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The potential lipid biomarker 5-HETE for acute exacerbation identified by metabolomics in patients with idiopathic pulmonary fibrosis

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Abstract

Background and Objective: Acute exacerbation (AE) is often the fatal complication of idiopathic pulmonary fibrosis (IPF). Emerging evidence indicates that metabolic reprogramming and dysregulation of lipid metabolism are distinctive characteristics of IPF. However, the lipid metabolic mechanisms that underlie the pathophysiology of AE-IPF remain elusive.

Methods: Serum samples for pilot study were collected from 34 Controls, 37 stable IPF (S-IPF) cases and 41 AE-IPF patients. UHPLC–MS/MS was utilized to investigate metabolic variations and identify lipid biomarkers in serum. ELISA, quantitative PCR and western blot were employed to validate the identified biomarkers.

Results: There were 32 lipid metabolites and 5 lipid metabolism pathways enriched in all IPF patients compared to Controls. In AE-IPF versus S-IPF, 19 lipid metabolites and 12 pathways were identified, with 5-hydroxyeicosatetraenoic Acid (5-HETE) significantly elevated in AE-IPF. Both in internal and external validation cohorts, the serum levels of 5-HETE were significantly elevated in AE-IPF patients compared to S-IPF subjects. Consequently, the indicators related to 5-HETE in lipid metabolic pathway were significantly changed in AE-IPF patients compared with S-IPF cases in the lung tissues. The serum level of 5-HETE was significantly correlated with the disease severity (CT score and PaO_2/FiO_2 ratio) and survival time. Importantly, the receiver operating characteristic (ROC) curve, Kaplan–Meier analysis and Multivariate Cox regression analysis demonstrated that 5-HETE represents a promising lipid biomarker for the diagnosis and prognosis of AE-IPF.

Conclusion: Our study highlights lipid reprogramming as a novel therapeutic approach for IPF, and 5-HETE may be a potential biomarker of AE-IPF patients.

KEYWORDS
5-Hydroxyeicosatetraenoic acid (5-HETE), acute exacerbation (AE), idiopathic pulmonary fibrosis (IPF), lipid metabolism

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic, irreversible interstitial pulmonary disease of unknown aetiology, characterized by gradual scarring of the lung parenchyma, leading to impaired lung function and respiratory failure.[1] Currently, there is a paucity of knowledge regarding the pathophysiology underlying IPF, which poses a significant obstacle to the development of effective treatments.[2] Despite the conduct of numerous clinical trials, the therapeutic

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options available for IPF remain limited, with only two antifibrotic agents having received approval for use.[3] It is noteworthy that a minority of patients experience acute exacerbations of IPF (AE-IPF), characterized by episodes of rapid clinical respiratory deterioration, new radiographic opacities on chest imaging and the absence of an identifiable aetiology.[4] AE-IPF is difficult to predict or prevent and precedes approximately half of all IPF-related deaths, with a mean survival time of 3–4 months.[5,6] Numerous factors have been associated with the risk of AE-IPF, such as pulmonary hypertension, gastroesophageal reflux, emphysema and extended mechanical ventilation.[7–9] Nonetheless, there is a lack of established international guidelines for AE-IPF, and the underlying pathophysiology of AE-IPF remains incompletely comprehended.[10] Therefore, the identification of promising biomarkers for AE-IPF patient stratification is urgently needed.

Evidence indicates that the dysregulated lipid metabolism plays a contributory role in the pathogenesis and subsequent progression of IPF.[11] Lipids constitute a broad class of molecules, encompassing fatty acids, phospholipids, sterols and lipoproteins, each of which performs specific functions within the cell.[12,13] Elevated concentrations of fatty acids, coupled with significantly reduced levels of carnitine, have been identified in the lungs of patients with IPF.[14] This leads to lipid accumulation and downregulation of β-oxidation in alveolar macrophages.[14] Moreover, the concentration of sphingosine 1-phosphate (S1P), lysophosphatidic acid (LPA) has been observed to elevate in serum or bronchoalveolar lavage (BAL) samples of patients with IPF.[15–17] However, the plasma lipid profiles of patients with IPF, especially those with AE, remain inadequately characterized.

5-hydroxyeicosatetraenoic acid (5-HETE) is a metabolite derived from arachidonic acid through the lipoxygenase pathway.[18] The selective synthesis of 5-HETE is reliant upon the activity of its synthesizing enzyme, LOX5.[18] LOX5 is predominantly expressed in cells involved in innate immune responses, inflammation and allergy such as neutrophils, eosinophils, B lymphocytes, monocytes, macrophages and mast cells, while it is also expressed in other cells albeit at lower levels.[19] The excessive production of 5-HETE increases the expression of VEGF and LTB4, thereby implicating in various pathological processes, including inflammation, vascular biology and cancer progression.[20–25] Nevertheless, the precise contribution of 5-HETE to the pathogenesis and progression of pulmonary fibrosis in humans remains elusive. In this study, we utilized UHPLC–MS/MS to identify lipid metabolic profiles in individuals with AE-IPF, stable IPF (S-IPF) and Controls, and explored the potential of the characteristic metabolite 5-HETE as a biomarker for the diagnosis and prognosis of AE-IPF.

SUMMARY AT A GLANCE

This study highlights the critical role of lipid metabolism in AE-IPF. The identification of 5-HETE as a promising lipid biomarker in AE-IPF patients may provide new insights into the disease's pathophysiology, offering significant potential for improved diagnosis, prognosis and therapeutic strategies for AE-IPF.

METHODS

Subjects

For pilot study cohort, the serum samples of Controls ($n = 34$), patients with S-IPF ($n = 37$) and AE-IPF ($n = 41$) were obtained from the Nanjing Drum Tower Hospital (Nanjing, China) from January 2016 to December 2018; for more information on the outpatient visit volume at Nanjing Drum Tower Hospital, see Appendix S2 in the Supporting Information. For internal and external validation cohorts, serum samples were obtained from Controls ($n = 25$ and $n = 20$, respectively), patients with S-IPF ($n = 28$ and $n = 30$, respectively) and AE-IPF ($n = 26$ and $= 27$, respectively), at Nanjing Drum Tower Hospital, during the two separate time periods: January 2016 to December 2018 and January 2018 to December 2020, respectively. Serum samples from all patients with AE-IPF were collected at the onset of their first acute exacerbation event. The vital status was determined by conducting a telephone follow-up or reviewing medical records. The deadline of follow-up was November 20, 2019 in internal validation cohort and October 31, 2021 in the external validation cohort, respectively. The survival duration was defined as the time interval between the date of sample collection and the date of either death or confirmation of vital status. Lung tissue specimens of Controls (normal lung tissue adjacent to cancer), S-IPF and AE-IPF were collected through surgical lung biopsy or lung transplantation from Nanjing Drum Tower Hospital.

Statistical analysis

Categorical variables were presented numerically and as percentages, while continuous variables were presented as mean ± SD. Categorical parameters were compared using the chi-square (χ^2) or Fisher's exact test. Continuous variables were assessed with the independent samples t-test, Kruskal–Wallis test, Mann–Whitney U test, or one-way ANOVA. Correlations between 5-HETE and clinical variables were evaluated using Spearman's or Pearson's correlation, depending on variable distribution. Survival curves were constructed using Kaplan–Meier methods and compared with the log-rank test.

The other relevant materials and methods were provided in Appendix S1 in the Supporting Information.

RESULTS

Clinical characteristics of study cohort

The pilot study cohort consisted of 112 participants, including 34 Controls, 41 patients with AE-IPF and 37 patients with S-IPF. The clinical characteristics of these participants are shown in Table 1. The white blood cell (WBC) count and C-reactive protein (CRP) level was significantly higher in both the AE-IPF and S-IPF groups compared to the Control group. In addition, patients with AE-IPF had a decreased PaO_2/FiO_2 ratio compared to those with S-IPF. Simultaneously, the HRCT scores of AE-IPF patients were significantly elevated in comparison to those of S-IPF patients. Notably, significantly lower levels of the crucial lipid metabolism molecule high-density lipoprotein (HDL) and Apo AI were observed in both the AE-IPF and S-IPF groups in comparison to the Control group. Similar results were found for albumin (ALB) levels in the AE-IPF, S-IPF and Control groups.

In both internal and external validation cohorts, clinical characteristics significantly matched those of the pilot study. Compared to the S-IPF group, the AE-IPF group demonstrated significantly elevated HRCT scores and markedly lower PaO_2/FiO_2 ratios in both validation cohorts. Laboratory analyses in validation cohorts revealed differences between the S-IPF and AE-IPF groups in WBC, Apo AI and ALB, consistent with the findings from the pilot study. In internal validation, differences were observed in the number of patients receiving antifibrotic therapy for over 3 months between the S-IPF and AE-IPF groups. Furthermore, the AE-IPF group showed significantly higher prior corticosteroid usage rate compared to the S-IPF group in both validation cohorts. Detailed clinical characteristics can be seen in Table 2.

TABLE 1 Baseline clinical characteristics of Control, S-IPF and AE-IPF in pilot study cohort.

	Control	S-IPF	AE-IPF	*p* value
Gender (M/F)	25/9	30/7	31/10	0.735
Age (years)	66.53 ± 7.53	68.7 ± 6.97	69.24 ± 6.88	0.270
Smoking (Y/N)	—	18/19	21/20	0.820
WBC ($*10^9$)	6.40 ± 1.63	6.48 ± 1.83	10.20 ± 3.74	**<0.001**
CRP (mg/L)	0.98 ± 1.44	3.83 ± 2.30	47.68 ± 74.58	**<0.001**
TG (mmol/L)	1.22 ± 0.66	1.65 ± 1.37	1.14 ± 0.48	**0.040**
TC (mmol/L)	4.50 ± 0.65	4.46 ± 0.81	4.38 ± 1.01	0.826
LDL (mmol/L)	2.59 ± 0.48	2.42 ± 0.93	2.91 ± 1.04	0.107
HDL (mmol/L)	1.32 ± 0.29	1.28 ± 0.73	1.04 ± 0.33	**0.030**
Apo B (g/L)	0.88 ± 0.16	0.85 ± 0.17	0.94 ± 0.21	0.153
Apo AI (g/L)	1.34 ± 0.23	1.15 ± 0.32	0.97 ± 0.30	**<0.001**
ALB (g/L)	43.89 ± 1.45	39.87 ± 4.52	33.36 ± 7.70	**<0.001**
GLU (mmol/L)	5.24 ± 0.54	5.19 ± 1.33	6.86 ± 2.80	**<0.001**
HRCT scores	—	3.32 ± 1.25	6.76 ± 1.14	**<0.001**
PaO_2/FiO_2 ratio	—	359.2 ± 53.80	192.6 ± 91.78	**<0.001**

Serum metabolomic profiles of AE-IPF, S-IPF and Controls

Nontargeted metabolic profiling using UHPLC-MS/MS was employed to search for differentially expressed serum metabolites in AE-IPF, S-IPF and Controls. Following data processing and filtration, 16,876 and 18,280 metabolic peaks were detected in the positive and negative ESI models, respectively. Unsupervised PCA, OPLS-DA and permutation test were utilized to investigate intrinsic metabolic variations and data quality in the metabolic analysis.[29–31] The results of both PCA and OPLS-DA analyses, as well as permutation tests of the metabolic profiles, revealed statistically significant differences between IPF patients and Controls in the positive ESI mode (Figure S1A–C in the Supporting Information). In line with this, differences were also identified between patients with AE-IPF and those with S-IPF (Figure S1D–F in the Supporting Information). Similar results were observed in the negative ESI mode, wherein significant differences between IPF patients and Controls, as well as between AE-IPF patients and S-IPF patients were identified (Figure S2A–F in the Supporting Information). Taken together, these findings indicate significant alterations in metabolomic profiles of patients with IPF, particularly those with AE.

Metabolic profiling

To identify characteristic metabolic changes and potential biomarkers, volcano plot analysis was applied to the metabolomic profiling data. The results indicated that 457 metabolites were detected with a p-value <0.05, of which 208 metabolites were upregulated and 249 metabolites were downregulated in the IPF group compared to the Control group (Figure 1A). Moreover, to further investigate the overall characteristic metabolic alterations in IPF patients, KEGG pathway enrichment analysis was conducted on the differentially expressed metabolites. A total of 24 metabolic pathways were significantly enriched between the IPF and Control groups (Figure 1B). Interestingly, multiple pathways related to lipid metabolism were identified, including ABC transport, bile secretion, taurine and hypotaurine metabolism, cortisol synthesis and secretion and phenylalanine metabolism (Figure 1B). Additionally, 32 lipid metabolites were enriched in IPF group compared to Controls. On this basis, a bar chart was generated to present the top 15 upregulated or downregulated metabolites, which were selected as potential biomarkers of AE-IPF for further investigation in our study (Figure 1C).

Consistently, the volcano plot displayed a distinct overall separation between the AE-IPF and S-IPF groups (Figure 2A). Our findings revealed the detection of 407 metabolites ($p < 0.05$), of which 206 metabolites were upregulated and 201 metabolites were downregulated in the AE-IPF compared to the S-IPF group (Figure 2A). Notably,

TABLE 2 Baseline clinical characteristics of control, S-IPF and AE-IPF in internal and external validation cohorts.

	Internal validation cohort				External validation cohort			
Clinical variables	**Control**	**S-IPF**	**AE-IPF**	***p* value**	**Control**	**S-IPF**	**AE-IPF**	***p* value**
Gender (M/F)	18/7	23/5	21/5	0.639	17/3	24/6	25/2	0.406
Age (years)	67.88 ± 6.09	68.67 ± 7.06	68.54 ± 7.95	0.911	66.70 ± 10.84	69.73 ± 11.50	71.19 ± 8.04	0.331
Smoking (Y/N)	—	14/14	13/13	0.990	—	14/16	17/10	0.220
WBC ($*10^9$)	6.57 ± 1.72	6.21 ± 1.57	10.25 ± 3.78	**<0.001**	5.96 ± 1.34	7.30 ± 2.39	9.54 ± 3.32	**<0.001**
TG (mmol/L)	1.24 ± 0.70	1.64 ± 1.25	1.20 ± 0.52	0.142	1.26 ± 0.45	1.37 ± 0.75	1.29 ± 0.90	0.864
TC (mmol/L)	4.38 ± 0.83	4.46 ± 0.69	4.29 ± 1.13	0.800	4.49 ± 0.65	4.15 ± 1.13	4.62 ± 1.26	0.250
LDL (mmol/L)	2.62 ± 0.71	2.67 ± 0.44	2.51 ± 0.93	0.642	2.59 ± 0.48	2.42 ± 0.91	2.81 ± 1.09	0.273
HDL (mmol/L)	1.31 ± 0.28	1.27 ± 0.82	0.99 ± 0.32	0.082	1.27 ± 0.28	1.10 ± 0.41	1.14 ± 0.40	0.308
Apo B (g/L)	0.87 ± 0.17	0.86 ± 0.15	0.92 ± 0.21	0.424	0.79 ± 0.01	0.75 ± 0.22	0.90 ± 0.30	0.077
Apo AI (g/L)	1.35 ± 0.23	1.12 ± 0.27	0.92 ± 0.29	**<0.001**	1.49 ± 0.17	0.99 ± 0.27	0.89 ± 0.22	**<0.001**
ALB (g/L)	43.57 ± 1.43	40.10 ± 4.73	33.48 ± 3.72	**<0.001**	43.09 ± 2.11	37.72 ± 3.01	33.74 ± 3.44	**<0.001**
GLU (mmol/L)	5.28 ± 0.54	5.05 ± 1.23	6.86 ± 3.04	**0.002**	5.53 ± 0.44	5.76 ± 2.27	6.60 ± 3.00	0.230
HRCT scores	—	3.25 ± 1.11	6.69 ± 1.29	**<0.001**	—	3.93 ± 1.49	6.15 ± 1.35	**<0.001**
PaO_2/FiO_2 ratio	—	363.5 ± 44.28	180.2 ± 102.0	**<0.001**	—	332.4 ± 107.2	185.0 ± 73.70	**<0.001**
Antifibrotic therapy (≥3 months, Y/N)	—	10/17	3/23	**0.031**	—	19/11	11/15	**0.116**
Antifibrotic therapy (pirfenidone/nintedanib)	—	10/0	3/0	—	—	19/0	9/2	0.130
Duration antifibrotic therapy (months)	—	11.1 ± 15.62	2.5 ± 6.92	0.120	—	12.6 ± 15.15	8.7 ± 15.44	0.340
Prior corticosteroid usage (Y/N)	—	11/17	20/6	**0.004**	—	8/22	21/6	**<0.001**
Corticosteroid dosage (mg/day)	—	7.61 ± 16.21	29.54 ± 26.82	**<0.001**	—	6.33 ± 13.19	36.11 ± 33.92	**<0.001**
Survival duration (months)	—	30.50 ± 11.63	9.03 ± 12.49	**<0.001**	—	24.75 ± 8.13	13.09 ± 11.36	**<0.001**

Note: The bold values presented in Tables 1 and 2 represent P values less than 0.05, which are considered statistically significant.

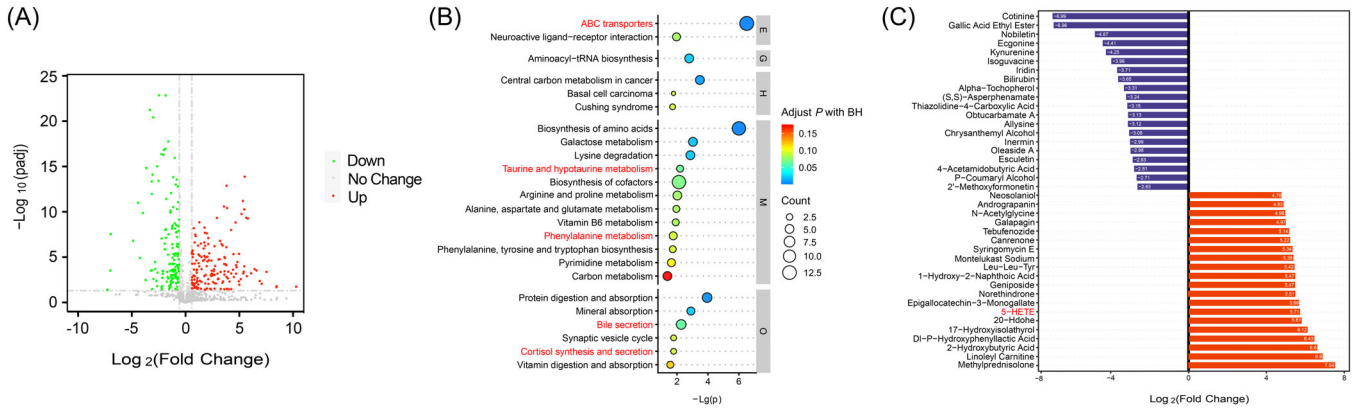

FIGURE 1 Metabolite differential abundance between all IPF patients and Controls. (A) The volcano plot with numbers of significantly (FDR <0.05) up or downregulated metabolites. Red: metabolites with upregulated differential expression, green: metabolites with downregulated differential expression, grey: metabolites with no significant differences. (B) The KEGG pathway enrichment plot of significantly (FDR <0.05) up or down-regulated metabolites. The colour of nodes shows the *p* value of the enrichment, and the size of the nodes indicates the number of matched metabolites. (C) The bar chart of top 15 differential metabolites. Red: upregulated differentially expressed metabolites, blue: downregulated differentially expressed metabolites.

the KEGG enrichment analysis indicated that half of the pathways (12 out of 24) were associated with lipid metabolism (Figure 2B). Moreover, 19 lipid metabolites exhibited distinctions between the AE-IPF and S-IPF groups. Combined with the findings from the comparison of IPF with Controls, these results suggested that lipid metabolism may play a crucial role in the development of AE-IPF. More importantly, 5-HETE, an important product of lipid metabolism, was the only metabolite among the top 15 that was elevated in both IPF compared to Controls and in AE-IPF

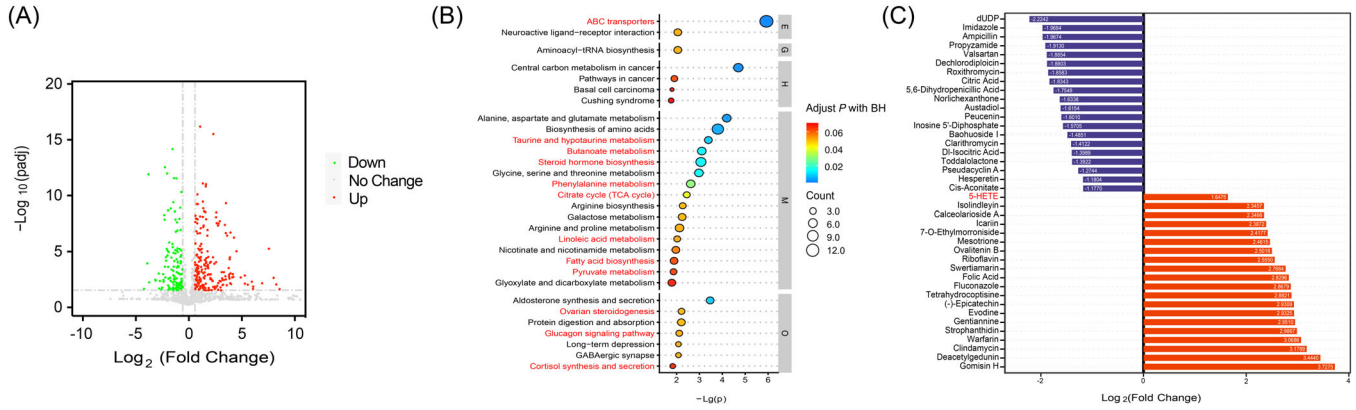

FIGURE 2 Metabolite differential abundance between AE-IPF patients and S-IPF patients. (A) The volcano plot with numbers of significantly (FDR <0.05) up or downregulated metabolites. Red: metabolites with upregulated differential expression, green: metabolites with downregulated differential expression, grey: metabolites with no significant differences. (B) The KEGG pathway enrichment plot of significantly (FDR <0.05) up or downregulated metabolites. The colour of nodes shows the *p* value of the enrichment, and the size of the nodes indicates the number of matched metabolites. (C) The bar chart of top 15 differential metabolites. Red: upregulated differentially expressed metabolites, blue: downregulated differentially expressed metabolites.

compared to S-IPF group (Figures 1C and 2C). Collectively, this data indicates a significant alteration in lipid metabolism in patients with AE-IPF and suggest that the downstream product 5-HETE may serve as a potential biomarker for AE-IPF.

Identification of potential biomarker 5-HETE

Next, we investigated whether there were significant alterations in the expression levels of 5-HETE and its related indicators in IPF patients. 5-HETE is a product of the cellular metabolism of phospholipids and arachidonic acid.[18] The specific synthesis of 5-HETE is reliant on the action of its synthesizing enzyme LOX5.[32] Elevated levels of 5-HETE stimulate the production of downstream effector molecules, such as VEGF, ABCA1 and ABCG1, ultimately promoting the progression of pulmonary fibrosis.[24,33,34] Thus, the enzyme-linked immunosorbent assay (ELISA) was used to assess the expression levels of 5-HETE in the serum of patients with Controls, S-IPF and AE-IPF in internal and external validation. In internal validation cohort, the findings revealed a significant downregulation of 5-HETE expression in patients with Controls ($n = 25$, 1001 ± 187.10 pg/mL) when compared to that of IPF ($n = 54$, 1520 ± 562.20 pg/mL) (Figure 3A). More importantly, significantly upregulated expression of 5-HETE was also observed in AE-IPF patients ($n = 26$, 1753 ± 629.50 pg/mL) as compared to those with S-IPF ($n = 28$, 1304 ± 391.20 pg/mL) (Figure 3B). Similar results were observed in the external validation cohort, showing a significant elevation of 5-HETE in IPF patients ($n = 57$, 1471 ± 323.90 pg/mL) compared to Controls ($n = 20$, 1000 ± 201.30 pg/mL), and in AE-IPF patients ($n = 27$, 1646 ± 304.40 pg/mL) compared to S-IPF patients ($n = 30$, 1313 ± 255.50 pg/mL) (Figure 3C,D).

To further verify the feasibility and specificity of 5-HETE as a biomarker for AE-IPF, we analysed 5-HETE levels in bronchoalveolar lavage fluid (BALF) from Controls, IPF, and AE-IPF patients, as well as in serum from Controls, Stable COPD (S-COPD), and AE-COPD patients. ELISA analysis confirmed a significant upregulation of 5-HETE expression in BALF of AE-IPF patients ($n = 5$, 116.1 ± 7.99 pg/mL) compared to those with S-IPF ($n = 6$, 43.36 ± 17.02 pg/mL) and Controls ($n = 6$, 9.59 ± 10.59 pg/mL) (Figure S3A in the Supporting Information). Interestingly, 5-HETE levels showed no statistical difference among Controls, S-COPD and AE-COPD patients (Figure S3B in the Supporting Information).

Consistent with this, the qPCR assay demonstrated that the upstream enzymes of 5-HETE, PLC and LOX5, as well as the downstream effector molecule VEGF, were upregulated in the IPF group (Figure S4A–C in the Supporting Information). In addition, the expression of the lipid transporters ABCA1 and ABCG1 was decreased in the IPF group, which is consistent with the previous observation of reduced levels of HDL in the serum of IPF patients (Figure S4D,E in the Supporting Information). Notably, the expression of PLC, LOX5, VEGF was also upregulated in AE-IPF as compared to S-IPF (Figure S4F–H in the Supporting Information). The expression of ABCA1 and ABCG1 was decreased in AE-IPF compared to S-IPF (Figure S4I,J in the Supporting Information). Moreover, the protein levels of LOX5 and VEGF were also upregulated in AE-IPF as compared to S-IPF and Controls (Figure S4K,L in the Supporting Information). Together, these findings suggest that the expression of 5-HETE is highest in patients with AE-IPF, followed by S-IPF patients, and lowest in Control subjects.

Diagnosis and prognosis value of 5-HETE in AE-IPF

To better understand the association between 5-HETE and IPF, bivariate correlation analysis was conducted to analyse

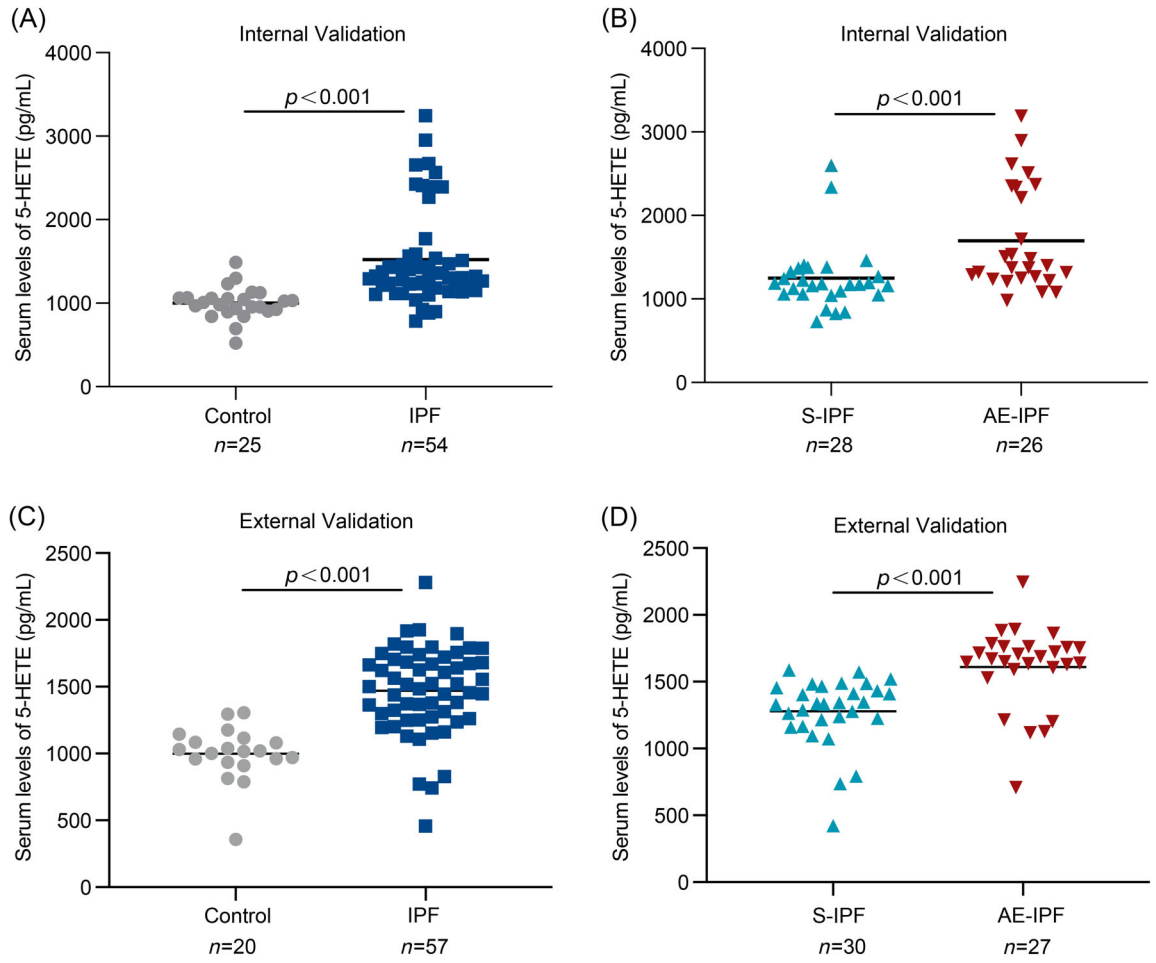

FIGURE 3 Expression levels of 5-HETE in AE-IPF patients. (A) In internal validation cohort, serum concentrations of 5-HETE were significantly elevated in all IPF patients ($n = 54$) compared with Controls ($n = 25$) ($p < 0.001$). (B) Serum 5-HETE level in AE-IPF subjects ($n = 26$) was significantly higher than that in S-IPF cases ($n = 28$) ($p < 0.001$). (C, D) The findings of 5-HETE serum levels in external validation cohort were similar to internal validation cohort (both $p < 0.001$).

the serum levels of 5-HETE and clinical variables in all IPF patients. As demonstrated in Table S2 in the Supporting Information, the serum levels of 5-HETE exhibited positive correlations with lactate dehydrogenase (LDH) and HRCT scores and negative correlations with ALB, PaO_2/FiO_2 ratio and survival duration in both the internal and external validation cohorts. These findings suggest the potential involvement of 5-HETE in the progression of IPF. Moreover, in internal validation, a duration of antifibrotic therapy exceeding 3 months demonstrated a positive correlation with survival time, and there was also a positive correlation observed between the duration of antifibrotic therapy and survival time. The detailed results can be seen in Table S3 in the Supporting Information.

Subsequently, ROC curve analysis and Kaplan–Meier analysis model were performed to evaluate the diagnostic and prognostic value of 5-HETE in AE-IPF. In internal validation cohort, ROC curve analysis revealed that 5-HETE serves as a promising biomarker for discriminating acute exacerbation from IPF patients, with an area under the ROC curve (AUC) of 0.768 (Figure 4A). Correspondingly, ROC curve analysis for mortality showed an AUC of 0.777 (Figure 4B). Additionally, Kaplan–Meier analysis in internal validation cohort identified higher mortality among patients with a serum 5-HETE level above 1302.0 pg/mL compared with those below this threshold ($p < 0.001$) (Figure 4C). Similarly, in the context of external validation cohort, ROC curves assessing the diagnostic and prognostic value of 5-HETE (with AUC of 0.843 and 0.740, respectively) (Figure S5A,B in the Supporting Information), along with Kaplan–Meier analysis ($p < 0.001$) (Figure S5C in the Supporting Information). Similar results were observed in integrated validation cohort (Figure S5D–F in the Supporting Information).

In multivariable Cox models, both in internal and external validation as well as integrated validation, LDH and 5-HETE were the independent predictors for overall survival in IPF patients (Table S4 in the Supporting Information). Additionally, all significant univariate COX analysis results in validation cohorts were presented in Table S5 in the Supporting Information. Collectively, these results suggested

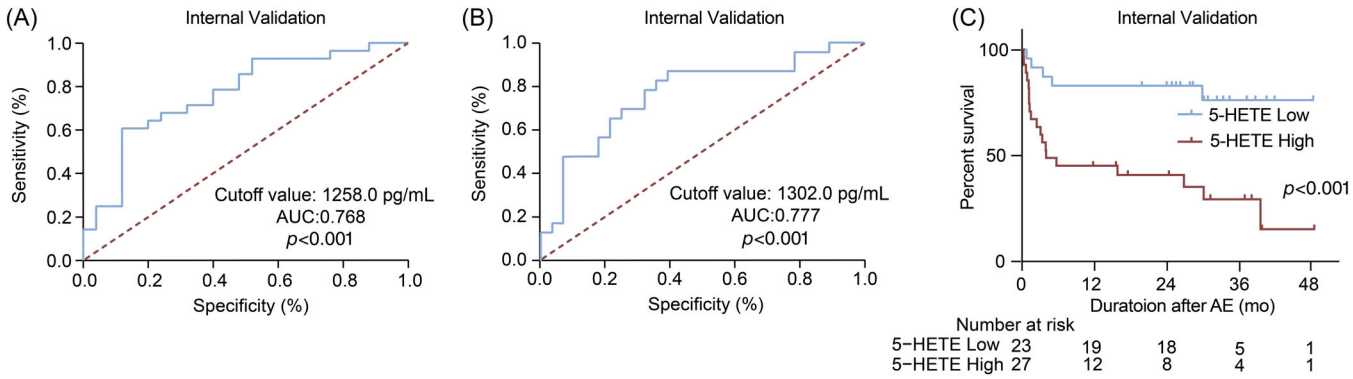

FIGURE 4 5-HETE is a potential metabolic biomarker for discriminating AE and predicting the survival of IPF patients. (A) ROC curve of 5-HETE for distinguishing AE-IPF subjects from S IPF patients in internal validation cohort (AUC: 0.768, $p < 0.001$, 95%CI: 0.641–0.895, cut-off value 1258.0 pg/mL). (B) ROC curve of 5-HETE for predicting the survival of IPF patients in internal validation cohort (AUC: 0.777, $p < 0.001$, 95%CI: 0.646–0.909, cut-off value 1302.0 pg/mL). (C) The survival of IPF patients with 5-HETE serum level above cutoff value 1302.0 pg/mL was significantly worse than those below that by Kaplan–Meier model in internal validation ($p < 0.001$).

that 5-HETE may serve as a potential biomarker for the diagnosis AE and prognosis of IPF.

Working model of 5-HETE's role in AE-IPF mechanism

As shown in Figure S6 in the Supporting Information, this model illustrates the potential role of 5-HETE in the pathogenesis of AE-IPF and highlights its involvement in inflammatory and fibrotic processes.

DISCUSSION

The pathobiology of AE-IPF involves a variety of factors, encompassing extensive inflammatory responses as well as cell apoptosis.[6] Evidence suggests that AE-IPF exhibits several similarities with acute respiratory distress syndrome (ARDS), including similar clinical, radiographic, pathophysiological alterations and triggering factors.[35] It remains uncertain whether the phenomena of AE constitute an inherent acceleration of the underlying fibrotic disease or a reactive response to external factors.[36] Nonetheless, several factors have been linked to a higher risk of AE-IPF, with the presence of physiologically or radiologically advanced disease serving as the most consistently replicated risk and prognostic factor for AE-IPF.[9,37–39] Infection, gastroesophageal reflux, cardiac disease, air pollution and pulmonary hypertension have been proposed as potential risk factors for the development of AE-IPF.[9,37,40,41] In addition, elevated serum concentrations of growth differentiation factor-15 (GDF-15), leptin, LDH, Krebs von den Lungen-6 (KL-6) and CRP have been identified as highly sensitive predictors of the incidence and poor prognosis of AE-IPF.[27,42–45] However, further investigation and clinical validation are required before these biomarkers can be deemed appropriate for implementation in clinical practice.

We performed a metabolomics analysis to elucidate the characteristic metabolic profiles and related pathways in AE-IPF patients, S-IPF patients and Controls. Our findings suggested significant alterations in lipid metabolism in the serum of patients with AE-IPF, and further identified 5-HETE as a potential biomarker for AE-IPF. A detailed metabolic profile using the bronchoalveolar lavage fluid (BALF) or lung tissue in the future would help to delineate the specific metabolic pathways involved in AE-IPF and further elucidate the disease mechanisms underlying this condition.

Previous research has demonstrated that metabolic dysfunction plays a significant role in the development of IPF.[11] Metabolomic abnormalities in alveolar epithelial cells, myofibroblasts, macrophages and fibroblasts have been shown to contribute to aberrant collagen synthesis and perturbed airway remodelling in lung fibrosis via various mechanisms.[15,46,47] However, the metabolic profile of AE-IPF remains poorly understood. Previous work has illustrated that glycolysis mediated by glucose transporter 1 (GLUT1) contributes to the exacerbation of pulmonary fibrogenesis during Streptococcus pneumoniae infection by activating the AIM2 inflammasome.[48] A recent study comparing BALF obtained from patients with S-IPF and those with AE-IPF revealed that the sequestration of altered extracellular lipids by foamy macrophages may play a critical role in the progression of fibrosis to AE.[49,50] In addition, studies have demonstrated the potential involvement of lipid metabolism in the pathogenesis of IPF and acute lung injury.[51,52] Lipid metabolites, namely phospholipids and arachidonic acid, exert a substantial impact on the onset of acute inflammatory responses as well as the advancement of pulmonary injury and fibrosis.[53,54] Consistently, our analysis of metabolomic profiles revealed significant dysregulation of various lipid metabolism pathways in patients with AE-IPF compared to those with S-IPF, including ABC transporters, citrate cycle, fatty acid biosynthesis, Pyruvate metabolism and so on. Interestingly, we also observed that the levels of HDL

and ALB gradually decreased in the serum of the Controls, S-IPF patients and AE-IPF patients. Based on these findings, we can conclude that significant dysregulation of lipid metabolism occurs in patients with AE-IPF, which may be attributed to the imbalances in lipid transport, synthesis and degradation.

5-HETE, a metabolite associated with acute inflammation, injury, allergy and tumorigenesis, has not been previously reported to have a role in the pathogenesis of AE-IPF.[20–23] 5-HETE was initially identified as a lipid mediator involved in the innate immune response to infections.[18] Mechanistically, 5-HETE participates in the activation of the innate immune system by stimulating effector cells to engage in various pro-defensive activities, including migration, degranulation and production of inflammatory cytokines and chemokines.[55] Furthermore, 5-HETE has been implicated in allergic reactions, including allergy and arthritis.[56] Recent research has demonstrated that 5-HETE induces bronchial smooth muscle contraction in isolated human bronchi, leading to increased airway hyperresponsiveness.[57] Additionally, 5-HETE has been shown to promote the migration and aggregation of neutrophils in the lungs.[58] Another significant role of 5-HETE is tumorigenesis, which is mediated by its ability to stimulate cellular proliferation and inhibit apoptosis.[59,60] It's worth noting that the synthesizing enzyme LOX5 for 5-HETE is also implicated in various pathological processes, including inflammation, vascular biology and cancer progression.[58] Acute inflammation, infection, cellular proliferation and apoptosis are closely associated with the development of AE-IPF.[4,7] These findings provide compelling theoretical support for the potential involvement of 5-HETE in the development of AE-IPF.

Consistent with this, we also demonstrated a significant upregulation of 5-HETE in AE-IPF, which may activate downstream signalling that further promote the acute exacerbation of IPF through the regulation of inflammation, proliferation and apoptosis. Intriguingly, we observed no significant difference in 5-HETE levels among Controls, S-COPD and AE-COPD patients, suggesting that 5-HETE may be specific to IPF rather than a general marker of immune-associated acute exacerbations in chronic lung diseases. In addition, since a higher proportion of AE-IPF patients received steroid therapy compared to IPF patients, this may affect their arachidonic acid metabolism and confound 5-HETE levels. A detailed comparison using prospective cohort samples in the future would help to delineate the complexity. Notably, recent studies have shown promise in using pharmacological LOX5 inhibitors to reduce 5-HETE synthesis for cancer prevention and treatment.[61,62] Given the correlation between 5-HETE and disease severity, it may serve as a diagnostic and prognostic biomarker, and a target for pharmacological intervention in AE-IPF.

Figure S6 in the Supporting Information depicted the potential mechanism of 5-HETE in AE-IPF. Upon stimulation, PLC selectively binds with arachidonic acid (AA). AA is then transported to activate LOX5, leading to its oxidation and conversion into 5-HPETE, which is further metabolized into 5-HETE. 5-HETE has the capability to activate downstream factors such as VEGF, thereby promoting neoangiogenesis and cell proliferation. Moreover, 5-HETE can be further converted to LTB4, which indirectly stimulate cellular inflammatory responses and the generation of chemokines, collectively contributing to the occurrence of acute exacerbations in IPF. Notably, LOX5 plays a potential role in inhibiting cholesterol efflux by downregulating ABCA1 and ABCG1 expression, leading to cholesterol accumulation.

Our study is constrained by a relatively small sample size, the absence of metabolomics study cohorts from two separately medical centres, or the data sequences encompassing the occurrence of AE in the same patients. Second, the functional role of 5-HETE in AE-IPF and the specific role of arachidonic acid or other eicosanoids in regulating 5-HETE have not yet been elucidated. We will conduct a detailed investigation into the biological functions and regulatory mechanisms of 5-HETE in the near future. Third, the samples of two validation cohorts were collected at different time points within the same medical centre, inevitably leading to variations in 5-HETE levels in two validation cohorts.

In conclusion, our study provided valuable insights into the lipid metabolism of AE-IPF, and 5-HETE may be a potential biomarker of AE-IPF patients.

AUTHOR CONTRIBUTIONS

Yichao Zhao: Conceptualization (equal); data curation (lead); investigation (equal); methodology (equal); project administration (equal); writing – original draft (lead). **Yanchen Shi:** Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); software (lead); writing – review and editing (supporting). **Ji Zhang:** Investigation (equal); supervision (equal); writing – review and editing (supporting). **Huizhe Zhang:** Formal analysis (supporting); investigation (supporting); methodology (supporting); writing – review and editing (supporting). **Zimu Wang:** Investigation (supporting); software (supporting); writing – review and editing (supporting). **Shufei Wu:** Investigation (supporting); software (supporting); writing – review and editing (supporting). **Mingrui Zhang:** Investigation (supporting); validation (supporting); visualization (supporting); writing – review and editing (supporting). **Mengying Liu:** Investigation (supporting); validation (supporting); writing – review and editing (supporting). **Xu Ye:** Investigation (supporting); resources (supporting); writing – review and editing (supporting). **Huimin Gu:** Investigation (supporting); validation (lead); writing – review and editing (supporting). **Cheng Jiang:** Investigation (supporting); resources (supporting); writing – review and editing (supporting). **Xiaoling Ye:** Investigation (supporting); resources (supporting); writing – review and editing (supporting). **Huihui Zhu:** Investigation (supporting); resources (supporting); writing – review and editing (supporting). **Qi Li:** Investigation (supporting); resources (supporting); writing – review and editing (supporting). **Xinmei Huang:** Conceptualization (lead); investigation (equal); methodology (equal); project

administration (equal); writing – original draft (equal). **Mengshu Cao:** Funding acquisition (lead); supervision (equal); visualization (lead); writing – review and editing (supporting).

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CONFLICT OF INTEREST STATEMENT

None declared.

DATA AVAILABILITY STATEMENT

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

HUMAN ETHICS APPROVAL DECLARATION

The research protocol was approved by the Medical Ethics Committee of Nanjing Drum Tower Hospital in accordance with the Declaration of Helsinki (1989) (No. 2016-160-01). All study participants provided written informed consent before enrollment.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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