.

Thus, on the lipid profile and IR, we observed that our results are in accordance with the literature, and in this sense, it can be seen that there may be a tendency to reduce fat deposition as the LPL activity increases, or vice versa, given that in the MRI analysis, we found that LPL presented a positive correlation with the SVI and negative correlation with the diameter of the vessel lumen of the ascending aorta (37, 40, 47).

In the comparison of LPL between controls and smokers, there was no statistically significant differences. For some years now, LPL has been investigated in smokers because smoking can induce a reduction in LPL activity in both adipose and muscle tissues, either by reducing TG hydrolysis and clearance or through hyperinsulinemia, which reduces TG hydrolysis and leads to an increase in its values in smokers (50). Thus, it can be observed that LPL may be associated with low weight in these individuals and with mass gain after smoking cessation (10, 25, 51).

However, LPL activity in smokers remains controversial (25, 52, 53). In general, LPL is the main enzyme that hydrolyzes circulating TG and releases FFA, which can be used as energy by the myocardium. Classic studies have already suggested that the greater FFA response in patients with AMI could be the result of a greater release of catecholamines after stimulation by nicotine; however, studies that have evaluated the lipid profile and its relationship with cardiac tissue are still scarce (54).

Myocardial fat is commonly seen on computed tomography and cardiac MRI in healthy adults or those with heart disease (55). The physiological and/or pathophysiological role of this fat deposition is still poorly understood; however, it may be related to conductivity changes resulting from increased oxidative stress and inflammation (42, 56).

The TG deposition determined by proton spectroscopy between smoking and control group was not significant and, in this sense, we did not find previous studies comparing TG deposition by spectroscopy specifically in smokers. However, in healthy individuals, a study by Van der Meer et al. (36) with 20 nonsmokers identified values of 0.4% ± 0.02%; Liu et al. (57) presented median values of 0.5 (0.3%–1.0%) in a sample of 92 individuals, of whom 62% were smokers; and Sai et al. (58) found mean values of 0.85% ± 0.40% in 37 participants, with no information on the number of smokers. When comparing our findings with the literature, we observed that in a sample with 50% smokers, the median TG value was 0.24 (0.12%–0.55%), a lower percentage than those found by the authors mentioned earlier.

In our sample, no significant differences were observed in individuals before and after smoking cessation in the TG deposition in the myocardium by proton spectroscopy; however, there was a reduction in the percentage values, showing a greater tendency of TG deposition in smokers than in nonsmokers, which corroborates the pathophysiology of tobacco-induced myocardial lipid peroxidation. Furthermore, it was identified that TG deposition was associated with smoking history and, only 1 month after smoking cessation, a reduction in its values was observed (59). Thus, we can speculate that the cessation time was not sufficient to detect a greater reduction in the TG values or that the power of the present study was not sufficient for this analysis.

Regarding the analysis of morphometric and functional variables after 1 month of smoking cessation, we identified only a reduction in the ESV and ESVI of the RV and a significant increase in the ESD of the LV. In relation to these findings, we can assume that some pathophysiological aspects may be associated. For example, the diastolic and systolic volumes refer to the resulting volume within the cavity at end diastole and end systole, respectively, and characteristically, we could interpret the

following findings as a consequence of reduced activation of β-adrenergic receptors in the heart and renin–angiotensin–aldosterone system, which would reduce preload by reducing volume retention. Thus, the efficiency of the right cavity identified in this study would be better after smoking cessation, with a consequent reduction in the ESV and ESVI.

As for the changes in the LV, our hypothesis was that in our sample, the smoker's heart would be working adaptively without many morphological changes that we could have identified, but after smoking cessation, this previous adaptation would have become more evident. This finding may be related to the Frank–Starling mechanism, in which the preload determines the force of contraction, that is, when there is an increase in myocardial fiber distension, both the tension generated and the contractility increase (60, 61). Our findings suggest that patients who smoke had adequate preload and systolic function before smoking cessation, and after smoking cessation, the change in volume would have been evident and consequently highlighted the adaptation of the geometry by increasing the LV ESD, which may have been associated with concentric remodeling.

Comparatively, in this context, we can identify clinical studies only with patients with different characteristics, including different comorbidities; nonetheless, these were analyzed using adjusted statistical models, which can be interpreted and partially compared with our findings. The Multi-Ethnic Study of Atherosclerosis (MESA), with more than 6,000 participants with cardiac MRI assessment for atherosclerosis risk factors, showed that the group of never-smokers had better cardiac ejection fraction and lower EDVI and LVMI; however, we cannot say that ex-smokers also have the same characteristics (62). Thus, residual myocardial changes may occur after smoking cessation, as in the study by Rosen et al. who found slight changes in LV function after smoking cessation (63).

Regarding inflammation, in this study, evaluated by CRP, no statistical differences were observed between the groups of smokers and nonsmokers. In general, smokers have higher CRP values than individuals who have never smoked (64); however, as CRP is an indicator of acute inflammation, caution should be exercised when interpreting the available data on CRP levels in patients with chronic conditions or without exacerbation of CRP underlying disease, especially when excluding patients with recent infections and/or inflammation (65).

Thus, our results confirm the findings in the literature on the lipid profile and IR of smokers; however, their associations with myocardial fat deposition, assessed by MRI, were weak (10, 37–39, 66, 67). In addition to the sample size, we can speculate that physiologically, the human heart contains fat deposits that may vary in different individuals. For example, the amount of adipose tissue and its range in muscle tissue can be influenced by advancing age; there is more fat deposition in men than in women and more prominently in white people, Asians, black people, and Hispanics (33, 68). Another well-discussed aspect is that myocardial fat deposition is better observed in individuals with cardiopathies, such as healed myocardial infarction, arrhythmogenic RV dysplasia, cardiac lipoma, cardiomyopathy with muscular dystrophy, hypertrophic cardiomyopathy, and dilated cardiomyopathy (45, 49, 68, 69).

This study has some limitations that need to be reinforced. First, there is a need for further studies to confirm our findings because the sample size was small and was composed only of young smokers. Second, we did not confirm the nicotine levels to assess the impact of smoking on possible mechanisms involved. Third, this was a cross-sectional study that cannot assert causality. Fourth, there was no follow-up of smokers to assess the evolution of cardiac functions and long-term outcomes.

5. Conclusion

Active smoking has a direct influence on cardiac morphometric characteristics and myocardial fat deposition. Cardiac function and lipid profile can be modified early after smoking cessation in young smokers.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee from Botucatu Medical School. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

All authors made a significant contribution to the work reported and have agreed on the journal to which the article has been submitted. AB, TG, IG, PA, and ST contributed to conception and design of the study. AB and TG organized the database. ST and SP performed the statistical analysis. RP wrote the first draft of the manuscript. PM, and EF wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Giuseppe Guida,
University of Turin, Italy

REVIEWED BY
Akira Yamasaki,
Tottori University, Japan
Jesús Miguel García-Menaya,
University Hospital of Badajoz, Spain

*CORRESPONDENCE
Zhihong Chen
✉ chen.zhihong@zs-hospital.sh.cn
Huayin Li
✉ li.huayin@zs-hospital.sh.cn

†These authors have contributed equally to this work

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Peripheral neutrophils and oxidative stress-associated molecules for predicting the severity of asthma: a cross-sectional study based on multidimensional assessment

Ruolin Mao[1,2†], Zhilong Jiang[1†], Zhihui Min[3], Gang Wang[4], Min Xie[5], Peng Gao[6], Lei Zhu[7], Huayin Li[1*] and Zhihong Chen[1*]

[1]Department of Respiratory and Critical Care Medicine, Shanghai Institute of Respiratory Disease, Zhongshan Hospital, Fudan University, Shanghai, China, [2]Department of Respiratory and Critical Care Medicine, Shanghai Chest Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [3]Research Center of Zhongshan Hospital, Fudan University, Shanghai, China, [4]Department of Respiratory and Critical Care Medicine, Clinical Research Center for Respiratory Disease, West China Hospital, Sichuan University, Chengdu, China, [5]Department of Respiratory and Critical Care Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [6]Department of Respiratory Medicine, The Second Affiliated Hospital of Jilin University, Changchun, China, [7]Department of Respiratory and Critical Care Medicine, Huadong Hospital, Fudan University, Shanghai, China

Objectives: This study aims to explore the relationship between the severity of asthma and neutrophils and related oxidative stress-associated molecules in peripheral blood and induced sputum.

Methods: A total of 67 subjects were included in this study, namely, 25 patients with severe asthma and 42 patients with non-severe asthma. Clinical data, induced sputum and peripheral blood were collected. Lung function and molecules related to oxidative stress in induced sputum and peripheral blood of asthma patients were detected. The relationship between neutrophils and asthma severity was analyzed. HDAC2 mRNA and protein expression levels and HDAC2 activity were also analyzed. Multivariate logistic regression was performed to select statistically significant variables.

Results: The absolute value of neutrophils and percentage of neutrophils were higher in the severe asthma patients. These two values were used to predict the severity of asthma by ROC analysis, with the best cutoff values being 4.55×10^9/L (sensitivity 83.3%, specificity 64.0%) and 55.15% (sensitivity 54.8%, specificity 88.0%). The ROS concentration of neutrophils in the induced sputum samples and the 8-iso-PGF2α concentration in the peripheral blood samples were higher in the severe asthma group ($P = 0.012$; $P = 0.044$), whereas there was reduced

HDAC2 protein activity in PBMCs ($P < 0.001$). A logistic equation and a nomogram were created to give a precise prediction of disease severity.

Conclusion: Oxidative stress is increased in severe asthma patients. Peripheral blood neutrophils and 8-iso-PGF2α can be used as biomarkers to predict the severity of asthma. A prediction model was created for evaluating asthma severity.

KEYWORDS

severe asthma, neutrophil, oxidative stress, 8-iso-prostaglandin F2alpha, histone deacetylase 2

1 Introduction

Severe asthma (SA) patients comprise a subpopulation of patients with asthma who require treatment with medications for Global Initiative for Asthma (GINA) steps 4–5 to maintain disease stability. Although only 5–10% of all asthma patients have SA, it is associated with significant morbidity and mortality and with substantial psychological and socioeconomic burdens (1).

Asthma is a chronic airway inflammatory disease that involves a variety of inflammatory cells and cytokines, such as eosinophils, neutrophils, mast cells, and T cells, which have obvious heterogeneity and complexity (2, 3). Neutrophils are involved in both the inflammatory response and oxidative stress, and their role in the occurrence and development of asthma has received increasing attention in recent years (4, 5). Understanding the relationship between airway inflammation and clinical asthma outcomes in real life is of great importance to better understand the disease and choose the appropriate treatment (6).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can be generated during the aggregation and activation of inflammatory cells and play an important role in the occurrence and development of asthma (7, 8). In addition, previous data have demonstrated that an imbalance between oxidant-antioxidant and impaired airway macrophage function is associated with the severity of disease (9).

Accordingly, there is an urgent need to identify the featured characteristics in populations with SA and the relationship between these characteristics and neutrophils and oxidative stress. This study was designed to explore the sociodemographic and clinical characteristics of SA patients and the effects of systemic and airway neutrophils on asthma.

2 Material and methods

2.1 Study design and subjects

This was a cross-sectional study. Adult subjects (≥ 18 years old) diagnosed with asthma were recruited from the clinics of Zhongshan Hospital, Fudan University, from August 2020 to December 2021.

All the subjects had been previously diagnosed with asthma, and the diagnosis was confirmed by clinicians according to the GINA guidelines based on a history of variable respiratory symptoms and confirmed variable expiratory airway limitation. All the recruited patients were in a stable condition, which was defined as no exacerbation or respiratory tract infection for at least 1 month before enrollment. We excluded subjects who were pregnant or breastfeeding or had other chronic respiratory diseases or chronic unstable diseases of other systems or malignancies.

This study was conducted in accordance with the Declaration of Helsinki. The institutional review board of Zhongshan Hospital, Fudan University reviewed and approved this study (No. 2018-196). All included patients gave written informed consent prior to participation.

2.2 Definition and assessment of SA

According to the American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines (10, 11), SA is defined as asthma that requires treatment with suggested medications for GINA steps 4–5 asthma for the previous year or systemic corticosteroid (CS) use for ≥ 50% of the previous year to prevent uncontrolled asthma or asthma that remains uncontrolled despite this therapy. Based on the severity of asthma, all the included participants were classified into the SA group or the non-severe asthma (NSA) group.

2.3 Data collection and clinical assessments

Multidimensional assessments, including sociodemographic characteristics, asthma duration, smoking history, allergen detection results, comorbidities, medication use and acute asthma exacerbation, were performed for all included patients. Asthma control was assessed using the Asthma Control Questionnaire (ACQ) (12), the Mini Asthma Quality of Life Questionnaire (Mini-AQLQ) (13), and the Asthma Control Test (ACT) (14).

The participants also underwent spirometry, FeNO tests, routine blood tests and serum total IgE detection. The FeNO test was performed using a FeNO analyzer (NIOX MINO, Aerocrine AB, Sweden). After the FeNO test, pulmonary function and bronchial dilation tests were performed using spirometry (Master Screen-PFT, Jaeger, Germany). Routine blood tests were performed with a hematology analyzer (Sysmex XE-2100 Fully Automatic Hematology Analyzer, Sysmex, Japan). Serum total IgE was

measured by immunoassay (Hitachi 7600 automated biochemistry analyzer, Hitachi, Japan), with a minimum detectable level of IgE of 1.0 IU/mL.

2.4 Sputum induction and processing

Sputum induction was performed with routine standard methods. Briefly, subjects underwent spirometry and then gargled and blew their nose. Ten minutes after 100 μg of inhaled albuterol, a 7-min sputum induction was performed using 4.5% saline atomized by an ultrasonic nebulizer (Cumulus, Heyer, Germany). After gargling and blowing the nose again, the subjects coughed sputum into a clean petri dish. If the subjects had no sputum or too little sputum, the previous step was repeated until sufficient qualified specimens were obtained or the total atomization time reached 21 min. Sputum was induced with 0.9% saline for safety if the FEV1%pred was $< 40\%$ at baseline, and the procedure was stopped if the FEV1 declined more than 15% versus baseline.

The sputum samples were processed with plug selection and 0.1% dithiothreitol (DTT) treatment within 2 h. Cytospins were prepared using the centrifugation-smear method (SORVALL Stratos, Thermo Fisher Scientific, USA) and stained (May-Grunwald Giemsa), and then cell counting and classification (eosinophils, macrophages, neutrophils, and lymphocytes) were performed independently by two well-trained lab researchers. The eligibility criteria were epithelial cells $< 20\%$.

2.5 Definitions of inflammatory phenotype and neutrophil type

Asthma can be classified into four inflammatory phenotypes according to the ratio of neutrophils and eosinophils in qualified induced sputum specimens: eosinophilic type (eosinophils $\geq 3\%$, neutrophils $< 60\%$), neutrophilic type (eosinophils $< 3\%$, neutrophils $\geq 60\%$), mixed granulocytic type (eosinophils $\geq 3\%$, neutrophils $\geq 60\%$) and paucigranulocytic type (eosinophils $< 3\%$, neutrophils $< 60\%$) (15, 16).

To facilitate the analysis of the effect of airway neutrophils on asthma, neutrophilic and mixed granulocytic types were combined as the neutrophil-dominant type, and eosinophilic and paucigranulocytic types were combined as the non-neutrophil-dominant type.

2.6 Oxidative stress biomarker detection

Serum and induced sputum supernatant were collected, and the concentrations of myeloperoxidase (MPO), neutrophil elastase (NE), 8-iso-prostaglandin F2alpha (8-iso-PGF2α) and superoxide dismutase (SOD) were detected by using an ELISA kit (Shanghai Weiao Biotech Co., Ltd., Shanghai, China).

The neutrophil and ROS concentrations of neutrophils in induced sputum were detected by flow cytometry. The neutrophils were labeled with mouse anti-CD66b-PE antibody (BD Pharmingen, USA) and mouse anti-CD11b-FITC antibody (BD Pharmingen, USA). ROS were tested by the CellROX deep red flow cytometry assay kit (Life Technology, USA). Flow cytometry was performed with 405 nm, 488 nm and 633 nm laser wavelengths, and the average fluorescence intensity of ROX Deep Red dye in neutrophils represented the ROS concentration.

2.7 Histone deacetylase 2 (HDAC2) expression detection

HDAC2 mRNA expression was detected by qRT–PCR in induced sputum cells and peripheral blood leucocytes. Peripheral blood samples were treated with erythrocyte lysate to obtain peripheral blood leukocytes. Total RNA was extracted from cells by using TRIzol reagent (Invitrogen, USA), and a micro ultraviolet spectrophotometer (Nano Drop 2000, Thermo Fisher Scientific, USA) was used to measure the concentration and purity of total RNA. A real-time PCR System (LightCycler96, Roche, Switzerland) and SYBR Green Master Mix (QIAGEN, Germany) were used for qRT–PCR analysis. GAPDH was used as an internal control for HDAC2. The PCR primers were as follows: HDAC2, forward: 5′-ctgttaattgggctggagga-3′, reverse: 5′-aattcaaggatggcaagcac-3′, and GAPDH, forward: 5′-gcgagatccctccaaaatcaa-3′, reverse: 5′-gttcacacccatgacgaacat-3. The relative expression levels of HDAC2 were calculated by using the $2-\triangle\triangle$ CT method.

HDAC2 protein expression in peripheral blood mononuclear cells (PBMCs) was detected by Western blot assay. PBMCs were isolated from peripheral blood by using lymphocyte isolation fluid (Dakewe Biotech Co., Ltd., Shenzhen, China). Proteins of whole-cell extracts were extracted from PBMCs by using RIPA buffer supplemented with protease inhibitors (Shanghai Weiao Biotech Co., Ltd., Shanghai, China), and a BCA kit (Shanghai Jingke Chemical Technology Co., Ltd., Shanghai, China) was used to detect the protein concentration. Proteins were separated on an SDS–PAGE gel by electrophoresis. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes. After incubation with 5% bovine serum albumin, the PVDF membranes were incubated with primary antibodies, namely, rabbit anti-HDAC2 antibody (1:1,800 dilution, Abcam, UK) and mouse anti-GAPDH antibody (1:2,000 dilution, Abcam, UK), at 4°C overnight. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The bands were detected by using a Supersensitive Enhanced Chemiluminescence Substrate Kit (Shanghai Weiao Biotech Co., Ltd., Shanghai, China). GAPDH was used as an internal control. Bands were visualized with a GelDoc EZ imager (BioRad, USA).

2.8 HDAC2 activity detection

Serum was collected, and HDAC2 activity was detected with a human HDAC2 assay kit (ABNOVA, USA).

2.9 Statistical analyses

Descriptive analysis of variables is presented as *n* (%) for categorical data, and continuous data are presented as the mean

with standard deviations. We compared continuous variables using one-way ANOVA appropriately and categorical variables using chi-square tests among participants of two groups. In addition, *post hoc* Bonferroni comparisons were performed to explore differences between groups, in which the cutoff significance was set at α/n ($\alpha = 0.05$; n is the number of comparisons).

To study the influence of neutrophils on asthma severity, we differentiated SA or NSA as an outcome, drew receiver operating characteristic (ROC) curves with the absolute value or percentage of neutrophils as independent variables, and took the maximum Youden index as the basis for determining the cutoff value.

We examined the associations between each variable and asthma severity through univariate logistic regression models. The top 5 variables associated with asthma severity in the univariate analysis were included in multivariate logistic regression models, and a model was established to investigate factors associated with SA. After checking the ROC and calibration curve of the prediction regression model that basically met the requirements, a nomogram model was established to assess the probability of SA.

Linear regression models were established to investigate independent factors associated with neutrophils and each other. Variables associated with neutrophils were included in adjusted multivariate models, and the adjusted rate ratio (aRR) with 95% confidence interval (CI) was calculated. According to the analysis content, age, sex, body mass index (BMI) and smoking status might be regarded as potential confounders of regression analysis.

Data analyses were performed with SPSS 23.0 software (SPSS, Inc., USA) and R 3.4.1 (MathSoft, USA). Two-sided $P < 0.05$ was considered statistically significant.

3 Results

3.1 SA patients were more likely to develop AE and have more serious pulmonary function injury

Of the 67 participants included in our study, 25 were SA patients, and 42 were NSA patients. In the NSA group, patients controlled their disease with regular or intermittent inhalations, with a maximum therapeutic dose of Seretide 250 (salmeterol/fluticasone 50 ug/250 ug) or Symbicort 160 (budesonide/formoterol 160 ug/4.5 ug) one inhalation at a time, twice a day. No systemic steroids were used. In the SA group, patients regularly used inhaled drugs over a long period of time, the lowest dose was Seretide 500 (salmeterol/fluticasone 50 ug/500 ug) or Symbicort 320 (budesonide/formoterol 320 ug/9 ug) one inhalation at a time, twice a day. A total of 44% (11/25) patients had long-term systemic steroid use, 36% (9/25) patients took montelukast sodium orally on a long-term basis. None of the patients are currently using biologics agents. The sociodemographic and clinical characteristics of patients from the SA and NSA groups are shown in Table 1.

There was no significant difference in sex, age or BMI between the two groups, but the asthma duration in the SA group was significantly longer than that in the NSA group (27.48 ± 20.13 years vs. 15.86 ± 16.76 years, $P = 0.015$). The SA group patients were also more likely to develop acute exacerbation (AE) in the last year (1.32 ± 1.19 times vs. 0.33 ± 0.84 times, $P < 0.001$). The vast majority of asthma patients in both groups had never smoked (80.00 vs. 83.33%) and had other allergic diseases (92.00 vs. 83.33%).

The pulmonary function test (PFT) results for the two groups showed significant differences in FEV1%pred (51.52 ± 19.13% vs. 71.72 ± 24.64%, $P = 0.001$), FEV1/FVC (52.61 ± 11.54% vs. 66.10 ± 15.01%, $P < 0.001$) and DLCO%pred (89.95 ± 22.90% vs. 101.21 ± 15.77%, $P = 0.032$). The results showed that the impaired ventilation and diffusion function of the SA patients were significantly more serious than those of the NSA patients. However, there were no significant differences in the FeNO results ($P > 0.05$).

Unlike the ACQ results (13.38 ± 5.86 vs. 9.93 ± 5.09, $P = 0.017$), there was no significant difference in the ACT and Mini-AQLQ results.

3.2 Neutrophils in the peripheral blood of the SA patients were significantly increased

Blood samples were collected from 25 SA patients and 42 NSA patients, and the absolute value (5.24 ± 1.87 10^9/L vs. 3.64 ± 1.25 10^9/L, $P < 0.001$) and percentage (62.93 ± 7.95% vs. 54.84 ± 7.57%, $P < 0.001$) of neutrophils in the SA group were significantly higher than those in the NSA group, and there was no significant difference in eosinophil levels. The results are shown in Figures 1A1, A2 and Table 1.

3.3 Neutrophil-dominated airway inflammation in sputum was more likely to present as SA

Induced sputum collection was attempted in all the participants, and samples were obtained from 19 SA patients and 23 NSA patients. After sputum quality screening, the induced sputum samples from 15 SA patients and 21 NSA patients met the eligibility criteria (a percentage of epithelial cells < 20%).

The neutrophils in induced sputum were slightly higher in patients in the SA group (51.51 ± 22.09% vs. 40.04 ± 18.37%, $P = 0.108$). After classifying the inflammatory phenotype based on sputum cell counts, the proportion of the neutrophilic type in the SA group was higher than that in the NSA group (20.00 vs. 4.76%, $P = 0.456$), and the proportion of the neutrophil-dominant type in the SA group was also higher (40.00 vs. 19.05%, $P = 0.166$). Due to the relatively small number of qualified sputum samples, the results were not significantly different. The results are shown in Figures 1B, C.

After adjusting for the four covariables of age, sex, BMI and smoking status, the neutrophil-dominant type and asthma severity were analyzed by multivariate logistic regression, and a positive relative risk was found (RR = 9.26, CI [1.05, 81.70], $P = 0.045$). This means that the risk of SA was 9.26 times higher in those with neutrophil-dominant asthma than in those with non-neutrophil-dominant asthma. The results are shown in Figure 1D.

TABLE 1 Sociodemographic and clinical characteristics of asthma patients with different severities.

	Severe asthma	Non-severe asthma	χ^2/t	*P*-value
	n = 25	*n* = 42		
Male, *n* (%)	12 (48.00)	22 (52.38)	0.120	0.729
Age, year	58.6 ± 9.41	53.95 ± 13.99	1.452	0.151
BMI, kg/m^2	24.34 ± 3.62	24.26 ± 3.49	0.078	0.938
Asthma duration, year	27.48 ± 20.13	15.86 ± 16.76	2.505	0.015
Smoking status, *n* (%)			1.187	0.552
Never	20 (80.00)	35 (83.33)		
Ever	3 (12.00)	6 (14.29)		
Current	2 (8.00)	1 (2.38)		
Allergen detection, *n* (%)			1.908	0.385
Positive	11 (44.0)	15 (35.7)		
Negative	8 (32.0)	10 (23.8)		
Undetected	6 (24.0)	17 (40.5)		
With other allergic diseases, *n* (%)	23 (92.00)	35 (83.33)	1.012	0.314
AE in last year, time	1.32 ± 1.19	0.33 ± 0.84	3.913	< 0.001
FVC%pred	75.33 ± 17.02	85.39 ± 23.37	1.813	0.075
FEV1%pred	51.52 ± 19.13	71.72 ± 24.64	3.400	0.001
FEV1/FVC	52.61 ± 11.54	66.10 ± 15.01	4.427	< 0.001
DLCO%pred	89.95 ± 22.90	101.21 ± 15.77	2.201	0.032
Bronchial dilation test, *n* (%)			2.382	0.497
Positive	8 (32.00)	9 (21.43)		
Probable positive	6 (24.00)	13 (30.95)		
Negative	9 (36.00)	19 (45.24)		
Undetected	2 (8.00)	1 (2.38)		
FeNO, ppb	58.41 ± 44.17	55.73 ± 39.24	0.238	0.813
ACT	18.52 ± 4.07	19.83 ± 4.07	1.258	0.213
ACQ	13.38 ± 5.86	9.93 ± 5.09	2.452	0.017
Mini-AQLQ	70.48 ± 12.80	70.48 ± 14.48	0.001	0.999
Blood Neu, 10^9/L	5.24 ± 1.87	3.64 ± 1.25	4.151	< 0.001
Blood Eos, 10^9/L	0.27 ± 0.24	0.34 ± 0.31	0.987	0.327
Blood Neu, %	62.93 ± 7.95	54.84 ± 7.57	4.088	< 0.001
Blood Eos, %	3.45 ± 2.95	5.46 ± 4.83	1.872	0.066
IgE, IU/ml	326.20 ± 478.46	288.38 ± 550.29	0.281	0.779
Blood 8-iso-PGF2α, pmol/ml	162.41 ± 307.32	55.14 ± 45.47	2.056	0.044
Blood NE, pg/ml	1, 877.35 ± 1, 920.51	1, 868.83 ± 1, 605.74	0.019	0.985
Blood SOD, ng/ml	133.39 ± 25.64	114.35 ± 33.03	2.400	0.019
Blood MPO, ng/ml	120.42 ± 36.65	148.63 ± 35.75	2.985	0.004

3.4 High level of neutrophils in peripheral blood can predict SA

Receiver operating characteristic (ROC) analysis showed that both the absolute value and percentage of neutrophils were predictive parameters for SA. The AUCs of the two were 0.777 (CI [0.660, 0.893]) and 0.762 (CI [0.646, 0.879]), and the best cutoff values were 4.55 × 10^9/L (sensitivity 83.3%, specificity 64.0%) and 55.15% (sensitivity 54.8%, specificity 88.0%), respectively. The results are shown in Figure 2.

3.5 Composite clinical indexes, including blood neutrophils, predict the probability of SA

By multivariate logistic regression, we found that decreased DLCO%pred and an increased absolute value of neutrophils in blood were statistically significant for predicting SA, and the results are shown in Figure 3A. Then, we obtained the multivariate logistic regression model: Ln[pr(Severe Asthma)/1-pr(Severe Asthma)] = −0.133–0.049 × DLCO%pred + 0.565 × blood

absolute value of neutrophils −0.132 × blood percentage of eosinophils + 0.004 × blood 8-iso-PGF2α + 0.018 × blood SOD. The ROC of the prediction regression model was 0.8612 (CI [0.7546, 0.9678]). The calibration curve also basically agreed with the statistical requirements. Then, a nomogram for assessing the probability of SA was developed and is shown in **Figure 3B**.

3.6 Oxidative stress damage was more severe in SA patients

There was no significant difference in induced sputum neutrophils between the SA and NSA groups, but the ROS concentration of neutrophils in the SA group was significantly higher (39,331.50 ± 20,101.99 a.u. vs. 18,357.25 ± 4,646.68 a.u., $P = 0.012$). The results are shown in **Figures 4A, B**.

The 8-iso-PGF2α concentration of neutrophils in the SA group was higher in both peripheral blood (162.41 ± 307.32 pmol/ml vs. 55.14 ± 45.47 pmol/ml, $P = 0.044$) and induced sputum (239.92 ± 234.13 pmol/ml vs. 106.39 ± 97.78 pmol/ml, $P = 0.022$). In addition, the SOD concentration in the peripheral blood of SA patients was also higher (133.39 ± 25.64 ng/ml vs. 114.35 ± 33.03 ng/ml, $P = 0.019$). There was no significant difference in the NE concentration between the two groups. The results are shown in **Figures 4C, D**.

3.7 MPO was lower in SA patients

The MPO concentration in the SA group was lower in peripheral blood (120.42 ± 36.65 ng/ml vs. 148.63 ± 35.75 ng/ml, $P = 0.004$), and there was no significant difference in induced sputum (5.47 ± 2.61 ng/ml vs. 4.98 ± 2.72 ng/ml, $P = 0.579$). MPO concentration in peripheral blood was significantly positively correlated with SOD concentration ($\beta = 0.470$, $P = 0.002$), while the correlation between the two was not obvious in induced sputum. The neutrophils in peripheral blood or induced sputum increased, but the MPO concentration in blood decreased ($P < 0.001$, $P = 0.013$), no significant relationship with MPO concentration in induced sputum. And the MPO concentration in peripheral blood was negatively correlated with NE concentration ($\beta = -0.004$, $P = 0.041$). MPO concentration in induced sputum was positively correlated with 8-iso-PGF2α concentration ($\beta = 31.43$, $P = 0.003$), and no significant relationship in peripheral blood.

3.8 HDAC2 activity was impaired in SA patients

Peripheral blood cells and induced sputum cells were collected and assessed for HDAC2 mRNA via qRT–PCR. There was no significant difference in gene expression between the two groups ($P > 0.05$). HDAC2 protein expression and activity were detected in PBMCs. The results showed that the activity of HDAC2 in SA patients was significantly lower than that in NSA patients (1.517 ± 0.338 vs. 5.133 ± 0.319, $P < 0.001$). The results are shown in **Figure 5**.

4 Discussion

Airway inflammation is a common pathological feature of asthma patients. In addition to eosinophils, the role of neutrophils in the occurrence and development of asthma has received increasing attention in recent years (17). Some studies have found that neutrophilic inflammation in the acute phase of asthma can be observed and is associated with glucocorticoid resistance in SA patients and that induced sputum neutrophil levels increase in some patients with chronic persistent asthma (18–23). The neutrophils in induced sputum are also correlated with the degree of airway obstruction and severity of asthma (18, 24).

Neutrophils contain and release a powerful arsenal of mediators, including several granular enzymes, ROS and neutrophil extracellular traps (25). The occurrence and development of asthma is related to Th2 inflammation, and studies on human and animal respiratory virus infections have confirmed that neutrophils can promote Th2 inflammation through DNA extracellular traps (26–28).

Peripheral blood cell counting is a regular clinical lab test that is simple and non-invasive and can be widely carried out in primary medical institutions. Based on the absolute value of eosinophils and neutrophils in peripheral blood, Nadif et al. (29) classified asthma patients into 4 categories and found that a substantial number of asthma patients (56.2%) had the eosinophil-low pattern (< 250 eosinophils/mm^3). Although some studies have confirmed that neutrophils are significantly active in the peripheral blood of SA patients (30) and that the autophagy and extracellular DNA traps of peripheral blood neutrophils could enhance asthma severity by damaging the airway epithelium and triggering inflammatory responses of airway epithelial cells and peripheral blood eosinophils (31), the role of neutrophils in the peripheral blood in the diagnosis of asthma and the judgment of its severity have not yet been determined.

In our study, we found that peripheral blood neutrophils can be used as biomarkers for predicting SA, and it is believed that asthma patients with absolute values greater than 4.55 × 10^9/L and proportions higher than 55.15% are likely to have SA, with a sensitivity of 83.3%/54.8% and specificity of 64.0%/88.0%, respectively. In addition, a multivariate logistic regression was performed by introducing the variables selected in the univariate regression model, and the statistically significant variables, such as peripheral blood cells, lung function, plasma oxidative stress biomarker 8-iso-PGF2α and SOD, were identified. Finally, a logistic equation and a nomogram were created to give a precise prediction of disease severity for a given patient. This has not been reported in previous studies.

The concentration of the lipid membrane peroxidation damage product 8-iso-PGF2α can reflect the level of oxidative stress injury. The peroxidation reaction of membrane lipids can lead to fatty acid chain rupture, aggravate airway inflammation and cause tissue damage and morphological changes (32). Lipid peroxidation is also believed to be closely related to asthma, and increased concentrations of 8-iso-PGF2α and its congeners have been found in the exhaled condensate, induced sputum, peripheral blood and urine of asthma patients (33–36). Its concentration in the blood of asthma patients increased significantly in the acute exacerbation stage of asthma and decreased in the remission stage and was

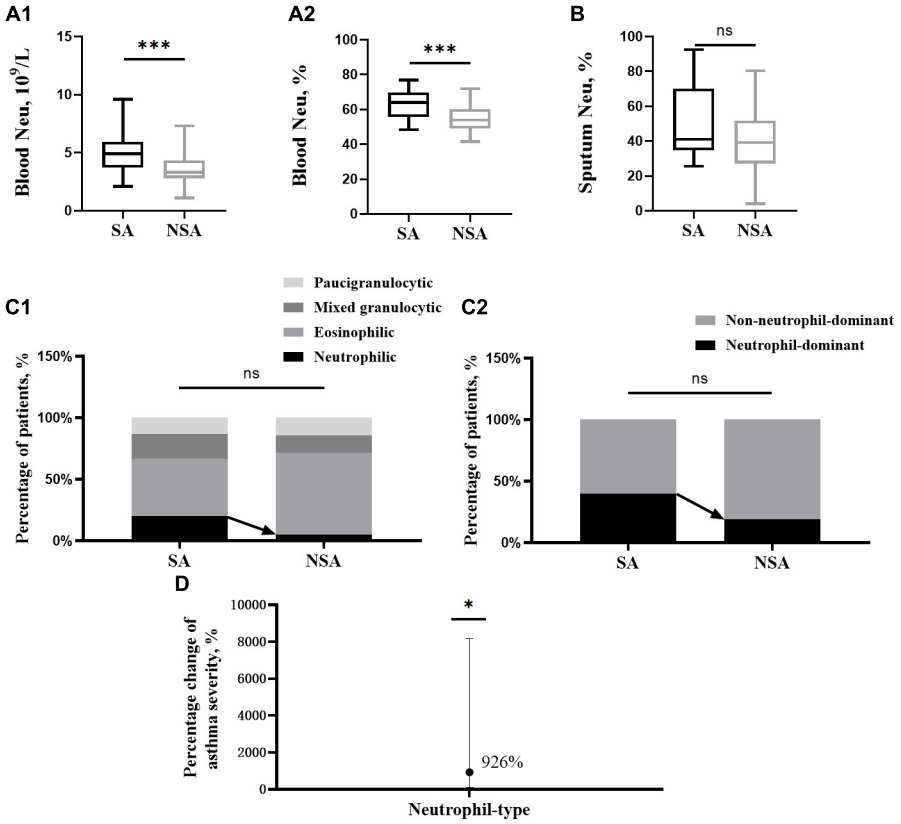

FIGURE 1
Differences in neutrophils and associated phenotypes in patients with asthma of different severities. This figure shows *t*-test results of neutrophils in peripheral blood **(A1,A2)** and induced sputum **(B)** and the percentage of inflammatory phenotype **(C1)** and neutrophil type **(C2)** between the severe (black block) and non-severe (gray block) asthma groups. The effect of neutrophil type on asthma severity **(D)**. ns: $P > 0.05$, *: $P < 0.05$, ***: $P < 0.001$.

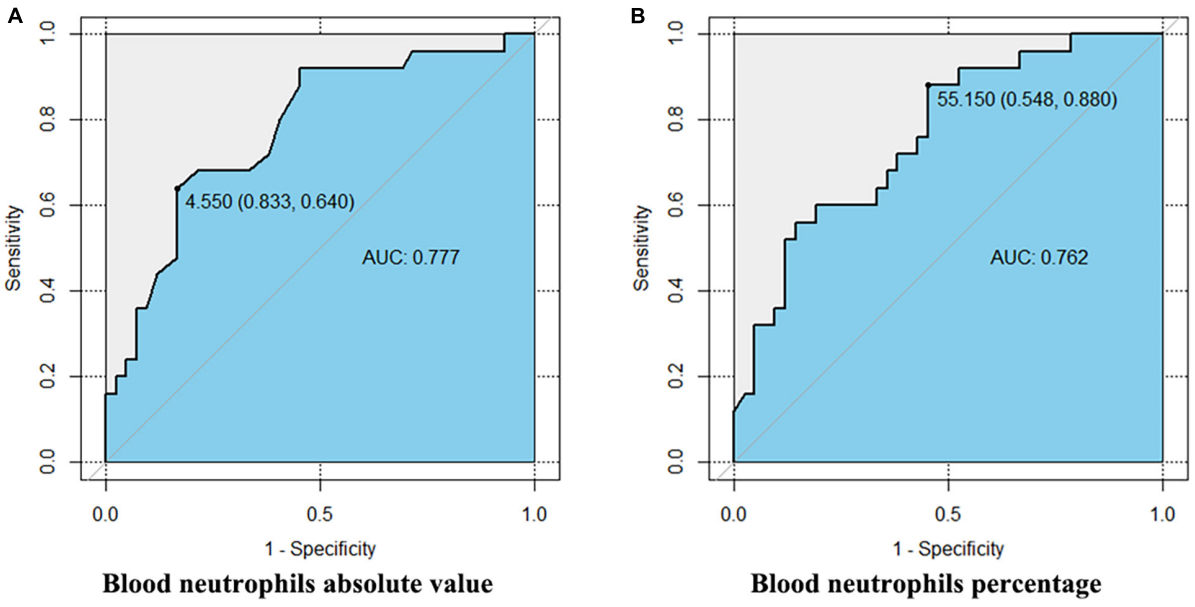

FIGURE 2
The ROC of peripheral blood neutrophils predicting severe asthma. This figure shows the Delong test results of the absolute value **(A)** and percentage **(B)** of peripheral blood neutrophils and severe asthma. The AUCs of the two are 0.777 (CI [0.660, 0.893]) and 0.762 (CI [0.646, 0.879]), and the best cutoff values are 4.55 × 10^9/L (sensitivity 83.3%, specificity 64.0%) and 55.15% (sensitivity 54.8%, specificity 88.0%), respectively.

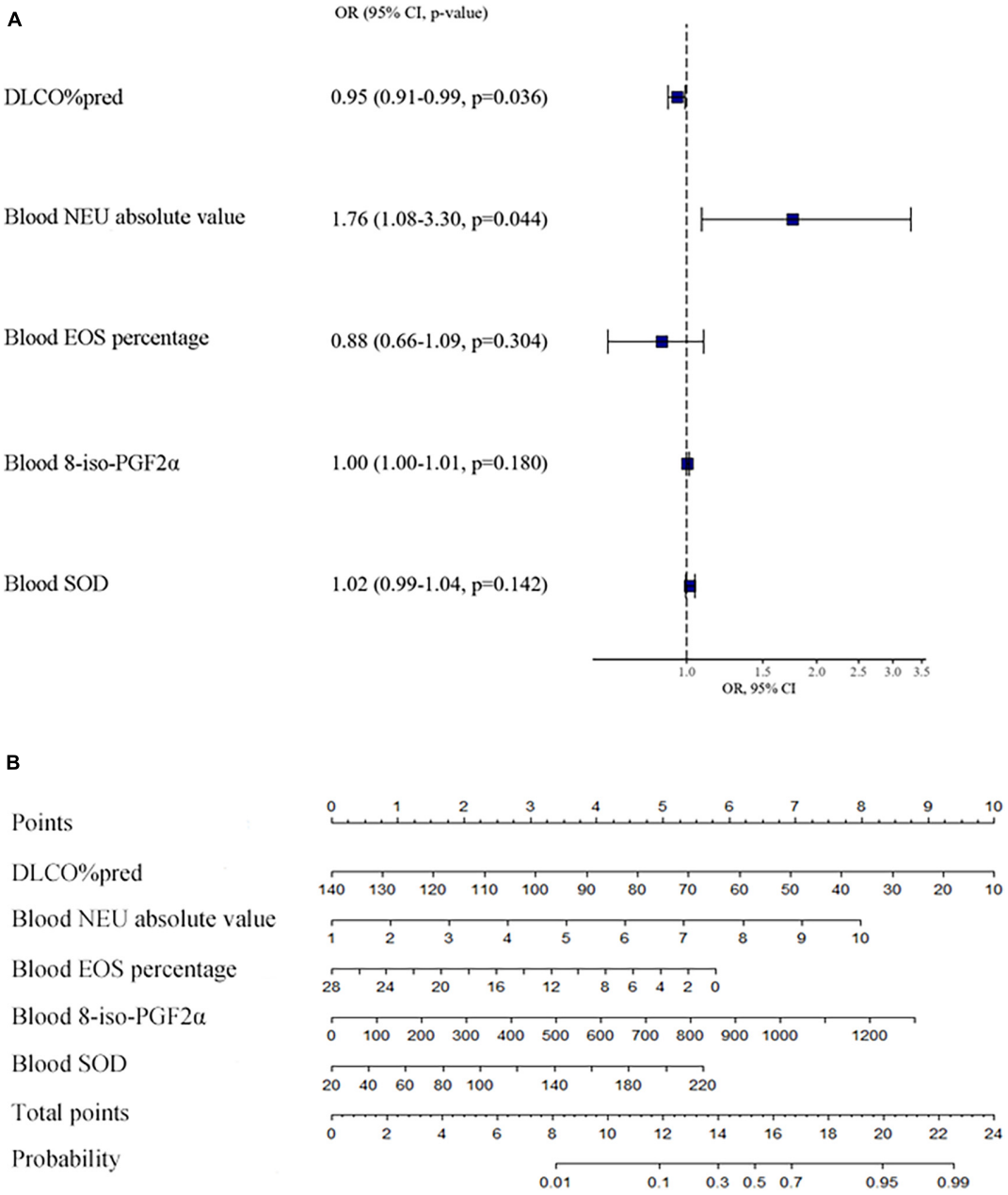

FIGURE 3
Influence of various factors on asthma severity and the nomogram for assessing the probability of severe asthma. This figure **(A)** shows the multivariate logistic regression model of factors on asthma severity. This figure **(B)** shows the nomogram of the multivariate logistic regression model of the probability of severe asthma. We can see a total of 8 horizontal lines with scales, the first one is "points." We could evaluate the variables of horizontal lines 2-6 according to patient's situation, and draw a vertical line upward, respectively, to the first horizontal line, and then get the points of each item. All the points are then added together to get the "total points," which is marked on the seventh horizontal line. Finally, draw a vertical line down to the eighth horizontal line, to estimate the "probability" that the patient will have severe asthma. DLCO: carbon monoxide diffusion capacity, NEU: neutrophil, EOS: eosinophil, 8-iso-PGF2α:8-iso-prostaglandin F2alpha, SOD: superoxide dismutase.

significantly higher than that of healthy people (37). In our study, this result is consistent with previous literature, that is, both in peripheral blood and induced sputum, the level of 8-iso-PGF2α in patients with SA was significantly increased.

Interestingly, an antioxidant substance, SOD, increased with the increase in oxidative damage products. SOD is the main enzymatic antioxidant, including three isozymes, among which extracellular SOD is the main SOD in the extracellular range and is highly expressed in lung tissues (37). We were also doubtful about the increase of MPO in NSA group, and it was negatively correlated with neutrophils in peripheral blood. To verify whether there is an enhanced oxidation reaction in patients with SA, ROS in induced sputum were directly measured by flow cytometry. The results showed that ROS in the SA group was significantly increased, indicating that an enhanced oxidation reaction did exist.

Antioxidation is a cellular and cellular product defense system established by the body to resist oxidative substances and prevent bodily damage. Some studies have found that the concentration of

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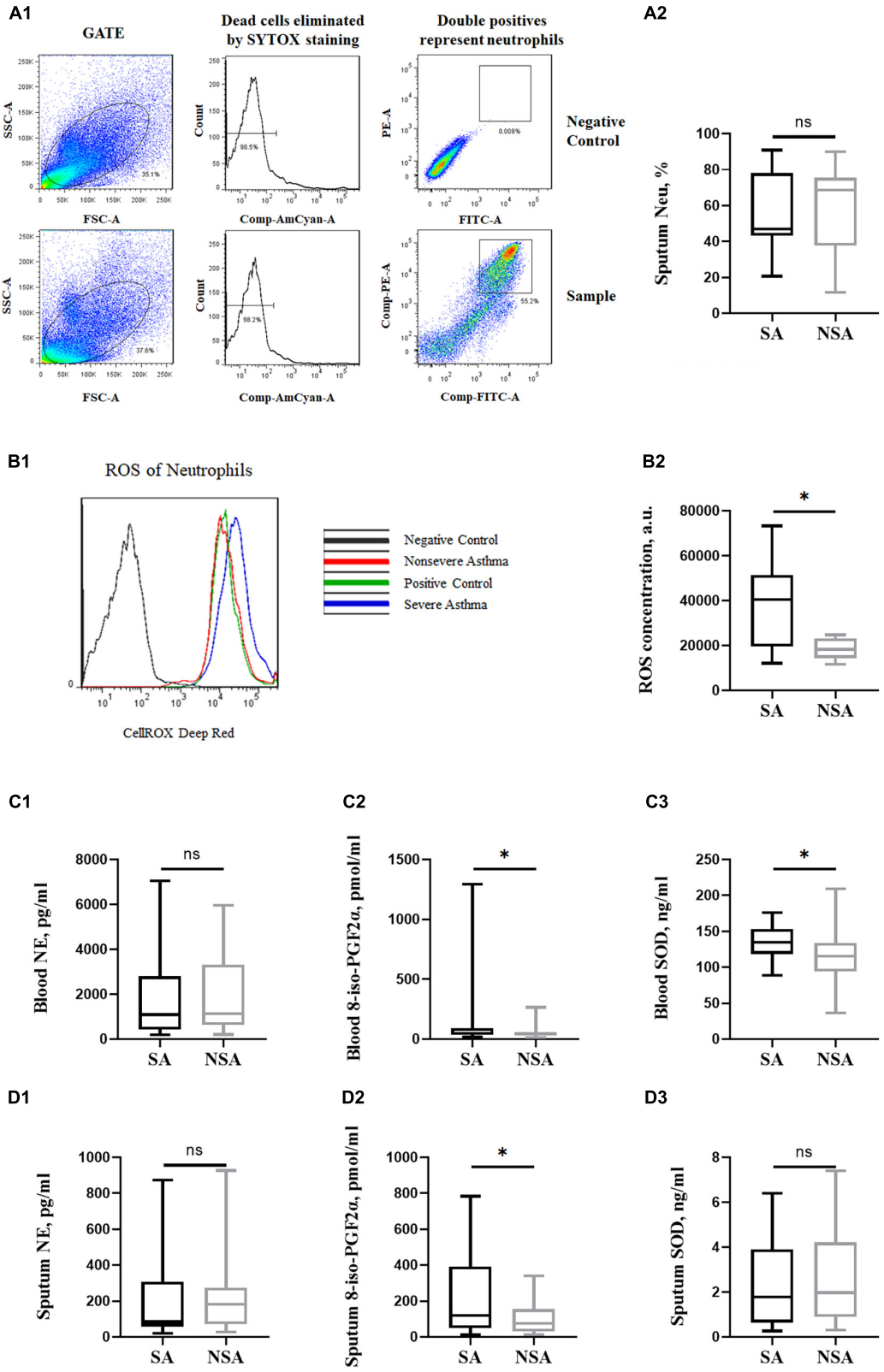

FIGURE 4
Oxidative stress of patients with asthma of different severities. This figure shows the flow cytometry of neutrophils and ROS concentration in induced sputum **(A1,B1)**. *T*-test results of neutrophils and ROS concentrations **(A2,B2)** and NE, 8-iso-PGF2α and SOD concentrations in peripheral blood **(C1–C3)** and induced sputum **(D1–D3)** between the severe (black block) and non-severe (gray block) asthma groups. ns: $P > 0.05$, *: $P < 0.05$.

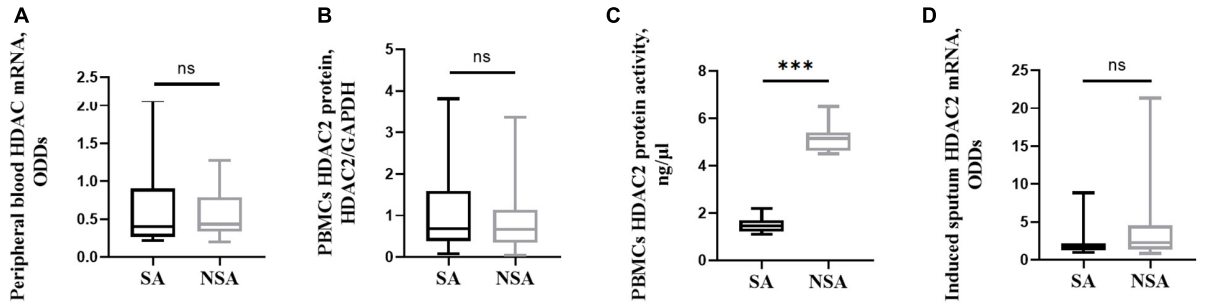

FIGURE 5
HDAC2 expression and activity in patients with asthma of different severities. This figure shows *t*-test results of the expression of peripheral blood HDAC2 mRNA **(A)**, and PBMCs HDAC2 protein **(B)**, PBMCs HDAC2 protein activity **(C)**, as well as the induced sputum HDAC2 mRNA **(D)** between the severe (black block) and non-severe (gray block) asthma group. ns: $P > 0.05$, ***: $P < 0.001$. PBMCs: peripheral blood mononuclear cells.

total glutathione (including glutathione and oxidized glutathione) in induced sputum and alveolar bronchial lavage in NSA patients is significantly higher than that in healthy people (38, 39). Ammar et al. (40) also found that increased SOD activity was one predictor of poorly controlled asthma. Based on the above, we believe that there is a compensatory effect of antioxidant activity in asthma patients and that when oxidative activity is increased, the antioxidant capacity is increased, even beyond the baseline capacity. However, the compensatory protection of antioxidant activity did not surpass the oxidative damage, so the increase in the oxidative stress product 8-iso-PGF2α was more pronounced. The mechanisms that determine the compensatory increase in antioxidant activity in asthma patients still need further study.

Myeloperoxidase (MPO) is often considered an indicator of neutrophil activity. The release of MPO during the activation of neutrophils is related to airway hyperreactivity (41), which can catalyze the reaction between hydrogen peroxide and chloride to produce hypochlorous acid with higher activity and stronger toxicity, which is considered to be a significant marker of inflammatory response (42), while nitrite formed by nitric oxide oxidation is an important substrate for MPO production (43). However, according to our results, we believe that MPO concentration cannot be used to determine the activation intensity of neutrophils in the body. A similar view has been found in studies of childhood asthma (44), which suggest that serum MPO is not involved in the assessment of inflammatory processes in childhood asthma, and the measurement of serum MPO appears to have no role in assessing the involvement of neutrophils in asthma.

As a protease, HDAC2 plays an important role in the modification of chromosome structure and regulation of gene expression and is related to the deacetylation of a variety of transcription factors, receptors and histones. HDAC2 is widely expressed and exists in the nucleus, and its decreased activity is correlated with hormone resistance (45). Oxidative stress can affect the expression and activity of HDAC2 protein in a variety of ways, resulting in hormone resistance (46), which may adversely affect the treatment of asthma. In SA patients, HDAC2 activity and expression are decreased, which may lead to a decreased glucocorticoid treatment effect and an enhanced inflammatory response (47, 48). In our study, HDAC2 activity was significantly reduced in the SA group. The mRNA expression of HDAC2 both in peripheral blood and in sputum showed a decreasing trend (due to the lower sample size, there was no statistical significance). These results suggest that HDAC2 expression may be impaired in asthma patients with neutrophilic and mixed granulocytic inflammatory phenotypes, leading to insensitivity to glucocorticoid therapy. They also suggest that the diagnosis and treatment of asthma, especially neutrophil-dominant asthma, need further exploration from the aspect of HDAC2 impairment. Until now, roxithromycin, 1,25-dihydroxyvitamin D3, clarithromycin and other drugs have been proven to be beneficial to improve the activity of HDAC2 in asthma animal experiments (49–51), which may become a new choice for asthma treatment in the future.

Inevitably, there are many biomarkers associated with neutrophilic asthma that we did not detect at this time, of which interleukin (IL)-17 is currently receiving a lot of attention. Th17 cells/IL-17 plays an important role in host defense and hyperimmune responses against pathogenic bacteria accompanied by the recruitment of neutrophils (52). Th17-associated inflammation usually contributes to the neutrophilic phenotype asthma, which is often characterized by greater severity, airflow obstruction, and steroid resistance (19, 52). IL-17-related cytokines expression was amplified in bronchial/nasal mucosa of neutrophilic asthma prone to exacerbation, suggesting a pathogenic role of IL-17F infrequent exacerbators (53). Animal models and human clinical studies have found that IL-17 exerts these effects through a variety of pathways. Östling et al. (54) found the activation of thromboxane B2 pathway in IL-17-high asthma patients. Hong et al. (55) found that the blood lipocalin-2 (LCN2) and serum amyloid A (SAA) levels may be associated with a type 17 asthma subtype, which are steroid-resistant IL-17A target genes in airway cells, and IL-17A–Act1/CEBPB axis is an important regulatory mechanism of LCN2 and SAA. Although Nascimento et al. (56) showed that treatment with IL-17A antibody contributed to the control of Th1/Th2/Th17 inflammation, chemokine expression, extracellular matrix remodeling, and oxidative stress in a murine model of lipopolysaccharide-exacerbated asthma. However, two clinical trials of humanized anti-IL-17A monoclonal antibodies, secukinumab and CJM112 failed to improve asthmatic symptoms in severe asthma patient (57). What's more, the studies of brodalumab, a humanized monoclonal antibody that binds to IL-17RA, was stopped because of the relative lack of efficacy in the initial asthma clinical trial and a questionable safety issue (58). These results highlight that studies on the association

between ILA-17 and neutrophil-dominant phenotype of severe asthma still need to be continued, which will be one of our future research directions.

5 Conclusion

In conclusion, our research suggests a role for neutrophilic-type airway inflammation in the pathogenesis of SA. The relationship among infiltrating neutrophils, oxidative stress damage and dysfunctional HDAC2 might contribute to glucocorticoid resistance. A prediction model for evaluating disease severity based on multidimensional clinical variables has been created, which does not require observing patients' status for a long time. Because of the complexity and heterogeneity of the pathogenesis of SA, the underlying mechanisms involved in neutrophilic-type SA need further study.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Institutional Review Board of Zhongshan Hospital, Fudan University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

ZC and HL conceived the writing structure. RM, ZJ, and ZM enrolled the patients and collected the raw data. RM, GW, MX, PG, and LZ reviewed or analyzed the data statistically. RM, ZC, and HL wrote and modified the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Zhihong Chen,
Fudan University, China

REVIEWED BY
Gioele Castelli,
University of Padua, Italy
Shi Chen,
Hunan Normal University, China
Mihir P. Rupani,
National Institute of Occupational Health (ICMR), India

*CORRESPONDENCE
Zhao-Hui Tong
✉ tongzhaohuicy@sina.com

†These authors have contributed equally to this work

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The systemic immune-inflammation index is significantly associated with the severity of silicosis: a 9-year retrospective study in Beijing

Han-Yu-Jie Kang[1†], Si-Yu Cao[1†], Shuai Shao[1], Li-Rong Liang[2] and Zhao-Hui Tong[1*]

[1]Department of Respiratory and Critical Care Medicine, Beijing Institute of Respiratory Medicine, Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China, [2]Department of Clinical Epidemiology, Beijing Institute of Respiratory Medicine and Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China

Background: Silicosis shows an increasing trend with the development of new industries. However, the potential biomarkers for predicting the disease severity are lacking. A novel inflammatory marker, the systemic immune-inflammation Index (SII), has not been studied in silicosis.

Methods: In this retrospective study, we used data from a big database platform of a tertiary general hospital in Beijing, which was established based on the electronic medical records of the hospital. The clinical data of adult patients diagnosed with silicosis at the Department of Occupational Medicine and Toxicology from 2013 to 2022 were collected. The data extracted from the database were in de-identified form. Only patients with a first diagnosis of silicosis and without conditions that might affect the parameters of routine blood tests were included in the analysis. Analyses were performed to assess the relationship between SII and the advanced stage of silicosis.

Results: A total of 246 participants were included in the study. Most of the patients were exposed to silica particles during excavation and digging ($n = 149$, 60.6%). SII level was significantly higher in patients with advanced stages of silicosis. A multivariate logistic regression analysis revealed that a higher SII level was associated with the advanced stage of silicosis [odds ratio (OR) = 1.002; 95% confidence interval (CI): 1.000–1.003, $p < 0.001$] after adjusting for all covariates. The best cutoff value of SII was 444.1. The results of the subgroup analysis also showed a significant correlation between SII level over 444.1 and the advanced stage of silicosis in groups stratified by gender, history of smoking, and duration of silica exposure. Moreover, our results showed a significant but weak negative correlation between the level of SII and some lung function parameters in silicosis.

Conclusion: Higher SII is associated with the advanced stage of silicosis and impaired lung function. More long-term, large-scale studies are needed to confirm these findings.

KEYWORDS

occupational disease, silicosis, severity, systemic immune-inflammation index, lung function

Introduction

Silicosis is a disease characterized by progressive pulmonary fibrosis caused by long-term inhalation of free silica dust in many industries (1). The formation of silicotic nodules and diffuse interstitial pulmonary fibrosis characterizes it (1). Silicosis is an occupational disease with relatively high morbidity and mortality (2). Globally, 2.65 million cases of silicosis were reported in 2019 (3). Silicosis is rising with new industries, such as artificial stone mesa manufacturing and jewelry polishing (2). There are still no effective treatments, and lung transplantation is often needed in the late stage (4). Therefore, it is essential to explore markers associated with the severity of the disease to help easily and quickly identify advanced-stage silicosis, which may be helpful to guide clinical management. It has been shown that the incidence of silicosis may be influenced by many factors, such as duration of dust exposure, cumulative total dust exposure, and genetic variants (5, 6). However, markers that may be associated with the severity of silicosis are very limited.

The systemic immune-inflammation index (SII) is an index that represents the body's systemic immune-inflammatory response based on the peripheral neutrophil, platelet, and lymphocyte counts (7).

Given the ease of access to these routine blood indicators, SII has received much attention in recent years. Many studies have explored the correlation between SII and the prognosis of some serious diseases, such as cancer, sepsis, and cardiovascular disease (8–10). According to a recent study, SII greater than 500 is a marker of pulmonary interstitial involvement in connective diseases (11). There is a close relationship between immunity and inflammation with the development of silicosis (2). Persistent inflammation and progressive worsening of lung fibrosis are characteristics of silicosis (12). However, little is known about the association between SII and the severity of silicosis.

Therefore, the focus of this study was to assess the association between SII and the severity of silicosis and further explore the predictive value of SII for the severity of silicosis, with the aim of helping the management of silicosis.

Methods

Research design and study population

This was a retrospective study using the big data platform of Beijing Chao-Yang Hospital, which was based on electronic medical records. We included patients diagnosed with silicosis at the Department of Occupational Medicine and Toxicology, Beijing Chao-Yang Hospital, between 2013 and 2022. Silicosis was diagnosed through multidisciplinary discussions on the basis of the occupational history of exposure to silica dust and the radiological criteria based on the International Labor Organization classification (13). If a patient had more than one hospitalization, only the one for the first diagnosis of silicosis was included for analysis. Patients with occupational lung diseases other than silicosis were not included. We also excluded patients who had diseases (pneumonia, tuberculosis, other infectious diseases, lung cancer) or were using drugs (immunosuppressive drugs) that could affect the parameters of routine blood tests. In addition, patients lacking information on silicosis staging, the duration of silica exposure, smoking history, and routine blood tests were excluded.

A total of 246 patients were included in the analysis. The study was approved by the Research Ethics Board of Beijing Chao-Yang Hospital (2023-ke-357). Data in the big data platform were de-identified. The patient's personal information was not identifiable, so informed consent was not required. The study adhered to the STROBE Guidelines, and the checklist is presented in Supplementary Table S1.

Classification of the stage of silicosis by chest radiograph

Silicosis was classified into three stages based on the International Labor Organization staging classification system (13) by multidisciplinary discussions. In short, posterior chest radiographs showed that each lung field was separated into three zones, namely upper, middle, and lower. The patients were classified as stage I when the distribution affected two or more zones, the greatest density of tiny opacities was ≥1/0, and pleural plaques were seen. The patients were classified as stage II when the distribution covered more than four zones and the highest density of small opacities was ≥2/1. The patients were classified as stage III when the biggest opacity measured ≥20 × 10 mm in diameter, or when the distribution affected four or more zones with aggregation of tiny or large opacities and the highest density of small opacities was ≥3/2. The advanced stage of silicosis was defined as stages II and III.

Data collection

Clinical records of the patients during hospitalization were obtained from the database. Specifically, demographic characteristics, including age, gender, duration of silica exposure, history of smoking, history of alcohol intake, and comorbidities, were collected. In addition, we extracted data on the patients' vital signs and symptoms on admission, silicosis stage, laboratory tests, lung function parameters, and outcome indicators (length of hospital stay and hospital mortality) from the database.

Calculation of the SII index

We collected neutrophil, platelet, and lymphocyte counts from admission routine blood tests to assess the SII index. The following formula was used to calculate the SII index: (neutrophil count × platelet count)/lymphocyte count (7, 14).

Covariates

The study adjusted for covariates that could affect the correlation between SII and the severity of silicosis. In detail, many variables,

Abbreviations: SII, Systemic immune-inflammation index; OR, Odds ratio; CI, Confidence interval; CRP, C-reactive protein; ROC, Receiver operating characteristic; AUC, Area under a receiver operating characteristic curve; PAF, Platelet-activation factor; NLR, Neutrophil-to-lymphocyte ratio; PLR, Platelet-to-lymphocyte ratio; CTD-ILD, Interstitial lung disease secondary to connective tissue diseases.

including baseline characteristics (such as age, gender, duration of silica exposure, history of smoking, and history of smoking ≥10 pack-years), and laboratory tests [such as neutrophil count, lymphocyte count, platelet count, and C-reactive protein (CRP)], were considered.

Statistical analysis

For continuous variables, normally distributed data are presented as the mean ± standard deviation, and non-normally distributed data are presented as the median with an interquartile range. For categorical variables, data are presented as frequencies (percentages). Student's *t*-test or Mann–Whitney U test was used for comparing continuous variables. Categorical variables were compared by the Chi-square test or Fisher's exact test.

The receiver operating characteristic (ROC) curve was used to access the optimal cutoff value of the SII according to the Youden index. Before multivariate analysis, bivariate analyses were performed. The variables with a *p*-value lower than 0.05 and other covariates that could be associated with advanced silicosis were included in the multivariate logistic regression model for adjustment. Then, multivariate logistic regression analysis was performed to investigate the association between SII and the advanced stage of silicosis. Sensitivity analyses were performed by subgroup analyses, which were further used to explore the association between SII and severe silicosis in different subgroups. The subgroups were stratified by gender, smoking history, and silica exposure duration. The interaction test assessed the heterogeneity of the association between the subgroups. ROC curves were used to judge the sensitivity of markers to identify the advanced stage of silicosis. The Pearson r test or Spearman test was used to explore the correlation between the variables. All statistical analyses were performed using SPSS version 26.0 (SPSS Inc., Chicago, IL, United States) and the statistical software package R. A $p < 0.05$ was considered statistically significant.

Results

Patient characteristics

After the selection process presented in Figure 1, a total of 246 eligible patients were included in the study. More than 50% of the patients were workers with an occupational history of exposure to silica dust during excavation and digging (149, 60.6%), followed by polishing and buffing (53, 21.5%), handling raw materials (24, 9.8%), and rock blasting and sand blasting (20, 8.1%). The median age was 61 years (IQR, 54–70), and the median duration of silica exposure was 20 years (IQR, 12–25). Most of the patients were male (84.1%), and only a small portion of the patients in this study were female (15.9%). Among these patients, 106 (43.1%) had a smoking history. A significant smoking history of ≥10 pack-years was reported by 77 (31.3%) patients. Only 57 (23.2%) patients had a history of alcohol intake. The main symptoms of these patients on admission were coughing sputum ($n = 159$, 64.6%), cough ($n = 131$, 53.3%), and dyspnea ($n = 106$, 43.1%). Although coughing sputum was the most obvious symptom, it was not caused by a chest infection, given that we excluded patients with infectious diseases. The symptom may have been related to silicosis itself, possibly due to silica dust irritation. The median length of hospital stay was 11 days (IQR, 8–13 days), and the hospital mortality was only 2.0% (5/246). Advanced stage silicosis (stage II-III silicosis) was present in 169 (68.7%) subjects. The characteristics of the 246 patients are summarized in Table 1.

Compared with the early stage of silicosis (stage I silicosis), the patients in the advanced stage of silicosis (stage II-III silicosis) had a longer duration of silica exposure ($p = 0.020$). More male genders were also found in the group of advanced stage of silicosis ($p = 0.011$). There were 26 subjects (33.8%) in stage I silicosis (early stage) and 80 subjects (47.3%) in stage II-III silicosis (advanced stage) with a history of smoking ($p = 0.046$). Especially patients with a history of smoking ≥10 pack-years were significantly more frequent ($p = 0.007$) among patients with stage II-III silicosis

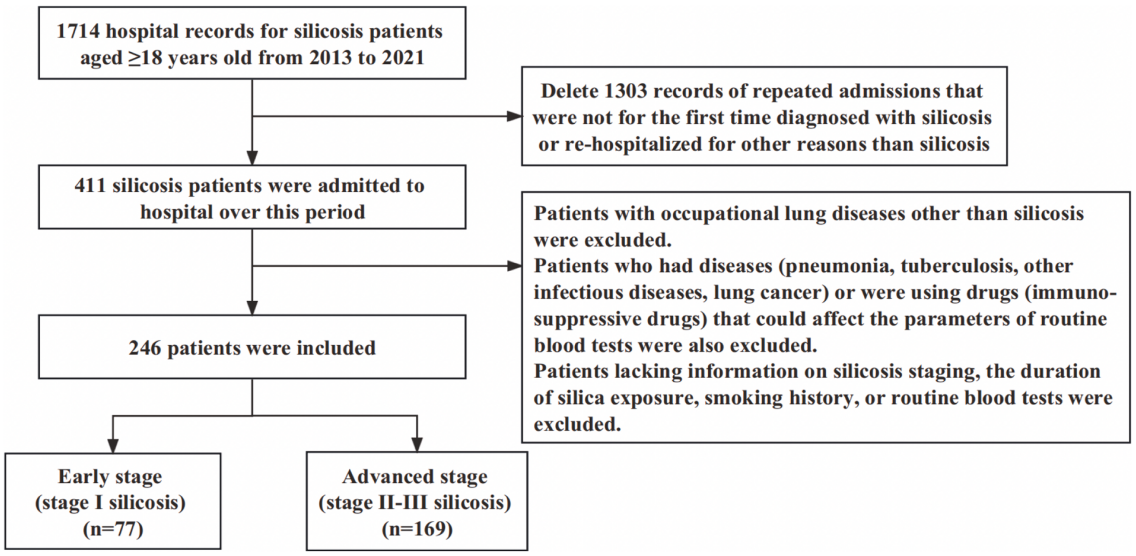

FIGURE 1
Flowchart of the participants selection.

(36.7%) compared to those with stage I silicosis (19.5%). The neutrophil counts ($p = 0.012$) and platelet counts ($p = 0.042$) were higher in patients with advanced stages of silicosis, whereas lymphocyte count was lower ($p = 0.002$). SII level was significantly higher in patients in the advanced stages of silicosis relative to those in the early stages ($p < 0.001$). More details of the comparison between the patients with stage I silicosis and stage II-III silicosis are shown in Table 1.

TABLE 1 Characteristics of patients with silicosis according to early (stage I silicosis) versus advanced (stage II-III silicosis) stage.

	Overall ($n = 246$)	Stage I silicosis ($n = 77$)	Stage II-III silicosis ($n = 169$)	p-value
Baseline characteristics				
Age, years	61.0 (54.0–70.0)	63.0 (58.5–69.0)	61.0 (52.5–71.5)	0.163
Male, n (%)	207 (84.1%)	58 (75.3%)	149 (88.2%)	0.011
Duration of exposure (years)	20.0 (12.0–25.0)	17.0 (11.0–23.5)	21.0 (12.5–26.0)	0.020
History of smoking, n (%)	106 (43.1%)	26 (33.8%)	80 (47.3%)	0.046
History of smoking ≥10 pack-years	77 (31.3%)	15 (19.5%)	62 (36.7%)	0.007
History of alcohol intake, n (%)	57 (23.2%)	15 (19.5%)	42 (24.9%)	0.354
Comorbidities, n (%)				
Diabetes	35 (14.2%)	10 (13%)	25 (14.8%)	0.707
Hyperlipidemia	6 (2.4%)	3 (3.9%)	3 (1.8%)	0.317
Hypertension	81 (32.9%)	19 (24.7%)	62 (36.7%)	0.063
Coronary heart disease	2 (0.8%)	1 (1.3%)	1 (0.6%)	0.567
Stroke	7 (2.8%)	4 (5.2%)	3 (1.8%)	0.135
On hospital admission				
Heart rate, bpm	80 (76–90)	80 (72–84)	80 (76–93)	0.068
Systolic blood pressure, mmHg	125 (120–138)	121 (118–130)	125 (120–140)	0.064
Diastolic blood pressure, mmHg	75 (70–80)	73 (70–80)	77 (70–80)	0.530
Symptoms				
Chest pain, n (%)	7 (2.8%)	2 (2.6%)	5 (3.0%)	0.618
Chest distress, n (%)	26 (10.6%)	6 (7.8%)	20 (11.8%)	0.339
Coughing sputum, n (%)	159 (64.6%)	53 (68.8%)	106 (62.7%)	0.353
Cough, n (%)	131 (53.3%)	38 (49.4%)	93 (55.0%)	0.408
Hemoptysis, n (%)	12 (4.9%)	1 (1.3%)	11 (6.5%)	0.067
Dyspnea, n (%)	106 (43.1%)	35 (45.5%)	71 (42.0%)	0.613
Shortness of breath, n (%)	43 (17.5%)	9 (11.7%)	34 (20.1%)	0.106
Laboratory tests				
White blood cell, 10^9/L	6.28 (5.19–7.70)	6.06 (4.95–7.14)	6.39 (5.24–7.82)	0.126
Neutrophil, 10^9/L	4.03 (3.18–5.01)	3.81 (2.86–4.57)	4.23 (3.35–5.44)	0.012
Lymphocyte, 10^9/L	1.54 (1.16–1.99)	1.70 (1.38–2.14)	1.50 (1.06–1.86)	0.002
Monocyte, 10^9/L	0.43 (0.34–0.55)	0.42 (0.30–0.51)	0.44 (0.34–0.56)	0.097
Platelet, 10^9/L	214 (175–261)	203 (173–247)	222 (176–270)	0.042
Hemoglobin, g/L	133 (118–145)	134 (125–145)	133 (115–146)	0.296
CRP, mg/L	0.74 (0.28–1.75)	0.38 (0.25–1.32)	0.84 (0.33–2.23)	0.134
SII, 10^9/L	529.7 (396.1–927.6)	415.2 (289.5–676.4)	569.8 (438.1–1060.5)	<0.001
Outcome				
Length of hospital stay, days	11.0 (8.0–13.0)	10.0 (8.0–13.0)	11.0 (8.0–13.5)	0.127
Hospital mortality, n (%)	5 (2.0%)	1 (1.3%)	4 (2.4%)	0.501

The enumeration data indicators were described by frequency/percentage and compared by the Chi-square or Fisher' s exact test. The continuous variables with normal distribution were described by mean ± standard deviation, and median and interquartile ranges were used to describe the non-normal distribution variables. Student' s t-test compared variables with normal distribution, and variables with non-normal distribution were compared by Mann–Whitney test. Significance was set as $p < 0.05$.

SII and the severity of silicosis

We created a multivariate logistic regression model to explore the factors that may be associated with the advanced stage of silicosis. Baseline characteristics associated with the advanced stage of silicosis by bivariate analysis and other potential confounding factors were included in the multivariate analysis. The details of adjusting for confounding factors are presented in Table 2. In the multivariate logistic regression, the SII level (OR = 1.002, 95% CI: 1.000–1.003, $p < 0.001$), male (OR = 3.909, 95% CI: 1.319–11.581, $p = 0.014$), duration of silica exposure (OR = 1.038, 95% CI: 1.003–1.075, $p = 0.031$) and history of smoking ≥10 pack-years (OR = 2.112, 95% CI: 1.075–4.147, $p = 0.030$) were significantly associated with the advanced stage of silicosis.

To further explore the relationship between the SII index and the advanced stage of silicosis, we performed ROC curve analysis to determine the optimal cutoff value of the SII. The results revealed that the optimal cutoff value of SII was 444.1 for the advanced stage of silicosis. Thus, we classified the patients with an SII value greater than 444.1 into the high SII group, while all other patients were defined as the low SII group. Similarly, a multivariate logistic regression model was used. As shown in Table 3, the multivariate analysis showed that high SII levels were significantly associated with the advanced stage of silicosis (OR = 5.110, 95% CI 2.807–9.302, $p < 0.001$). In addition, a history of smoking ≥10 pack-years (OR = 2.249, 95% CI 1.125–4.496, $p = 0.022$) and a longer duration of silica exposure (OR = 1.044, 95% CI 1.007–1.082, $p = 0.018$) were also associated with more severe silicosis (stage II-III silicosis) in the study.

Subgroup analysis

In Table 4, subgroup analyses based on the factors that may influence the severity of silicosis in the above regression models were performed to explore the association between the SII and severe silicosis. The results of the subgroup analysis indicated that the positive correlation between SII and the advanced stage of silicosis was significant in all of the subgroups stratified by gender, history of smoking, and duration of silica exposure. The interaction test suggested no significant effects of different genders, history of smoking, and duration of silica exposure on the positive relationship between the SII and the advanced stage of silicosis (p for interaction >0.05). More information about the subgroup analysis of the confounding factors is shown in Supplementary Tables S2–S7.

Correlations between SII and pulmonary function test

Of these patients, a total of 56 underwent pulmonary function tests. We found that lung function parameters, such as FVC (% pred), FEV1 (% pred), FEV1/FVC %, and DLCO (% pred), were significantly different between the advanced stages and early stages of silicosis. The results are shown in Table 5. These parameters negatively correlated with the level of SII (Figure 2). The results revealed the relatively poor pulmonary function in silicosis, which is possibly related to higher SII levels. Given the obvious difference in lung function parameters between the advanced and early stages of silicosis, the association may be more due to the higher SII levels in more advanced stages of silicosis. Hence, the findings again suggested a correlation between the SII and disease severity.

Predictive power of high SII levels for the severity of silicosis

The ROC curve was used to evaluate the sensitivity and specificity of high SII levels to predict the advanced stages of silicosis. Although we found that high SII levels had a higher area under a receiver operating characteristic curve (AUC) for identifying more severe silicosis than a history of smoking ≥10 pack-years and duration of

TABLE 2 The logistic regression model revealed the association between the systemic immune-inflammation index and the stage of silicosis.*

Variables	OR (95% CI)	p
SII	1.002 (1.000–1.003)	<0.001
Male	3.909 (1.319–11.581)	0.014
Duration of exposure (years)	1.038 (1.003–1.075)	0.031
History of smoking ≥10 pack-years	2.112 (1.075–4.147)	0.030
History of smoking	0.559 (0.139–2.246)	0.413
Age	0.980 (0.953–1.007)	0.144
Neutrophil counts	0.612 (0.329–1.139)	0.122
Lymphocyte counts	0.704 (0.136–3.652)	0.676
Platelet counts	1.003 (0.989–1.017)	0.668
CRP	0.938 (0.733–1.200)	0.610

OR, odds ratio; CI, confidence interval.
*Whole Model Test < 0.001.
*$n = 246$.
*The model adjusted for age, gender, duration of silica exposure, history of smoking, history of smoking ≥10 pack-years, neutrophil counts, lymphocyte counts, platelet counts, and CRP.

TABLE 3 The logistic regression model revealed the association between higher SII (SII ≥ 444.1) and the advanced stage (stage II-III silicosis) of silicosis.*

Variables	OR (95% CI)	p
SII ≥444.1	5.110 (2.807–9.302)	<0.001
Duration of exposure (years)	1.044 (1.007–1.082)	0.018
History of smoking ≥10 pack-years	2.249 (1.125–4.496)	0.022
Male	3.201 (0.882–11.626)	0.077
Age	0.976 (0.947–1.006)	0.110
History of smoking	0.766 (0.294–1.998)	0.586
Neutrophil counts	1.046 (0.823–1.330)	0.713
Lymphocyte counts	0.613 (0.321–1.172)	0.139
Platelet counts	1.002 (0.997–1.008)	0.401
CRP	0.986 (0.793–1.225)	0.896

OR, odds ratio; CI, confidence interval.
*Whole Model Test < 0.001.
*$n = 246$.
*The model adjusted for age, gender, duration of silica exposure, history of smoking, history of smoking ≥10 pack-years, neutrophil counts, lymphocyte counts, platelet counts, and CRP.

TABLE 4 Subgroup analysis of the association between higher SII and advanced stage (stage II-III silicosis) of silicosis.*

Subgroup	No of participants	OR (95% CI)	*p*	*p* for interaction
Gender				0.753
Male	143	4.793 (2.435, 9.435)	<0.001	
Female	16	5.625 (1.359, 23.274)	0.017	
History of smoking				0.536
≥10 pack-years	56	4.308 (1.318, 14.081)	0.016	
<10 pack-years	103	5.571 (2.763, 11.232)	<0.001	
Duration of exposure (years)				0.759
>20 years	77	6.469 (2.493, 16.786)	<0.001	
≤20 years	82	4.747 (2.121, 10.626)	<0.001	

*The model adjusted for age, gender, duration of silica exposure, history of smoking, history of smoking ≥10 pack-years, neutrophil counts, lymphocyte counts, platelet counts, and CRP.

TABLE 5 Lung functions of patients with silicosis according to early (stage I silicosis) versus advanced (stage II-III silicosis) stage.

	Overall (*n* = 56)	Stage I silicosis (*n* = 28)	Stage II–III silicosis (*n* = 28)	*p*-value
FVC (% pred)	90.2 ± 22.3	97.0 ± 19.0	83.3 ± 19.0	0.020
FEV1 (% pred)	79.4 ± 27.8	89.8 ± 23.2	69.0 ± 28.5	0.004
FEV1/FVC %	83.8 ± 13.8	88.0 ± 8.5	79.5 ± 16.7	0.023
DLCO (% pred)	81.4 ± 16.8	87.3 ± 11.3	75.4 ± 19.3	0.007

silica exposure, we still thought that a single factor could not be sufficient for stratification of the severity of silicosis. Thus, we further established new ROC curves based on the combined markers. As shown in Figure 3, the ROC analysis demonstrated that the combination of high SII levels with the other two variables had the highest AUC of 0.757 (95% confidence interval 0.692–0.822; $p < 0.001$) for identifying the more severe silicosis.

Discussion

To our knowledge, this is the first study to demonstrate the association between the SII and the severity of silicosis. We found that the patients with advanced stages of silicosis (stage II-III silicosis) had a higher level of SII than the patients with early stages of silicosis (stage I silicosis). Our findings revealed that high SII levels significantly correlated with the advanced stage of silicosis, even after adjusting for various covariates among the patients with silicosis.

Inflammation is a driving factor in the development of silicosis (12). The potential mechanism is that macrophage necrosis after macrophage phagocytosis of silica particles drives inflammation (1). More macrophages again phagocytose the released silica dust, and the repeated process leads to persistent inflammation, thereby contributing to fibrosis (1). Many studies have revealed the relationship between immunity and silicosis. Recently, a multi-omics study based on the silicosis mouse model has emphasized the importance of immune cell chemotaxis, an essential biological process during silicosis development (15). Moreover, peripheral blood T-cell dysregulation has been shown in silica-exposed workers (16). Studies on mouse models have also suggested that T-lymphocyte subsets play an important role in promoting the progression of silicosis (17, 18). Furthermore, increased neutrophil infiltration has been found in the silicosis mouse model (19). Another study showed that mitoDNA could activate neutrophils via TLR9 and cause severe inflammatory responses in the lung tissues of mice with silicosis (20). Although there are few studies about platelets in silicosis, platelet-activation factor (PAF) concentrations have been found to be significantly higher in the plasma of silicosis patients (21). Furthermore, the antagonist of PAF has been shown to improve silica-induced pulmonary fibrosis in animal models (22). Overall, these studies have suggested the close correlation of neutrophils, platelets, and lymphocytes with the pathogenesis of silicosis.

Several studies have explored the correlation of some inflammatory markers obtained from routine blood tests, such as neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR), with silicosis. The NLR and PLR have been found to be higher in patients with silicosis than in unexposed controls and patients exposed to silica without silicosis (23). Studies also indicated their potential value in predicting the prognosis of patients with silicosis (24, 25). In special populations with silicosis, such as engineered stone silicosis patients, these indicators could help to evaluate the disease progression (26). As a new marker, the SII can comprehensively integrate the association between neutrophils, platelets, and lymphocytes, compared with NLR and PLR. Some studies have explored its relation to fibrosis-related diseases, such as interstitial lung disease secondary to connective tissue diseases (CTD-ILD) (11) and liver fibrosis (27). Silicosis is also a fibrotic lung disease (28). Our study explored the relationship between the SII, a novel marker, and silicosis for the first time. The results suggested that higher SII levels, especially with SII values greater than 444.1, were significantly associated with more severe silicosis, even after adjusting for many covariates. However, it is worth noting that the clinical application of the SII is somewhat limited by the fact that

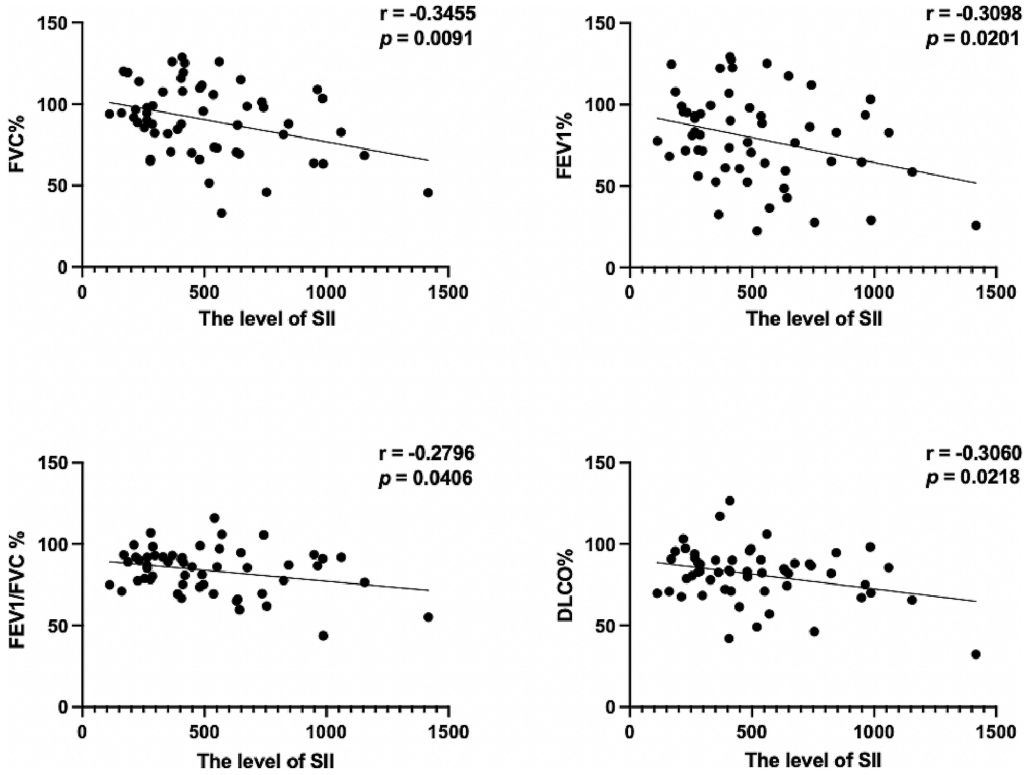

FIGURE 2
Correlations between SII and pulmonary function test.

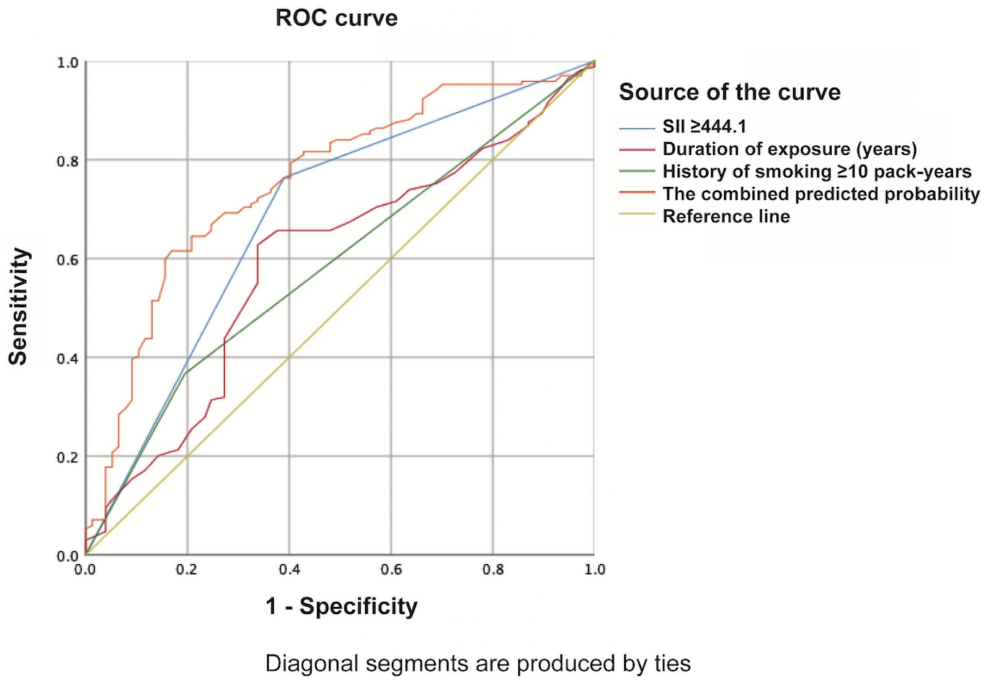

FIGURE 3
ROC curve of the combination of high SII levels with the other two variables evaluating the severity of silicosis.

blood-based inflammatory indexes are not specific, as they may elevate with respiratory infection or other inflammatory conditions. In addition, tuberculosis, a common complication of silicosis (29, 30), also may potentially alter the inflammatory markers. Therefore, in order to avoid the nonspecific effects of SII, we set strict inclusion and exclusion criteria for the patients' enrollment, excluding

pneumonia, tuberculosis, other infectious diseases, and any condition that could affect hematologic indexes, so as to make the results of the association between SII levels and the severity of silicosis more objective.

Predicting the severity of silicosis by only one factor is of limited value. Our study showed that in addition to higher SII, the duration of silica exposure and a history of smoking ≥10 pack-years were associated with the advanced stages of silicosis. It is well-known that the duration of silica exposure is an important factor in the development of silicosis (1, 5) because exposure duration reflects, to some extent, the cumulative dose of silica (1). As for smoking history, recent animal experimental data have shown that smoking can aggravate inflammation and pulmonary fibrosis of silicosis (31, 32). The findings from these animal studies suggest that cigarette smoking may be associated with the severity of silicosis, thereby supporting our study results. Previous studies have shown an association between smoking and the risk of idiopathic pulmonary fibrosis (33). The proteomic approach also has revealed that smoking exposure leads to a significant dysregulation of many molecular pathways related to interstitial lung diseases (34). As for silicosis, some studies also suggested that cigarette smoking could increase the risk of death in individuals with silica dust exposure (35, 36). This further suggests the harm of smoking, emphasizing the importance of quitting smoking. In addition, our study showed that there were more male patients in the advanced-stage silicosis group, possibly because adult males have more potential for occupational exposure as the primary workforce. Our findings further suggest that the combined indicators, including a higher SII level (SII ≥ 444.1), longer duration of silica exposure, and heavy smoking history, may be better suited for identifying more severe silicosis. This study examined the value of the SII in predicting disease severity, and the diagnostic value of the SII should be further investigated in the future. The diagnostic accuracy of the SII needs to be compared with that of chest radiography-based diagnosis of silicosis to help explore the early and quick diagnosis marker of silicosis.

Previous studies have indicated that lung function impairment increases with silicosis progression (1).

Our study similarly showed impaired pulmonary function in the advanced stage of silicosis relative to the early stage. Lung function in patients with silicosis might present with obstructive, restrictive, or even mixed ventilatory disorders (37–39). The obstructive disorder may be related to the direct inflammatory stimulation of airways induced by inhaled silica particles (40). Restrictive ventilatory disorder may be associated with the progression of interstitial fibrosis (41). As for inflammatory markers, such as NLR and PLR, their correlations with lung function parameters are controversial. A previous study revealed a negative correlation of NLR and PLR with FVC and of NLR with FEV1/FVC (24). However, a recent study has only found an association of NLR with DLCO (23). In contrast, our results showed a significant negative correlation, although weak, between the SII and lung function parameters, such as FVC (% pred), FEV1 (% pred), FEV1/FVC %, and DLCO (% pred). However, correlation does not necessarily mean causation. We rather think that the association was more due to the higher SII values in the more severe stages of silicosis. This finding again suggests that the level of SII might be indicative of more severe stages of silicosis with lung function progression. More importantly, as higher SII levels were related to the advanced stage of silicosis, these patients should be managed and followed up regularly to prevent the development of lung function impairment.

There were still several limitations to the study. First, given the nature of single-center studies, the results may have selection bias. To reduce selection bias to some extent, strict inclusion and exclusion criteria and rigorous study methodology were developed to ensure that the patients included in the study and the results were representative. Second, due to the limited data in the database, more clinical records were lacking. Third, given the limited sample size, there is currently no significant difference between the SII levels of stage II and III silicosis (this part of the data is not presented); the sample size will be expanded in the future to explore the subgroups of different stages for more detailed analyses, making the results more convincing. Therefore, prospective large-scale multicenter studies will need to confirm our findings in the future.

Conclusion

In summary, our findings showed that higher SII levels are associated with more severe silicosis, and the best cutoff value of SII is 441.1. There is a significant but weak negative correlation between SII levels and lung function parameters. The SII, a comprehensive and easily accessible potential biomarker, might be used to help identify the severity of silicosis routinely. However, given the non-specificity of the inflammatory markers in general and the limitation of the single-center retrospective study, more long-term, large-scale studies are required to validate these findings.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by The Research Ethics Board of Beijing Chao-Yang Hospital (project approval number: 2023-ke-357). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

H-Y-JK: Conceptualization, Data curation, Methodology, Writing – original draft. S-YC: Data curation, Methodology, Writing – original draft. SS: Data curation, Writing – original draft. L-RL: Methodology, Writing – review & editing. Z-HT: Conceptualization, Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2024.1351589/full#supplementary-material

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EDITED BY
Suzana Erico Tanni,
São Paulo State University, Brazil

REVIEWED BY
Konstantinos Bartziokas,
Independent Researcher, Trikala, Greece
Renata Ferrari,
São Paulo State University, Brazil

*CORRESPONDENCE
Ting Yang
✉ zryyyangting@163.com

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Efficacy and safety of a music-therapy facilitated pulmonary telerehabilitation program in COPD patients: the COPDMELODY study protocol

Minghui Shi[1,2,3,4,5], Lulu Yang[6], Shiwei Qumu[1,2,3,4], Jieping Lei[7], Ke Huang[1,2,3,4], Ruoxi He[1,2,3,4], Hongtao Niu[1,2,3,4], Fen Dong[7], Siyuan Wang[8], Jiaze He[1,2,3,4] and Ting Yang[1,2,3,4]*

[1]National Center for Respiratory Diseases, Beijing, China, [2]Institute of Respiratory Medicine, Chinese Academy of Medical Sciences, Beijing, China, [3]National Clinical Research Center for Respiratory Diseases, Beijing, China, [4]Department of Pulmonary and Critical Care Medicine, Center of Respiratory Medicine, China-Japan Friendship Hospital, Beijing, China, [5]Capital Medical University, Beijing, China, [6]Fangzhuang Community Health Service Center, Capital Medical University, Beijing, China, [7]Department of Clinical Research and Data Management, Center of Respiratory Medicine, China-Japan Friendship Hospital, Beijing, China, [8]Department of Rehabilitation Medicine, China-Japan Friendship Hospital, Beijing, China

Despite considerable evidence for the benefit in chronic obstructive pulmonary disease (COPD), the implementation of pulmonary rehabilitation (PR) is insufficient. However, music therapy may help address this gap due to its unique benefits. Therefore, we aimed to develop a music-therapy facilitated pulmonary telerehabilitation program based on rhythm-guided walking, singing, and objective telemonitoring. A supervised, parallel-group, single-blinded, randomized controlled clinical trial will be conducted, including 75 patients with COPD anticipated to be randomized in a 1:1:1 ratio into three groups. The intervention groups will receive a 12-week remotely monitored rehabilitation program, while the usual care group will not receive any rehabilitation interventions. Of the two intervention groups, the multi-module music therapy group will contain rhythm-guided walking and singing training, while the rhythm-guided walking group will only include music tempo-guided walking. The primary outcome is the distance of the incremental shuttle walking test. Secondary outcomes include respiratory muscle function, spirometry, lower extremity function, symptoms, quality of life, anxiety and depression levels, physical activity level, training adherence, and safety measurements. The results of this study can contribute to develop and evaluate a home-based music-facilitated rehabilitation program, which has the potential to act as a supplement and/or substitute (according to the needs) for traditional center-based PR in patients with stable COPD.

Clinical trial registration: https://classic.clinicaltrials.gov/, NCT05832814.

KEYWORDS

chronic obstructive pulmonary disease, telerehabilitation, music therapy, randomized controlled trial, pulmonary rehabilitation

1 Introduction

Chronic obstructive pulmonary disease (COPD) is the leading cause of morbidity and premature mortality globally, surpassing cancer, with the third-highest number of deaths in China, resulting in overwhelming pressure on healthcare systems (1). Moreover, living with COPD is often a daily struggle marked by reduced physical activity, further increasing disease progression, and creating a vicious circle (2). Psychologically, patients with COPD tend to suffer from increased stress and anxiety due to dyspnea and muscle dysfunction (3, 4).

Despite significant evidence on exercise capacity, symptoms and prognosis, the implementation of conventional pulmonary rehabilitation (PR) for COPD is insufficient, mainly due to inadequate resources, high cost, travel distance, lack of time, and low self-efficacy (5). Nonetheless, various telerehabilitation programs have been developed to overcome these barriers (6, 7). However, they play a limited role in reversing patients' low interests in exercises and rejection of rehabilitation due to fear of dyspnea and fatigue during training. Moreover, it is difficult to ensure the exact intensity of home-based exercises without professional equipment and real-time supervision (8, 9) and telerehabilitation programs with minimal equipment support are limited.

Recent studies have highlighted that music-facilitated rehabilitation not only benefits the interrelated physical and psychological consequences of patients but also improves motivation and training adherence. Music therapy (MT) targeting COPD includes passive (listening to music during aerobic exercises) and active elements (singing) (10, 11). Using rhythms to control the walking speed could offer an easy, economical way to ensure the exercise intensity at home. It can also be regarded as distractive auditory stimulus therapy, helping with dyspnea and fatigue during exertion (12). Additionally, singing promotes adaptation to breath control and training related respiratory muscles with less cost and more interest (13). Therefore, integrating tele-PR and MT may help overcome the previously mentioned barriers. However, there is a lack of relevant home-based studies as most of the existing studies were conducted in the hospital or community. Moreover there is limited evidence on the integrated effects of singing and music-guided aerobic exercises, with most studies only applying one kind of intervention (14).

Therefore, to identify a helpful and easy-to-use rehabilitation mode, our study aimed to develop a home-based, music-facilitated rehabilitation program for patients with COPD, using music tempo-guided walking exercises and breathing exercises, such as singing. Based on a thorough literature review, this is the first study to combine the two forms of MT for home-based PR and apply objective recording and supervision of training implementation using wearable sensors (a sports wristwatch that monitors step counts and walking distance) for music-facilitated tele-PR. This study protocol describes the process to investigate the efficacy and safety of the developed MT-facilitated telerehabilitation program (including rhythm-guided walking and singing).

The primary aim of this study is to explore the efficacy of this home-based, MT- facilitated rehabilitation program (including rhythm-guided walking and singing). The secondary aim is to explore whether addition of singing training results in improvements compared with rhythm-guided walking alone. Therefore, we hypothesize that (1) our MT-facilitated tele-PR mode could benefit patients with COPD in terms of exercise capacity, respiratory musculature, and symptoms; and (2) singing training could result in greater improvement compared with rhythm-guided walking alone.

2 Methods and analysis

2.1 Study design

This study will use a multi-center, prospective, three-arm, randomized controlled trial design. The study will include patients with COPD enrolled in a multi-module MT rehabilitation group (multi-module MT group), a rhythm-guided walking rehabilitation group (rhythm-guided walking group), and a usual care group. The study period will last for 12 weeks, and the outcomes will be measured at baseline (V0), 4 weeks (V1), 8 weeks (V2), and the end of intervention (V3). A Consolidated Standards of Reporting Trials (CONSORT) flow diagram is illustrated in Figure 1. The study protocol will be reported according to the Standard Protocol Items: Recommendations for Intervention Trials 2013 (SPIRIT) (15) guidelines and the intervention procedures are described according to the CONSORT 2010 Checklists (16). The trial is registered at ClinicalTrials.gov (registration number: NCT05832814). The study schedule and assessments are summarized in Table 1.

2.2 Study population and recruitment

This multi-center study will be conducted at the China-Japan Friendship Hospital. Patients will be recruited from the China-Japan Friendship Hospital, Beijing Tiantan Hospital, Beijing Luhe Hospital, and the Second Affiliated Hospital of Xi'an Jiaotong University, Qingdao Municipal Hospital. Advertising strategies will include flyers within the hospital and professional recommendations. Potential eligible patients who are interested in participation will be invited to have either a face-to-face or telephone meeting wherein the researchers will explain the study in detail and allow time for questions. Potential patients will be asked about medical history and undergo a pulmonary function test to ensure final eligibility. The inclusion and exclusion criteria are summarized in Table 2. Patients who meet the eligibility criteria will be invited to sign informed consent forms and complete baseline outcome assessment.

2.3 Randomization, allocation concealment, and blinding

All patients will be randomized in a 1:1:1 ratio (adhering to block randomization within each center) into three groups: Multi-module MT, rhythm-guided walking, and usual care groups using a table of random numbers generated in the SPSS statistical package held by an independent statistical analyst. Group information will be stored in an opaque envelope and sealed thereafter. After obtaining informed consent and baseline assessment, the study coordinator will unseal the envelope to obtain the random numbers and grouping information for each participant.

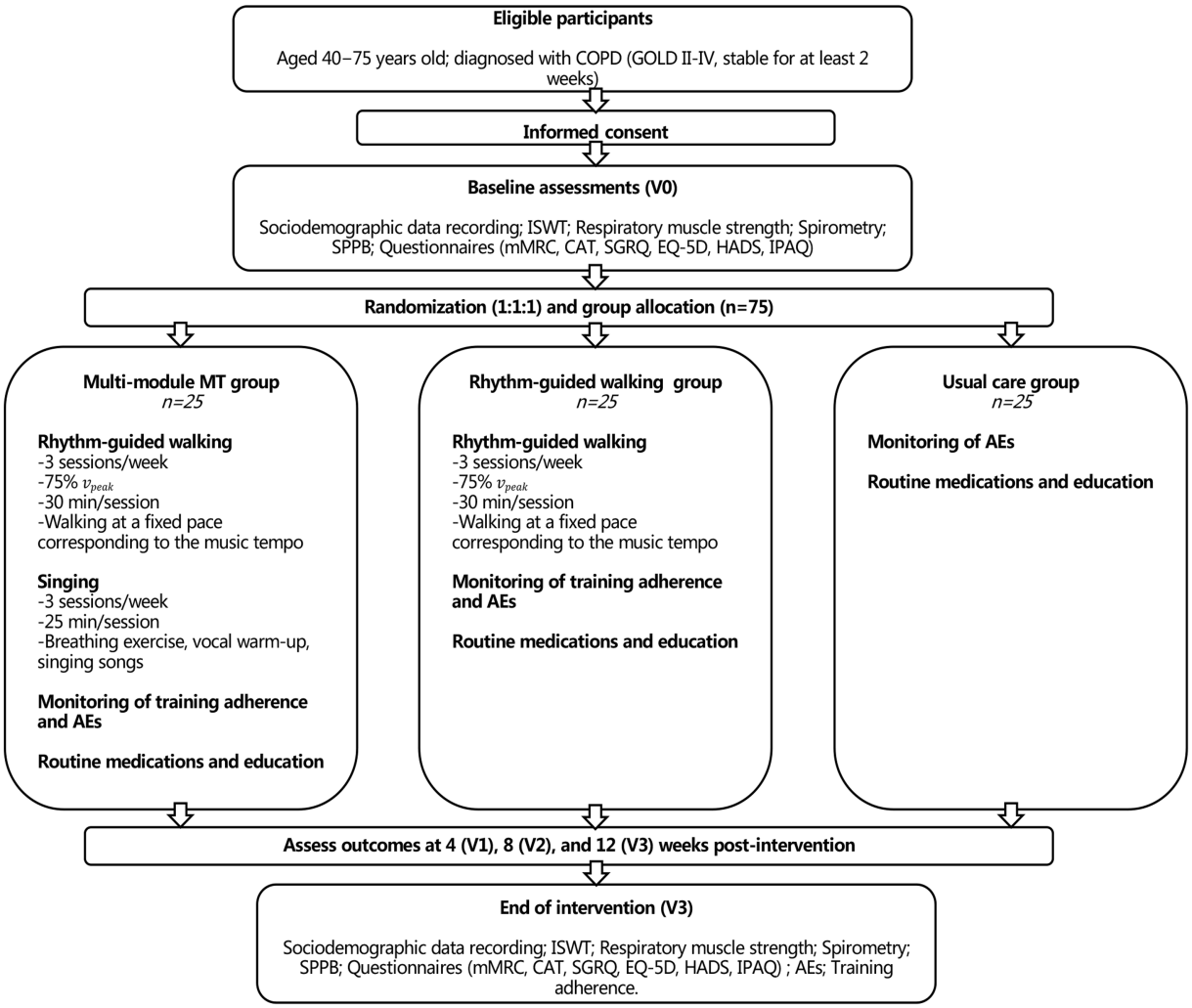

FIGURE 1
Flow diagram of study design.

Due to the nature of the intervention, neither the participants nor the exercise instructors will be blinded. However, pulmonology and rehabilitation nurses conducting outcome assessments and statistical analysts will be blinded to the assignments.

2.4 Interventions

All patients will continue taking routine medications, such as bronchodilators and steroid inhalers, according to their respective conditions and will maintain their regular treatment visits throughout the study period. Additionally, patients in the two intervention groups will receive two different forms of PR programs conducted by a multi-disciplinary team consisting of pulmonologists, rehabilitation physicians, pulmonology and rehabilitation nurses, music therapists, and research assistants (Tables 3, 4). The 12-week PR program is based on exercise, facilitated with music.

Home-based rehabilitation prescriptions will be conveyed by a software installed on the smartphone, containing different function modules based on the intervention group. Each patient will be provided a secure user account to log into the software.

2.4.1 Multi-model MT group

Participants in the multi-model MT group will undergo two types of training: rhythm-guided walking (aerobic exercises) and singing (respiratory muscle training). Additionally, their softwares will contain two function modules: the "rhythm-guided walking" module which includes melodies with various rhythms (60–120 bpm) that can match individual walking speeds and the "singing" module which includes various songs suitable for patients to sing, which are selected and recorded by music therapists.

The participants will undertake three walking sessions of at least 30 min per week. The exercise intensity (in the form of walking speed) will be prescribed individually, according to the ISWT results. The peak walking speed (v_{peak}) will be assessed by the ISWT, and the targeted training speed will be set at 75%, according to the American College of Sports Medicine. A music rhythm (i.e., stride frequency) matching the target speed will be calculated using the following formula:

TABLE 1 Summary of study schedule.

	Enrolment	Baseline (V0)	4 weeks (V1)	8 weeks (V2)	12 weeks (V3)
Eligibility screening					
Informed consent	√				
Randomization		√			
Allocation		√			
Interventions					
Multi-module music therapy		←	—	—	→
Rhythm-guided walking		←	—	—	→
Usual care		←	—	—	→
Assessments					
Demographic characteristics		√			
ISWT		√	√	√	√
MIP; MEP		√	√	√	√
Spirometry		√			√
SPPB		√	√	√	√
mMRC; CAT		√	√	√	√
SGRQ; EQ-5D		√	√	√	√
HADS		√	√	√	√
IPAQ		√	√	√	√
Training adherence			√	√	√
AEs			√	√	√

ISWT, incremental shuttle walking test; MIP, maximal inspiratory pressure; MEP, maximal expiratory pressure; SPPB, Short Physical Performance Battery; mMRC, Modified Medical Research Council; CAT, COPD assessment test; SGRQ, St. George's Respiratory Questionnaire; EQ-5D, EuroQoL-5D Questionnaire; HADS, Hospital Anxiety and Depression Scale; IPAQ, International Physical Activity Questionnaire; AE, adverse event.

TABLE 2 Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
1. Adults aged 40–75 years	1. Acute myocardial infarction within 4 weeks, unstable angina, uncontrolled atrial or ventricular arrhythmia, and heart failure
2. Diagnosed with COPD of GOLD II-IV	2. Hypertrophic cardiomyopathy, severe valvular heart disease, severe aortic stenosis
3. Current outpatient, stable for at least 2 weeks prior to the intervention enrolment	3. Acute pulmonary embolism, uncontrolled asthma, respiratory failure
4. Able to use a smartphone	4. Comorbidity precluding exercise training (e.g., orthopedic, neurological, or cognitive conditions)
5. Able to understand the purpose of the clinical trial and voluntarily participate with signed informed consent	5. Presence of malignant tumor
	6. Involved in pulmonary rehabilitation programs within the past 12 months

$$\text{Stride frequency}\left(\text{steps}\cdot\text{min}^{-1}\right)=0.75\times v_{\text{peak}}\left(\text{km}\cdot\text{h}^{-1}\right)\times 100\times\text{steps per shuttle}/60 \quad (1)$$

$$\text{Music rhythm}\left(\text{beats}\cdot\text{min}^{-1}\right)=\text{stride frequency}\left(\text{steps}\cdot\text{min}^{-1}\right) \quad (2)$$

Patients will be asked to follow this individualized music rhythm and walk at a fixed pace to maintain a constant speed. During the aerobic exercise, patients will need to open the "rhythm-guided walking" module on the software, to play the melodies with the prescribed specific tempo on a loop. Patients will need to regulate their stride frequency corresponding to the tempo to achieve adequate exercise intensity. During this, they will be asked to wear a sports wristwatch displaying their walking distance.

TABLE 3 Multi-module MT rehabilitation program.

Aerobic training	
◆ Rhythm-guided walking	Exercise at an intensity of 75% of the patients' peak speed evaluated by ISWT
	Walking at a fixed pace following the music tempo matched with the targeted speed
Duration	30 min
Respiratory muscle training	
◆ Breathing exercise ◆ Vocal warm-up ◆ Singing songs	Breathing control, pursed lips breathing, and abdominal breathing Introducing 'primal sounds' such as Hey, Ho, Ha, etc. Choosing appropriate songs fit for patients in terms of phrase lengths, breath points, lyrics, melodic challenge, and range
Duration	25 min

MT, music therapy; ISWT, incremental shuttle walking test.

TABLE 4 Rhythm-guided walking rehabilitation program.

Aerobic training	
◆ Rhythm-guided walking	Exercise at an intensity of 75% of the patients' peak speed evaluated by ISWT
	Walking at a fixed pace following the music tempo matched with the targeted speed
Duration	30 min

ISWT, incremental shuttle walking test.

Additionally, the participants will undertake three singing sessions of at least 25 min per week, following the prescriptions conveyed by the software. One session includes 10 min of breathing exercises and vocal warm-up with a focus on awareness of supporting musculature during inhalation and exhalation, as well as subconscious vocal release before singing songs. It rounds up with 15 min of singing songs to improve the strength, endurance, and flexibility of the respiratory muscles. During the respiratory muscle training, patients need to open the "sing" module to follow the guiding audios and videos.

2.4.2 Rhythm-guided walking group

Participants in the rhythm-guided walking group will perform only music-facilitated walking exercises as previously described in the multi-module MT group.

2.4.3 Usual care group (waiting-list control group)

During the study period, patients in the usual care group will not receive any rehabilitation intervention during the 12-week study period. However, they will be invited to access the music-facilitated PR program once the follow-up test is complete, thus, establishing a waiting-list control group.

2.5 Outcome measures

Table 1 illustrates the items to be measured and the time window for data collection. The primary outcome is the exercise capacity measured by ISWT (relative change in the ISWT distance seen at 4-, 8-, and 12-weeks post-intervention compared with baseline) in all three subgroups. The ISWT will be determined according to the recommendations of the European Respiratory Society/American Thoracic Society (17). In addition, as an externally paced maximal exercise test, walking speed will be controlled by a series of pre-recorded signals. The walking speed increases progressively until the participant can no longer continue.

The secondary outcomes are:

1 Respiratory muscle function: This will be assessed by the maximal inspiratory and expiratory pressures using the Gio Digital Pressure Gauge (18).
2 Pulmonary function test: This will be assessed by spirometry performed using automated equipment as the guideline (19).
3 Lower Extremity Function: This will be assessed using the Short Physical Performance Battery (SPPB), an objective tool for measuring lower extremity physical performance status. The SPPB is based on three timed tasks: standing balance, walking speed, and chair-stand tests (20).
4 Symptoms: Chronic activity-related dyspnea will be assessed using the modified Medical Research Council dyspnea scale (mMRC). The respiratory health status will be assessed using the COPD Assessment Test (CAT).
5 Health-related quality of life (HRQoL): This will be measured using St. George's Respiratory Questionnaire (SGRQ) (21, 22) and the EuroQoL-5D Questionnaire (EQ-5D) (23). These are simple, generic HRQoL instruments widely used as patient-reported outcome measures.
6 Anxiety/depression rates: This will be evaluated in the hospital using the Hospital Anxiety and Depression Scale (HADS) which comprises seven items each for the anxiety and depression subscales. The HADS is a validated measure for assessing anxiety and depression symptoms and is recommended for patients with COPD (24, 25).
7 Physical Activity (PA) level: Daily PA will be measured using the International Physical Activity Questionnaire (IPAQ) adopted in Chinese, which presents acceptable reliability and high repeatability values (26).
8 Training adherence: Patient training adherence is defined as the percentage of the total number of completed training sessions. The supervising hospital staff will record patient adherence.
9 Safety measurements: All adverse events (AEs) that occur during the study will be recorded and evaluated for relevance to the intervention. AEs include exacerbations, exercise injuries, and falls.

2.6 Data management and quality control

Patients will be required to practice rhythm-guided walking or singing until they master it on their first hospital visit. Telephone calls or video conferences by rehabilitation instructors will be integrated at the start of the program to ensure proper execution. After each training session, participant performance will be automatically transferred to the digital platform to facilitate the identification and verification of adherence to the prescribed exercise plan. Research assistants will verify training completion every day, record training adherence, and remind the patients to exercise.

To decrease measurement error, each participant will be assessed by a single nurse during different visits. Study data will be maintained on a password-protected platform and backed up to a secure external hard drive, with access restricted to authorized researchers and staff. Automatic plausibility controls will be set to detect any inconsistencies or inaccuracies during data entry.

2.7 Statistical analysis

All statistical analyses will be performed using full intention-to-treat analyses, with scores on the dependent variables for dropouts carried-over using the multiple imputation method. Independent samples *t*-tests, chi-square tests, and F-tests will be used to analyze differences between dropouts and completers regarding baseline characteristics.

All three groups will be examined using a two-sample Student's *t*-test for normal distributions or a Wilcoxon test for non-normal distributions (pre and postscores). The differences among the three groups at each time point (4, 8, and 12 weeks) will be analyzed using the Student's *t*-test or Mann–Whitney U test. A two-way analysis of variance with repeated measures will be used to determine the effects of time and group on the dependent variables.

All data will be analyzed using SPSS V.21.0 (IBM) software packages. Statistical significance is set at two-sided $p < 0.05$.

2.7.1 Sample size calculation

Considering the lack of previous studies combining music-guided exercise with singing, we did not perform a formal sample size calculation. Nevertheless, we calculated a sample size based on relevant studies applying similar rehabilitation interventions (27, 28). We used ISWT (the primary outcome) to calculate the sample size, as exercise capacity was one of the most sensitive outcome measures to detect the benefit of rehabilitation. We assumed that the mean changes in ISWT of multi-model MT group, rhythm-guided walking group and usual care group are 40 m, 27 m, and -15 m, respectively (27, 28), and the combined standard deviations (SD) are 35 m, 38 m, and 40 m, respectively. Based on a rate of type-I error of $\alpha = 0.05$, a power of 80% (rate of type-II error of $\beta = 0.1$), and a possible dropout rate of 50%, each group will require at least 18 patients.

$$n = \frac{\psi^2\left(\frac{\sum S_i^2}{k}\right)}{\sum\left(\overline{X_i} - \bar{X}\right)(k-1)}$$

Therefore, 75 participants are anticipated to be enrolled into this study.

3 Discussion

This trial aims to establish a music-facilitated telerehabilitation program using wearable sensors and web applications for COPD. Unlike common telerehabilitation modes, the program creatively includes music tempo-guided PA and breathing exercises in the form of singing, to develop a helpful and attractive rehabilitation mode.

Supplementing tele-PR with music therapy is the main feature of this study. Compared to traditional aerobic exercise, music-guided endurance training can help patients exercise more by decreasing or delaying the perception of exercise-induced dyspnea and fatigue (12). Additionally, music-guided respiratory training (i.e., singing) could help patients learn how to control respiratory function, including training and coordinating the related inspiratory and expiratory musculature (29) in a better psychological status. Moreover, MT is indicated to help patients overcome the fear of dyspnea and fatigue related to physical activity, thereby improving their adherence to PR.

Notably, various parts of PR may play distinct roles in benefiting patients' functions. For example, systematic endurance training can significantly improve exercise capacity through its direct effect on peripheral muscles (30, 31), while respiratory training may contribute more to correct the imbalance between respiratory muscle function and load and relieve dyspnea (32). Similar to the relationship between conventional physical exercise and respiratory training, music-guided endurance exercise combined with singing training has the potential to provide additional benefits through various physiological mechanisms compared to individual applications. Unlike most relevant studies which focus on only one kind of MT intervention, our research designed a home-based rehabilitation program with the integration of music-guided endurance exercise and singing training to maximize the clinical benefits. Additionally, by comparing the difference between the two intervention groups, we aim to offer more evidence on whether singing could bring additional improvements compared with rhythm-guided walking alone, especially in respiratory muscle function.

As a country with a large territory and population, China has an unequal healthcare system. Moreover, there is high variability in access to PR, which means that PR is almost absent in primary healthcare institutions, leaving a wide gap between numerous patients with COPD and limited rehabilitation resources (33). However, recent guidelines support the development of home-based PR, telehealth, and mHealth interventions in which objective, timely, and reliable monitoring of treatment implementation is deemed important. Remote technologies and wearable activity trackers could strengthen contact with patients, increase participation rates, and long-term adherence to healthy behaviors (34). Furthermore, although the digital literacy of patients with COPD is considered a barrier to PR participation, data from a recent study revealed encouraging results with the successive use of smartphones and wearable technology by an older respiratory population (35).

Therefore, there is an urgent need for innovative, effective, and safe PR strategies with easier accessibility and better compliance. The results of this study can contribute to develop and evaluate a

home-based music-facilitated rehabilitation program, which has the potential to function as a supplement and/or substitute (according to the needs) for traditional center-based PR in patients with stable COPD. However, undoubtedly, barriers to clinical research will ensue during the program's implementation stage. Nonetheless, we have designed the study to minimize such barriers. First, convincing participants to comply on the visit plan across the 12-week period is a potential challenge, especially for the usual care group. Therefore, we will inform the patients that those in the control group will also be given the opportunity to receive the same intervention upon completion of the study. For patients in the intervention groups, researchers will confirm the exercise performances every day through the platform and provide reminders when necessary. Additionally, for our secondary aim, we may not be able to detect statistical significance in distance of ISWT when comparing the multi-model MT group with the rhythm-guided walking group in this pilot study, however, we do expect a better respiratory muscle function and mental health which are more sensitive to the addition of singing training.

Ethics statement

The studies involving humans were approved by the institutional review board at the China-Japan Friendship Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MS: Writing – review & editing, Writing – original draft. LY: Writing – review & editing, Conceptualization. SQ: Writing – review & editing, Conceptualization. JL: Writing – review & editing, Methodology. KH: Writing – review & editing, Conceptualization. RH: Writing – review & editing. HN: Writing – review & editing. FD: Writing – review & editing. SW: Writing – review & editing. JH: Writing – review & editing. TY: Writing – review & editing, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Zhihong Chen,
Fudan University, China

REVIEWED BY
Rodrigo Torres-Castro,
University of Chile, Chile
Marlies Van Dijk,
University Medical Center Groningen, Netherlands

*CORRESPONDENCE
Haiyan Ge
✉ haiyange@hotmail.com

†These authors have contributed equally to this work

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Risk factors for anxiety and its impacts on acute exacerbation in older patients with chronic obstructive pulmonary disease

Yan Mou†, Lin Shan†, Yunhuan Liu†, Yue Wang, Zhengming He, Xiangyang Li, Huili Zhu and Haiyan Ge*

Department of Pulmonary and Critical Care Medicine, Huadong Hospital, Fudan University, Shanghai, China

Background: Anxiety is common in patients with chronic obstructive pulmonary disease (COPD), especially in older patients with the definition of age over 60 years old. Few studies have focused on anxiety in older COPD patients. This study aimed to analyze the risk factors of anxiety in older COPD patients and the impacts of anxiety on future acute exacerbation.

Methods: The general information, questionnaire data, previous acute exacerbation and pulmonary function were collected. Hamilton Anxiety Rating Scale (HAMA) was used to evaluate the anxiety of older COPD patients. The patients were followed up for one year, the number and the degrees of acute exacerbations of COPD were recorded.

Results: A total of 424 older COPD patients were included in the analysis. 19.81% (N = 84) had anxiety symptoms, and 80.19% (N = 340) had no anxiety symptoms. There were increased pack-years, more comorbidities, and more previous acute exacerbations in older COPD patients with anxiety compared to those without anxiety ($P < 0.05$). Meanwhile, a higher modified Medical Research Council (mMRC), a higher COPD assessment test (CAT) score and a shorter six-minute walking distance (6MWD) were found in older COPD patients with anxiety ($P < 0.05$). The BODE index, mMRC, CAT score, comorbidities and acute exacerbations were associated with anxiety. Eventually, anxiety will increase the risk of future acute exacerbation in older COPD patients (OR = 4.250, 95% CI: 2.369–7.626).

Conclusion: Older COPD patients with anxiety had worsening symptoms, more comorbidities and frequent acute exacerbation. Meanwhile, anxiety may increase the risk of acute exacerbation in the future. Therefore, interventions should be provided to reduce the risk of anxiety in older COPD patients at an early stage.

KEYWORDS

COPD, anxiety, Hamilton Anxiety Rating Scale (HAMA), comorbidity, acute exacerbation

1 Introduction

Chronic obstructive pulmonary disease (COPD) is one of the most frequent respiratory diseases among middle-aged and old individuals, contributing to significant global morbidity and mortality (1). The annual death toll associated with COPD reaches approximately 3 million, and it is predicted to rise to over 4.5 million by 2030 worldwide (2). There are currently 99.9 million people with COPD in China, and the prevalence of COPD in people over 40 years old and over 60 years old are 13.7 and 27%, respectively (3). With the increasing levels of air pollution and aging population, COPD is expected to become the primary economic burden of chronic diseases in the future (4). Therefore, it is crucial for the society to display special concern on COPD.

Recently, there has been growing attention toward comorbidities in individuals with COPD (5). Comorbidity prevalence is quite high among COPD patients: more than half have one or two comorbidities; while around 15.8% have three or four comorbidities; additionally, about 6.8% suffer from five or more comorbidities (6). Anxiety is a common comorbidity observed in individuals with COPD. In the general adult population of China, anxiety was found to have a prevalence of 5.3% according to the Hospital Anxiety and Depression Scale (HADS) and 5.6% based on the Diagnostic and Statistical Manual of Mental Disorders fourth edition (DSM-IV) scale (7, 8). The prevalence of anxiety ranged from 10 to 55% for inpatients and 13–46% for outpatients among patients with COPD (9).

Patients with COPD often experience poor mental health and older COPD patients are more likely to develop mental health especially anxiety (10). There are many risk factors for anxiety in COPD patients, such as continued smoking, poor knowledge, loneliness, and low social status (11, 12). As COPD progressing and age increasing, patients experience increased dyspnea, decreased physical function, and limited physical and social activities which leads to more severe anxiety symptoms (13). They often faced accelerated health deterioration, increased risk of adverse events, reduced quality of life, and experienced frequent acute exacerbation (14). However, there have been limited clinical trials conducted in this age group. In this study, we attempted to identify the risk factors of anxiety in older COPD patients and the impacts of anxiety on future acute exacerbation.

2 Materials and methods

2.1 Study design and participants

This study involved 424 older COPD patients who visited pulmonary outpatient clinic at fifteen hospitals in Shanghai from June 2017 to December 2020 (ChiCTR2000030911). All the patients were in a stable condition and randomly admitted. Written informed consent was obtained. The study was approved by the Ethics Committee of Huadong Hospital.

Inclusion criteria were as follows: (1) primary diagnosis of COPD according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (1). The forced expiratory volume in the first second of forced vital capacity (FEV1/FVC) < 0.7 after inhaling bronchodilators (BD) confirmed persistent airflow limitation (15); (2) participants with age $\geq$ 60 years; (3) signed written informed consent in the study. Exclusion criteria were as follows: (1) history of COPD acute exacerbation within one month prior to enrollment; (2) history of respiratory infection within one month prior to enrollment; (3) mental disorders (e g, schizophrenia, cognitive disorder, senile dementia, or Alzheimer) impairing capacity for informed consent; (4) missing follow-up information.

2.2 Demographic data

All COPD patients were required to complete a structured questionnaire and were given a thorough physical examination. All data were collected by physicians. The frequency and severity of acute exacerbation in the previous year were recorded at the first visit. Patients were followed up for one year. Demographic characteristics and clinical features were recorded. Comorbidity included diseases of respiratory system (asthma, allergic rhinitis, lung cancer, pulmonary embolism and bronchiectasis), cardiovascular system (angina, arrhythmia, hypertension and heart failure), metabolism system (diabetes, osteoporosis and metabolism syndrome), nervous system (stroke, subarachnoid and dementia), digestive system (peptic ulcer, digestive tumor and liver disease) and other diseases like connective tissue disease, peripheral vascular disease, lymphoma, leukemia, and anxiety.

2.3 Assessment of anxiety

The Hamilton Anxiety Rating Scale (HAMA) was widely used to screen anxiety in the general hospital. All participants were assessed by the same physician. All items of the HAMA were scored on a scale of 0–4 points. The HAM-A included 14 items covering two types of symptom factors which were psychic anxiety factors and somatic anxiety factors. The psychic anxiety factors were as follows: anxiety mood, tension, fears, insomnia, difficulties in concentration and memory, depression mood and behaviors during the interview. The somatic anxiety factors included somatic symptoms concerning seven symptoms: muscle, sensory, cardiovascular, respiratory, gastrointestinal, genito-urinary and other autonomic nervous system symptoms. HAMA $\geq$ 14 was defined as COPD with anxiety (16).

2.4 Definition of acute exacerbation

An acute exacerbation of chronic obstructive pulmonary disease (AECOPD) defines as an acute worsening of respiratory symptoms that result in additional therapy (1). Exacerbation events are classified as mild [treated with short acting bronchodilators (SABDs) only], moderate (relieved by SABDs plus antibiotics, with or without oral corticosteroids), or

severe (refer to acute exacerbation requiring hospitalization, emergency admission or ICU transferring) (17). The number of total exacerbations, mild, moderate, or severe exacerbations in the previous year and in the following-up one year were documented.

2.5 Assessment of pulmonary function

Spirometry was obtained from a Jaeger Toennies spirometer (Höchberg, Germany) according to the American Thoracic Society (ATS) guidelines (1). Each patient completed the spirometry test and bronchodilator reversibility test (BDR). The parameters including FEV1/predicted post BD, FEV1/FVC post BD and residual volume/total lung capacity (RV/TLC) % were recorded. The spirometry tests were performed by professional technicians and the results were interpreted by two physicians. COPD severity was evaluated according to the severity of airflow obstruction. GOLD1: FEV1 $\geq$ 80% predicted means mild; GOLD2: 50% $\leq$ FEV1 $<$ 80% predicted means moderate; GOLD3: 30% $\leq$ FEV1 $<$ 50% predicted means severe; GOLD4: FEV1 $\leq$ 30% predicted means very severe.

2.6 Assessment of COPD symptoms and health-related quality of life

The BODE index, a multidimensional grading system, is based on the body-mass index (B), the degree of airflow obstruction (O) evaluated by FEV1, the grade of dyspnea (D) assessed by the modified Medical Research Council (mMRC) dyspnea score, and the exercise capacity (E) assessed by the six-minute walking distance test (6MWD). The total scores of the BODE index ranged from 0 to 10 points (higher scores indicated more severity). The BODE index predicted death and other poor outcomes in COPD (18). mMRC dyspnea score was used to estimate the impact of dyspnea in everyday activities. The COPD assessment test (CAT) and St. George's respiratory questionnaire (SGRQ) were used to evaluate health-related quality of life (HRQL) (19). 6MWD was carried out to evaluate exercise capacity of COPD patients (20). The evaluation was done by professional physicians.

2.7 Statistical analyses

All statistical analyses were performed by a commercially software program (SPSS 22.0 for Windows; SPSS, Chicago, IL, USA). Continuous variates were presented as mean $\pm$ standard deviation for the normally distributed data or median (25th and 75th percentile) for the non-normally distributed data, while categorical variates were presented as n or n (%). Student's t-test was used for normally distributed data, while the Mann Whitney u test was used for non-normally distributed data. The categorical variates were analyzed by chi-square test. We used Logistic regression to evaluate risk factors of anxiety in COPD patients. We used Logistic regression and Poisson regression to predict the effect of anxiety on future exacerbation. $P < 0.05$ was considered statistically significant.

TABLE 1 Baseline characteristics of the 424 subjects.

Characteristics	COPD
Age (years) [M (P25, P75)]	70 (64–78)
Gender (male) [n (%)]	380 (89.60)
BMI (kg/m2) [M (P25, P75)]	23.52 (22.03–24.91)
Pack-years [M (P25, P75)]	
Never smoker	NA
Former/current smoker	30 (15–50)
COPD course (years) [M (P25, P75)]	8 (5–10)
mMRC	
Grade 0 [n (%)]	29 (6.8)
Grade 1 [n (%)]	156 (36.8)
Grade 2 [n (%)]	139 (32.8)
Grade 3 [n (%)]	82 (19.3)
Grade 4 [n (%)]	18 (4.2)
SGRQ [M (P25, P75)]	36 (31.00–51.75)
CAT score [M (P25, P75)]	11.5 (8.25–17.00)
6 MWD (m) [M (P25, P75)]	310 (295.50–340.00)
Pulmonary function	
FEV1/predict post BD (%)[M (P25, P75)]	31.30 (28.00–52.40)
FEV1/FVC post BD (%)[M (P25, P75)]	63.00 (51.00–66.50)
RV/TLC% [M (P25, P75)]	43.00 (41.00–108.00)
Comorbidities [n (%)]	368 (86.79)
Previous exacerbations [n (%)]	238 (56.13)

COPD, chronic obstructive pulmonary disease; BMI, body mass index; FEV1, forced expiratory volume in 1 s; BD, bronchodilator; FVC, forced vital capacity; CAT, the COPD assessment test; mMRC, modified Medical Research Council; SGRQ, St. George's respiratory questionnaire; 6MWD, six-minute walking distance; NA, not applicable.

3 Results

3.1 General characteristics of older COPD patients

A total of 424 older COPD patients were included to analyze the relation between anxiety and its associated factors in older COPD patients. There were 380 (89.60%) males and 44 females (10.40%) with a median age of 70 years. The median pack-years were 30 (15–50). The median FEV1/predict post BD was 31.30 (28.00–52.40) %. The median score on SGRQ was 36 (31.00–51.75), while the median 6MWD (m) was 310 (295.50–340.00). Among the 424 patients, 86.79% had one or more comorbidities, and 56.13% had at least one exacerbation in the previous one year (Table 1).

TABLE 2 Comparison of baseline data between COPD with anxiety and COPD without anxiety.

Characteristics	Group 1 COPD with anxiety	Group 2 COPD without anxiety	*p*-value
Number of subjects [*n* (%)]	84 (19.81)	340 (80.19)	–
Gender (male/female) [*n*]	76/8	304/36	0.775
Age (years) [M (P25, P75)]	68 (63–77)	70 (65–78)	0.263
BMI (kg/m2) [M (P25, P75)]	23.41 (21.54–24.42)	23.61 (22.06–25.00)	0.314
Pack-years [M (P25, P75)]	40 (22.50–57.25)	30 (12.50–47.13)	0.008*
COPD courses (years) [M (P25, P75)]	8 (3–12)	8 (6–10)	0.517
mMRC			
Grade 0 [*n* (%)]	0 (0)	29 (8.53)	< 0.0001*
Grade 1 [*n* (%)]	13 (14.94)	143 (42.06)	
Grade 2 [*n* (%)]	35 (41.67)	104 (30.59)	
Grade 3 [*n* (%)]	24 (28.57)	58 (17.06)	
Grade 4 [*n* (%)]	12 (14.29)	6 (1.76)	
SGRQ score [M (P25, P75)]	37.5 (27.50–45.75)	36 (31.00–65.00)	0.073
CAT score [M (P25, P75)]	17.5 (13.75–22.25)	11 (8–14)	< 0.0001*
6 MWD (m) [M (P25, P75)]	276 (210–336)	320 (300–362)	< 0.0001*
Comorbidity			
One or no comorbidity [*n* (%)]	19 (22.62)	204 (60.00)	< 0.0001*
More than one comorbidity [*n* (%)]	65 (77.38)	136 (40.00)	
Exacerbation			
With exacerbation [*n* (%)]	68 (80.95)	170 (50.00)	< 0.0001*
Without exacerbation [*n* (%)]	16 (19.05)	170 (50.00)	

COPD, chronic obstructive pulmonary disease; BMI, body mass index; SGRQ, St. George's respiratory questionnaire; mMRC, modified Medical Research Council. CAT, the COPD assessment test; 6MWD, six-minute walking distance. *Results were considered significant when $p < 0.05$.

3.2 Comparison of baseline data between older COPD patients with anxiety and older COPD patients without anxiety

Older COPD patients in our analysis were divided into two groups (Table 2). Group 1, Older COPD patients with anxiety; Group 2, Older COPD patients without anxiety. Table 2 showed the baseline data and comparisons between the two groups. Group 1 included 84 older COPD patients with anxiety: 76 (90.48%) males and 8 females (9.52%) were included, while the average age was 68 (63–77) years. Group 2 consisted of 340 older COPD patients without anxiety: 304 (89.41%) males and 36 females (10.59%) were included, while the average age was 70 (65–78) years. There were increased pack-years, more comorbidities, and more acute exacerbations in older COPD patients with anxiety. They were statistically different. There were statistically differences in mMRC, CAT score and 6MWD between the two groups. However, there were no statistically differences in gender, BMI, COPD courses, SGRQ score between the two groups.

3.3 Possible factors of anxiety in terms of symptoms, disease severity, and exercise capacity

Higher COPD severity evaluated by BODE index was associated with a higher risk of anxiety in older COPD patients. Degree of dyspnea, evaluated by mMRC had association with the risk of anxiety. The higher the CAT score, the higher the risk of anxiety. CAT score in severe and very severe COPD was 3.547 times of that in mild and moderate COPD. However, 6MWD were not related to anxiety (Table 3).

3.4 Related factors of anxiety in terms of comorbidities and acute exacerbations

Older COPD patients with more than one comorbidity had greater risk of anxiety than those with none or one comorbidity with the odds ratio of 5.671 (95% CI: 3.193–10.07).

TABLE 3 Possible factors associated with anxiety in older COPD patients.

Characteristics	Risk of anxiety	
	Unadjusted OR (95% CI)	Multivariable adjusted OR (95% CI)
BODE index	2.492 (1.828–3.397)*	2.493 (1.828–3.398)*
mMRC	2.417 (1.839–3.176)*	2.462 (1.860–3.259)*
CAT score	3.63 (2.212–5.959)*	3.547 (2.129–5.909)*
6MWD (m)	0.991 (0.987–0.995)	0.990 (0.986–0.995)

Results are from logistic regression analysis of relations between possible factors associated with anxiety. Multivariable adjustment included age, gender, BMI, COPD course and smoking history. *Results were considered significant when $p < 0.05$.

TABLE 4 Relations between comorbidities and exacerbations and anxiety in older COPD patients.

Indicators	Unadjusted OR (95% CI)	*p*-value	Multivariable adjusted OR (95% CI)	*p*-value
Comorbidities ≤ 1	1.00	–	1.00	–
> 1	5.132 (2.945–8.942)	$p < 0.001$*	5.671 (3.193–10.07)	$p < 0.001$*
Exacerbations < 1	1.00	–	1.00	–
≥ 1	4.25 (2.369–7.626)	$p < 0.001$*	4.004 (2.204–7.273)	$p < 0.001$*

Results are from logistic regression analysis of relations between comorbidities, previous exacerbations and the risk of anxiety. Results were expressed as odds ratio (95% confidence interval). Multivariable adjustment included age, gender, BMI, COPD course and smoking history. *Results were considered significant when $p < 0.05$.

Compared with older COPD patients without acute exacerbation in the previous year, the odds ratio of anxiety in older COPD patients with acute exacerbation was 4.004 (95% CI: 2.204–2.273) (Table 4).

3.5 Anxiety associated with the increased risk of future acute exacerbation

Anxiety was associated with increased risk of various degrees of future exacerbation from the aspect of both incidence and frequency. Unadjusted odds ratio (95% CI) of future exacerbation for older COPD patients with anxiety was 4.250 (2.369–7.626) compared to those without anxiety. Corresponding unadjusted odds ratios (95% CI) were 2.653 (1.526–4.611) and 2.006 (1.221–3.297) for moderate and severe exacerbations in one year (Table 5). Meanwhile, Unadjusted incidence-rate ratio (95% CI) for total acute exacerbation was 2.000 (1.572–2.545) in older COPD patients with anxiety compared to those without anxiety. Corresponding incidence-rate ratios (95% CI) were 2.285 (1.422–3.669) and 2.080 (1.350–3.203) for moderate and severe acute exacerbations within one year (Table 6).

Multivariable adjusted odds ratio (95% CI) of future acute exacerbation in older COPD patients with anxiety was 4.029 (2.217–7.322) compared to those without anxiety. Multivariable adjusted odds ratios (95% CI) were 2.563 (1.443–4.552) and 1.84 (1.100–3.077) for moderate and severe acute exacerbations in one year (Table 5). Meanwhile, Multivariable adjusted incidence-rate ratio (95% CI) for total acute exacerbation was 1.954 (1.537–2.485) in older COPD patients with anxiety compared to those without anxiety. Corresponding incidence-rate ratios (95% CI) were 1.969 (1.200–3.233) and 1.915 (1.227–2.989) for moderate and severe acute exacerbations within one year after additional adjustment for potential confounders (Table 6).

4 Discussion

Chronic obstructive pulmonary disease (COPD) usually coexists with various comorbidities. Anxiety, an important comorbidity of COPD, is frequently under-diagnosed and significantly impacts the prognosis of COPD patients, especially in older COPD patients.

In our analysis, the prevalence of anxiety in older COPD patients was 19.81%. However, different studies had reported various prevalence rates. For instance, a cross-sectional study conducted in Shanghai included 275 mild COPD patients from urban communities and found that 7.6% had anxiety (21). Another study evaluated 491 Chinese COPD patients by Hospital Anxiety and Depression Scale (HADS) and reported an anxiety prevalence rate of 10% (22). The China Pulmonary Health Study (CPH) revealed that anxiety affected approximately 10.79% COPD patients (23). This discrepancy could be attributed to differences sample size, methodological design, participant sources, screening instruments, and severity levels of COPD (24).

Chronic obstructive pulmonary disease (COPD) primarily affected older populations and exhibited male predominance; this trend was also evident in our cohort where there were more male participants. However, no gender differences were observed between the two groups. Our findings indicated that COPD patients with anxiety tended to have higher pack-years, have greater comorbidities, and experience more frequent exacerbations. Additionally, COPD patients with anxiety exhibited higher levels of dyspnea (mMRC), worse health status (CAT score), and less exercise capacity (6MWD). Our analysis indicated that the BODE index, mMRC score and CAT score were associated with anxiety ($P < 0.05$).

It is worth noting that anxiety negatively impacts COPD. On one hand, the symptoms of COPD, such as gradually increasing dyspnea, cough, and expectoration, may be the main cause of anxiety in COPD patients (25). On the other hand, other comorbidities such as lung cancer, cardiovascular disease, and gastroesophageal reflux disease contribute to the occurrence of anxiety in COPD patients. Our study revealed that an increased comorbidities was associated with a higher risk of anxiety (OR 5.671; 95% CI: 3.193–10.07). Acute exacerbation of COPD is associated with increased mortality rate (26, 27). 25% of patients experiencing acute exacerbation was required for ICU admission, further increasing the economic burden of COPD (28). Additionally, frequent acute exacerbation severely worsened patients' quality of life. A previous study conducted by our group identified that anxiety, angina, and hypertension were independent risk factors for acute exacerbation within a year (29, 30). In our study, we found that acute exacerbation in the previous year were related to anxiety and increased the risk of anxiety (OR 4.004; 95% CI: 2.204–7.273). We also discovered that older COPD patients with anxiety increased the risk of future exacerbation in one year, especially moderate and severe acute exacerbation compared to those without anxiety.

Older COPD patients with anxiety have worse dyspnea symptoms, more comorbidities, and experience more frequent

TABLE 5 Anxiety in relation to incidence of different levels of acute exacerbations in COPD.

Anxiety	Incidence of exacerbation in 12 months							
			Unadjusted OR (95% CI)				Multivariable adjusted OR (95% CI)	
	Total	Mild	Moderate	Severe	Total	Mild	Moderate	Severe
No	1	1	1	1	1	1	1	1
Yes	4.250 (2.369–7.626)	1.686 (0.978–2.908)	2.653 (1.526–4.611)	2.006 (1.221–3.297)	4.029 (2.217–7.322)	1.537 (0.880–2.686)	2.563 (1.443–4.552)	1.84 (1.100–3.077)
	$p < 0.001$*	$p = 0.06$	$p = 0.001$*	$p = 0.006$*	$p < 0.001$*	$p = 0.131$	$p = 0.001$*	$p = 0.02$*

Results were from logistic regression analysis of the relations between anxiety and incidence of exacerbation in one months. Results were expressed as odds ratio (95% confidence interval). Multivariable adjustment included age, gender, BMI, COPD course, smoking history and exacerbations in the previous year. *Results were considered significant when $p < 0.05$.

TABLE 6 Anxiety in relation to frequency of different levels of acute exacerbations in COPD.

Anxiety	Acute exacerbation in 12 months							
			Unadjusted IRR (95% CI)				Multivariable adjusted IRR (95% CI)	
	Total	Mild	Moderate	Severe	Total	Mild	Moderate	Severe
No	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Yes	2.000 (1.572–2.545)	1.495 (0.922–2.424)	2.285 (1.422–3.669)	2.080 (1.350–3.203)	1.954 (1.537–2.485)	1.353 (0.839–2.181)	1.969 (1.200–3.233)	1.915 (1.227–2.989)
	$p < 0.001$*	$p = 1.492$	$p = 0.001$*	$p = 0.001$*	$p < 0.001$*	$p = 0.215$	$p = 0.007$*	$p = 0.004$*

Multivariable Poisson regression model was used for the frequency of future acute exacerbation in one year. Results were expressed as incidence-rate ratios (95% confidence interval). Multivariable adjustment included age, gender, BMI, COPD course, smoking history and exacerbations in the previous year. *Results were considered significant when $p < 0.05$.

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acute exacerbations. Therefore, early diagnosis of COPD with anxiety is very important. However, the current scales for the diagnosis and assessment of anxiety are professional and complex. Fortunately, respiratory physicians are sensitive to clinical indicators. If there was a possibility that respiratory physicians can evaluate COPD patients with anxiety through clinical indicators, they would transfer them to psychologists as soon as possible for further treatment including psychotherapy, medications, and exercise. That would improve treatment compliance, improve symptoms, and reduce acute exacerbations of older COPD with anxiety.

There are some limitations to consider regarding our study findings. Firstly, the data on acute exacerbations were obtained from the medical records of COPD patients. Considering that some patients may have sought treatment from other hospitals during acute exacerbation episodes, there was a possibility of underreporting the frequency of acute exacerbations. Additionally, it should be noted that different assessment tools for evaluating anxiety may yield different results. In our study, HAMA was used to assess anxiety in older COPD patients.

5 Conclusion

In summary, our study found that older COPD patients with anxiety had worse symptoms, more comorbidities and more frequent. In addition, our study also found anxiety can increase the risk of future acute exacerbation in older COPD patients. In COPD management, routine screening for psychiatric symptoms should be an integral part of clinical work to reduce the risk of anxiety in older COPD patients at an early stage.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by Ethic Committee of Huadong Hospital (protocol code 20180064). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YM: Methodology, Project administration, Writing – original draft, Writing – review and editing. LS: Methodology, Investigation, Writing – review and editing. YL: Software, Validation, Writing – original draft. YW: Data curation, Resources, Writing – review and editing. ZH: Methodology, Software, Writing – review and editing. XL: Validation, Writing – review and editing. HZ: Visualization, Writing – review and editing. HG: Project administration, Supervision, Writing – review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Suzana Erico Tanni,
São Paulo State University, Brazil

REVIEWED BY
Watchara Boonsawat,
Khon Kaen University, Thailand
Konstantinos Bartziokas,
Independent researcher, Trikala, Greece

*CORRESPONDENCE
Zhigang Pan
✉ pan.zhigang@zs-hospital.sh.cn

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Screening performance of COPD-PS scale, COPD-SQ scale, peak expiratory flow, and their combinations for chronic obstructive pulmonary disease in the primary healthcare in Haicang District, Xiamen City

Xueting Shen[1], Hua Yang[1], Chengdian Lan[2], Fen Tang[3], Qinfei Lin[4], Yingjie Chen[5], Jinxiang Wu[6], Xionghua Chen[7] and Zhigang Pan[2]*

[1]Department of General Medicine, Zhongshan Hospital, Fudan University, Shanghai, China, [2]Department of General Medicine, Xiamen Branch, Zhongshan Hospital, Fudan University, Xiamen, China, [3]Health Bureau of Haicang, Xiamen, China, [4]Department of General Medicine, Shitang Community Health Service Center, Xiamen, China, [5]Department of General Medicine, Songyu Community Health Service Center, Xiamen, China, [6]Department of General Medicine, Xinyang Community Health Service Center, Xiamen, China, [7]Department of General Medicine, Dongfu Community Health Service Center, Xiamen, China

Objectives: This study aimed to evaluate the screening performance of COPD-PS questionnaire, COPD-SQ questionnaire, peak expiratory flow (PEF), COPD-PS questionnaire combined with PEF, and COPD-SQ questionnaire combined with PEF for chronic obstructive pulmonary disease (COPD).

Methods: This was a cross-sectional study. We distributed self-designed surveys and COPD screening scales (COPD-PS questionnaire and COPD-SQ questionnaire) to residents who underwent physical examination in five community health centers in Haicang District, Xiamen City, from February 2023 to May 2023, and measured their lung function and PEF with a portable device. We used logistic regression to obtain the coefficients of COPD-PS questionnaire, COPD-SQ questionnaire, and PEF, and plotted the receiver operating characteristic curves of each tool for diagnosing COPD and moderate-to-severe COPD. We evaluated and compared the optimal cut-off points and scores of sensitivity, specificity, Youden index, and area under the curve (AUC) values, and assessed the screening efficiency of different methods.

Results: Of the 3,537 residents who completed the COPD-SQ questionnaire, COPD-PS questionnaire, and spirometry, 840 were diagnosed with COPD. We obtained the coefficients of COPD-PS questionnaire combined with peak expiratory flow (PEF), and COPD-SQ questionnaire combined with PEF, by logistic regression as −0.479-0.358 × PEF +0.321 × COPD-PS score and −1.286-0.315 × PEF +0.125 × COPD-SQ score, respectively. The sensitivity of diagnosing COPD by COPD-SQ questionnaire, COPD-PS questionnaire, PEF, COPD-PS questionnaire combined with PEF, and COPD-SQ questionnaire combined with PEF were 0.439, 0.586, 0.519, 0.586, 0.612 respectively, and the specificity were 0.725, 0.621, 0.688, 0.689, 0.663 respectively, with ROC values of 0.606 (95%CI: 0.586–0.626), 0.640 (0.619–0.661), 0.641 (0.619–0.663), 0.678 (0.657–0.699),

0.685 (0.664–0.706) respectively. The sensitivity of diagnosing GOLD II and above by COPD-SQ questionnaire, COPD-PS questionnaire, PEF, COPD-PS questionnaire combined with PEF, and COPD-SQ questionnaire combined with PEF were 0.489, 0.620, 0.665, 0.630, 0.781 respectively, and the specificity were 0.714, 0.603, 0.700, 0.811, 0.629 respectively, with ROC values of 0.631 (95%CI: 0.606–0.655), 0.653 (0.626–0.679), 0.753 (0.730–0.777), 0.784 (0.762–0.806), 0.766 (0.744–0.789) respectively.

Conclusion: Our study found that the accuracy of COPD screening by COPD-SQ questionnaire and COPD-PS questionnaire can be improved by combining the results of PEF. The screening performance of COPD-SQ questionnaire combined with PEF is relatively better. In future research, further studies are needed to optimize the performance of screening tools and understand whether their use will affect clinical outcomes.

KEYWORDS

chronic obstructive pulmonary disease (COPD), screening test, COPD-SQ questionnaire, COPD-PS questionnaire, peak expiratory flow (PEF), primary healthcare (PHC), diagnosis

1 Introduction

Chronic obstructive pulmonary disease (COPD) is a common, preventable, and treatable chronic airway disease that causes irreversible airway obstruction and progressive lung function decline (1). Patients with COPD often remain undiagnosed for about 3.6 ± 4 years after the onset of symptoms, leading to clinical deterioration such as worsening lung function, increased symptoms, or acute exacerbations (2). Early diagnosis of patients with irreversible obstruction by spirometry, irrespective of their symptoms, is essential to prevent further lung damage and improve their quality of life. Spirometry is the "gold standard" for diagnosing COPD and evaluating its severity, progression, prognosis, and treatment response (3). However, spirometry is not widely available or accessible in many settings, and it requires time, effort, and proper patient cooperation to obtain accurate results (4). Therefore, a simple, easy-to-use, low-cost and time-efficient screening method is needed to identify patients at high risk of COPD who would benefit from spirometry testing (5). Family doctors can provide follow-up care and treatment to control symptoms and slow down disease progression for patients with early COPD screened out. Patients with moderate or severe COPD, however, need more frequent medical monitoring and intervention because they have higher rates of lung function decline, acute exacerbation, and mortality than those in the early stage. Therefore, screening for this type of patient is more important.

Martinez et al. developed a three-level screening strategy based on the "COPD assessment in primary care to identify undiagnosed respiratory disease and exacerbation risk questionnaire" (CAPTURE), which can reduce the workload while ensuring the screening efficiency, and has reference value[6]. However, the CAPTURE questionnaire performed poorly in primary care settings in China (6, 7). However, the CAPTURE questionnaire performed poorly in primary care settings in China (8) The "Expert Consensus on COPD Screening in County-level Areas of China (2020)" recommended that different regions and units can choose COPD-PS or COPD-SQ for COPD screening according to their own needs (8). However, the criteria for selecting the suitable questionnaire based on "own needs" are unclear, and primary care physicians face a challenge in making this decision. Moreover, the current screening tools and methods are mainly applied for research purposes, and their effectiveness in real-world settings is not well reported.

Therefore, we performed questionnaire screening and PFT among residents who underwent physical examination in Haicang District, Xiamen City. Our aim was to compare the screening performance of COPD-PS questionnaire, COPD-SQ questionnaire, peak expiratory flow (PEF), COPD-PS questionnaire plus PEF, and COPD-SQ questionnaire plus PEF for detecting COPD by PFT.

2 Materials and methods

2.1 Participants

This study is a cross-sectional study. The study population comprised residents who underwent physical examinations at five community health service centers (Shitang, Dongfu, Songyu, Qiaonan, and Xinyang) located in Haicang District, Xiamen City, between February 2023 and May 2023. The inclusion criteria for this study required participants to be aged ≥40 years and permanent residents of Haicang District, Xiamen City.

The exclusion criteria were as follows: (1) Individuals who have suffered from myocardial infarction, stroke, or shock in the past three months. (2) Individuals with severe heart failure, severe arrhythmia, or unstable angina within the past four weeks. (3) Individuals who have experienced massive hemoptysis within the past four weeks. (4) Individuals who require medication for epilepsy. (5) Patients with uncontrolled primary hypertension (systolic blood pressure > 200 mmHg and/or diastolic blood pressure > 100 mmHg; 1 mmHg = 0.133 kPa). (6) Patients with aortic aneurysm. (7) Patients with severe hyperthyroidism. (8) Individuals with respiratory system diseases other than chronic obstructive pulmonary disease, such as

bronchiectasis, bronchial asthma, lung cancer, and respiratory infectious diseases. (9) Individuals who have recently undergone eye, ear, or cranial surgery. (10) Individuals with pneumothorax or giant bulla who are not ready for surgical treatment. (11) Pregnant women. (12) Individuals with psychiatric disorders or cognitive impairments.

2.2 Research tools

2.2.1 Portable spirometer

This study used a portable spirometer (X1, Xeek, Co., Ltd., Xiamen, China) to measure lung function. This device is a small and low-cost instrument that can measure lung function easily and portably. It is based on the physical principle of differential pressure transducer, and measures the flow and volume of gas exhaled or inhaled by patients through the respiratory pipeline. It can measure conventional ventilation function parameters, such as forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1), peak expiratory flow (PEF), etc., and can perform bronchodilation test. A previous study compared the consistency of this portable spirometer with a standard spirometer, and found no significant bias and good agreement for all parameters. It is suitable for clinical applications such as primary lung function screening, COPD diagnosis and treatment, lung function follow-up of patients in remote areas, etc. (9).

2.2.2 The "COPD population screener"

The COPD-PS questionnaire, developed by the American Clinical Practice Group in 2008, is the most common screening tool in primary health care settings in China (10). It consists of five items that assess objective factors (age and smoking status) and subjective symptoms (dyspnea, activity change, and cough/sputum). Each item is scored from 0 to 2 points, and the total score ranges from 0 to 10 points. A higher score indicates a higher likelihood of having COPD (11). Current guidelines and literature recommend pulmonary function screening for residents with a COPD-PS score of 5 or more to confirm the diagnosis of COPD (11, 12).

2.2.3 The "COPD self-screening questionnaire"

The COPD-SQ questionnaire is a screening tool for COPD adapted and validated by Chinese scholars for the Chinese population. It has seven questions that evaluate subjective symptoms (cough and dyspnea on weekdays) and objective factors (age, smoking status, body mass index, family history, and biomass fuel exposure) (13). The developers of the COPD-SQ questionnaire emphasized that biomass smoke is a major risk factor for female COPD, and it is important to include it in the screening questionnaire (13). Each item has different scores depending on the options, and the total score ranges from 0 to 28 points. A higher score indicates a higher probability of having COPD. PFT is advised for patients with a total score of 16 or more to confirm the diagnosis of COPD. The COPD-SQ questionnaire differs from the COPD-PS questionnaire by adding the assessment of biomass fuel exposure, which is a significant risk factor for COPD in Chinese women.

2.2.4 Peak expiratory flow

PEF is the maximum flow at the mouth achieved during an expiration, delivered with maximum force starting from the level of maximum lung inflation (14). It is a common measure of pulmonary ventilation function that correlates well with FEV1 measured by spirometry and reflects airway patency (14). PEF also has a good correlation with the St. George's Respiratory Questionnaire score and indicates the quality of life of patients (15). PEF only requires a short maximum expiration time and less operation skills, and has high repeatability and user compliance. It can be used as an effective tool for identifying COPD patients, monitoring and predicting COPD acute exacerbations (16).

2.3 Research methods

2.3.1 Questionnaire completion

The primary health care physicians in Haicang District, Xiamen City, were trained uniformly by Xiamen Hospital affiliated to Zhongshan Hospital of Fudan University.

With the help of the healthcare physicians, the residents filled out a self-designed general information questionnaire and the COPD-PS and COPD-SQ questionnaires on the spot. The healthcare physicians checked and collected the questionnaires. For those who had difficulty in completing the questionnaires, the healthcare physicians explained and filled them out for them.

2.3.2 Pulmonary function testing

We used a portable spirometer (model: X1, China Xeek company) to measure the lung function parameters (FEV_1, FVC, PEF, etc.) of the community residents, with the assistance of primary community physicians who had received professional training. The bronchodilation test was performed 15 min after inhaling salbutamol 200ug via a metered-dose inhaler. We followed the quality control standards of the American Thoracic Society (ATS) and performed up to eight tests before and after bronchodilation. We obtained at least two ATS-acceptable pulmonary function curves, and the variation of FEV_1 and FVC between the two best and largest tests was <0.2 L, or < 0.1 L when FVC < 1 L (17). We assigned the quality control level (A-F) according to the quality control standards, and only pulmonary function tests of level A, B, and C were included in the analysis. All subjects were asked to sit, pinch their nose, and use a disposable mouthpiece.

2.3.3 COPD diagnosis and grading criteria

According to the 2023 GOLD guidelines (1), COPD is diagnosed as $FEV_1/FVC < 0.70$ after bronchodilation test. The bronchodilation test was positive if FEV_1 increased by more than 15% or more than 0.2 L after inhaling salbutamol 200ug for 15 min, compared with baseline. Among patients with $FEV_1/FVC < 0.7$, the severity of COPD is graded according to the percentage of FEV_1 to the predicted FEV_1 (FEV1%pred), that is, FEV_1%pred≥80% is GOLD I (mild), 80%>FEV_1%pred≥50% is GOLD II (moderate), 50%>FEV_1%pred≥30% is GOLD III (severe), and FEV_1%pred<30% is GOLD IV (very severe).

2.3.4 The smoking index

The smoking index was obtained by multiplying the daily cigarette consumption/exposure and the smoking duration in years. Smoking index/secondhand smoke index ≤200 was mild smoke, 201–400 was moderate smoking smoke, and >400 was heavy smoking smoke (18).

2.4 Quality control

We trained the investigators and pulmonary function testers uniformly before conducting the formal research survey. They had to pass five qualified tests in the pre-survey. All operations (questionnaire, pulmonary function testing, review and revision, etc.) were signed by operators. We randomly selected 10% of the pulmonary function test results for review by two pulmonary function experts who agreed on the diagnosis. We uploaded all questionnaire data and pulmonary function results to the Xeek intelligent management platform cloud backup, which prevented any deletion or modification.

2.5 Statistics analysis

Descriptive analysis was employed to evaluate demographic characteristics, spirometric parameters, and questionnaire scores. Continuous variables were reported as mean ± standard deviation. The optimal cut-off point for the screening questionnaire was determined by selecting the value with the highest Youden's index. A multiple logistic regression was run with COPD as a dependent variable, while the independent variables included the combined COPD-PS scale with PEF and COPD-SQ scale with PEF. The receiver operating characteristic (ROC) curve was plotted for each screening method. Sensitivity, specificity, Youden's index, and the area under the receiver operating characteristic curve (AUROC) were calculated for the optimal cut-off value and previously recommended values. Statistical significance was set at $p < 0.05$. All analyses were performed by R v4.3.1 (R Core Team, Vienna, Austria).

3 Results

3.1 Demographic and related information

This study involved 4,216 residents, of whom 3,537 completed both a valid questionnaire survey and spirometry, yielding an effective rate of 83.9%. Figure 1 shows the flow chart of the study. A total of 840 subjects (455 males) were diagnosed with chronic obstructive pulmonary disease. Table 1 presents the demographic characteristics, spirometry results, and screening questionnaire results.

3.2 Multiple logistic regression analysis

We performed a multifactor logistic regression to examine the association between COPD presence and the COPD-PS score, the COPD-SQ score, and the PEF results. The results showed that lower PEF results, higher COPD-PS scores, and higher COPD-SQ scores were significantly associated with increased COPD risk. The regression equations were:

$$\left[\text{Logit}\left(\text{COPDPS} + \text{PEF}\right)\right] = -0.479 - \left(0.358 \times \text{PEF}\right) + \left(0.321 \times \text{COPDPS}\right)$$

$$\left[\text{Logit}\left(\text{COPDSQ+PEF}\right)\right] = -1.286 - \left(0.315 \times \text{PEF}\right) + \left(0.125 \times \text{COPDSQ}\right)$$

Tables 2, 3 show the logistic regression results of the COPD-PS score and the COPD-SQ score, respectively, in combination with the PEF.

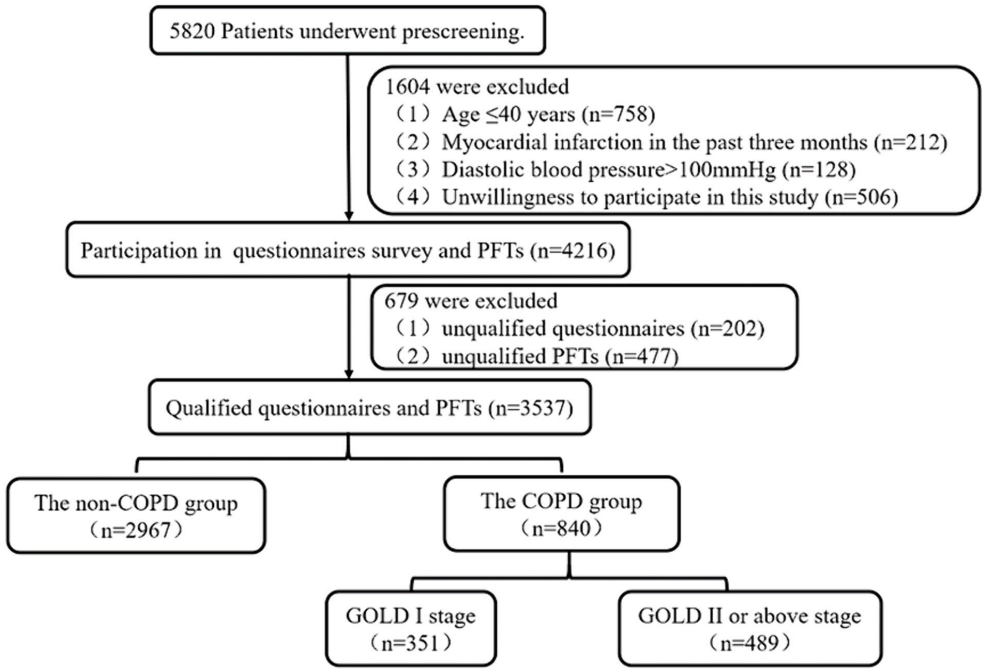

FIGURE 1
The flowchart of study. COPD, chronic obstructive pulmonary disease; GOLD, Global Initiative for Chronic Obstructive Lung Disease; PFTs, pulmonary function tests.

TABLE 1 Demographic and related information between the COPD group and the non-COPD group.

	The total (*n* = 3,537)	The COPD group (*n* = 840)	The non-COPD group (*n* = 2,697)	*p*	GOLD I (*n* = 351)	GOLD II, III and IV(n = 489)	*p*
Gender	–	–	–	0.000	–	–	0.000
Male	1,537	455	1,082	–	174	281	–
Female	2000	385	1,615	–	177	208	–
Age	66.29 ± 7.89	68.28 ± 6.86	65.67 ± 8.09	0.000	66.27 ± 7.92	66.29 ± 7.90	0.000
40–49	186	12	174	–	6	9	–
50–59	364	63	301	–	32	31	–
60–69	1887	427	1,460	–	185	242	–
70–79	1,030	315	715	–	125	190	–
≥80	60	23	47	–	3	17	–
BMI (kg/m^2)	24.65 ± 3.18	23.97 ± 3.04	24.85 ± 3.19	0.000	24.67 ± 3.18	23.94 ± 3.09	0.000
<18.5	62	17	45	–	5	12	–
18.5–24.9	1,468	432	1,036	–	186	246	–
25–29.9	1,507	310	1,197	–	128	182	–
>30	500	87	419	–	32	49	–
PFT	–	–	–	–	–	–	–
PEF, L/s	4.47 ± 1.54	3.90 ± 1.48	4.64 ± 1.51	–	4.49 ± 1.54	3.33 ± 1.25	–
FVC, L	2.69 ± 0.80	2.83 ± 0.81	2.64 ± 0.79	–	2.68 ± 0.80	2.46 ± 0.65	–
FEV_1, L	2.02 ± 0.60	1.80 ± 0.57	2.08 ± 0.59	–	2.02 ± 0.60	1.50 ± 0.43	–
FEV_1, %pred	84.56 ± 18.22	74.54 ± 19.20	87.68 ± 16.21	–	84.95 ± 18.12	61.96 ± 13.91	–
FEV_1/FVC, %	75.65 ± 9.67	63.18 ± 6.87	79.54 ± 6.65	–	76.02 ± 9.33	61.01 ± 0.08	–
GOLD stage	–	–	–	–	–	–	–
GOLD I	–	351	–	–	–	–	–
GOLD II	–	394	–	–	–	–	–
GOLD III	–	77	–	–	–	–	–
GOLD IV	–	18	–	–	–	–	–
Smoking index	–	–	–	0.000	–	–	0.000
Nonsmoker	2,913	623	2,290	–	283	340	–
Mild	97	31	66	–	9	22	–
Moderate	129	42	87	–	16	26	–
Severe	398	144	254	–	43	101	–
Secondhand smoking index	–	–	–	0.001	–	–	0.000
Nonsmoker	3,045	696	2,394	–	298	398	–
Mild	146	38	108	–	18	20	–
Moderate	97	22	75	–	6	16	–
Severe	249	84	165	–	29	55	–
Biomass Combustion	–	–	–	0.119	–	–	0.190
Yes	714	188	526	–	274	110	–
No	2,823	652	2,171	–	77	379	–
Family history of COPD	–	–	–	0.005	–	–	0.000
Yes	168	55	113	–	11	45	–
No	3,369	785	2,584	–	340	444	–
Previously diagnosis of COPD	–	–	–	0.000	–	–	0.000

(Continued)

TABLE 1 (Continued)

	The total (*n* = 3,537)	The COPD group (*n* = 840)	The non-COPD group (*n* = 2,697)	*p*	GOLD I (*n* = 351)	GOLD II, III and IV(n = 489)	*p*
Yes	36	21	15	–	2	19	–
No	3,501	819	2,682	–	349	470	–
Previously diagnosis of tuberculosis	–	–	–	0.001	–	–	0.087
Yes	36	17	19	–	8	9	–
No	3,501	823	2,687	–	343	480	–
Previously SARS-CoV-2 infected	–	–	–	0.000			0.001
Yes	2,642	571	2071	-	236	335	–
No	895	269	626	-	115	154	–
Complications	–	–	–	–			–
Hypertension	1,534	329	1,205	0.005	126	203	0.399
Diabetes	606	113	493	0.001	47	66	0.025
Coronary heart disease	67	25	42	0.357	9	16	0.026
Gout	59	17	42	0.008	7	10	0.609
Scale Scores	–	–	–	–	–	–	
COPD-PS	2.47 ± 1.26	2.87 ± 3.54	2.44 ± 1.27	0.000	2.45 ± 1.26	3.06 ± 1.46	
COPD-SQ	11.07 ± 3.92	12.61 ± 4.95	10.71 ± 3.96	0.000	11.03 ± 3.92	12.97 ± 4.00	

COPD, chronic obstructive pulmonary disease; BMI, body mass index; PFT, pulmonary function test; PEF, Peak expiratory flow; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; % pred, % predicted; GOLD, Global Initiative for Chronic Obstructive Lung Disease; COPD-PS, the "COPD Population Screener"; COPD-SQ, the "COPD Self-Screening Questionnaire" (COPD-SQ).

TABLE 2 Results of logistic regression analysis of COPD-PS score and PEF.

Variables	β	SE	Wald	OR	*p*
COPDPS	0.321	0.032	100.558	1.379 (1.295, 1.468)	0.000
PEF	−0.358	0.030	142.207	0.699 (0.659, 0.741)	0.000
Constant	−0.479	0.150	10.137	0.620	0.001

COPD-PS, the "COPD Population Screener"; PEF, Peak expiratory flow.

TABLE 3 Results of logistic regression analysis of COPD-SQ score and PEF.

Variables	β	SE	Wald	OR	*p*
COPDSQ	0.125	0.011	120.403	1.134 (1.109, 1.159)	0.000
PEF	−0.315	0.030	109.907	0.730 (0.688, 0.774)	0.000
Constant	−1.286	0.195	43.376	0.276	0.000

COPD-SQ, the "COPD Self-Screening Questionnaire" (COPD-SQ); PEF, Peak expiratory flow.

3.3 The performance of different screening methods for COPD

We calculated the sensitivity, specificity, Youden index and ROC of five screening methods for COPD: COPD-PS scale, COPD-SQ scale, PEF, COPD-PS scale combined with PEF and COPD-SQ scale combined with PEF. We reported the optimal cut-off values for each method. Table 4 shows the performance of different screening methods for COPD with different cut-off values. Figure 2 shows the ROC curves of each screening method.

3.4 The performance of different screening methods for moderate and above COPD

We evaluated the sensitivity, specificity, Youden index and ROC of five screening methods for COPD and reported the optimal cut-off values for each method. Table 5 shows the performance of different screening methods for moderate and above COPD with different cut-off values. Figure 3 shows the ROC curves of each screening method for diagnosing moderate and above COPD.

4 Discussion

This study aimed to evaluate the screening effects of COPD-PS questionnaire, COPD-SQ questionnaire, PEF, COPD-PS questionnaire + PEF and COPD-SQ + PEF for chronic obstructive pulmonary disease (COPD). The results showed that all five methods had some screening ability for COPD and moderate or above COPD among residents in Haicang District of Xiamen City, China, and that adding PEF improved the performance of the questionnaires. Among the five screening methods, COPD-SQ questionnaire combined with PEF had relatively better diagnostic ability for COPD. However, when screening patients with moderate or above COPD, the sensitivity of COPD-PS questionnaire + PEF and COPD-SQ questionnaire + PEF was (0.630 vs. 0.781), specificity was (0.811 vs. 0.629), Youden index was (0.441 vs. 0.410), and area under the receiver operating characteristic curve (AUROC) was (0.784 vs. 0.766). The purpose of this study was to find a suitable screening method for COPD, which required a balance between sensitivity and specificity. Sensitivity is the ability of the screening test to accurately identify patients with a specified disease,

TABLE 4 The performance of different screening methods for COPD.

	Optimal cut-off	Sensitivity	Specificity	Youden index	PPV	NPV	ROC (95%CI)
COPD-PS	2.5	0.439	0.725	0.164	0.332	0.806	0.606 (0.586–0.626)
COPD-SQ	11.5	0.586	0.621	0.207	0.325	0.828	0.640 (0.619–0.661)
PEF[$]	3.765	0.519	0.688	0.207	0.341	0.821	0.641 (0.619–0.663)
COPD-PS + PEF*	−1.058	0.586	0.689	0.275	0.37	0.842	0.678 (0.657–0.699)
COPD-SQ + PEF[#]	−1.079	0.612	0.663	0.275	0.361	0.846	0.685 (0.664–0.706)

$Since PEF is negatively correlated with COPD, the ROC calculation in this study used negative PEF; * COPD-PS + PEF = [Logit(COPD-PS + PEF)] = −0.479 − (0.358 * PEF) + (0.321 * COPD-PS); #COPD-SQ + PEF = [Logit(COPD-SQ + PEF)] = −1.286 − (0.315 * PEF) + (0.125 * COPD-SQ); COPD-PS, the "COPD Population Screener"; COPD-SQ, the "COPD Self-Screening Questionnaire" (COPD-SQ); PEF, Peak expiratory flow; PPV, Positive predictive value; NPV, Negative Predictive Value.

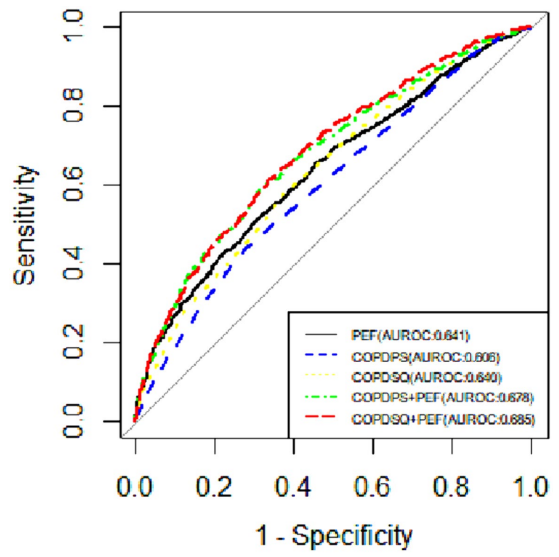

FIGURE 2
The ROC curves of different screening methods for diagnosing COPD. COPD-PS, the "COPD Population Screener"; COPD-SQ, the "COPD Self-Screening Questionnaire" (COPD-SQ); PEF, Peak expiratory flow.

and specificity is the ability of the screening test to accurately identify patients without the disease. High sensitivity results mean that there are few false negative results and missed cases. When the disease is serious and treatable in the pre-clinical stage, sensitivity is usually increased at the expense of specificity to increase the potential screening value (19). Therefore, although COPD-PS questionnaire + PEF had higher Youden index and AUROC than COPD-SQ questionnaire, we suggest using COPD-SQ questionnaire + PEF to screen patients with COPD and moderate or above COPD, considering the preference of screening test for high sensitivity, until further research can potentially optimize the performance of screening tools.

Pulmonary function test (PFT) is the "gold standard" for diagnosing COPD, but it is time-consuming, labor-intensive, and requires trained professionals. Therefore, most COPD screening uses a two-stage strategy: first, a screening questionnaire to assess the risk factors of patients and divide them into "high-risk" and "low-risk" groups; second, a PFT for the high-risk group to confirm the diagnosis of COPD. This strategy is simple, convenient, and more cost-effective than questionnaire or PFT alone. In China, common COPD screening questionnaires include COPD-PS, COPD-SQ, CAPTURE, and COPD-MH. Among them, COPD-PS is currently the most widely used (12, 20). However, some studies in Beijing and Shanghai found that COPD-SQ had a better screening effect than COPD-PS, which was consistent with this study. However, some studies in Beijing and Shanghai found that COPD-SQ had a better screening effect than COPD-PS (17, 21), which was consistent with this study. Moreover, the Chinese National Chronic Obstructive Pulmonary Disease Screening Program also recommends using COPD-SQ for COPD screening in primary healthcare center (18). The CAPTURE questionnaire was developed by Martinez et al. in the US, and it covers exposure to risk factors, respiratory problems, environmental effects, life and work impacts, fatigue, and respiratory diseases (22). Pan et al. conducted a large-scale multicenter study based on primary health care institutions in China, and found that the Youden index of CAPTURE was lower than that of COPD-SQ (0.220 vs. 0.326), suggesting that COPD-SQ had a better screening performance (23). COPD-MH is a questionnaire developed by Shi Jindong et al. based on primary community hospitals in Minhang District in 2022. The results showed that COPD-MH had a better screening effect than both COPD-SQ and COPD-PS (17). However, since COPD-MH was released recently and only studied in Minhang District in Shanghai, its screening effect needs to be further verified. The two-stage screening method also has some limitations. First, the questionnaire may not capture all the characteristics of COPD patients, especially other risk factors in the early stage of COPD. Second, the questionnaire may involve patients recalling past habits, which are subjective and may produce recall bias. In summary, the two-stage screening method is an effective method for COPD screening, but it needs to be constantly adjusted and optimized in practical application.

PEF is a simple, reliable, and low-cost method of lung function testing that measures the highest flow rate during forced expiration and reflects the degree of airflow limitation. PEF are smaller and more portable than spirometers, making them convenient, feasible, and reproducible for screening and monitoring COPD. Previous studies have shown that PEF can predict and detect COPD hospitalization exacerbations (24–27). COPD screening questionnaires assess the likelihood of COPD by asking patients about their symptoms and signs to assess the likelihood of COPD, but this assessment may be biased by subjective factors. PEF, as an objective indicator of lung function, can directly measure the expiratory flow rate and reflect the degree of airflow limitation. Therefore, combining screening questionnaires with PEF can

TABLE 5 The performance of different screening methods for moderate and above COPD.

	Optimal cut-off	Sensitivity	Specificity	Youden index	PPV	NPV	ROC (95%CI)
COPD-PS	2.5	0.489	0.714	0.203	0.215	0.897	0.631 (0.606–0.655)
COPD-SQ	11.5	0.620	0.603	0.223	0.200	0.908	0.653 (0.626–0.679)
PEF	3.725	0.665	0.700	0.365	0.262	0.929	0.753 (0.730–0.777)
COPD-PS + PEF	−0.844	0.630	0.811	0.441	0.349	0.932	0.784 (0.762–0.806)
COPD-SQ + PEF	−1.122	0.781	0.629	0.410	0.252	0.947	0.766 (0.744–0.789)

COPD-PS, the "COPD Population Screener"; COPD-SQ, the "COPD Self-Screening Questionnaire" (COPD-SQ); PEF, Peak expiratory flow.

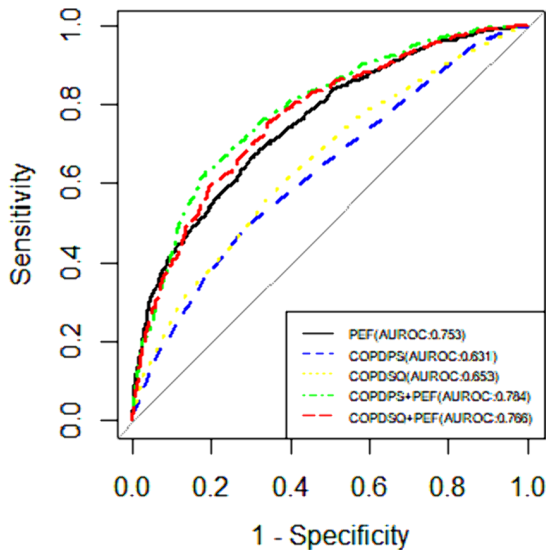

FIGURE 3
The ROC curves of different screening methods for diagnosing moderate and above COPD. COPD-PS, the "COPD Population Screener"; COPD-SQ, the "COPD Self-Screening Questionnaire" (COPD-SQ); PEF, Peak expiratory flow.

provide more comprehensive and accurate information and improve the screening performance. In 2016, Martinez et al. applied CAPTURE questionnaire and PEF to screen COPD in 346 subjects in US pulmonary and primary care clinics, and constructed a three-level screening strategy of "Questionnaire-PEF-Spirometry" (28). They found that combining PEF increased the AUROC of CAPTURE questionnaire from 0.795 to 0.906. In 2023, they expanded the study and applied CAPTURE questionnaire and PEF to screen COPD in 4658 subjects in primary care clinics. They also found that combining PEF increased the AUROC of CAPTURE questionnaire indeed (28). However, Yang et al. conducted CAPTURE questionnaire, COPD-SQ questionnaire and PEF in residents aged 35 years and above in Beijing primary health care institutions, and found that using COPD-SQ questionnaire alone had the best screening performance, while combining PEF reduced the screening performance of both questionnaires (7). They attributed this contradiction to the epidemic situation or the poor cooperation between the subjects and the spirometry examiners. This study and Martinez et al.'s study both suggested that PEF can be combined with screening questionnaires to improve the screening ability of primary health care institutions for COPD. In future studies, we hope to further explore the value of PEF in COPD screening, diagnosis, follow-up and prognosis, and provide more valuable information for early, comprehensive, individualized treatment and management of COPD.

This study has important significance for screening COPD in primary health care institutions. Spirometry is often difficult to perform in these settings due to the lack of professional personnel and equipment. Therefore, the screening strategy of COPD-SQ questionnaire combined with PEF can provide a simple, fast, low-cost, and efficient method for primary health care institutions, which can help to improve the diagnosis and treatment of COPD, and enhance the prognosis and quality of life of patients. In the future, training and education for medical staff and patients in primary health care institutions can be strengthened to improve their understanding and mastery of PEF usage methods and significance.

This study has limitations. First, the results of screening tools reflect the clinical characteristics of primary health care cohort in Haicang District of Xiamen City. However, the applicability of COPD screening tools may vary in different regions, different age groups, different severity levels, different risk factors, etc. Second, FEV_1/FVC ratio decreases with age increase, using a fixed cut-off point $FEV_1/FVC < 0.70$ to define COPD may overestimate the risk of COPD in elderly subjects (29, 30). In future studies, we suggest conducting research on more regions and larger samples to verify the results of this study. In addition, further optimization of screening tools' performance is needed, as well as understanding whether their use will affect clinical outcomes.

5 Conclusion

Our study found that the accuracy of COPD screening by COPD-SQ questionnaire and COPD-PS questionnaire can be improved by combining the results of PEF. The screening performance of COPD-SQ questionnaire combined with PEF is relatively better. In future research, further studies are needed to optimize the performance of screening tools and understand whether their use will affect clinical outcomes.

Data availability statement

The datasets presented in this article are not readily available because all data from this project is required to be handled confidentially. To adhere to the confidentiality guidelines provided by our funding agency, interested parties are welcome to contact us via

email at shelly1019@126.com where upon proper recording and with the necessary permissions, we can then share the data.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Xiamen Branch, Zhongshan hospital, Fudan University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

XS: Writing – original draft, Writing – review & editing, Conceptualization, Formal analysis. HY: Writing – review & editing. CL: Data curation, Formal analysis, Writing – original draft. FT: Data curation, Supervision, Writing – original draft. QL: Data curation, Supervision, Writing – original draft. YC: Data curation, Supervision, Writing – original draft. JW: Writing – original draft, Data curation. XC: Data curation, Supervision, Writing – original draft. ZP: Conceptualization, Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Zhihong Chen,
Fudan University, China

REVIEWED BY
Dai Jinghong,
Nanjing University, China
Roberto Carbone,
University of Genoa, Italy

*CORRESPONDENCE
Yi Huang
✉ huangliur@163.com
Jianming Zheng
✉ Jmzheng1962@smmu.edu.cn
Yuchao Dong
✉ dongyc1020@aliyun.com

†These authors have contributed equally to this work and share first authorship

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Identification of CFH and FHL2 as biomarkers for idiopathic pulmonary fibrosis

Xingchen Liu[1†], Meng Yang[2†], Jiayu Li[3†], Hangxu Liu[2], Yuchao Dong[2*], Jianming Zheng[1*] and Yi Huang[2*]

[1]Department of Pathology, The First Affiliated Hospital of Naval Medical University, Navy Medical University, Shanghai, China, [2]Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Naval Medical University, Navy Medical University, Shanghai, China, [3]Department of Breast and Thyroid Surgery, The First Affiliated Hospital of Naval Medical University, Navy Medical University, Shanghai, China

Background: Idiopathic pulmonary fibrosis (IPF) is a fatal disease of unknown etiology with a poor prognosis, characterized by a lack of effective diagnostic and therapeutic interventions. The role of immunity in the pathogenesis of IPF is significant, yet remains inadequately understood. This study aimed to identify potential key genes in IPF and their relationship with immune cells by integrated bioinformatics analysis and verify by *in vivo* and *in vitro* experiments.

Methods: Gene microarray data were obtained from the Gene Expression Omnibus (GEO) for differential expression analysis. The differentially expressed genes (DEGs) were identified and subjected to functional enrichment analysis. By utilizing a combination of three machine learning algorithms, specific genes associated with idiopathic pulmonary fibrosis (IPF) were pinpointed. Then their diagnostic significance and potential co-regulators were elucidated. We further analyzed the correlation between key genes and immune infiltrating cells via single-sample gene set enrichment analysis (ssGSEA). Subsequently, a single-cell RNA sequencing data (scRNA-seq) was used to explore which cell types expressed key genes in IPF samples. Finally, a series of *in vivo* and *in vitro* experiments were conducted to validate the expression of candidate genes by western blot (WB), quantitative real-time PCR (qRT-PCR), and immunohistochemistry (IHC) analysis.

Results: A total of 647 DEGs of IPF were identified based on two datasets, including 225 downregulated genes and 422 upregulated genes. They are closely related to biological functions such as cell migration, structural organization, immune cell chemotaxis, and extracellular matrix. CFH and FHL2 were identified as key genes with diagnostic accuracy for IPF by three machine learning algorithms. Analysis using ssGSEA revealed a significant association of both CFH and FHL2 with diverse immune cells, such as B cells and NK cells. Further scRNA-seq analysis indicated CFH and FHL2 were specifically upregulated in human IPF tissues, which was confirmed by *in vitro* and *in vivo* experiments.

Conclusion: In this study, CFH and FHL2 have been identified as novel potential biomarkers for IPF, with potential diagnostic utility in future clinical applications. Subsequent investigations into the functions of these genes in IPF and their interactions with immune cells may enhance comprehension of the disease's pathogenesis and facilitate the identification of therapeutic targets.

KEYWORDS

CFH, FHL2, biomarker, IPF, machine-learning strategies

1 Introduction

IPF is a chronic progressive lung disease distinguished by the excessive accumulation of mesenchymal cells and extracellular matrix, resulting in an irreversible decline in lung function (1). Predominantly affecting individuals in middle to older age groups, the incidence rate of IPF is steadily rising by 11% annually (2). Due to the insidious onset of IPF, the difficulty of imaging and histology to distinguish IPF from other usual interstitial lung diseases (ILDs), and the absence of reliable laboratory tests contribute to delayed diagnoses in most patients, typically occurring during the intermediate to advanced disease stages (1, 3). Because of delayed diagnosis and limited treatment options, individuals with IPF experience a median survival time of 2 to 5 years, with a five-year survival rate of 30%, which is inferior to that of many malignancies (4, 5).

At present, the pathogenesis of IPF is not fully understood, and it is mainly related to repetitive damage and repair dysregulation of alveolar epithelial cells. Epithelial apoptosis and impaired function of progenitors (AT2 cells) lead to the inability to complete the normal re-epithelialization process, induce fibroblasts to proliferate and transform into myofibroblasts. These activated myofibroblasts then deposit extracellular matrix (ECM) components, such as collagen, and exert contractile forces to facilitate wound healing (6). Scar foci formation triggers restrictive ventilation disorders and gas exchange disorders, ultimately leading to respiratory failure and death (7). Studies have found that immunity and inflammation are closely related to IPF, in which both innate and adaptive immunity are activated (8). In IPF, the damaged pulmonary epithelial cells released chemokines and cytokines, which leads to the recruitment and activation of innate immune cells such as neutrophils and macrophages, further activate the adaptive immune system (B cells and T cells) (9). Single-cell analysis showed that increased alveolar macrophages, dendritic cells (DCs), and memory T cells were present in IPF lungs and had possessed an activation profile indicating increased IFN-γ signaling and upregulation of adaptive immunity (10). Multicellular interactions between the activated innate and adaptive immune cells and lung fibroblasts may be crucial for the pathologic mechanisms of IPF and need further, which required further research.

Currently, there is a lack of effective treatments for IPF. Only two recommended drugs (nintedanib and pirfenidone) that have been approved for IPF but can only delay the decline in lung function and cannot stop the progression of the disease (11, 12). Numerous clinical trials investigating the efficacy of anti-inflammatory drugs for IPF have yielded unfavorable outcomes, including potential harm to patients (13, 14).

In summary, IPF remains a fatal disease characterized by a lack of timely diagnosis and efficacious treatment modalities. Therefore, this article aims to find the biomarkers with significantly altered expression levels in IPF patients through bioinformatics analysis, evaluate their diagnostic efficacy, and explore the relationship between the key genes that may be found and immune cell infiltration, so as to offer novel perspectives for the development of targeted immunotherapies for IPF.

2 Materials and methods

2.1 Dataset collection

Data analysis procedures of our study are shown in Figure 1. Gene expression profiles of GSE150910 and GSE32537 were downloaded from the Gene Expression Omnibus (GEO) database. GSE150910 dataset includes RNA-sequencing results of 103 IPF lung samples and 103 unaffected control lung samples. GSE32537 dataset includes transcriptional profiles on lung tissue from 119 IPF subjects and 50 non-diseased controls. The scRNA-seq data, accession number GSE132771, was obtained from GEO based on GPL24676 platform. We used sequencing results of three IPF patient lungs and three normal human lungs.

2.2 Identification of DEGs

We conducted the differential expression analysis by comparing IPF lung samples to normal lung samples in the R computing environment using limma package. Genes were regarded as differentially expressed with the threshold of FDR-adjusted p-value <0.05 and $|\text{Log2foldchange (FC)}| \geq 0.585$. FDRs were estimated with Benjamini-Hochberg procedure. Visualization of DEGs including volcano plots, heatmaps for top 50 DEGs, and Venn diagram was achieved by using ggplot2 package, Pheatmap package, and VennDiagram package in R, respectively.

2.3 Functional enrichment analysis

After identifying the overlapping DEGs between the above two datasets, we performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis via the clusterProfiler package to determine function and pathway enrichment of DEGs. Enrichment is achieved by comparing GO terms or pathways present in DEGs using three annotation databases of GO terms (BP, Biological Process; CC, Cellular Component; and MF, Molecular Function) or KEGG pathway database. The GO enrichment was performed for all DEGs together and for up- and down-regulated genes separately. Only terms with p-values and q-values<0.05 were considered significantly enriched. The results were visualized with GOplot R package.

2.4 Machine learning algorithms

In order to reduce the risk of bias, three machine learning algorithms were applied in this study to select characteristic genes of IPF: RF, Random Forest, LASSO logistic regression, The Least Absolute Shrinkage and Selection Operator, and WGCNA, Weighted Gene Co-expression Network Analysis. All statistical analyses were implemented through the randomForest package, glmnet package, and WGCNA package of R software, respectively. The results of the three algorithms were intersected by Venn diagrams to finalize the possible key genes for further study.

2.5 Diagnostic efficacy assessment

The expression levels of key genes identified were compared between the IPF group and the control group in two gene databases separately. If the differential expression is significant (p-value <0.05 and $|\text{Log2FC}| \geq 0.585$), the diagnostic effectiveness of key genes was evaluated by plotting the ROC curve and calculating the AUC via R

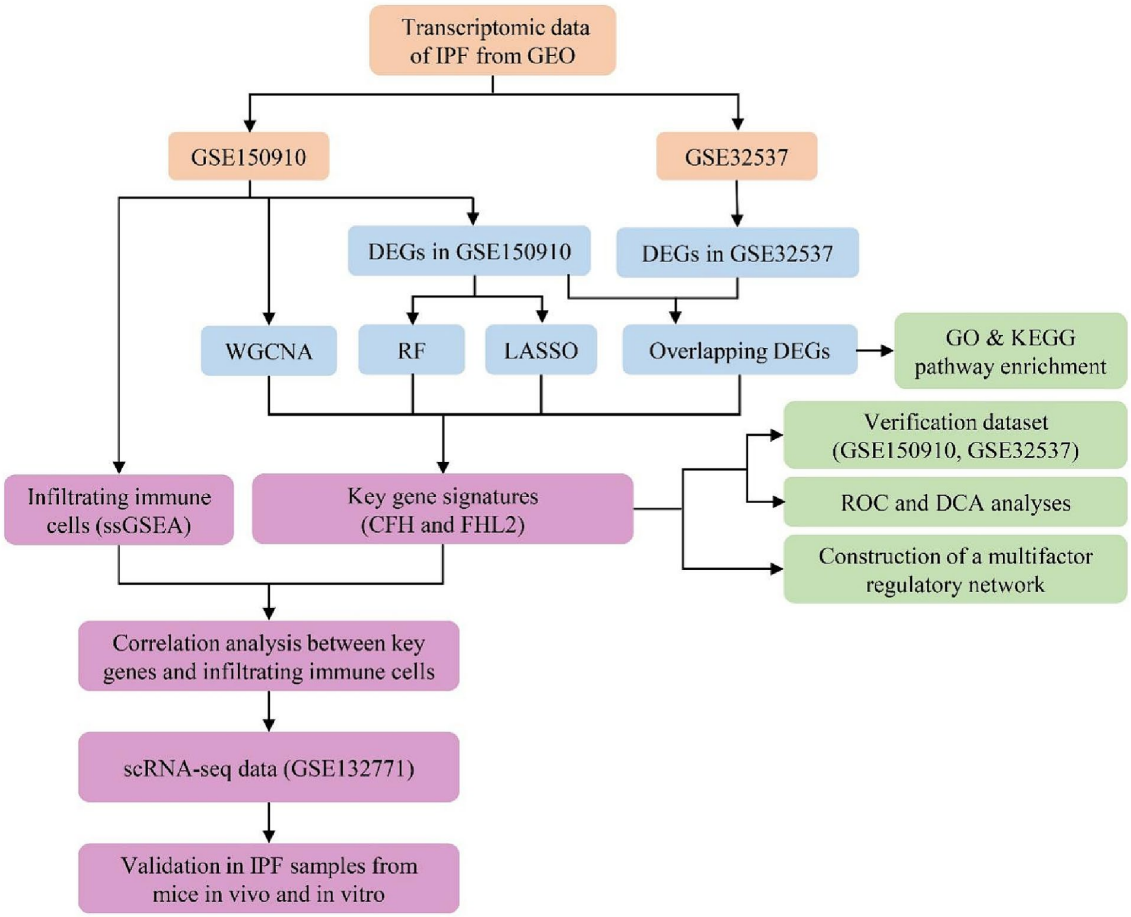

FIGURE 1
Flowchart of the study.

software pROC package. These genes were considered to be poorly diagnostic if the AUC is less than 0.7, moderately diagnostic if 0.7–0.9, and well diagnostic if greater than 0.9. In addition, we compared the predicted clinical assessment effects of key genes by constructing decision curve analysis (DCA).

2.6 Mapping gene regulatory network

Gene expression is subject to multiple regulators. We analyzed microRNAs (miRNA), long non-coding RNA (lncRNA), and transcription factor (TF) associated with the expression of key genes through mirDIP, starbase, and hTFtarget to find regulators that may lead to differential expression. The visualization process and the search for common regulators between key genes were completed by Cytoscape software.

2.7 Immune cell infiltration

First, we analyzed the intra-group similarity and inter-group variability between the IPF group and the control group by principal component analysis (PCA) to evaluate the sample data quality. ssGSEA was used to calculate the abundance of immune cell infiltrated in each sample from GSE150910 and plot heat map. The differences in Infiltrated immune cell set and function between the IPF and the control group were compared by ssGSEA and box plots were drawn. Spearman rank correlation coefficient calculation was used to screen infiltrating immune cells closely related to key genes. $p < 0.05$ was considered statistically significant.

2.8 Single-cell RNA sequencing analysis

The barcodes data, gene features data, and gene count matrix data of GSE132771 preprocessed by Cellranger (10X Genomics) were downloaded from the GEO database. We conducted the differential expression analysis by comparing IPF lung samples to normal lung samples in the R computing environment using the Seurat V4.1.0 package. Firstly, cells were subjected to quality control based on the following criteria: a gene count per cell >500, a percentage of mitochondrial genes <20%, and a red blood cell gene proportion < 3%. Then the data were normalized by SCTransform function (15) and integrated with reciprocal principal component analysis (rPCA) approach (16). Subsequently, PCA, cluster analysis, and Uniform Manifold Approximation and Projection (UMAP) were performed using the RunPCA, FindClusters, and RunUMAP functions, respectively. Additionally, cell annotation was performed using the

SingleR V1.4.1 package with the HumanPrimaryCellAtlasData as the reference dataset (17).

2.9 Isolation, culture and treatment of primary lung fibroblasts

Lung tissue from euthanized post-natal 4 weeks SD rats were quickly extracted (trachea and excess tissue removed) and rinsed with PBS before being infiltrated in serum-free medium. Then we minced lung tissue into 1–3 mm pieces with sterile ophthalmic forceps in a dish and transferred pieces to serum-free medium containing Dispase II (Sigma-Aldrich, 4,942,078,001), DNase I (Sigma-Aldrich, D5025). After incubating at 37°C for 45 min with gentle shaking every 15 min, add 10% FBS to stop digestion. Digestion products passing through a 70 μm filter were centrifuged at 1500 rpm for 5 min. Added Red Cell Lysis Buffer (Beyotime, C3702) to resuspend, incubated at room temperature for 5 min, and then centrifuged at 1500 rpm for 5 min. After resuspending cells with DMEM with 10% FBS (Gibco, United States), 100 U/mL of penicillin and 100 μg/mL of streptomycin, PLFs were plated at a seeding density of 5×10^5–1×10^6/well with 2 mL complete medium in 6-well plate. All steps were performed on ice or at 4°C unless stated otherwise. Cells were cultured at 37°C in a 5% CO2 incubator with regular feeds and passaged at 80% fusion using 0.25% of trypsin–EDTA in a 1:3 ratio. PLFs were divided into three groups comprising a control group of cells without treatment, a group with TGFβ1 10 ng/mL treatment for 48 h, and a group with TGFβ1 10 ng/mL treatment for 72 h.

2.10 Bleomycin-induced mouse lung fibrosis model

10 wild-type SPF-grade male C57BL/6J mice (6 weeks) were purchased from Shanghai Bikai Keyi Biotechnology Co. and randomly divided into two groups, a control group ($n = 5$) and a bleomycin group ($n = 5$). After gas anesthesia, the mice were intratracheally injected with normal saline (total volume 50 μL) or 5 mg/kg Bleomycin (selleck, S1214). Lung tissue samples from the mice were collected at 21 days.

2.11 Hematoxylin and eosin, Masson staining and immunohistochemistry

H&E staining and Masson staining was performed as previously described (18). Briefly, lung specimens were fixed with 4% paraformaldehyde for 24 h. Then the samples were dehydrated, paraffin embedded and cut into 3 μm sections. Sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome stain to assess gross morphology and collagen deposition, respectively. For IHC, after dewaxing and hydration, epitope retrieval was performed with 10 mM citrate buffer. Then sections were blocked with 1% BSA for 1 h at ambient temperature before incubated with primary antibody at 4°C overnight. Then the sections were rewarmed for 45 min on the next day and incubated with secondary antibodies for 30 min at room temperature (Zsbio, pv8000), followed by detection using the DAB detection kit (OriGene Technologies, ZLI-9017). The primary antibodies and secondary antibodies used were as follows: anti-CFH (Abclonal, A13686; 1:100), anti-FHL (proteintech, 21,619-1-AP; 1:100) and anti-α-SMA (Abcam, ab7817; 1:100).

2.12 Quantitative real-time PCR

Total RNA was extracted using the RNA extraction Kit (Fastagen, 220,010). Reverse transcription was performed using cDNA Synthesis Kit (Vazyme, R312-01). The reverse transcription conditions were 37°C for 15 min and 85°C for 5 s. The RT-PCR were performed using HiScript RT superMix for qPCR (Vazyme, R122-01) and the reaction conditions were initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. Primers and reagents used were as follows: Fhl2(rat), Forward: 5′- TCTGACCCCACAGGTTGCTG-3′; Reverse: 5′- TCACAGGTGTTGGCATAGAGC-3′. Cfh(rat), Forward: 5′- GTGTAAAGCCCCGAAGTCAAC-3′; Reverse: 5′- GGAGGG CAGAATCTTTTCTCATT-3′. Acta2 (rat), Forward: 5′- GTGTTCAGA GAGGGTGAGCC-3′; Reverse: 5′- TCAGGTTGGTCCTCTGGTCT-3′. Gapdh (rat), Forward: 5′- GCATCTTCTTGTGCAGTGCC-3′; Reverse: 5′- GATGGTGATGGGTTTCCCGT-3′.

2.13 Western blot

Samples were lysed with RIPA buffer on ice and centrifuged at 16000 g for 15 min to extract protein. Protein concentrations were measured using the BCA Protein Assay kit (Thermo, 23,227). These protein samples were separated by electrophoresis using 8% or 15% SDS-PAGE at 100 V for 20 min and 120 V for 100 min. Proteins were electrostatically transferred to NC membrane and blocked with 5% BSA for 120 min. The primary antibodies were incubated overnight at 4°C and the secondary antibody for 60 min at room temperature. Finally, the labeled protein bands were developed with developing solution and scanned. All experiments were repeated three times. The antibodies used were as follows: GADPH (Abcam, 16,891; 1:1000), anti-CFH (Abclonal, A13686; 1:1000), anti-FHL (proteintech, 21,619-1-AP; 1:1000) and anti-α-SMA (Abcam, ab7817; 1:1000).

2.14 Statistical analyses

All statistical analyses were performed using GraphPad Prism 8.0. A two-tailed unpaired student *t*-test was used to determine significance. One-way analysis of variance (ANOVA) with a Bonferroni post-test was used to compare differences among multiple groups. $p < 0.05$ was considered as statistically significant.

3 Results

3.1 Identification of DEGs

Differential expression analysis showed that there were 1950 differentially expressed genes (DEGs) in the GSE150910, including 739 genes down-regulated in the IPF group and 1,211 genes up-regulated (Figures 2A,D). Meanwhile, there were 1,259 DEGs in the GSE32537, consisted of 477 down-regulated genes and 782

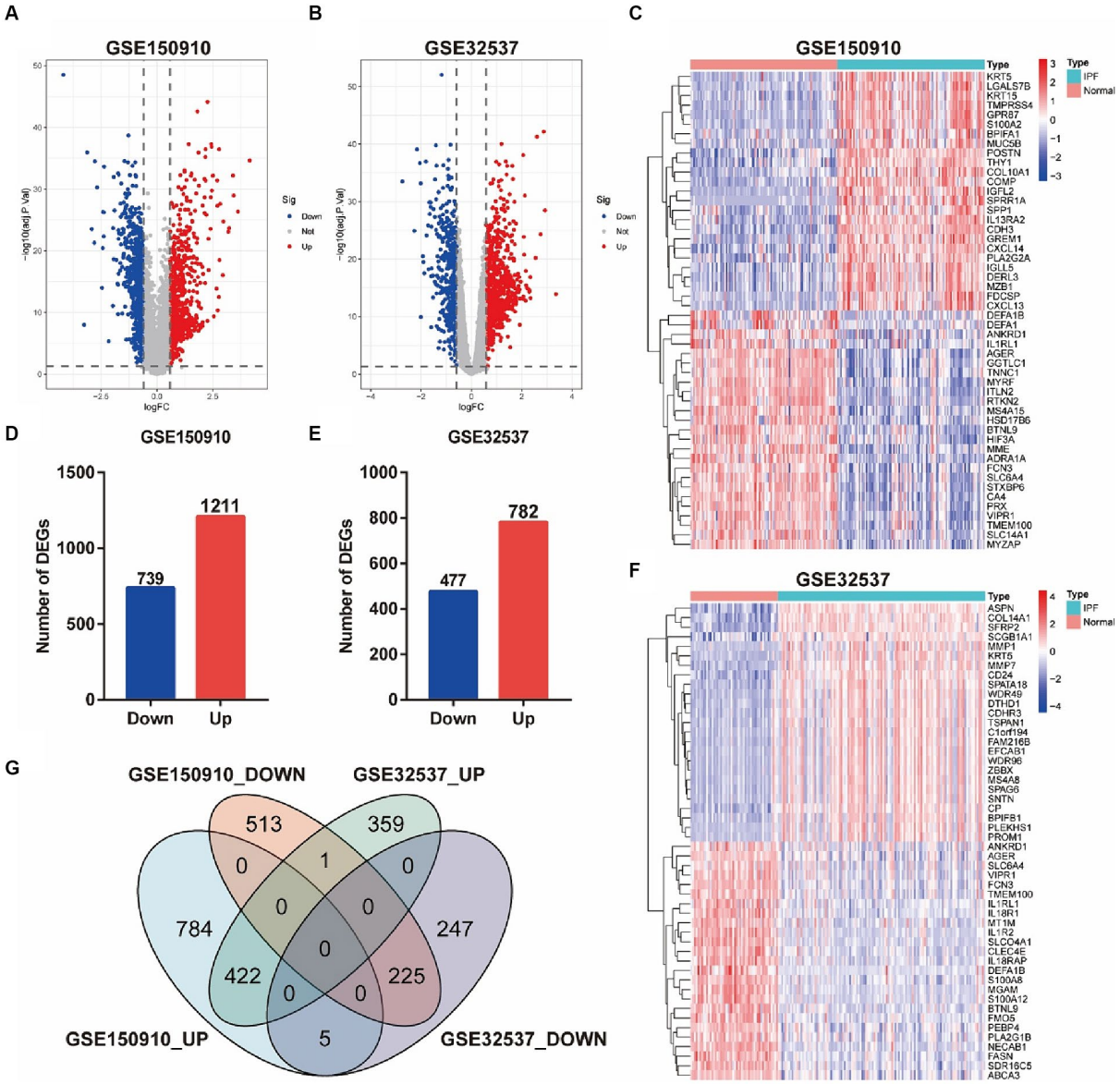

FIGURE 2
Identification of DEGs between IPF and normal samples. **(A,B)** Volcano plots of genes in GSE150910 **(A)** and GSE32537 **(B)**. Red dots represent up-regulated genes, blue dots represent down-regulated genes, and gray dots represent genes with no significance (|log2FC| > 0.585 and FDR < 0.05). **(C,F)** Heatmaps of top 25 up- and down-regulated DEGs in GSE150910 **(C)** and GSE32537 **(F)**. x-axis represents each sample, and y-axis represents each gene. Legend on the top right represents the log fold change of the genes. Red and blue colors represent relative increase or decrease in gene expression. **(D,E)** Number of DEGs in GSE150910 **(D)** and GSE32537 **(E)**. **(G)** Venn diagram of DEGs from the two datasets.

up-regulated genes (Figures 2B,E). The comparison of gene expression between the IPF group and the control group is shown by the volcano plot (Figures 2A,B). The top 25 up-regulated genes and the top 25 down-regulated genes in the GSE150910 or GSE32537 were presented in the heat maps, respectively, (Figures 2C,F). The Venn diagrams exhibited a total of 225 overlapping down-regulated genes and 422 overlapping up-regulated genes between the two datasets (Figure 2G).

3.2 Functional enrichment analysis

A biological functional classification of overlapping DEGs was performed by GO enrichment analysis. In the BP category, the significantly enriched terms included ameboidal-type cell migration, cell-substrate adhesion, microtubule-based movement, external encapsulating structure organization, extracellular structure organization, extracellular matrix organization, tissue migration, epithelial cell migration, and cell chemotaxis (Figure 3A). Meanwhile the significantly enriched terms in CC contained collagen-containing extracellular matrix, cell–cell junction, and external side of plasma membrane (Figure 3C). As for MF, the significantly enriched terms comprised extracellular matrix structural constituent, glycosaminoglycan binding, sulfur compound binding, integrin binding, and heparin binding (Figure 3E). Subsequently, GO enrichment analysis were conducted on the up-regulated and down-regulated overlapping DEGs, respectively (Figures 3B,D). Among the

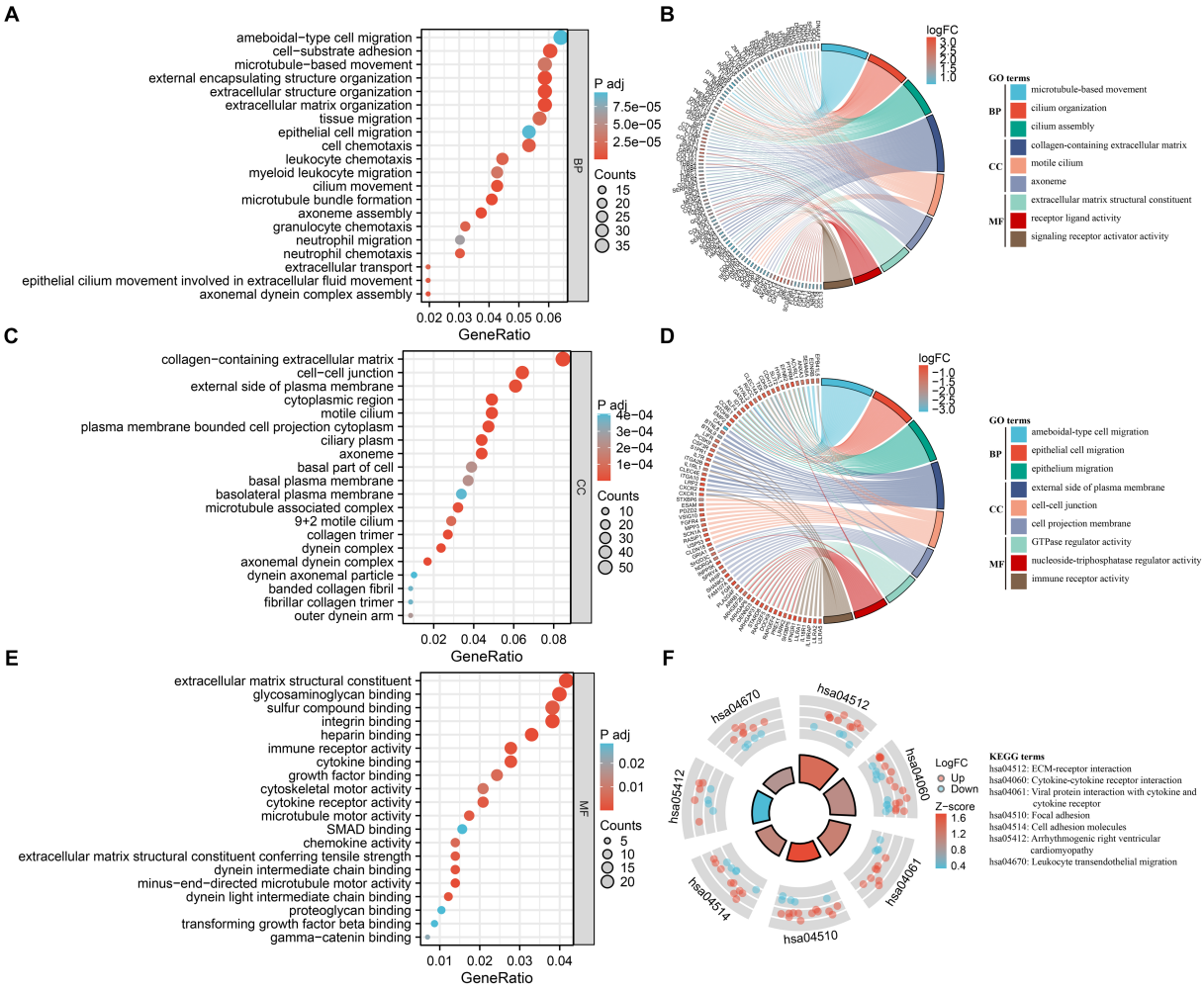

FIGURE 3
Functional enrichment analysis of DEGs. **(A,C,E)** GO analysis of DEGs. Bubble charts indicate enriched GO terms associated with DEGs in IPF sorted by: BP **(A)**, CC **(C)**, and MF **(E)**. x-axis represents gene ratios, and y-axis represents GO terms. Circle size represents gene count and color represents adjusted *p*-value. **(B,D)** Chord plots demonstrate enriched GO terms of up-regulated DEGs **(B)** and down-regulated DEGs **(D)**. The colored squares next to each gene indicate the logFC values shown in the legend on the top right. Each DEG is connected to their respective GO terms by ribbons, and the color of the GO term corresponds to the ribbon. **(F)** KEGG pathway enrichment analysis of DEGs. The outside ring shows the expression levels (logFC) of each gene in KEGG pathway. Red dots represent up-regulated DEGs, and blue dots represent down-regulated DEGs. The inside ring is a bar plot, with the height indicating the significance of the pathway enrichment and color indicating z-score shown in the legend on the right.

up-regulated DEGs, the predominantly enriched go terms included microtubule-based movement, cilium organization, and cilium assembly in BP, collagen-containing extracellular matrix, motile cilium, and axoneme in CC, and extracellular matrix structural constituent, receptor ligand activity, and signaling receptor activator activity in MF. In the down-regulated DEGs, the remarkably enriched go terms included ameboidal-type cell migration, epithelial cell migration, and epithelium migration in BP, external side of plasma membrane, cell–cell junction, and cell projection membrane in CC, and GTPase regulator activity, nucleoside-triphosphatase regulator activity, and immune receptor activity in MF. Then pathway analyses were performed by mapping genes to KEGG pathways. The result showed that the most abundant pathways included ECM-receptor interaction, cytokine-cytokine receptor interaction, viral protein interaction with cytokine and cytokine receptor, and focal adhesion (Figure 3F).

3.3 Feature selection

Three machine learning algorithms were combined to analyze the data from GSE150910 and screen for key genes by taking their intersections. 38 genes were identified by RF algorithm (Figures 4A,B) and 30 genes were screened out by LASSO regression algorithm (Figures 4C,D). In the WGCNA analysis, the network was constructed with 9 as the soft threshold based on the scale-free topology model fit index and the mean connectivity (Figures 4E,F). We identified 10 modules that were significantly co-expressed (Figure 4G) and explored the correlation between each module and IPF through a heat map (Figure 4H). The result showed that the MEdarkmagenta module had the highest positive correlation with IPF, so we further screened 38 genes that were highly correlated with IPF from the MEdarkmagenta module (Figure 4I). The Venn diagram showed that CFH and FHL2

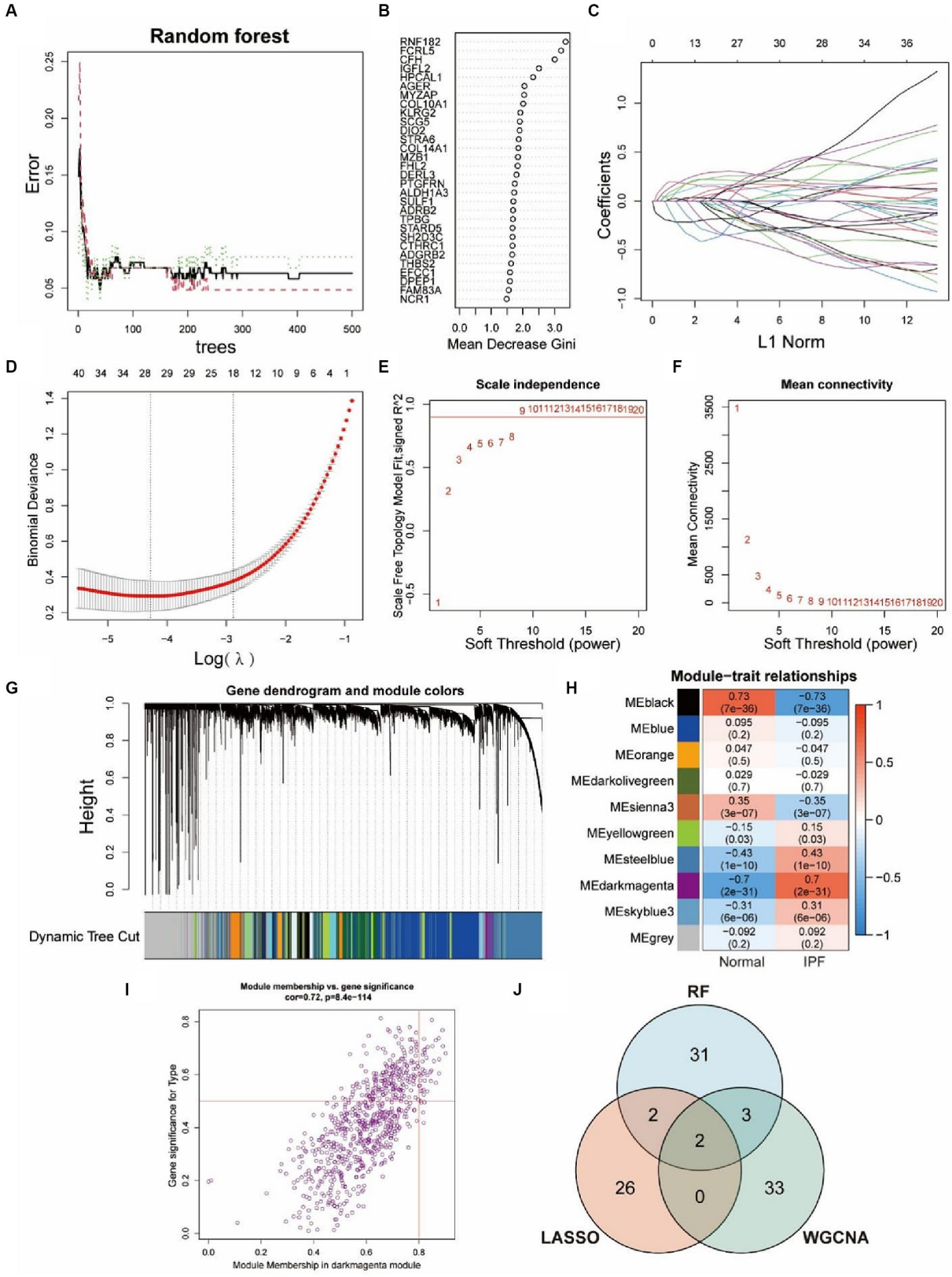

FIGURE 4
Identification of key genes via three machine-learning algorithms. **(A,B)** Key genes selection via RF algorithm. Distribution of out-of-bag (OOB) error rate at various values of trees **(A)**. Variable importance assessed in terms of the mean decrease Gini is computed using the OOB error **(B)**. A higher mean decrease in Gini coefficient indicates higher variable importance. **(C,D)** Key genes selection via LASSO algorithm. LASSO coefficient profile of 29 genes, different colors represent different genes **(C)**. Selection of the optimal parameter (lambda) in the LASSO model, and generation of a coefficient

(Continued)

FIGURE 4 (Continued)
profile plot **(D)**. **(E–I)** Key genes selection via WGCNA algorithm. Network topology analysis of various soft-threshold powers **(E,F)**. Horizontal axis represents soft threshold power, and vertical axis represents scale free topology model fit index **(E)** or mean connectivity **(F)**. Clustering dendrogram of DEGs related to IPF **(G)**, with dissimilarity based on topological overlap, together with assigned module colors. Module-trait associations **(H)**. Each row corresponds to a module, and each column corresponds to a trait. Each cell contains corresponding correlation and *p*-value. The table is color-coded by correlation according to the color legend. Gene significance for IPF in the MEdarkmagenta module **(I)**. One dot represents one gene in the module. Venn diagram shows the intersection of key genes obtained by three indicated algorithms **(J)**.

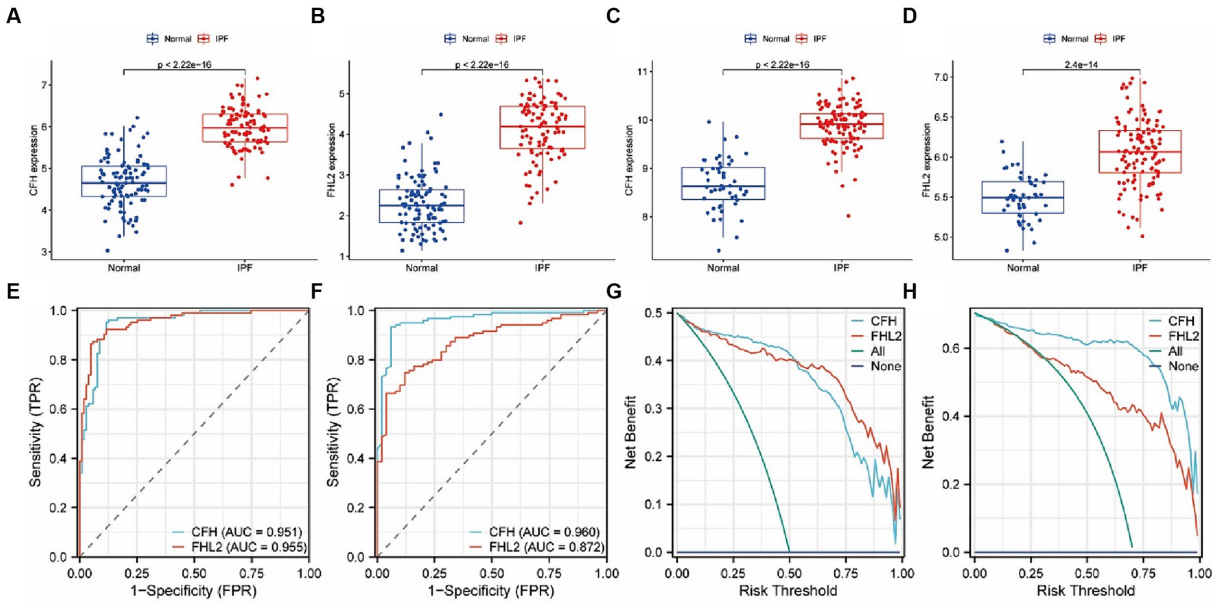

FIGURE 5
the expression levels of CFH and FHL2 in two datasets. **(A–D)** Box plots of the expression of CFH and FHL2 between IPF and normal samples in GSE150910 **(A,B)** and GSE32537 **(C,D)**. **(E,F)** The ROC curves of CFH and FHL2 in GSE150910 **(E)** and GSE32537 **(F)**. **(G,H)** The decision curves of CFH and FHL2 in GSE150910 **(G)** and GSE32537 **(H)**. CFH, complement factor H; FHL2, four and a half LIM domains 2.

were the overlapping key genes screened by three algorithms ultimately (Figure 4J).

3.4 Diagnostic value of key genes

By comparing the expression levels of CFH and FHL2 between the IPF group and the control group in the GSE150910 and GSE32537 datasets, the significant high expression of the two key genes in the disease group was verified ($p < 0.01$) (Figures 5A–D). The ROC curves indicated that both CFH and FHL2 exhibited strong diagnostic capabilities, with CFH achieving an AUC of 0.951 or 0.960, and FHL2 an AUC of 0.955 or 0.872 (Figures 5E,F). In the DCA curves, the net benefit of CFH method or FHL2 method was higher than that of the two extreme curves (all treatment or no treatment) within a large risk threshold range, which meant the two diagnostic methods had good clinical utility (Figures 5G,H).

3.5 Gene regulatory network

By integrating lncRNA/miRNA/TF that interacts with two key genes, we constructed a gene multifactor regulatory network. In this network, CFH was regulated by a total of 18 miRNAs and 7 TFs, while FHL2 was regulated by 98 miRNAs, 2 lncRNAs and 34 TFs. Notably, the regulatory network revealed the presence of TFs that co-regulate two key genes, including RAD21, LMNB1, FOXA2, FOXA1, SPI1, and CTCF (Figure 6).

3.6 Assessment of immune cell infiltration

The existing literature suggested that immunity is closely related to IPF, so we further explored the relationship between immune cell infiltration and two key genes. First, we confirmed by PCA that the samples in the IPF group and the control group from the GSE150910 dataset were well separated (Figure 7A). Then heatmap of infiltrating immune cells in GSE150910 was achieved (Figure 7B). Next, we compared the composition of 23 types of infiltrated immune cells between the IPF group and the control group. Box plot displayed that the IPF group had a higher proportion of activated B cell, immature B cell, and CD56bright NK cell (all $p < 0.01$), and a lower proportion of mast cell, monocyte, neutrophil, γδT cell, Tfh, Th17, and Th2 (all $p < 0.001$) (Figure 7C). By comparing the immune function score, it was found that APC co-stimulation, DC, macrophage, mast cell, B cell, Th, Tfh, Th1, and T cell co-stimulation was increased and aDC, pDC, neutrophil, NK cell, APC co-inhibition, cytolytic activity, Th2, and type1 IFN response was decreased in the IPF group (all $p < 0.001$)

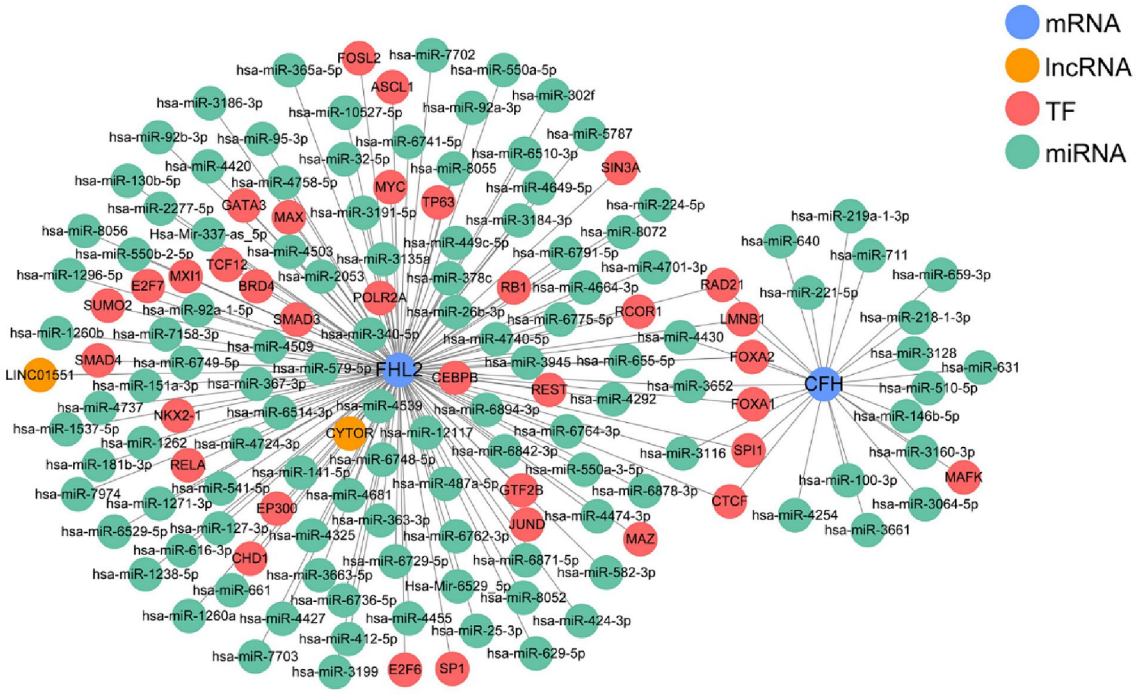

FIGURE 6
The multifactor regulatory network based on CFH and FHL2.

(Figure 7D). The correlation analysis between genes and immune cells demonstrated that both FHL2 and CFH were positively correlated with activated B cell, CD56bright NK cell, CD56dim NK cell and Treg, and negatively correlated with mast cell, monocyte, and Th17. Furthermore, FHL2 displayed a positive correlation with immature DC and a negative correlation with γδT cell and Th2 (all $p < 0.01$) (Figure 7E).

3.7 Single cell gene expression

We have more precisely localized the cell populations expressing CFH and FHL2 mRNA in IPF by single cell analysis. After a series of pre-processing of the data from the GSE132771, we performed a cluster analysis of the cells based on the similarity of the gene expression profile (Figures 8A,B). Among the 10 cell identities identified by clustering, the proportions of B cells, fibroblasts and endothelial cells in the IPF group were significantly increased, while epithelial cells, macrophages, neutrophils and NK cells were significantly decreased (Figure 8C). The expression levels of CFH and FHL2 in the IPF group were significantly upregulated in fibroblasts marked by ACTA2 and COL1A1, confirming that the two key genes obtained in this study are closely related to fibrosis induced by fibroblasts ($p < 2^{\wedge}10–16$) (Figures 8D,E).

3.8 Evaluation of CFH and FHL2 expression *in vivo* and *in vitro*

To verify the reliability of the results of bioinformatics analysis in IPF, we firstly established a IPF mouse model by bleomycin. H&E and Masson staining showed that 21 days after bleomycin administration, there was a significant increase in the deposition of collagen. Also, the expression of a widely accepted markers of fibroblast, α-smooth muscle actin (α-SMA) had a significant increase, as well as that of CFH and FHL2 (Figure 9A). WB analysis also showed that the content of α-SMA, CFH and FHL2 in the bleomycin-treated group increased compared with these in the control group (Figure 9B). Furthermore, we investigated whether CFH and FHL2 was involved in TGFβ1-induced fibroblasts activation. PLFs were treated with TGFβ1 (10 ng/mL) or PBS as control. And TGFβ1 treatment increased the expression of α-SMA, CFH and FHL2 both by WB analysis and qRT-PCR (Figures 9C,D). Collectively, these data indicated that CFH and FHL2 are involved in the pulmonary fibrosis and activation of fibroblasts induced by bleomycin and TGFβ1, respectively.

4 Discussion

Given that the underlying pathogenesis is not fully understood and the safe and effective therapeutic approaches remain elusive, currently there is an unmet clinical need for patients with IPF. In this study, we investigated the characteristics of the gene transcriptome in IPF patients compared to healthy individuals through comprehensive bioinformatics analysis, and identified a total of 422 up-regulated DEGs and 225 down-regulated DEGs. Functional enrichment analysis of overlapping DEGs revealed an enrichment of terms related to cell migration, structural organization, immune cell chemotaxis, extracellular matrix, etc., which is consistent with the existing literature. Notably, we further reduced the number of markers by taking the intersection of DEGs screened by the three machine learning algorithms, improving the specificity and sensitivity of the

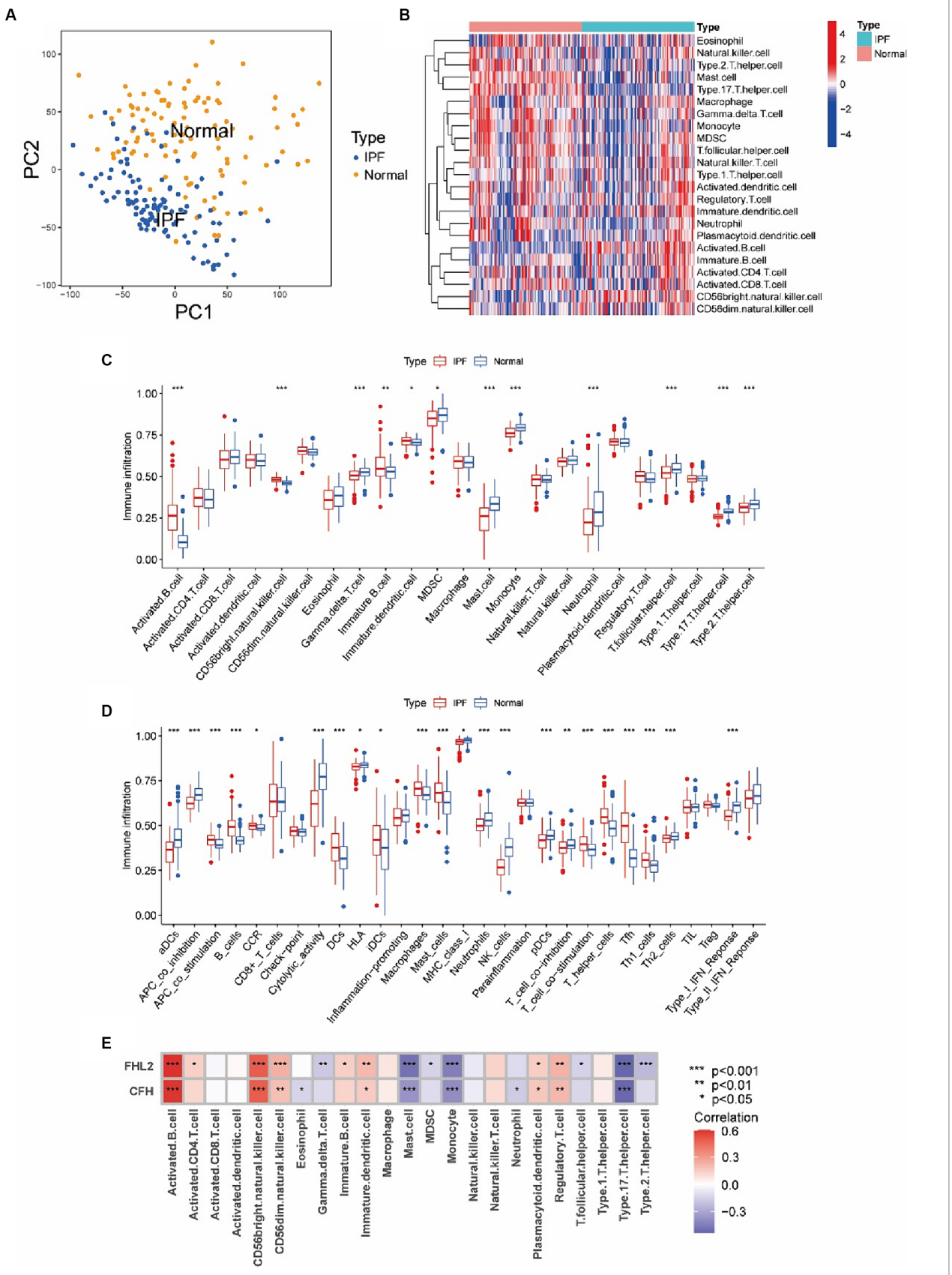

FIGURE 7
Assessment of immune cell infiltration and its relationship to key genes in IPF. **(A)** Principal component analysis (PCA) cluster plot of gene expression profile between IPF and normal samples in GSE150910. **(B)** Heatmap of infiltrating immune cells in GSE150910. x-axis represents each sample, and

(Continued)

FIGURE 7 (Continued)
y-axis represents each cell types. Legend on the top right represents the log fold change of cell counts. Red and blue colors represent high and low cell counts. **(C,D)** Box plots of the proportion of 23 types of immune cells **(C)** and 29 types of immune functions **(D)** between IPF and normal samples. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. **(E)** Correlations between CFH, FHL2, and infiltrating immune cells. Each cell is color-coded for logFC value of the correlation according to legend on the right, and significance is indicated.

features of IPF. Through this process, CFH and FHL2 were eventually identified as key genes with significantly altered expression in the disease, and their accurate and safe diagnostic efficacy was confirmed by ROC curves and DCA, which was further supported by both *in vivo* and *in vitro* models.

Actin alpha 2, smooth muscle (ACTA2) encodes one of six different actin proteins (19). Collagen type I alpha 1 chain (COL1A1) encodes the pro-alpha1 chains of type I collagen whose triple helix comprises two alpha1 chains and one alpha2 chain (20). These two genes, ACTA2 and COL1A1, are currently considered to be the main markers of pathological fibrosis in IPF (21). However, their diagnostic specificity is limited due to the potential expression of these molecules in normal cells such as smooth muscle cells and pericytes, in addition to pathogenic fibroblasts that overproduce extracellular matrix (22). While in this study, the results of single-cell analysis revealed that the upregulation of CFH and FHL2 in the IPF group was predominantly localized in fibroblasts, suggesting that these two molecules may be able to serve as precise indicators of IPF fibroblast foci in IPF.

The human CFH gene is located on chromosome 1q32 within the RCA (complement activation regulation) gene cluster and encodes a 155 kDa glycoprotein called complement factor H (23). CFH protein consists of 20 short consensus repeats (SCRs) and is mainly synthesized by the liver before being secreted into the plasma. In addition to hepatic production, various cell types including myofibroblasts, peripheral blood lymphocytes, RPE cells, glomerular mesangial cells, and podocytes have been shown to express CFH extrahepatically, potentially contributing to localized concentration increases (24). CFH is an important negative regulator of complement alternative pathway (AP) through three distinct mechanisms: competitive binding to C3b with factor B to inhibit the formation of C3 convertase, displacement of C3b from the formed C3 convertase to accelerate the decay of complement activators, and functioning as a cofactor of factor I to facilitate the degradation and inactivation of C3b. Additionally, CFH binds autologous cells by interacting with sialic acid and heparin-like glycosaminoglycan polyanions on the surface of host cells, thereby shielding them from damage. The deficiency of CFH has been shown to be associated with a variety of diseases, such as membranoproliferative glomerulonephritis, atypical hemolytic uremic syndrome (aHUs), age-related macular degeneration (AMD), etc. (25–27). However, the relationship between CFH and IPF has not yet been reported. In this study, CFH was found to be highly expressed in lung fibroblasts from IPF samples by single-cell analysis and confirmed by experiments. Whether the change in the expression level of CFH is the result of inflammatory regulation disorder or a way for fibroblasts to protect themselves from immune damage needs to be further explored.

The FHL2 gene encodes a member of the four-and-a-half-LIM-only protein family, characterized by two highly conserved zinc finger domains, each with four cysteines bound to a zinc atom, which regulate transcription factor activity and cytoskeletal proteins. Due to its structural properties, FHL2 interacts with a wide range of proteins and participates in a variety of cellular processes, such as transcriptional regulation, cell differentiation, proliferation, migration, apoptosis, and signal transduction (28). Normally, FHL2 is only highly expressed in cardiac tissue, but as an early-response gene protein, FHL2 expression is upregulated during tissue remodeling. For example, FHL2 is difficult to detect in normal skin, but its expression is significantly increased during the repair process after skin injury, especially in the migration and proliferation phases. Notably, FHL2 is predominantly expressed in myofibroblasts during this period, indicating a close association with its functional role. It stimulates fibroblast migration in a RAC-dependent manner, regulates matrix assembly, and acts as a transcriptional cofactor to support the expression of α-SMA and ECM proteins (29–33). Consequently, elevated levels of FHL2 may be implicated in the excessive wound healing and tissue remodeling observed in patients with IPF. Several studies have indicated that increased FHL2 expression could potentially serve as a distinguishing factor between individuals with IPF and healthy controls (34). The expression level of FHL2 was significantly and negatively correlated with percent diffusing capacity of the lungs for carbon monoxide (%DLCO), suggesting a potential role for FHL2 in stratifying patients based on disease severity (35). Experiments have shown that FHL2 inhibitors can significantly delay the progression of pulmonary fibrosis (36). Nevertheless, the relationship between FHL2 and immune dysregulation in IPF disease states has not been reported.

The relationship between IPF and immunity or inflammation is currently unclear. Some views suggest that aberrant immune activation plays a role in the development of IPF, while others argue that inflammation is a secondary characteristic of the disease, as evidenced by the limited efficacy of anti-inflammatory treatments in clinical trials. In this study, many DEGs in IPF samples were found to be associated with the chemotaxis and migration of a variety of immune cells as well as with the activity of immune receptors by functional enrichment analysis, confirming the close relationship between IPF and immunity. Further analysis showed that FHL2 and CFH were significantly positively correlated with B cells, and B cells were significantly enriched in IPF lung samples, suggesting a potential role for these genes in regulating B cell function in IPF. Previous studies have suggested that the binding of CFH and/or its related proteins to B lymphocytes may influence the migration of these cells and their role in adaptive immunity, further supporting the findings of this study (24, 37–39). Regarding FHL2, it is involved in the regulation of immune cell infiltration through direct interaction with various integrins, particularly the B2 subunit of the CD11a-d and CD18 integrin heterodimer receptor on immune cells. Additionally, FHL2 may indirectly influence immune cell attraction by modulating the expression of pro-inflammatory or anti-inflammatory cytokines (40).

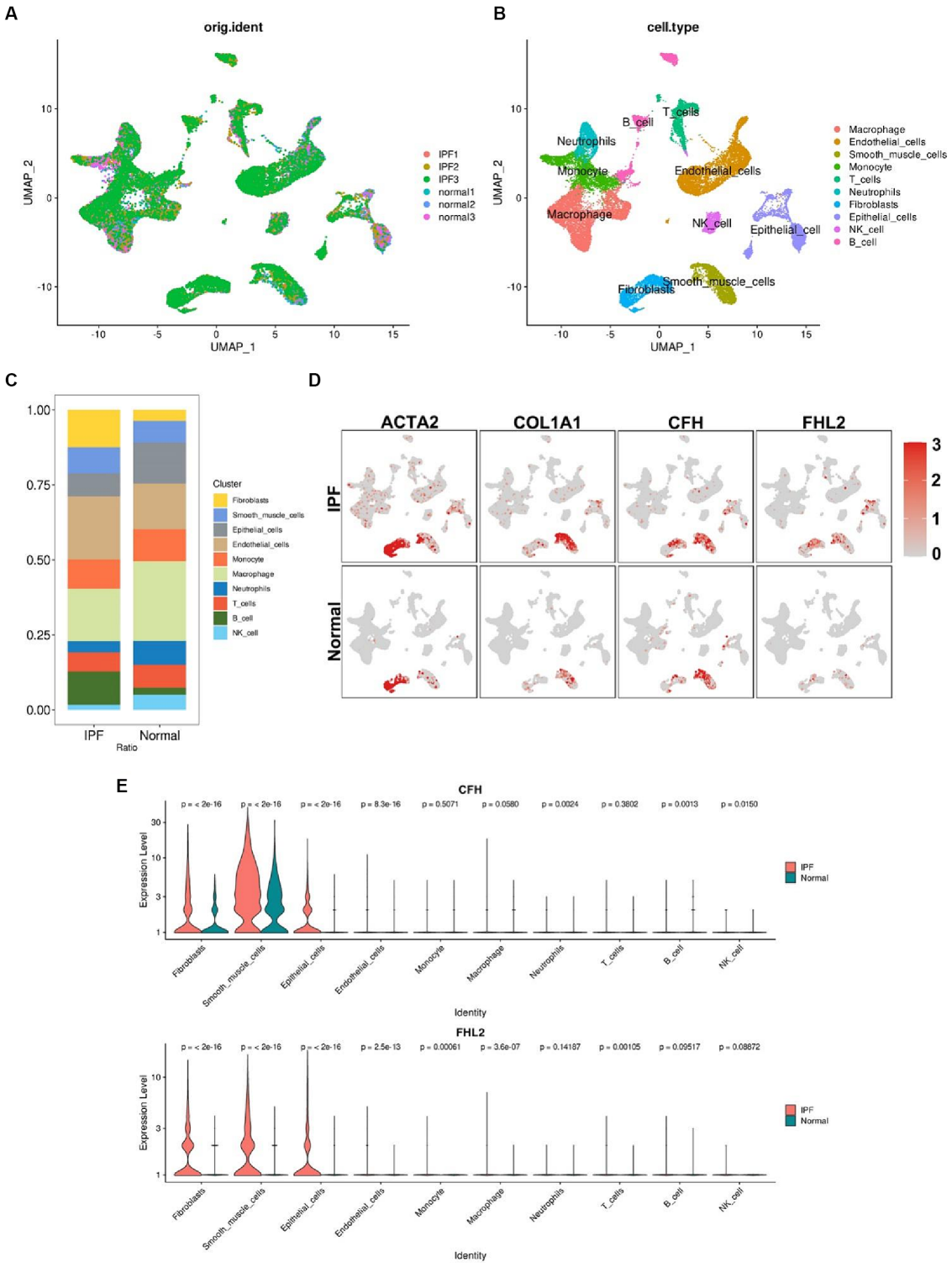

FIGURE 8
Single-cell RNA sequencing analysis. **(A)** Preprocessed single-cell data of GSE132771. **(B)** UMAP visualization of clustering revealing 10 cell clusters. **(C)** Comparison of the proportion of each cell cluster between IPF and normal group. The colors correspond to the cell types shown in the legend on the right. **(D)** Comparison of the expression levels of ACTA2, COL1A1, CFH, and FHL2 in different cell clusters between IPF and normal group. Red dots represent cells expressing the gene noted above. Shades of color correspond to the expression level shown in the legend on the right. **(E)** Violin plots compare the expression of CFH and FHL2 in different cell clusters between IPF and normal group with *p* value indicated above. CFH, complement factor H; FHL2, four and a half LIM domains 2; UMAP, Uniform Manifold Approximation and Projection.

This study also has some limitations. Firstly, the utilization of datasets from a public database without comprehensive clinical information hindered the ability to conduct prognostic analyses to ascertain the correlation between aberrant alterations in biomarker expression and poor patient prognosis. Further clinical studies are necessary to validate the diagnostic and stratification efficacy of

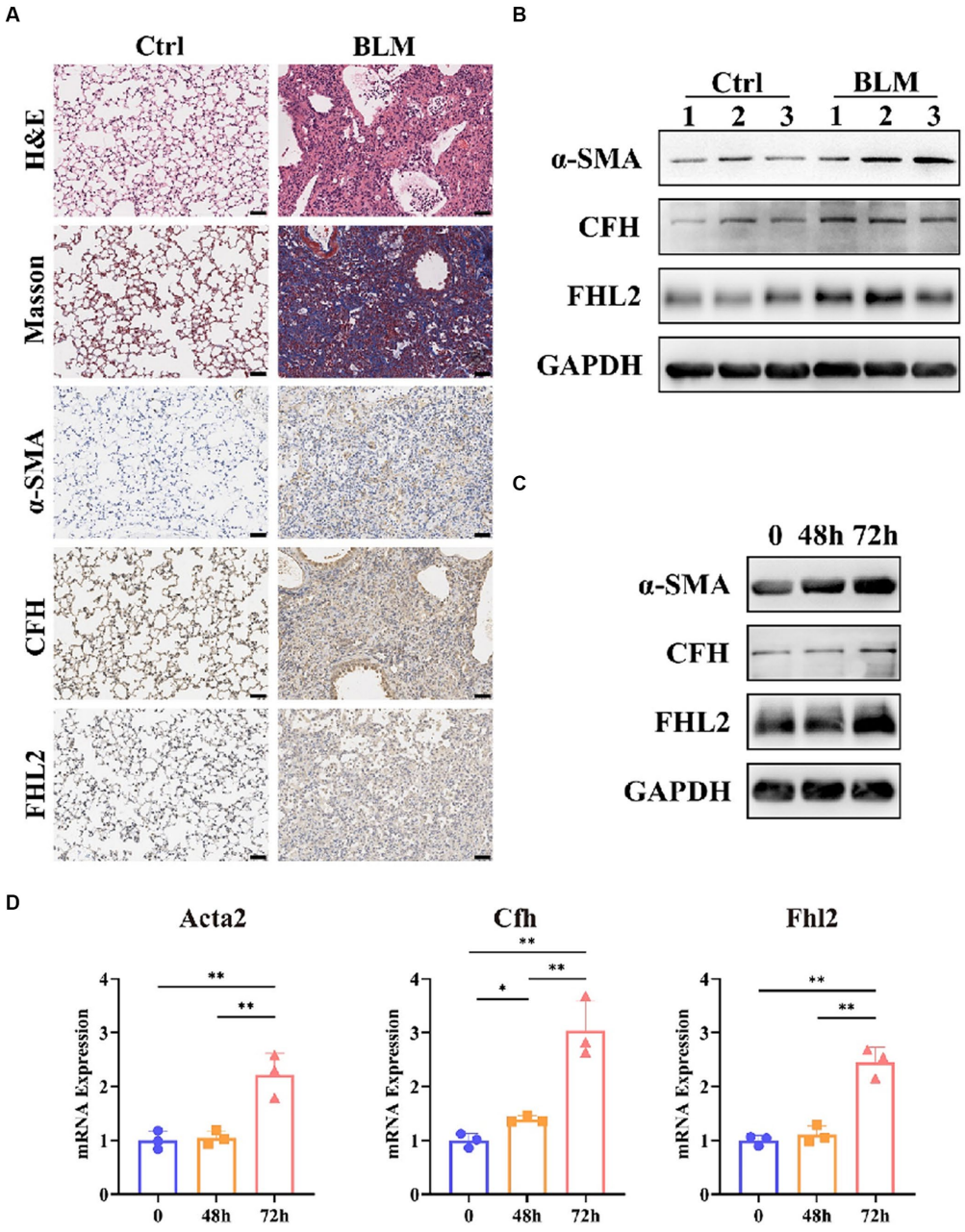

FIGURE 9
Expression of CFH and FHL2 in mouse and cellular IPF models. **(A)** HE and MASSON staining and immunohistochemical staining for α-SMA, CFH, and FHL2 in lung tissues of mice in control and bleomycin-induced groups. **(B)** Protein levels of α-SMA, CFH, and FHL2 in lung tissues of mice in control and bleomycin-induced groups assessed by Western blot. **(C)** Protein levels of α-SMA, CFH, and FHL2 in PLFs with or without TGF-β stimulation assessed by Western blot. **(D)** mRNA levels of Acta2, Cfh, and Fhl2 in PLFs with or without TGF-β stimulation assessed by qPCR. CFH, complement factor H; FHL2, four and a half LIM domains 2; α-SMA, alpha-smooth muscle actin; ACTA2, actin alpha 2, smooth muscle; PLFs, primary lung fibroblasts; TGFβ, transforming growth factor-β.

biomarkers. Furthermore, the molecular mechanism of biomarkers in the pathogenesis of IPF and their relationship with immunity have not been elucidated. Additional research is required to investigate and identify potential therapeutic targets for IPF.

In summary, CFH and FHL2 have been identified as promising novel biomarkers for IPF with strong diagnostic capabilities, suggesting their potential utility as diagnostic aids in the future. Further studies of the role of these two genes in IPF and their relationship with immune cells could help to understand the pathogenesis of IPF and provide potential therapeutic targets.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession

number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE150910 https://www.ncbi.nlm.nih.gov/geo/, GSE32537 https://www.ncbi.nlm.nih.gov/geo/, GSE132771.

Ethics statement

The animal study was approved by Committee on Ethics of Medicine, Navy Medical University, PLA. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XL: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Writing – original draft, Writing – review & editing. MY: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. JL: Data curation, Formal analysis, Investigation, Validation, Writing – original draft, Writing – review & editing. HL: Methodology, Software, Supervision, Writing – original draft, Writing – review & editing. YD: Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. JZ: Conceptualization, Funding acquisition, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. YH: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2024.1363643/full#supplementary-material

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EDITED BY
Zhihong Chen,
Fudan University, China

REVIEWED BY
Yeongkwon Son,
Desert Research Institute (DRI), United States
Basim Dubaybo,
Wayne State University, United States

*CORRESPONDENCE
Diane Rezende Batista
✉ diane.rezende@unesp.br

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Metal in biological samples from electronic cigarette users and those exposed to their second-hand aerosol: a narrative review

Diane Rezende Batista*, Liana Sousa Coelho, Suzana Erico Tanni and Irma de Godoy

São Paulo State University (Unesp), Medical School, Botucatu, Department of Internal Medicine, Pulmonology Division, São Paulo, Brazil

Introduction: Electronic nicotine delivery systems (ENDS) are gradually becoming more popular, particularly, among today's youth. Despite being marketed as safe by the tobacco industry, the notable absence of regulation in their composition is evident. Both the generated fluids and aerosol exhibit a wide variety of substances that are not yet fully identified. In addition to additives, the aerosol contains metals, the presence of which can be attributed to the excessive heating of metallic filaments used in vaporizing the liquid.

Objective: This review aimed to identify and describe studies that have assessed metal levels in biological samples obtained from electronic cigarette users and those exposed to their second-hand aerosol. This involved detailing the types and concentrations of metals identified and the biological samples in which the metals were detected.

Methods: Two independent researchers conducted searches in the MEDLINE and EMBASE databases to identify studies that measured the metal levels in human non-invasive biological samples from electronic cigarette users and second-hand exposure. Data were presented as a narrative review.

Results: In total, 18 articles were included in this review. Overall active and passive exposure to ENDS was related to higher levels of many metals, including lead and cadmium, in biological samples. ENDS users, in general, have lower metal concentrations in biological samples compared to the users of combustible cigarettes.

Conclusion: The exposure to primary and second-hand e-cigarette aerosol is related to higher metal concentrations in the biological samples. The adverse effects of this exposure on long-term users are yet to be determined.

KEYWORDS

e-cigarettes, electronic cigarettes, vape, metal, heavy metals, hair, urine

Introduction

Electronic nicotine delivery systems (ENDS) were quickly accepted and have become very popular since their introduction in the United States in 2006. Recently, Cooper et al. reported a significant number of e-cigarette users among high school students (14.1%) and middle school students (3.3%) (1). In some European countries,

the prevalence of e-cigarette use among teenagers has more than doubled in 4 years (2).

Although advertised as safe by the tobacco industry, there is no regulation on the form of use, concentration, and composition of aerosols in electronic devices. Furthermore, there are no guidelines on how manufacturers should report device characteristics and fluids available. The ban on the sales of menthol-flavored cigarettes became effective in Europe in 2020 (3) and in the USA in 2023 (4). However, there is no legislation for electronic cigarettes and hookahs. There are currently thousands of flavors for ENDS. In association with these flavors, fluid compositions for ENDS and the generated aerosols include a large, but not yet completely known, number of substances, whose effect and safety when used via inhalation are not defined.

In addition to additives, ENDS aerosol contains heavy metals. Metal aerosol in e-cigarettes are produced from vaporized fluid generated from the heating of metal filaments. These filaments are in general made from nichrome or kanthal (ferritic iron–chromium–aluminum alloy), so metals, such as silver (Ag), aluminum (Al), chromium (Cr), copper (Cu), iron (Fe), nickel (Ni), and zinc (Zn), are expected to be present in e-cigarette aerosols (5, 6). A systematic review published in 2020 (7) included 24 studies. In total, 12 studies detected metals/metalloids (Al, antimony, arsenic, cadmium, cobalt, Cr, Cu, Fe, lead, manganese, Ni, Se, tin, and Zn) in fluids and aerosols, and in 4 studies, metals were detected in samples of urine, saliva, serum, or blood from electronic cigarette users. An umbrella review (8) found evidence of these elements with considerable heterogeneity across the included studies. Two review studies examining EC users' urine and serum showed similar or higher levels of metals and metalloids compared to samples of users of combustible cigarettes or cigars.

Information on metal concentrations in biological samples from e-cigarette users is scarce. A systematic review by Zhao et al. (7) included few studies where metal concentrations were available. In our systematic review, we have included 18 studies that measured metal/metalloid levels in human biological samples from electronic cigarette users and those exposed to their second-hand aerosol.

Methods

For this narrative literature review regarding the identification and quantification of heavy metals in biological samples from electronic cigarette users, we performed a search of the MEDLINE and EMBASE databases in September and October 2023. The review was conducted according to Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) recommendations (Figure 1).

The search strategy was formulated by two authors and approved by the rest of the group. The search strategies employed for PubMed were as follows: (Electronic Nicotine Delivery System OR Electronic Cigarettes OR E-Cigs OR E Cigs OR E-Cig OR E Cig OR E-Cigarettes OR E Cigarettes OR E-Cigarette OR E Cigarette OR Electronic Cigarette OR Cigarette, Electronic OR Cigarettes, Electronic OR THC Vaping OR THC Vapings OR Vaping, THC OR Vapings, THC OR E-Cig Use OR E Cig Use OR E-Cig Uses OR Use, E-Cig OR ECig Use OR ECig Uses OR Use, ECig OR Vape OR Vapes OR E-Cigarette Use OR E Cigarette Use OR E-Cigarette Uses OR Use, E-Cigarette OR Nicotine Vaping OR Nicotine Vapings OR Vaping, Nicotine OR Vapings, Nicotine OR Ecigarette Use OR Ecigarette Uses OR Use, Ecigarette OR Uses, Ecigarette OR Electronic Cigarette Use OR Cigarette Use, Electronic OR Electronic Cigarette Uses OR Use, Electronic Cigarette OR E Cigarette Vapor OR Vapor, E-Cigarette OR Electronic Cigarette Vapor OR Cigarette Vapor, Electronic OR Vapor, Electronic Cigarette) AND (Heavy Metals OR Heavy Metal OR Metal, Heavy OR Metal OR Metals) and for EMBASE ('e cigarette' OR 'e cigarettes' OR 'electronic cigarettes' OR 'electronic nicotine delivery system' OR 'electronic nicotine delivery systems' OR 'electronic cigarette') AND ('metal, heavy' OR 'metals, heavy' OR 'heavy metal').

Two researchers independently conducted article selection. Initially, the selection was based on article titles and abstracts. Subsequently, selected articles underwent full-text reading to determine their inclusion or exclusion in the review. Any discrepancies between the researchers were resolved through consensus or, when necessary, after discussion with a third researcher. The articles were selected according to established inclusion criteria, which involved the analysis of heavy metals in non-invasive biological samples from electronic cigarette users. The following studies were excluded: (1) review articles, (2) *in vitro* sample studies, (3) airway model studies, and (4) studies using animal samples. No restrictions were applied regarding publication date, language, or availability of full text during the selection process.

Results

A total of 403 articles were identified in the selected databases. After removing duplicates, they were assessed based on the title and abstract. Review studies and those that did not meet the inclusion criteria, specifically those that did not address heavy metals and did not conduct biological sample analyses, were excluded. In total, 32 articles underwent comprehensive analysis of their full text. Following the exclusion of studies that did not meet inclusion criteria, 18 articles underwent detailed analysis and are described in this article.

Many studies have employed data from the Population Assessment of Tobacco and Health (PATH) Study, a longitudinal cohort study about tobacco use conducted among a sample of adults in the United States. Goniewicz et al. (11) analyzed data from users who only used e-cigarettes, who were dual users, and who never used any tobacco products from PATH Study Wave 1 (2013–2014). Urinary concentrations of Co, Pb, strontium, thallium, beryllium, Cd, and uranium were measured. A comparison between users who never used tobacco products and e-cigarette–only users showed Pb and Cd concentrations of approximately 19 and 23%, respectively, and were found to be lower in users who never used. The comparison between cigarette–only smokers and e-cigarette–only users showed Cd concentrations of 30% higher in in the first group. The comparison of the geometric mean of Pb and Cd concentrations between dual users and cigarette-only smokers did not differ.

In addition, from PATH Study Wave 1, Lizhnyak et al. (12) compared people who smoke against vape users and dual users split according to the frequency of cigarette and/or vape use. Urinary Cd levels were significantly different between people who frequently smoke and vape and people who frequently vape (0.33 vs. 0.28); between the group who infrequently smoke and vape (0.16) and people who smoke every day (0.31); and people who vape more than

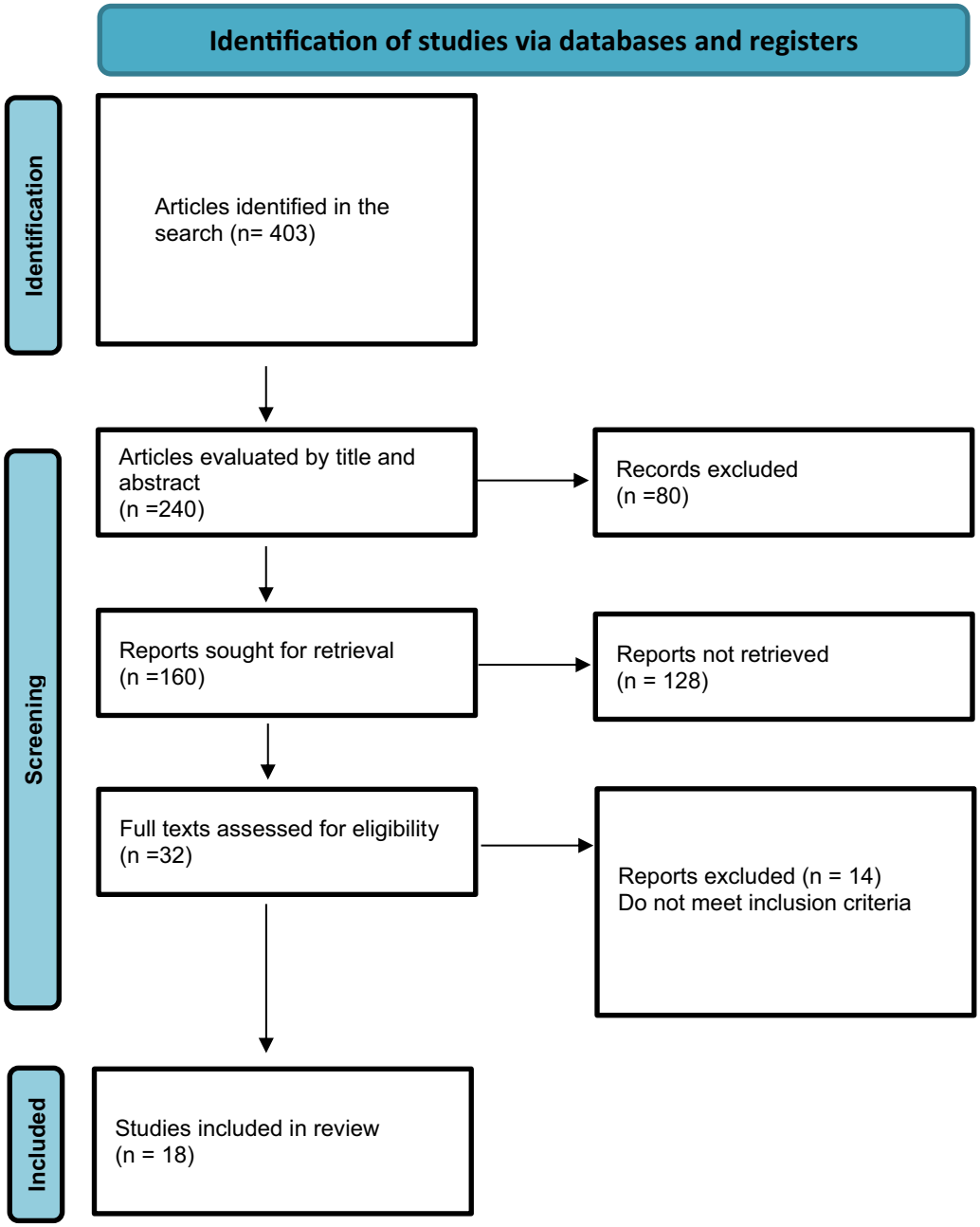

FIGURE 1
Preferred reporting items for systematic reviews and meta-analyses flow diagram of the process of including studies (9, 10).

smoke (0.29) and people who frequently vape (0.28). There were no differences between groups for urinary Pb levels.

Dai et al. (13) evaluated changes in urinary heavy metal levels (Co, Mn, Be, Cd, Pb, Sr., Tl, and U) when users transitioned between cigarette, dual use, and no use. Switching from exclusive cigarettes or dual use to e-cigarettes or no use was not associated with a decrease in heavy metal levels in urine. Switching from exclusive e-cigarette use to exclusive cigarette use or dual use at follow-up was not associated with an increase in heavy metal levels in urine. In a similar study (14), people who transitioned from exclusive smoking to dual use, no significant changes in Pb concentrations were observed. Pb levels showed a significant decrease among dual users who transitioned to exclusive ENDS use, while other transition groups did not exhibit significant changes.

To evaluate whether exposure to certain biomarkers could be associated with some respiratory symptoms, Dai et al. categorized the participants into three different groups at baseline: non-users, e-cigarette-only users, and dual e-cigarette/tobacco users. Those reporting exclusive e-cigarette use or dual use at baseline presented a higher prevalence of respiratory illness symptoms in the past 12 months compared to non-users. In relation to urinary Cd and Cr levels, there were no differences between groups and no association with respiratory illness symptoms (15).

Kaplan, B et al. (16) analyzed urine samples collected during PATH Study Waves 1, 2, and 3 for Pb, Co, Mn, Cd, Be, Sr., Tl, and U. Out of the 173 current ENDS users in Waves 1, 2, and 3, 50 were exclusive ENDS users who had never used any other ENDS, and the users of Waves 1, 2, and 3 had a history of using non-electronic tobacco products, both combustible and non-combustible types, and had transitioned to becoming exclusive ENDS users. In exclusive ENDS users who never used any tobacco products, urinary Cd concentrations remained consistent across Waves 1, 2, and 3 (0.25, 0.20, and 0.35, respectively, $p = 0.373$). For users who never used any tobacco products, Cd concentrations were found to be 0.22, 0.22, and 0.23, respectively. In Wave 3, Cd levels were significantly higher in ENDS users who had not used other tobacco products compared to non-users of ENDS ($p < 0.001$) and all ENDS users across all waves ($p < 0.001$). Those who never used any tobacco products showed consistent Cd, Pb, Be, and Tl urinary concentrations across the three waves. After adjusting for various factors, such as demographics, passive smoke exposure, and substance use, the study found that the geometric mean ratios (GMRs) for urinary Cd and Pb concentration in exclusive ENDS users, former non-electronic tobacco users who switched to ENDS, and all ENDS users were higher than those of never users For other metals, GMRs were not significantly different for exclusive ENDS users who never used non-electronic tobacco products compared to non-users. The authors concluded that current exclusive ENDS users, who had never used any other non-electronic tobacco products between 2013 and 2016, exhibited higher levels of Cd and Pb in urine compared to those who never used any tobacco products.

Nathan et al. (17) analyzed data from adults of at least 21 years who provided their urine samples for the PATH Study Wave (5). Participants were categorized into four groups based on their past 30-day use of ENDS and cigarette smoking. The study showed that the geometric mean levels for all three metals (Cd, Pb, and U) were significantly higher among all tobacco users compared to non-users. Specifically, in those dual users who smoked <10 cigarettes/day, Cd levels were significantly lower compared to smokers. However, the levels in dual users who smoked ≥10 cigarettes/day when compared to exclusive smokers showed no significant difference.

Dai et al. (18) evaluated racial and ethnic disparities by analyzing PATH Study Waves 1–5 data and did not find differences in heavy metal (Cd and Pb) concentrations among non-Hispanic (NH) White people, NH Black people, Hispanic/Latino people, and NH other people.

In Spain, 100 participants (50 vapers, 25 dual users, and 25 non-tobacco smokers) were recruited, and samples of urine, hair, and exhaled breath condensate (EBC) were collected. In urine samples, only median Cr and Sn levels were significantly lower in controls than in vapers and dual users. In contrast, in hair, median Cr and Cd levels were significantly higher in controls than vapers and dual users. EBC samples presented metal concentrations below or close to the detection limit for the studied metals; therefore, an analysis was not possible (19).

Prokopowicz et al. (20) evaluated 90 volunteers who were stratified according to their use of tobacco. Analysis of urinary samples for Ba, vanadium (V), Ag, Mn, Co, Ni, Cr, Sb, Cd, and Pb found no significant differences in urine concentrations of these elements between e-cigarette users, non-smokers, and smokers. The same group of authors (21) also evaluated exposure to Cd and Pb in 156 volunteers who switched from cigarette to EC use. Blood Cd concentrations adjusted for age and gender were 0.31 (0.26–0.36), 0.44 (0.37–0.52), 1.38 (1.11–1.72), and 1.44 (1.16–1.78) μg/L in non-smokers, e-cigarette users, dual users, and smokers, respectively. *Post hoc* analysis revealed significantly lower Cd concentrations between non-smokers and users of any kind of tobacco product. Blood Pb concentrations were only significantly different between the non-smoker and smoker groups ($p = 0.043$).

Serum metal levels in a group of 150 Romanian individuals showed that Cu, molybdenum (Mo), and Zn levels were significantly higher in cigarette smokers. In addition, cigarette smokers had the highest concentrations of Sb and Sr. On the other hand, the highest concentrations of Ag, Se, and V were detected in e-cigarette users. Ni levels showed no differences between groups (22).

Using data from the 2013 and 2016 Korea National Health and Nutritional Survey, Lee et al. (23) evaluated a total of 4,744 participants (2,162 men and 2,582 women) who were categorized into five groups according to smoking and ENDS use habits. Cigarette smoking in men and women and E-cigarette use in men are associated with a higher risk of higher blood Cd levels. In men, urinary Cd levels were significantly higher in E-cigarette users than in non-smokers (past-smokers $p = 0.017$; cigarette-smokers $p < 0.001$).

A study that recruited 64 E-cigarette users (50 E-cigarette smokers who had never smoked or had quit smoking at least 3 months earlier) and 14 dual users (who used combustible cigarettes at least weekly) showed that compared to dual users, only e-cigarette users had higher urine Ni and Cr levels (24).

In a cross-sectional study (25), which evaluated urine samples for metal evaluation, it was found that Zn concentrations were significantly higher in electronic cigarette users than in non-smokers, but Zn concentrations in electronic cigarette users were not different when compared to cigarette smokers (470.7 ± 223.6 μg/g, $p = 0.17$).

Based on data extracted from NHANES 2015–2016, in the analyses of heavy metals adjusted for sex, race/ethnicity, age, and poverty levels, the relationship between current or former e-cigarette use and metals did not achieve statistical significance. Nevertheless, individuals with a history of smoking were found to be more prone to elevated levels of blood lead and urinary cadmium compared to those who had not used either e-cigarettes or traditional cigarettes (26).

Amalia et al. (27) conducted an observational study to evaluate environmental and individual exposure to second-hand e-cigarette aerosol (SHA) in two household types: e-cigarette user homes and control homes. In total, 77 participants were included: 29 exclusive e-cigarette users (exposed), 29 non-users, and 21 controls. They found 27 metals in urine samples. The concentrations of all urinary biomarkers were similar between non-users and control participants. Of metal concentrations analyzed in urine, Co showed a higher geometric mean concentration in non-users compared to control participants. Non-users living with e-cigarette users had significantly higher urine Co levels than non-users living in control homes. The authors concluded that e-cigarette use at home created bystander exposure to SHA, irrespective of the features related to the use of e-cigarettes. The same group of researchers (28) evaluated a family unit comprising an e-cigarette user, a pregnant woman who delivered an infant during the study, and their 3-year-old son. They

found that metals were present in the urine and hair of all three participants, and also in the saliva of the adults, cord blood at the time of delivery, and breast milk. Several metals were identified in the urine, saliva, and hair samples of e-cigarette users, including Al, Cr, Ni, Cu, ZN, Sn, and Pb; however, Al was not found in urine. Metals were identified in cord blood and breast milk. Evaluation of samples from the 3-year-old revealed that the metals present in his urine and hair resembled those identified in samples from the pregnant woman, albeit generally in lower concentrations. Metals found at elevated concentrations in samples from the child, in contrast to those from the mother, included Zn in urine and Cr and Sn in hair. This research provided the first indications of involuntary exposure to e-cigarette aerosols in vulnerable populations, including children and pregnant women.

Table 1 lists heavy metals found in biological samples of electronic cigarette users.

Discussion

This review shows that exposure to ENDS, active and passive, is associated with higher levels of several metals in biological samples. In addition, ENDS users, in general, present lower biosample metal concentrations compared to combustible cigarette users. Several metals have been evaluated in urine, blood, exhaled breath condensate, and hair samples. The adverse effects of the metals detected in biological samples from the reviewed studies are presented in Supplementary Table 1.

Nine (50%) of the studies included in this review are derived from the PATH Study, a longitudinal cohort study of tobacco use in a national sample of US adults, which evaluated metal concentrations in urine samples from different Waves. The following metal concentrations were evaluated: Be, Cd, Co, Pb, Sr., Tl, and U. Higher urinary Pb and Cd concentrations were found in e-cigarette smokers than in non-smokers by Goniewicz et al. (11) and Kaplan et al. (16). Nathan et al. (17) showed a significantly higher concentration of Cd, Pb, and U in smokers and ENDS users than non-users and dual exposure (cigarette smoke/ENDS) is associated with higher Cd levels and that there is an exposure–response relationship with the number of cigarettes per day. From the PATH Study Wave 1, Lizhnyak et al. (12) also found significantly higher urinary Cd levels in those who frequently smoke and vape than in those who only vape. Those who infrequently smoke and vape presented a significantly lower Cd concentration than people who smoke only cigarettes every day or frequently vaped and infrequently smoked. The urinary levels of Pb were similar in these groups. While PATH Study Waves were being conducted, there were changes in the generation of ENDS products; during the first waves, the earlier-generation products were predominant, while more recent-generation products were available during the most recent PATH Waves. However, the results are concordant over time.

TABLE 1 Metals identified in electronic smoking devices.

Human samples devices	Metal
Urinary concentrations	Pb, Cd, Be, Ni, Cr, and Co
Blood	Cd, Se, and V
Saliva	Cr, Ni, and Pb
Hair	Cr, Ni, and Pb

Pb, lead; Be, beryllium; Cd, cadmium; Co, cobalt; Cr, chromium; Se, selenium; V, vanadium; Ni-nickel.

Other small-scale studies in the USA and other countries produced results in line with the PATH-derived studies. Data from Romania showed significantly higher Cu, Mo, and Zn values in cigarette smokers than in non-smokers and EC users. Sb and Sr. concentrations were highest in cigarette smokers. In contrast, the highest concentrations of metals, such as Ag, Se, and V, were found in e-cigarette users (22). Sakamaki-Ching et al. (25), in the USA, showed that Se concentrations were significantly higher in electronic cigarette users than non-smokers and cigarette smokers, and Zn concentrations were significantly higher in electronic cigarette users than non-smokers. Lee et al. (23) analyzed data from the 2013 and 2016 Korea National Health and Nutritional Surveys and showed that regular cigarette smoking in men and women and ENDs use in men are associated with a higher risk of elevated blood Cd levels. In men, urinary Cd levels in electronic cigarette users were significantly higher than in non-smokers. A comparison between electronic cigarette smokers and dual users showed that exclusive e-cigarette users had higher urine levels of Ni and Cr (24).

Transition to different forms of smoking was also evaluated in participants of Waves 1 and 2 of the PATH Study (13). The transition from sole smoking to dual use showed no significant changes in Pb concentrations. However, there was a significant decrease in Pb level in dual users who transitioned to exclusive ENDS users. Prokopowicz et al. (21) also evaluated the exposure to Cd and Pb in 156 volunteers, and *ad hoc* analysis revealed significant differences in Cd concentration between non-smokers and electronic cigarette users and between non-smokers or electronic cigarette users and dual users or smokers. The only significant difference in blood Pb concentrations was observed between the non-smoker and smoker groups. The authors hypothesized that the exposure to Cd and probably to Pb could be significantly reduced by completely switching to ECs and quitting conventional cigarette smoking.

The influence of racial and ethnic disparities was also evaluated in PATH Study Waves 1–5 participants, with results showing no differences in Cd and Pb concentrations for non-Hispanic (NH) White people, NH Black people, Hispanic/Latino people, and NH other people (18).

One study that examined a sample subset from the PATH Study Waves 1 and 2 designed to evaluate exposure and respiratory symptoms found no differences between groups (non-users, exclusive e-cigarette users, and poly e-cigarette/tobacco users) in terms of urinary Cd and Cr levels, and also showed no association with respiratory symptoms (15). In addition, in contrast with most of the PATH-derived studies, data extracted from the NHANES 2015–2016 showed lower mean blood Pb and Cd levels in e-cigarette users (with or without dual use) when compared to sole e-cigarette users (current or former). Similar results were shown for Ba and Sb levels. Lower median levels in current or former e-cigarette users failed to reach statistical significance (26). Prokopowicz et al. (20) found no significant differences in urinary Ba, V, indium (In), Ag, Mn, Co, Ni, Cr, Sb, Cd, and Pb in people smoking different combinations of conventional and e-cigarettes.

Evaluation of environmental and individual exposure to second-hand e-cigarette aerosol (SHA) (27) showed that non-users living with ENDs users had significantly higher urine Co levels than non-users

residing in control homes. Although the concentration is considered low, it is a marker of exposition.

The authors concluded that using e-cigarettes at home leads to exposure to SHA regardless of the characteristics of e-cigarette use. The same group of researchers, Ballbè et al. (28), evaluated homes with an e-cigarette user, a pregnant woman who delivered an infant during the study, and their 3-year-old son. Several metals were identified in the samples of e-cigarette users in urine, saliva, and hair, including Al, Cr, Ni, Cu, ZN, Sn, and Pb. However, Al was not found in urine. In the pregnant woman, several metals were found in cord blood and breast milk. Assessment of samples from the 3-year-old showed that metals found in his urine and hair were similar to those found in the pregnant woman, but usually at lower concentrations. Concentrations of Zn in urine and Cr and Sn in hair from the 3-year-old were higher than those in the mother. This study provided the first evidence of passive exposure to e-cigarette aerosols by people from vulnerable populations, such as children and pregnant women.

It is important to show that results may vary depending on the biological samples collected. A small study of urine, hair, and exhaled breath condensate samples showed that only median Cr levels in urine were significantly lower in controls than in vapers and dual users, whereas levels in hair were significantly higher in controls than in vapers and dual users. Exhaled breath condensate samples presented metal concentrations below or close to the detection limit for the metals studied, thus an analysis was not possible (19).

This review has some limitations. Half the data were derived from the PATH Study Waves, and data about the ENDS generation and other possible types of exposure to metals were not available in the different waves. In addition, most of the studies used self-reported data to classify categories of smokers. However, most of the results confirmed higher levels of metals in electronic cigarette users than non-smokers, with other smaller studies reinforcing this finding.

In conclusion, this review consistently shows that exposure to primary and second-hand e-cigarette aerosol is associated with higher concentrations of metals in biological samples in electronic cigarette users than non-smokers. It also shows that conventional combustible cigarette users have similar or higher metal levels than electronic cigarette users. Although the adverse effects of this exposure for long-term users are yet to be determined, further research related to the chemical characteristics of electronic cigarettes and their consequences in humans is urgently needed.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

DB: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. LC: Data curation, Writing – original draft, Writing – review & editing. ST: Writing – review & editing. IG: Conceptualization, Data curation, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2024.1349475/full#supplementary-material

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EDITED BY
Suzana Erico Tanni,
Sao Paulo State University, Brazil

REVIEWED BY
António Gil Castro,
University of Minho, Portugal
Wayne Robert Thomas,
University of Western Australia, Australia

*CORRESPONDENCE
Renata Kelly da Palma
✉ rekellyp@hotmail.com

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Effect of photobiomodulation in the balance between effector and regulatory T cells in an experimental model of COPD

Auriléia Aparecida de Brito[1,2], Karine Zanella Herculano[2,3], Cristiano Rodrigo de Alvarenga-Nascimento[2], Cintia Estefano-Alves[1], Cinthya Cosme Gutierrez Duran[1], Rodrigo Labat Marcos[1], José Antonio Silva Junior[1], Maria Cristina Chavantes[1], Stella Regina Zamuner[1], Flávio Aimbire[4], Laia Lladó-Pelfort[5], Albert Gubern[5,6], Anna Fàbrega[5,6,7], Renata Kelly da Palma[3,7,8,9]* and Ana Paula Ligeiro de Oliveira[1]

[1]Universidade Nove de Julho, São Paulo, Brazil, [2]Departament of Research Development and Innovation, Innovative Health System Health Management (HIS Medicine and Technology), São Paulo, Brazil, [3]Departament of Surgery, Faculty of Veterinary, University of São Paulo, São Paulo, Brazil, [4]Translational Medicine, Federal University of São Paulo-UNIFESP, São José dos Campos, Brazil, [5]Department of Basic Sciences, Faculty of Health Sciences at Manresa, University of Vic-Central University of Catalonia (UVic-UCC), Manresa, Spain, [6]Faculty of Medicine, University of Vic-Central, Manresa, Spain, [7]Tissue Repair and Regeneration Laboratory (TR2Lab), Institute for Research and Innovation in Life and Health Sciences in Central Catalonia (Iris-CC), Vic, Spain, [8]Faculty of Health Sciences at Manresa, University of Vic-Central University of Catalonia (UVic-UCC), Manresa, Spain, [9]University Center of Anápolis, Anápolis, Brazil

Introduction: Currently, Chronic Obstructive Pulmonary Disease (COPD) has a high impact on morbidity and mortality worldwide. The increase of CD4+, CD8+ cells expressing NF-κB, STAT4, IFN-γ and perforin are related to smoking habit, smoking history, airflow rate, obstruction and pulmonary emphysema. Furthermore, a deficiency in $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) may impair the normal function of the immune system and lead to respiratory immune disease. On the other hand, the anti-inflammatory cytokine IL-10, produced by Treg cells and macrophages, inhibits the synthesis of several pro-inflammatory cytokines that are expressed in COPD. Therefore, immunotherapeutic strategies, such as Photobiomodulation (PBM), aim to regulate the levels of cytokines, chemokines and transcription factors in COPD. Consequently, the objective of this study was to evaluate $CD4^+$STAT4 and $CD4^+CD25^+Foxp3^+$ cells as well as the production of $CD4^+$IFN- γ and $CD4^+CD25^+$IL-10 in the lung after PBM therapy in a COPD mice model.

Methods: We induced COPD in C57BL/6 mice through an orotracheal application of cigarette smoke extract. PMB treatment was applied for the entire 7 weeks and Bronchoalveolar lavage (BAL) and lungs were collected to study production of IFN- γ and IL-10 in the lung. After the last administration with cigarette smoke extract (end of 7 weeks), 24 h later, the animals were euthanized. One-way ANOVA followed by NewmanKeuls test were used for statistical analysis with significance levels adjusted to 5% ($p < 0.05$).

Results: This result showed that PBM improves COPD symptomatology, reducing the number of inflammatory cells (macrophages, neutrophils and lymphocytes), the levels of IFN-γ among others, and increased IL-10. We also observed a decrease of collagen, mucus, bronchoconstriction index, alveolar enlargement, CD4+, CD8+, CD4+STAT4+, and CD4+IFN-γ+ cells. In addition, in the treated group, we found an increase in CD4+CD25+Foxp3+ and CD4+IL-10+ T cells.

Conclusion: This study suggests that PBM treatment could be applied as an immunotherapeutic strategy for COPD.

KEYWORDS

COPD, photobiomodulation (PBM), T-cells, lung, cytokines, Foxp3

1 Introduction

According to WHO data (1), the main causes of human mortality, accounting for more than 68% of deaths, are chronic non-communicable diseases, many of which are related to smoking. The use of tobacco products, particularly cigarette smoke, represents the most important preventable public health problem for developed countries. These diseases include cardiovascular conditions (particularly acute myocardial infarction), cancer, stroke and chronic obstructive pulmonary disease (COPD). COPD, despite being considered a preventable and treatable condition, has become a major public health problem over the last few decades. Currently, it is among the top 3 leading causes of mortality and morbidity worldwide, with 90% of the deaths occurring in low- and middle-income countries (LMICs) (2).

The most frequent chronic symptoms in COPD include cough, sputum production, dyspnea and/or exacerbations, providing evidence of airflow impairment triggered by an abnormal inflammatory response and structural changes (2). COPD differentially encompasses several clinical phenomena. On one side, chronic bronchitis, mainly characterized by increased mucus secretion causing luminal obstruction of small airways and epithelial remodeling, among others (3). On the other side, emphysema, characterized by alveolar enlargement and alveolar wall destruction (4). Both conditions contribute to the remodeling and narrowing of small airways and destruction of the lung parenchyma. Thus, such structural changes determine that airflow limitation is largely irreversible.

COPD results from a complex not yet completely understood interaction between environmental and genetic factors over the lifetime of the individual. Regarding environmental exposure, smoking has been identified as the major determining factor in high-income countries whereas household air pollutants are considered to have a higher impact in LMICs. Air pollution is another key risk factor with global impact. Regarding the genetic traits several mutations have been described as internal risk factors (1). In fact, its pathophysiology is mainly triggered by chronic exposure to cigarette smoke and/or other irritants or pollutants, which activates a chronic inflammation state characterized by exacerbated recruitment of several types of immune cells, such as neutrophils, macrophages, $CD8^+$ T (Tc1-T-helper 1 cells) and $CD4^+$ T (Th1- type-1 cytotoxic T-cells and Th17) lymphocytes, into the lung parenchyma (5). Consequently, patients with COPD present increased numbers of macrophages in several clinical samples like bronchoalveolar lavage (BAL), cytology and sputum. This condition is attributed to the exacerbated recruitment of circulating monocytes in response to elevated chemokine levels, particularly those of CCL2 and CXCL1 (6). Interestingly, macrophages from these patients release more inflammatory mediators (i.e., IL-1β, IL-6, TNF-α, CXCL1, CXCL8, CCL2, LTB4) and reactive oxygen species (ROS) than those from individuals without COPD (7–9). Moreover, transcription factor STAT-4 is critical for the differentiation of Th1/Tc1 and the production of interferon (IFN)-γ. In this sense, Th1 cells and IFN-γ cytokine are increased in the airways of smokers with COPD (10). On the other hand, the anti-inflammatory cytokine IL-10, produced by Treg cells and macrophages (11), inhibits the synthesis of several pro-inflammatory cytokines that are expressed in COPD. IL-10 levels have been shown to be reduced in the sputum of COPD patients (12).

Therefore, the best pharmacologycal strategies for COPD treatment is to use anti-inflammatory compounds. Unfortunately, most currently available drugs have proven to be ineffective or characterized by unacceptable toxicity (13, 14). In the last 10 years photobiomodulation (PBM) therapy has emerged as a promising approach for the treatment of lung diseases (15). This strategy consists in the use of electromagnetic waves within the spectral range of red to near infrared (660–1,000 nm) to modulate cell functions. The outcomes depend on the administered dose, being able to inhibit or stimulate cell functions (16–20). It is noteworthy that several studies point to an anti-inflammatory role of PBM therapy on pulmonary inflammation by reducing edema, neutrophil influx, TNF-α production and increasing lung IL-10 levels (15, 21, 22). Indeed, in experimental models of COPD, data indicate the augmentation of IL-10 levels together with increased expression of this molecule in the lung after PBM (21).

The aim of this study is to enhance our understanding of PBM therapy at the molecular and cellular level, using a known experimental model of COPD, with the goal of improving management and treatment of this chronic and disabling disease.

2 Material and methods

2.1 Animals

The study was approved by the Ethics Committee on Animal Use (CEUA- AN006/2013) of Universidade Nove de Julho. Sixty healthy male C57Bl/6 mice, ~7 weeks old, weighing an average of 25.0 g, were used for the study. These specimens came from the breeding sector of the animal facility at Universidade Nove de Julho, where they were kept in good health in ventilated racks, with five animals per box, in a room with controlled environmental conditions (temperature: 22 ± 3°C, 12-h light-dark cycle and relative humidity between 30 and 70%).

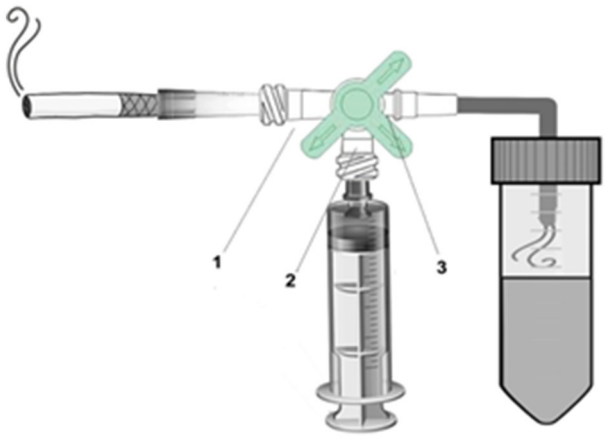

FIGURE 1
Cigarette smoke extract device. 1. Cigarette valve. 2. Syringe valve. 3. Conical tube valve.

2.2 Experimental groups

All mice were placed in a common box and divided randomly into three groups containing ten animals each: (1) control group, which consisted of non-manipulated mice; (2) PMB group, with animals exposed to PBM therapy; (3) COPD group, which consisted of animals who had been orotracheally administered cigarette smoke extract; and (4) COPD + PBM group, mice with induced COPD by cigarette extract orotracheal administration which were exposed to PBM therapy.

2.2.1 Model of COPD induced by cigarette smoke extract

The technique was adapted according to He et al. (23). A 20 mL plastic syringe equipped with a 3-way stopcock and a 50 mL conical tube was used to bubble smoke from 1 cigarette through 4.0 mL of deionized water (Figure 1). For disease induction, 30 μL of cigarette extract were administered orotracheally 3 times a week (Monday, Wednesday, and Friday) for 7 weeks to each animal. In order to allow access to the orotracheal administration route, animals were adequately immobilized after being anesthetized with 2% xylazine (0.06 ml/100 g) + 10% ketamine (0.08 ml/100 g).i.m. All animals belonging to experimental groups 3 (COPD) and 4 (COPD + PBM) were exposed to this protocol. After the last administration with cigarette smoke extract (end of 7 weeks), 24 h later, the animals were euthanized.

2.2.2 Model of PBM-treated mice

On day 35, the animals were irradiated with a diode laser, with a power of 100 mW and a wavelength of 660 nm, irradiating an area of 0.045 cm^2 with an energy density of 3 J. We based on the parameters of the previous study on the effect of PBM in an experimental model of asthma (22). One hour following the cigarette extract orotracheal injection, each mouse in group 4 (COPD + PBM) was given a 60-s direct application at three distinct regions: one underneath the trachea, and the other two in the right and left lung lobes, for a total of 180 s of exposure per mouse, once a day. Animal hair in this region was not removed. PBM was used for the full 7 weeks.

2.3 Assessment of pulmonary inflammation in bronchoalveolar lavage

Following the experiments, all groups were euthanized by giving them xylazine (10 mg/kg i.p.) and ketamine (100 mg/kg i.p.). They were also exsanguinated for blood collection, had their tracheas cannulated, had their tracheostomies, and their lungs were cleaned with 3 × 0.5 ml of phosphate buffered saline (PBS) after the extraction (24). For 5 min, the recovered lavage volume was centrifuged at 1,600 rpm and 4°C. The supernatant was kept at −70°C in preparation for ELISA cytokine analysis. The cell button was reconstituted in 1 milliliter of phosphate-buffered saline (PBS) and utilized in the production of a cytospin slide (Thermo Scientific), stained with Instant Prov (Newprov), and BAL cell determination using a Neubauer chamber (total cells). Differential cells were to do slides in cytospin and stained with Instant Prov.

2.4 Evaluation of cytokines, inflammatory mediators and chemokines in BAL

BAL sample collection was carried out as abovementioned. The levels of IL-1β, IL-6, KC/CXCL1, IFN-γ, TNFα, IL-10, GM-CSF, MCP-1, and LTB4 were evaluated in the BAL supernatant by ELISA, using the Biolegends and R&D kit Systems. A SpectraMax i3 (Molecular Devices) spectrophotometer with an adjusted absorbance of 450 nm was used for plate reading. The threshold for ELISA tests were: IL-1β is 31.3–2,000 pg/mL, TNF-α is 7.8–500 pg/mL, IL-6 is 7.8–500 pg/mL, IFN-γ is 31.3–2,000 pg/mL, MCP-1/CCL2 is 7.8–500 pg/mL, GM-CSF is 7.8–500 pg/mL, KC/CXCL1 is 15.6–1,000 pg/mL, LTB_4 is 10.3–2,500 pg/mL, and IL-10 is 31.3–2,000 pg/mL.

2.5 Flow cytometry

Following lung extractions, lung tissue was broken up into tiny fragments and incubated for 30 min at 37°C with constant stirring in 2 mg/ml collagenase IV and 1 mg/ml deoxyribonuclease I (DNAse) (Sigma). Following this time frame, we introduced Hank's balanced solution (HBSS) together with EDTA to decelerate the material's digestion. After massing and filtering the lung fragments through a 40-mm sieve, the contents were centrifuged for 10 min at 1,500 rpm, and the pellets were then resuspended in PBS buffer. Following the incubation period, the samples were resuspended in 200 μl of the same buffer after being cleaned with PBS containing 0.01% BSA and sodium azide. The samples were acquired in the BD Accuri flow cytometer and analyzed in the CSampler software (Becton Dickinson—BD®, East Rutherford, NJ, USA).

Treg cell phenotyping was carried out using anti-CD4 FITC and anti-CD25 PE, as well as the transcription factor [anti-Foxp3

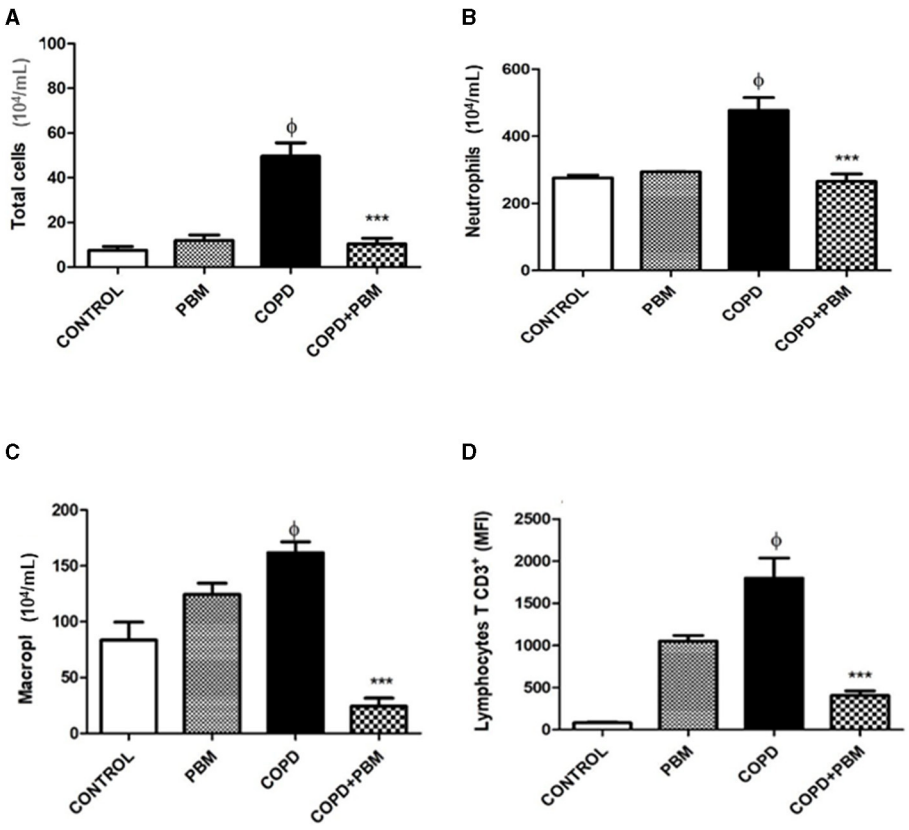

FIGURE 2
Effect of PBM with no total number of cells **(A)** and no number of neutrophils **(B)**, macrophages **(C)**, and lymphocytes **(D)** on BALF. The results refer to the use of 10 mice in each experimental group and five mice for control group. After the last administration with cigarette smoke extract (end of 7 weeks), 24 h later, the animals were euthanized. Values expressed as mean and standard deviation. $^{\phi}p < 0.001$ when compared to the control group; $^{***}p < 0.001$ when compared to the COPD group.

Percp], and IL-10 (anti-IL-10 APC) (Becton Dickinson—BD®, East Rutherford, NJ, USA) was characterized. The cells were incubated at 4°C for 20 min. Following the incubation period, the samples were resuspended in 200 ml of the same buffer after being cleaned with PBS containing 0.01% BSA and sodium azide. The BD Accuri flow cytometer was used to collect the samples, and the CSampler program (Becton Dickinson—BD®, East Rutherford, NJ, USA) was used for analysis.

The acquired cells were treated for 30 min at 4°C with a 1:100 concentration of eBioscienc® anti-CD16/32 monoclonal antibody to inhibit Fc receptors in order to assess the expression of surface molecules. The cells were then treated for 30 min at 4°C with fluorochrome conjugated monoclonal antibodies (FITC, PE, PercP, or APC) specific for the compounds of interest. The following Biolegend® monoclonal antibodies were used: 0.5 mg/106 cells of anti-CD3, 0.5 mg/106 cells of anti-CD4, 0.5 mg/106 cells of anti-CD25, 0.5 mg/106 cells of anti-Foxp3, and 0.5 mg/106 cells of anti-IL10. A BD Accuri flow cytometer (Becton Dickinson—BD®, East Rutherford, NJ, USA) was used to collect the samples. Following that, CD3+/CD4+ and CD3+/CD8+ lymphocyte populations were gated separately. Following that, STAT4+ and IFN-γ+ were gated in CD4+cells, and this population also included CD25+Foxp3+ and IL-10+. Each marker's mean fluorescence intensity (MFI) was measured and utilized in a statistical analysis. Ten mice were used in each experimental group for the sake of the results. Consequently, each sample yielded 20,000 occurrences. For the animals in the Control, COPD, and COPD + PBM groups, representative MFI histograms were acquired. One animal from each category is represented by the data.

2.6 Histology assessment of airway inflammation and remodeling and mucus production

In order to evaluate how different treatments affected the amount of collagen fibers in the airway wall, left lungs were removed, preserved in 10% formalin, and then embedded in paraffin. Sections of 4 μm thickness were made.

Collagen fibers were detected on slides stained with Picrosirius for the assessment of airway remodeling and inflammation. Vieira et al. (24) described a morphometric technique that was used for quantitative analysis. The NIH, Maryland, USA, Image Pro Plus software (version 4.5) was used to evaluate morphological characteristics. Every animal's five airways were examined.

Periodic acid Schiff stain was used on slides to measure mucus production. The morphometric method was followed

to achieve quantification (24). With the use of Image Pro Plus software (version 4.5, NIH, Maryland, USA), morphological parameters were assessed. Each animal's five full airways were measured at a magnification of 1,000 ×. The bronchial epithelium's area of interest (mm^2) was first determined, and the mucus area (mm^2) was then computed:

Therefore, the unit of mucus quantification in the airways is in mm^2/mm^2.

2.7 Measurement of the mean linear intercept (Lm)

The mean linear intercept (Lm) is an index used to determine emphysema by measuring the mean diameter of the distal airspaces. Fifteen fields were counted per slide at 200X magnification. Using the reticulum superimposed on the lung parenchyma in the most peripheral regions of the parenchyma the number of times the intercepts crossed the alveolar walls were counted. The fifteen fields for each slide were averaged and the Lm was calculated using the following equation: Lm = 2,500 μm/average of the number of times the intercepts crossed the alveolar walls. The value of 2,500 μm was determined by measuring the used reticle with ruler manufactured by Zeiss. The size of each line segment was measured using the ruler under a microscope with the reticle at 200X magnification (25). The sum of all the reticulum segments resulted in the value 2,500 μm.

2.8 Statistical analysis

Data were analyzed using GraphPad Prism 5.1 software (California, USA). The normality of data distribution was assessed using the Kolmogorov-Smirnov test. Data with parametric distribution were submitted to the One-way ANOVA test, followed by the Newman-Keuls test for group comparison. Data with non-parametric distribution were submitted to the One-way ANOVA on Ranks test, followed by the Dunn's test for group comparison. Graphs were generated using the GraphPad Prism 5.1 software (California, USA).

3 Results

3.1 Quantification of cells present in BAL

The results obtained from the quantification of cells in BAL samples are represented in Figure 2. We found a significant increase in the total number of cells (A), neutrophils (B), macrophages (C) and CD3+ T lymphocytes (D) in the COPD group when compared to the control group. Contrarily, when comparing the COPD group exposed to PBM therapy (COPD + PBM) with the COPD group, we observed a significant decrease in all cell types in the former group.

3.2 Quantification of cytokines, inflammatory mediators and chemokines in BALF supernatant

The supernatants obtained from BAL samples were used for quantification of several immune-related molecules. The results obtained are shown in Figure 3. Three different patterns may be considered. The first pattern is described regarding the levels of IL-1β, TNF-α, IL-6, IFN-γ, MCP-1/CCL2, GM-CSF, and KC/CXCL1 for which we noted a consistent and significant increase of ∼2-fold in the COPD group in comparison with the control group; only in the case of KC/CXCL1 a lower although still significant increase of ∼1.4-fold was observed. Moreover, in all these cases, BPM exposure of healthy mice did not lead to any significant change. In the COPD + BPM group, a significant reduction in all these molecules was observed, almost reaching control levels.

The second pattern refers to the LTB4 levels. An approximate 2-fold increase observed for the COPD-group in comparison to control animals was followed by a small but significant reduction in the COPD + BPM mice. However, we did observe that BPM exposure in healthy mice lead to LTB4 partially increased levels.

Finally, the third and contrary pattern was observed for IL-10: there was a reduction in the levels of this anti-inflammatory cytokine in the COPD group when compared with the control group, followed by a partial reversion toward basal levels. As observed for LTB4, healthy PBM-treated mice showed intermediate values.

3.3 Analyze of airways mucus and collagen deposition

Regarding the production of mucus, we noticed a significant increase in the COPD group when compared to the control group. In the COPD+PBM there was a significant reduction of mucus production when compared to the COPD group (Figures 4A, B).

The collagen deposition also showed an increase in the COPD group compared to the control group and a significant reduction in COPD + PBM group when compared to COPD (Figures 5A, B).

3.4 Emphysema determination

Alveolar enlargement data showed a significant increase in the COPD group when compared to the control group. Contrarily, a significant reduction was detected in the COPD + PBM group (Figure 6) compared to the COPD group.

The bronchoconstriction index showed an increase in the COPD group compared to the control group and a significant reduction in the COPD + PBM group (Figure 6).

3.5 Quantification of CD4+ and CD8+ T lymphocytes in BAL

The results of the quantification of CD4+ (A) and CD8+ (B) lymphocytes in BAL demonstrated a significant

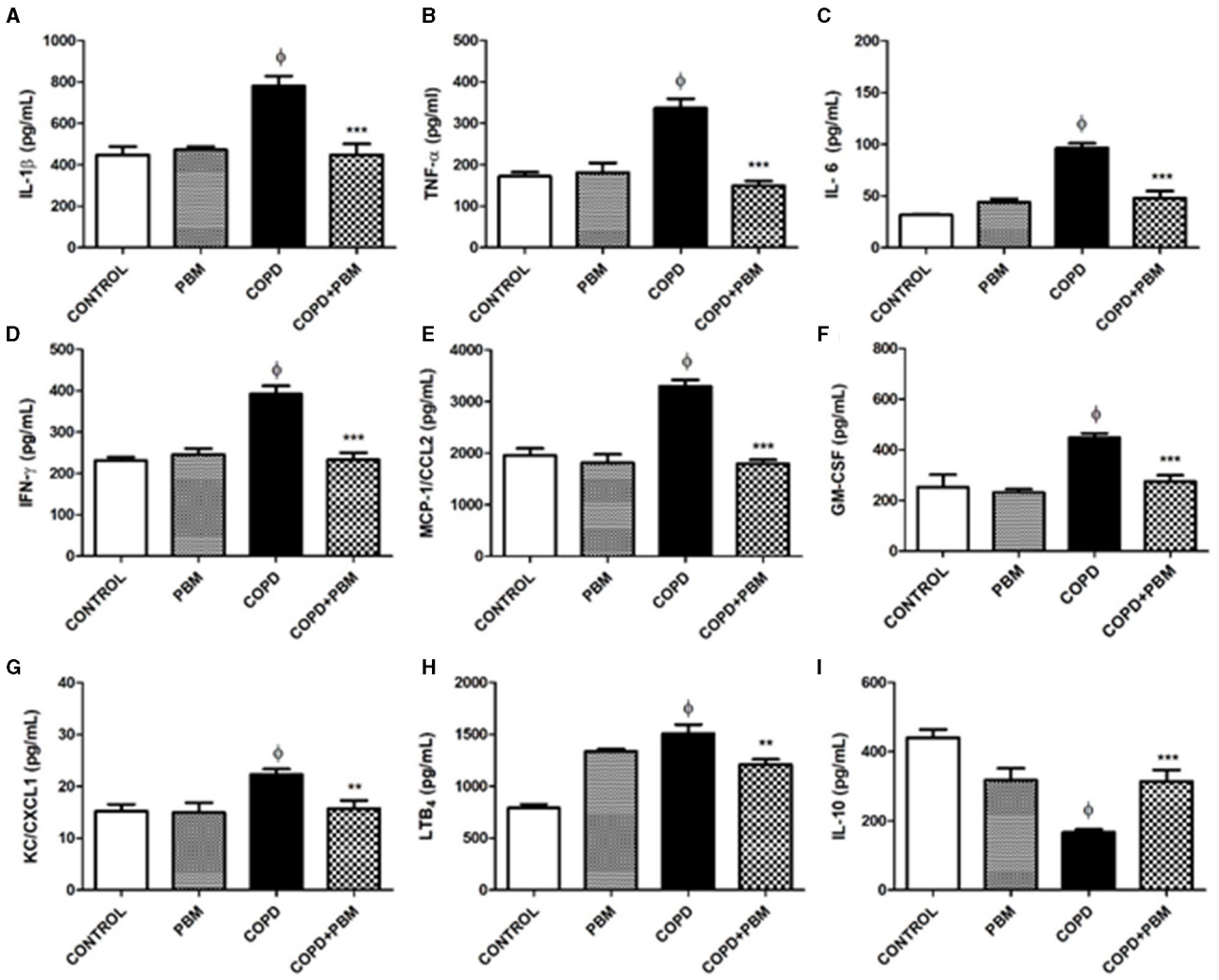

FIGURE 3
Effect of PBM on IL-1α **(A)**, TNF-β **(B)**, IL-6 **(C)**, IFN-γ **(D)**, MCP-1 **(E)**, GM-CSF **(F)**, KC/CXCL1 **(G)**, LTB4 **(H)**, and IL-10 **(I)** levels in BALF supernatant. The results refer to the use of 10 mice in each experimental group and five mice for control group. Values expressed as mean and standard deviation. $^{\phi}p < 0.001$ when compared to the control group; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ when compared to the COPD group.

increase in the COPD group compared to the control group (Figure 7). On the other hand, the COPD+PBM group had a reduction in CD4+ and CD8+ when compared to the COPD group.

3.6 Quantification of CD4+STAT-4+ and CD4+STAT-4+IFN-γ+ T lymphocytes in BAL

The results of CD4+STAT-4+ lymphocytes (Figure 8A) and CD4+IFN-γ+ (Figure 8B) in BAL showed a significant increase in the COPD group compared to the Basal group and a significant reduction in the PBM treated group. The histogram represents all groups evaluated (Figures 8C, D).

3.7 Quantification of CD4+CD25+Foxp3+ and CD4+CD25+IL10+ T lymphocytes in BAL

Data regarding the quantification of CD4+CD25+Foxp3+ (Figure 9A) and CD4+CD25+IL10+ (Figure 9B) T lymphocytes in BAL showed a significant reduction in the COPD group compared to the basal group and an increase in the PBM treated group. The histogram represents all groups evaluated (Figures 9C, D).

4 Discussion

The results obtained in the present study demonstrate that treatment with PBM irradiation significantly reduces chronic symptoms which typically emerge in a mouse COPD model

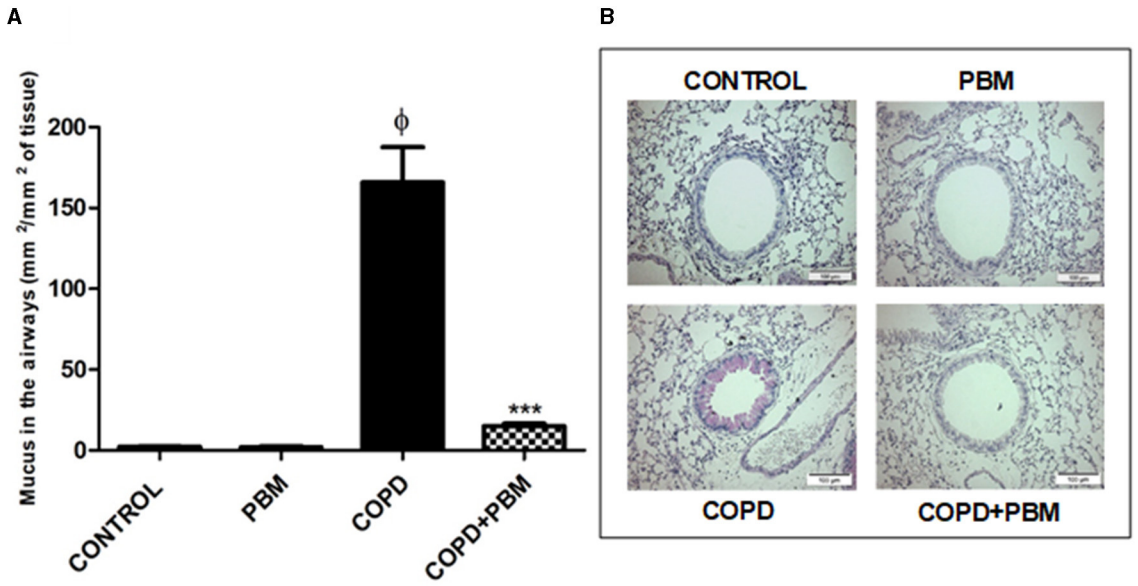

FIGURE 4
Effect of PBM on the production of mucus in the airways. **(A)**The results refer to the use of 10 mice in each experimental group and five mice for control group. Values expressed as mean and standard deviation. $^{\phi}p < 0.001$ when compared to the control group; $^{***}p < 0.001$ when compared to the COPD group. **(B)** The photomicrographs (x 200) represent all the evaluated groups.

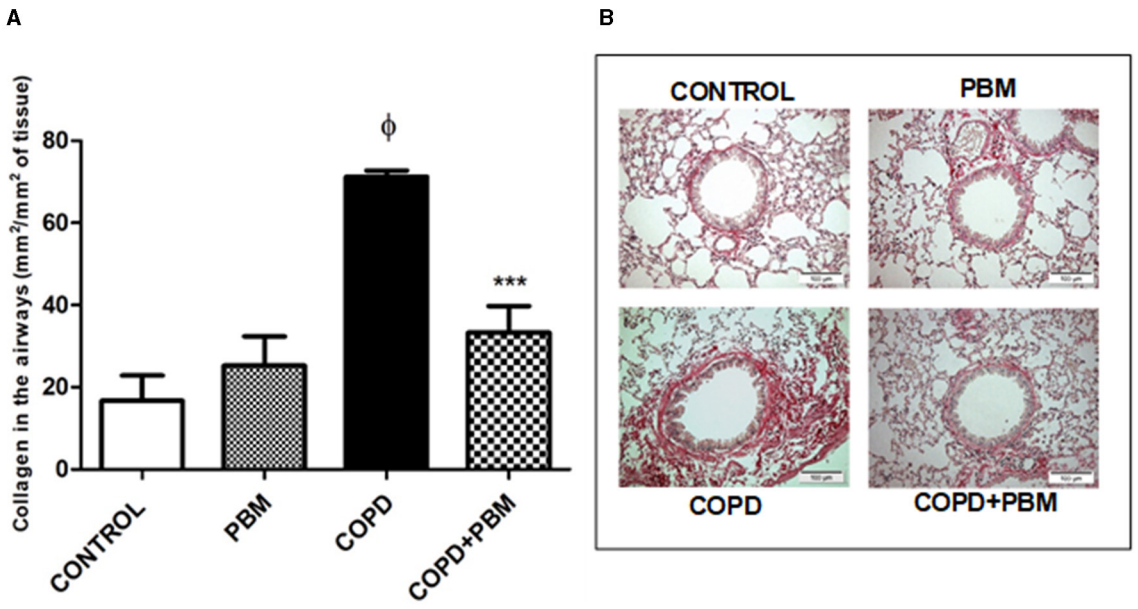

FIGURE 5
Effect of PBM on the collagen deposition in the airways. **(A)** The results refer to the use of 10 mice in each experimental group and five mice for control group. Values expressed as mean and standard deviation. $^{\phi}p < 0.001$ when compared to the control group; $^{***}p < 0.001$ when compared to the COPD group. **(B)** The photomicrographs (x 200) represent all the evaluated groups.

induced by orotracheal administration of cigarette smoke extract. The respiratory impairment observed in COPD affected organisms mainly include small-airway remodeling and narrowing as well as pulmonary emphysema. The latter condition refers to the observed pulmonary tissue destruction, particularly at the alveolar level (4, 26). Many of the parameters evaluated in this study, such as cellularity, secretion of pro-inflammatory cytokines, expression of the inflammatory transcription factor STAT4, were significantly reduced in BAL samples collected from COPD mice treated with PBM in comparison with the control and healthy group. On the other hand, the anti-inflammatory transcription factor Foxp3 as well as IL-10 levels were locally increased. This profile was associated with better maintenance of tissue integrity when compared to untreated COPD animals, evidenced by reduced mucus secretion, collagen deposition, and tissue damage. It is worth mentioning that

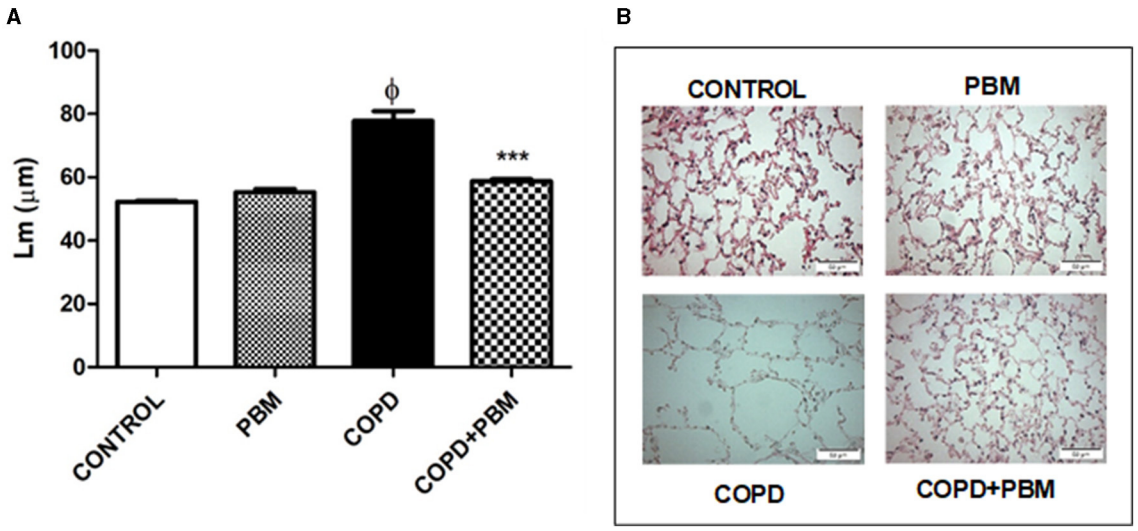

FIGURE 6
Effect of PBM on the alveolar enlargement in the airways. **(A)** The results refer to the use of 10 mice in each experimental group and five mice for control group. Values expressed as mean and standard deviation. $^{\phi}p < 0.001$ when compared to the control group; $^{***}p < 0.001$ when compared to the COPD group. **(B)** The photomicrographs (x 200) represent all the evaluated groups.

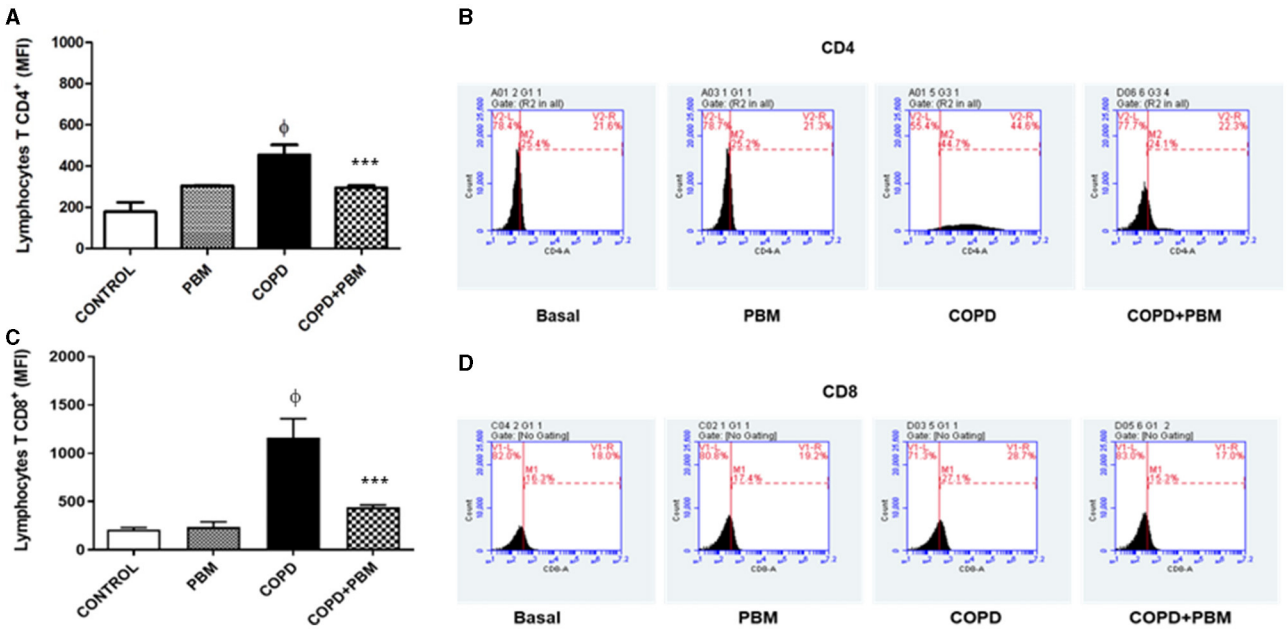

FIGURE 7
Effect of PBM therapy on lymphocytes T CD4+ **(A)** and lymphocytes T CD8+ **(C)** levels in BALF. The results refer to the use of 10 mice in each experimental group and 5 mice for control group. Values expressed as mean and standard deviation. The histograms represent all groups evaluated **(B, D)**. Values expressed as mean and standard deviation. $^{\phi}p < 0.001$ when compared to the control group; $^{***}p < 0.001$ when compared to the COPD group.

these characteristics have been proven to be associated with the mentioned tissue destruction and further decline in the patient's quality of life (26). In this regard, billions of dollars are spent each year on treating emphysematous patients around the world. Therefore, the need for a more efficient and cheaper therapeutic approach in the management of patients with COPD is of paramount importance.

Previous data have shown the benefits of a PBM therapy used in the mouse model of experimental COPD induced by cigarette smoke. One study showed that PBM therapy was able to reduce the levels of inflammation, airway remodeling and pulmonary emphysema, with the participation of the purinergic pathway (21). Another study previously carried out used PBM together with mesenchymal stem cells derived from human fallopian

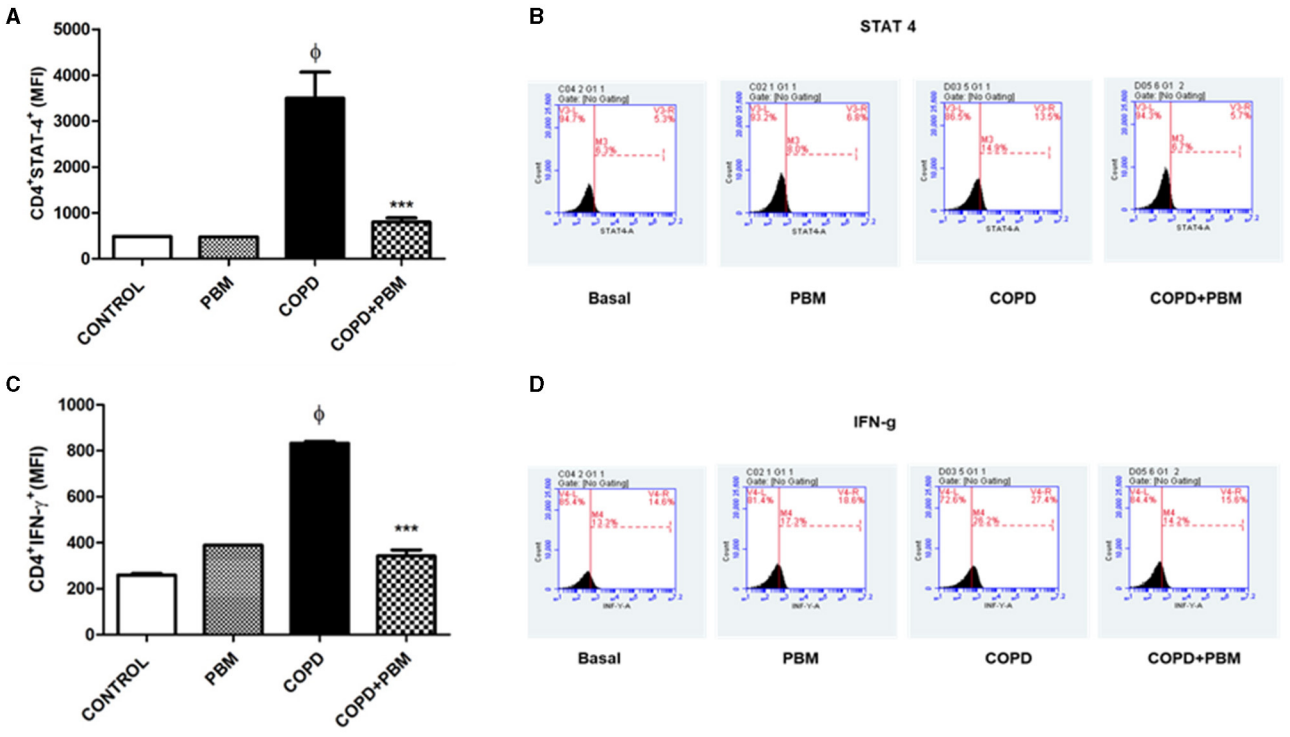

FIGURE 8
Effect of PBM therapy on CD4+IFN-y + **(A)** and CD4+STAT-4 +**(C)** levels in BALF. The results refer to the use of 10 mice in each experimental group and five mice for control group. Values expressed as mean and standard deviation. The histograms represent all groups evaluated **(B, D)**. Values expressed as mean and standard deviation. $^{\phi}p < 0.001$ when compared to the control group; $^{***}p < 0.001$ when compared to the COPD group.

tubes. There was a reduction in both lung inflammation and alveolar enlargement in sick animals treated with PBM. Many of the parameters evaluated, such as cellularity, secretion of proinflammatory cytokines, perivascular infiltrate and expression of transcription factors such as NF-KB were significantly reduced. Both treatments, PBM in combination with mesenchymal stem cells or PBM alone, were shown to maintain the integrity of the lung tissue, reduce collagen deposition as well as the peribronchial infiltrate (27).

In this context, we evaluated the presence of inflammatory cells in BAL samples. Overall, the data obtained indicated an increase in the number of macrophages, neutrophils and lymphocytes in the samples collected from animals with COPD. Diverse studies conducted elsewhere have also evidenced increased levels of all these cell types and others in COPD patients, highlighting that increased numbers of neutrophils and B lymphocytes are detected in the most severe cases. Moreover, macrophages are of great importance in this pathogenesis and are increased not only in individuals with COPD but also in smokers (4, 5).

Our results corroborate previous findings that demonstrate a reduction in the expression of STAT4, as well as in the concentrations of IL-8 and LTB4 in the lung, which are primarily responsible for attracting neutrophils (23). This suggests that the anti-inflammatory effect of laser irradiation on these mediators, and consequently the reduction in cell migration to the lung, could be attributed to the decreased expression of STAT4 and the inhibition of IL-8 and LTB4.

Exposure to cigarette smoke initially leads to a cellular infiltrate of blood-derived neutrophils and monocytes secreting pro-inflammatory and pro-fibrotic cytokines, such as IL-1β, IL-6, IL-12, TNF-α, and TGF-β, together with chemokines, lipid mediators and several other molecules. This scenario is accompanied by degradation of the extracellular matrix by MMP-1 (matrix metalloproteinase-1), secreted by alveolar macrophages, causing tissue destruction and emphysema (28). Chronic exposure, on the other hand, leads to an intense infiltrate of CD4 and CD8 T lymphocytes, and IFN-γ is probably the most important cytokine derived from T cells. We noticed that, in animals with COPD induced by cigarette smoke extract, INF-γ-producing Th1 cells, as well as those expressing STAT4, were increased, whereas Treg cells ($CD4^{+}CD25^{+}Foxp3^{+}$) were decreased.

Furthermore, granulocyte macrophage colony-stimulating factor (GM-CSF) has been connected to COPD and is involved in the differentiation and survival of neutrophils, eosinophils, and macrophages (29). In reaction to inflammatory stimuli, macrophages, epithelial cells, and T lymphocytes release GM-CSF primarily. Additionally, patients with COPD secrete GM-CSF from their alveolar macrophages, which may be crucial for improving neutrophil and macrophage survival in the airways (29). Increased neutrophil counts in the lung are correlated with elevated GM-CSF concentrations in the BAL of COPD patients, particularly during exacerbations.

In this study, we observed a significant increase in the number of CD4+ T lymphocytes in the COPD groups. Conversely, the

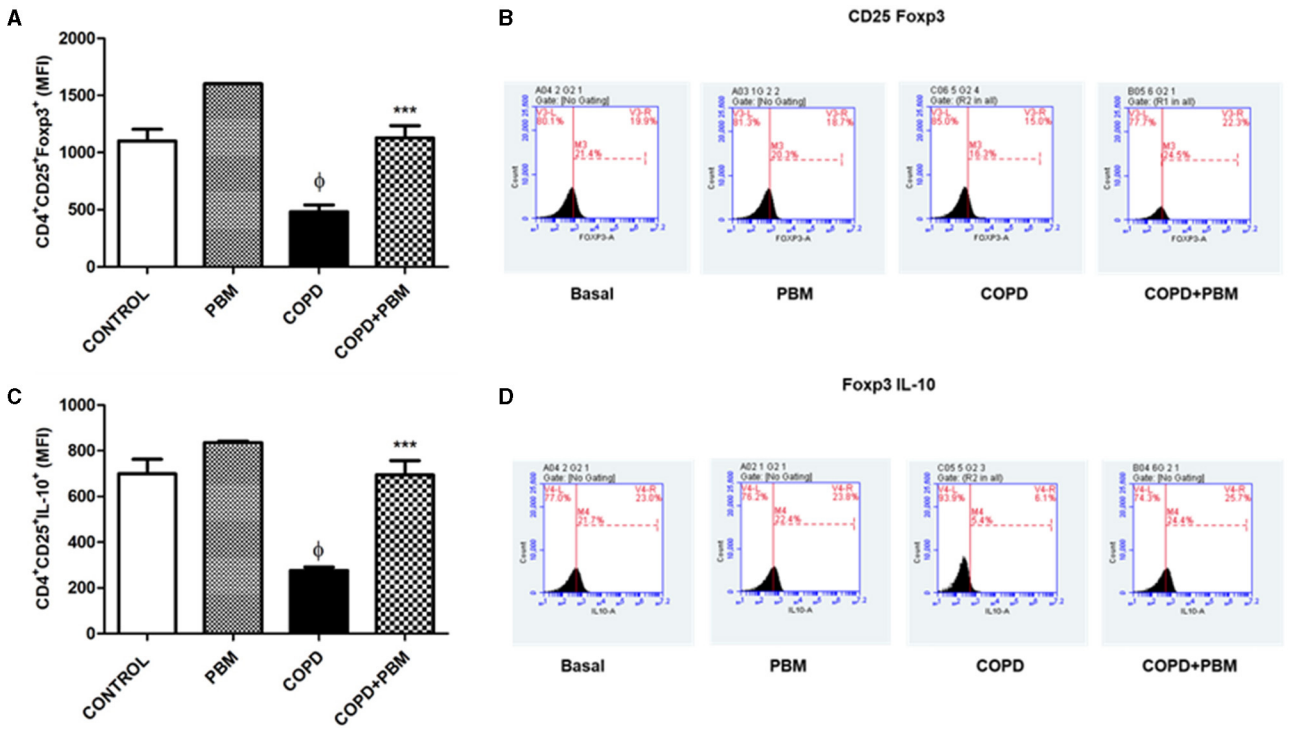

FIGURE 9
Effect of PBM therapy on CD4+CD25+FOXP3+ **(A)** and CD4+CD25+ IL-10+ **(C)** levels in BALF. The results refer to the use of 10 mice in each experimental group and five mice for control group. Values expressed as mean and standard deviation. The histograms represent all groups evaluated **(B, D)**. Values expressed as mean and standard deviation. $^{\phi}p < 0.001$ when compared to the control group; $^{***}p < 0.001$ when compared to the COPD group.

treated groups exhibited a decrease in CD4+ lymphocytes across all treatment cohorts. Other studies have reported that functional Treg cells suppressed the proliferation of activated CD4+ T cells (30), and their generation is primarily induced by the presence of TGF-β and IL-10 (31, 32). IL-10 inhibits the synthesis of numerous inflammatory proteins, including TNF-α, IL-1β, GM-CSF, chemokines, and metalloproteinases like MMP-9, which are highly expressed in COPD. Concentrations of IL-10 are reduced in the sputum of COPD patients (29).

In this context, therapeutic approaches capable of reducing this activation, could significantly contribute to maintaining pulmonary integrity. These findings suggest that PBM increased IL-10 levels produced by Treg cells in the treated group, thereby suppressing the proliferation of CD4+ T effector cells. Moreover, prior studies have indicated an inverse correlation between CD8+ T cells and lung function (33). Smoking directly disrupts the balance of the pro-/anti-inflammatory response by inhibiting CD8+ Treg cells (33). Nonetheless, further analysis of these cells is required to determine their regulatory functions or any potential relationship between cytotoxic CD8+ T cells and IL-10 production.

After analyzing these results, we propose that photobiomodulation holds the potential to reduce disease severity by upregulating IL-10 release. Several studies indicate a potential mechanism for the modulatory effects of PBM in the lungs through the release of IL-10 (34–36). Therefore, we urge other research groups to delve into the potential of laser therapy, given its promising outcomes in COPD. This underscores the necessity for further experiments, such as quantifying MMP-12 and MMP-9, characterizing the types of collagen present in lung parenchyma, and analyzing levels of IL-17, PGE2, TGF-β, ATP, and oxidative stress. Additionally, verifying the profile of regulatory CD8+ T cells is crucial, given their significant role in the pathophysiology of COPD. Furthermore, treatment given to the microbial component of COPD has not been evaluated, additional studies need to be carried out to discuss this aspect of the disease.

5 Conclusion

Our findings demonstrate that photobiomodulation therapy effectively reduces cell migration to the lung, levels of cytokines and chemokines, and alveolar enlargement. This reduction could potentially be attributed to the increased population of regulatory T cells (CD4+CD25+Foxp3+) producing IL-10 within the lung. Under this results, we suggest that these cells act by suppressing effector T cells (CD4+STAT-4+), known for producing IFN-γ. The significance of photobiomodulation therapy lies in its potential to regulate inflammation and treat pulmonary emphysema in individuals with COPD.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by the animal study was reviewed and approved by Nove de Julho University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AB: Methodology, Conceptualization, Formal analysis, Writing – original draft. KH: Methodology, Writing – review & editing. CA-N: Writing – review & editing. CE-A: Writing – review & editing. CD: Writing – review & editing. RM: Writing – review & editing, Supervision. JS: Writing – review & editing. MC: Writing – review & editing, Resources. SZ: Writing – review & editing, Data curation. FA: Writing – review & editing, Formal analysis, Methodology, Supervision. LL-P: Writing – review & editing, Writing – original draft. AG: Writing – review & editing. AF: Writing – review & editing. RP: Methodology, Writing – review & editing. AL: Conceptualization, Data curation, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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