ORIGINAL

Lysophosphatidic acid, ceramide 1-phosphate and sphingosine 1-phosphate in peripheral blood of patients with idiopathic pulmonary fibrosis

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Abstract : Idiopathic pulmonary fibrosis (IPF) is the most common idiopathic interstitial pneumonias. Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are signaling lipids that evoke growth factor-like responses to many cells. Recent studies revealed the involvement of LPA and S1P in the pathology of IPF. In this study, we determined LPA, S1P and ceramide 1-phosphate (C1P) in peripheral blood plasma of IPF patients, and examined correlation to the vital capacity of lung (VC), an indicator of development of fibrosis. Blood plasma samples were taken from eleven patients with IPF and seven healthy volunteers. The lipids of the sample were extracted and subjected to liquid chromatography-tandem mass spectrometry for analysis. Results showed that there is a significant negative correlation between VC and plasma LPA levels, indicating that IPF patients with advanced fibrosis had higher concentration of LPA in their plasma. Average of S1P levels were significantly higher in IPF patients than those in healthy subjects. Although it is not statistically significant, a similar correlation trend that observed in LPA levels also found between VC and S1P levels. These results indicated that plasma LPA and S1P may be associated with deterioration of pulmonary function of IPF patients. J. Med. Invest. 69 : 196-203, August, 2022

Keywords **:** *idiopathic pulmonary fibrosis, ceramide 1-phosphate, lysophosphatidic acid, sphingosine 1-phosphate, liquid chromatography-tandem mass spectrometry*

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common idiopathic interstitial pneumonias (IIPs) with different progression rates. IPF often has a very poor prognosis, with a 5-year survival rate of 30 % or below (1). IPF progression involves several physiological alterations to the lung. These include excess apoptosis of pulmonal epithelia, increased vascular permeability, recruitment of inflammatory cells, extravascular blood clotting, overproduction of fibrotic mediators, such as transforming growth factor (TGF), proliferation and accumulation of activated fibroblasts, and enhanced synthesis of extracellular matrix components, such as collagen (2). The factors that induce these biological and histological alterations in alveoli serve as potential targets for the treatment of interstitial pneumonias including IPF (3).

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are lipid mediators that regulate cellular functions, including apoptosis, migration and proliferation, via its specific G-protein coupled receptors. Six receptor types have been identified for LPA ($LPA_{1\text{-}6}$), while S1P has five receptor types ($S1P_{1\text{-}5}$) (4, 5). LPA is involved in various forms of fibrosis by enhancing vascular permeability and promoting the proliferation of fibroblasts and hepatic stellate cells (6, 7). The implication of LPA in interstitial pneumonia is evident from the fact that deletion of the gene encoding LPA_1 (8) or LPA-producing enzyme (ATX, lysophospholipase D) (9) ameliorates bleomycin-induced interstitial pneumonia. S1P is also an important mediator in the progression of interstitial pneumonia. Huang *et al*. have demonstrated the involvements of sphingosine kinase 1 (SphK1), a rate-limiting enzyme for S1P biosynthesis, and S1P lyase, an irreversible S1P-degrading enzyme, in the pathology of IPF (10, 11). In fact, several studies have shown increased levels of LPA or S1P in bronchoalveolar lavage fluid (BALF) and peripheral blood of IPF patients (8, 9, 12, 13). Based on these findings, the receptors for LPA or S1P, and the enzymes involved in the synthesis or degradation of these mediators are recognized as potential therapeutic targets for IPF (3, 6, 9-13).

Ceramide 1-phosphate (C1P) is a recently identified mediator that modulates several cellular functions, including cell survival

Abbreviations :
ATX, autotaxin ; BALF, bronchoalveolar lavage fluids ; C1P, ceramide 1-phosphate; FVC, forced vital capacity ; IIPs, idiopathic interstitial pneumonias ; IPF, idiopathic pulmonary fibrosis ; KL-6, Krebs von den Lungen-6 ; LC-MS/MS, liquid chromatography-tandem mass spectrometry ; LDH, lactate dehydrogenase ; LPA, lysophosphatidic acid ; SP-D, surfactant protein-D ; SphK, sphingosine kinase ; S1P, sphingosine 1-phosphate ; TGF-β, transforming growth factor-β ; VC, vital capacity.

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and cell migration (14). Recently, Baudiß *et al*. reported that C1P suppresses cigarette smoke-induced airway inflammation (15). LPS-induced lung injury is also reported to be ameliorated by C1P as it suppresses proinflammatory responses (16). These observations suggest that C1P plays a role in mitigating the inflammatory symptoms of lung disease. At present, the involvement of C1P in fibrotic alterations of lung tissue is unknown.

In this study, we examined LPA, S1P, and C1P levels in the peripheral blood plasma of IPF patients, and examined correlation with the vital capacity of lung (VC), an indicator of development of fibrosis (17). We found a significant negative correlation between LPA levels and VC, suggesting that LPA levels in the plasma of IPF patients with severe pulmonary status was higher than those in IPF patients with mild status. The average level of S1P in IPF patients was significantly higher than that in healthy controls. Although it is not statistically significant, a similar correlation trend as observed in LPA was found between S1P levels and VC.

PATIENTS AND METHODS

Patients and clinical test values

The protocols of this study were approved by the Ethics Committee of Tokushima University Hospital (No. 3068 and 4104). Informed consent was obtained from all participants. All outline methods were performed in accordance with the guidelines of the Declaration of Helsinki. Patients were diagnosed by two pulmonologists according to the International Guidelines for Diagnosis and Management of IIPs (18, 19). The subjects of this study comprised with eleven patients with IPF and seven healthy volunteers. The clinical test values obtained were vital capacity of lung (VC) and forced vital capacity of lung (FVC), as well as the serum concentrations of Krebs von den Lungen-6 (KL-6), surfactant protein-D (SP-D), and lactate dehydrogenase (LDH) (Table 1). The plasma of the patients was prepared from the peripheral blood, in which EDTA was used as anti-coagulant. The differences between the day of blood collection and that of clinical test for VC and FVC were maximum 14 days with most case 0–1 day. The highest score of emphysematous changes in the lung fields of IPF patients were 4 (two patients) with most case 0 as judged by Goddard method (20), indicating that all IPF patients showed slight or no pulmonary emphysema. Clinical test values and plasma concentrations of total LPA, total C1P and S1P in each subject are also presented (Supplementary Table 1).

Table 1. Details of subjects

	Healthy	Patients
		IPF
[a]No. of patients	7 (2)	11 (5)
[b]Smoker	0	4
Age	59.3 ± 15.3	72.9 ± 5.4
[c]VC (%)	-	69.2 ± 20.3
[c]FVC (%)	-	68.6 ± 19.4
[c]KL-6 (U/mL)	-	945 ± 321 (<500[d])
[c]SP-D (ng/mL)	-	272 ± 139 (<110[d])
[c]LDH (U/L)	-	238 ± 34 (124-222[d])

[a]Number of subjects (number of females). [b]Number of smokers who consumed one or more cigarettes per day or non-smoker with smoking history. [c]VC, vital capacity ; [c]FVC, forced vital capacity ; [c]KL-6, Krebs von den Lungen-6 ; [c]SP-D, surfactant protein-D ; [c]LDH, lactate dehydrogenase. [d]Values considered normal at Tokushima University Hospital.

Materials

N-lauroyl-ceramide 1-phosophate (C12 : 0 C1P), *N*-palmitoyl-ceramide 1-phosophate (C16 : 0 C1P) and *N*-lignoceroyl-ceramide 1-phosophate (C24 : 0 C1P), D-*erythro*s-phingosine 1-phosphate (S1P), and 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphate (17 : 0 LPA) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other reagents were of reagent grade, HPLC grade, or LC-MS grade.

Preparation of plasma lipid samples

Analyses of C1P, S1P and LPA in the plasma samples were conducted as reported previously (21-23). In a typical analysis, 0.1 mL of plasma was mixed with 1.0 mL of chloroform, 2.0 mL of methanol, and 0.7 mL of KCl aqueous solution (40 mg/mL) on ice. This was followed by sonication for 10–20 s. After adding 25 pmol of C12 : 0 C1P and 125 pmol of 17 : 0 LPA as internal standards to the mixture, 1.0 mL each of chloroform and water were added to the mixture for phase separation. After acidification of the aqueous layer to pH 2–3 using 5 M HCl, the mixture was centrifuged at 1,100 × g for 5 min. The chloroform layer was then withdrawn. Lipids were further extracted from the remaining water/methanol layer with 2.0 mL of chloroform. The combined chloroform layers were evaporated, and resulting lipids were dissolved in 0.8 mL of methanol, and filtered through a non-polar filter (Chromatodisc 4 N, 0.2 μm, Kurabo, Osaka, Japan). The filtrate was evaporated, and the resultant lipids were dissolved in 0.1 mL of methanol/formic acid (99 : 1, v/v) containing 5 mM ammonium formate. A total of 5 μL of 5 mM EDTA-2Na was added to this solution and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS).

LC-MS/MS

Lipid mediator analyses were performed using a triple quadrupole-linear ion trap hybrid mass spectrometer 4000 Q TRAP (Applied Biosystems/MDS Sciex, Concord, ONT, Canada) with multiple reaction monitoring using an Agilent 1100 liquid chromatograph (Agilent Technologies, Wilmington DE, USA) and HTSPAL autosampler (CTC Analytics AC, Zwingen, Switzerland) as described previously (21-23). The ions selected for multiple reaction monitoring transitions of LPA, C1P, and S1P are shown in Supplementary Fig. 1 and Supplementary Table 2. For the analyses of C1P and S1P, the extracted plasma lipids were separated using a Cadenza CD-C18 column (50 × 2 mm, 3 μm, Imtakt Corp., Kyoto, Japan) at 42℃ by elution with methanol/formic acid (99 : 1, v/v) containing 5 mM ammonium formate as the mobile phase, at an isocratic flow of 300 μL/min. For the analysis of LPA, the lipids were separated on a TSKgel ODS-100Z column (150 × 2 mm, 5 μm, Tosoh Corp., Tokyo, Japan) at 42℃ by elution with a methanol/water mixture (95 : 5, v/v) containing 5 mM ammonium formate as the mobile phase, at an isocratic flow of 200 μL/min.

Statistical analysis

Statistical analyses were performed using Student's *t*-tests. In all analyses, a *p*-value of <0.05 was considered statistically significant. The correlation coefficient was obtained by simple linear regression analysis.

RESULTS

Determination of molecular species of LPA, C1P, and S1P by LC-MS/MS.

Analytical parameters for the determination of molecular species of LPA, C1P and S1P by LC-MS/MS are summarized

in Supplementary Table 2. The structures of the ions used for multiple reaction monitoring transitions are shown in Supplementary Fig. 1. We used C12 : 0 C1P as an internal standard, and prepared standard curves for the determination of C16 : 0, C24 : 0, C24 : 1 C1Ps, and S1P, to correct the ionizing efficacies (Supplementary Fig. 2). The correction factor for C16 : 0 C1P, C24 : 0 C1P and S1P were 1/0.93 (≈ 1), 1/0.49 (≈ 2), and 1/0.41(≈ 2.5) respectively. We applied 1/0.49 (≈ 2) as a correction factor for C24 : 1 C1P.

Concentrations of LPA, S1P, and C1P in plasma of IPF patients.

Unsaturated LPAs, such as 18 : 2 LPA, 20 : 4 LPA, and 22 : 6 LPA are abundant molecular species in healthy human plasma. The composition and concentration of each LPA species in the blood plasma samples of this study were essentially comparable to those of our previous results (21) and results from other studies (24). We found that there were no significant differences in the concentration of LPA species between the control and IPF groups except for C16 : 1 LPA, a minor LPA species in blood (Fig. 1).

Long-chain C1P and very-long-chain C1P are reported to be the major species of C1Ps in plasma (25). We found that in the plasma of healthy subjects, the level of C16 : 0 C1P, C24 : 0 C1P, and C24 : 1 C1P were approximately 60, 10, and 50 pmol/mL, respectively. These values are compatible with those reported by Hammad *et al.* (25). We found that there were no significant differences in the concentration of any of C1P species between the control and IPF groups (Fig. 2).

The average value of plasma S1P level in healthy subjects was approximately 260 pmol/mL (Fig. 3). This is slightly lower than that reported by Milara *et al.* (13). The average value of S1P in IPF group was around 470 pmol/mL, which was 2 times higher than that of the healthy control group (Fig. 3). Our result is consistent with the previous study reported by Milara *et al.* (13), who showed that S1P levels in IPF patients were 1.5 times higher than those in healthy controls.

Correlation between pulmonary functions of patients and plasma lipid mediators

We found that there was a significant negative correlation between VC (FVC) values and total LPA levels in the plasma of IPF patients (Fig. 4), indicating that LPA levels in the plasma of IPF patients with severe pulmonary status was higher than those in IPF patients with mild status. Although it is not statistically significant, a similar trend was also observed in S1P levels. No significant correlation was observed between VC (FVC) and total C1P levels in plasma samples.

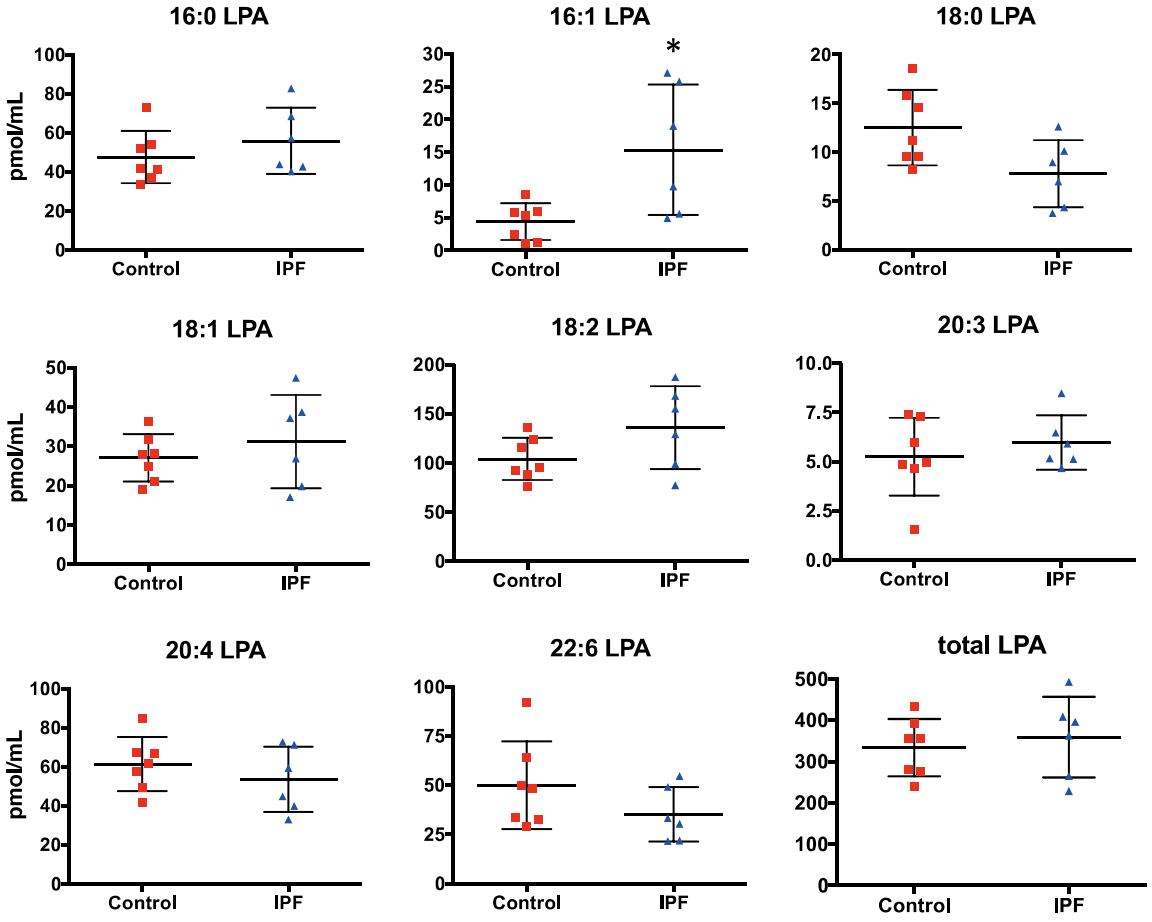

Fig 1. Concentrations of lysophosphatidic acid (LPA) species and total LPA in plasma from idiopathic pulmonary fibrosis (IPF) and healthy control groups.
Lipids from circulating blood plasma were extracted and subjected to LC-MS/MS for determination of LPA species with 17 : 0 LPA as an internal standard. Values are means ± S.D. Numbers of subjects were 7 for healthy controls and 6 for IPF patients. *$p < 0.05$ vs. healthy controls by Student's *t*-tests.

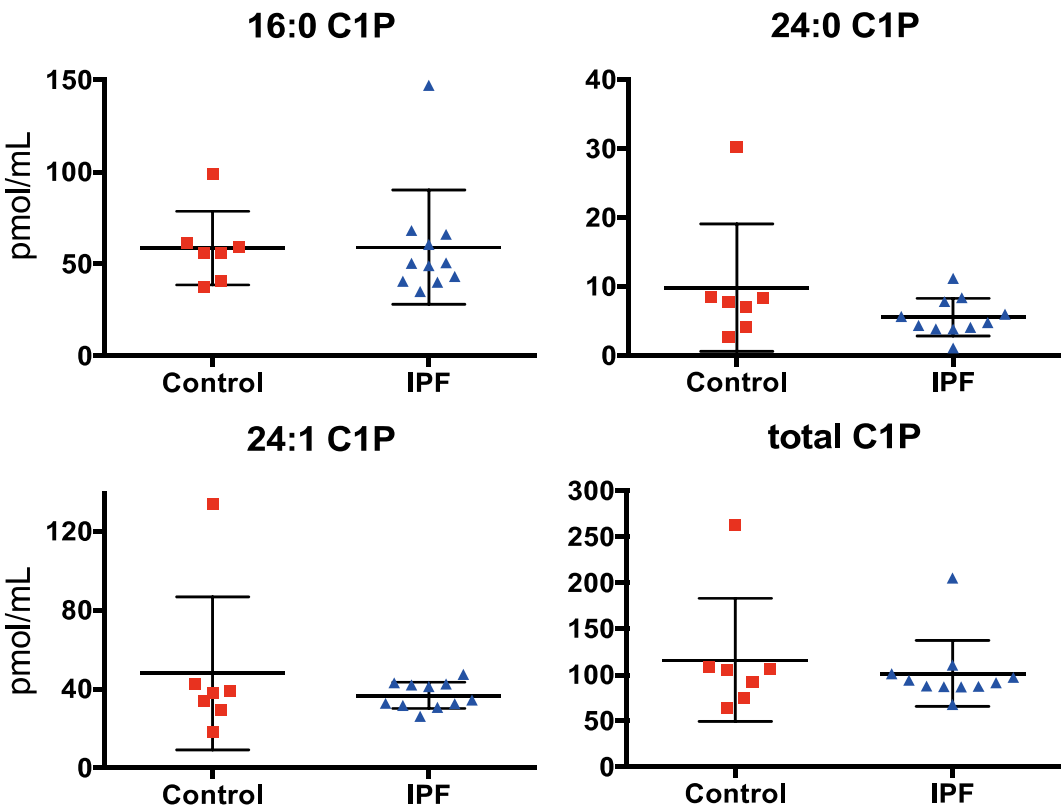

Fig 2. Concentrations of ceramide 1-phosphate (C1P) in plasma from idiopathic pulmonary fibrosis (IPF) and healthy control groups.
Lipids from circulating blood plasma were extracted and subjected to LC-MS/MS for determination of C1P with C12 : 0 C1P as an internal standard. Values are means ± S.D. Numbers of subjects are 7 for healthy controls and 11 for IPF patients. There is no significant difference between any of these two groups.

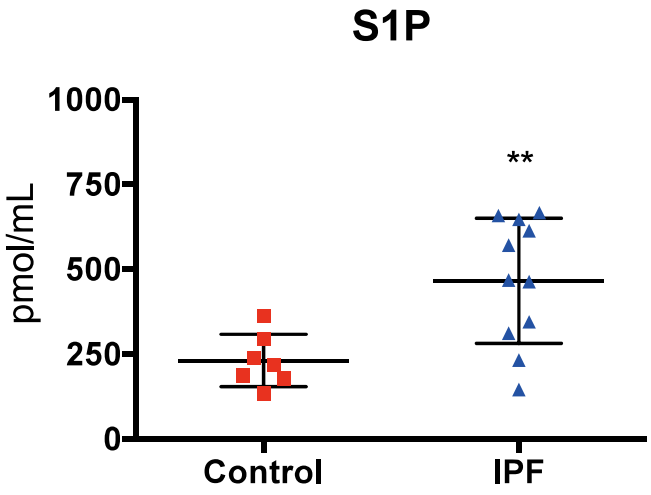

Fig 3. Concentrations of sphingosine 1-phospphate (S1P) in plasma from idiopathic pulmonary fibrosis (IPF) and healthy control groups.
Lipids from circulating blood plasma were extracted and subjected to LC-MS/MS for determination of S1P with C12 : 0 C1P as an internal standard. Values are means ± S.D. Numbers of subjects are 7 for healthy controls and 11 for IPF patients. $^{**}p < 0.01$, vs. healthy controls by Student's *t*-tests.

DISCUSSION

In this study, we examined the LPA levels in the circulating plasma of IPF patients using LC-MS/MS. Although average level of LPA in IPF patients was not significantly different from that of healthy controls, a significant negative correlation between VC or FVC and plasma LPA levels in patient samples was observed (Fig. 4). It has been known that FVC is reliable, valid and responsive measure of disease status in patients with IPF (17). Our results indicate a possibility that plasma LPA associates with deterioration of pulmonary function. It is important to examine the plasma LPA in larger number of patients with different stages of IPF for confirmation of this correlation.

C1P is a bioactive sphingolipid that promotes proliferation, anti-apoptosis, and migration in various types of cells (14). Recent studies have revealed that administration of C1P reduces inflammation in chronic obstructive pulmonary disease, asthma, and lung fibrosis (26). To our knowledge, available data on C1P levels in patients with lung disease does not exist. In this study, we established an analytical system that assessed human plasma C1P levels using LC-MS/MS. We found that there was no significant difference in C1P levels between IPF and healthy control groups.

S1P has been shown to promote the differentiation of fibroblasts into myofibroblasts which actively produce extracellular matrix components (4, 13). It has been shown that SphK1

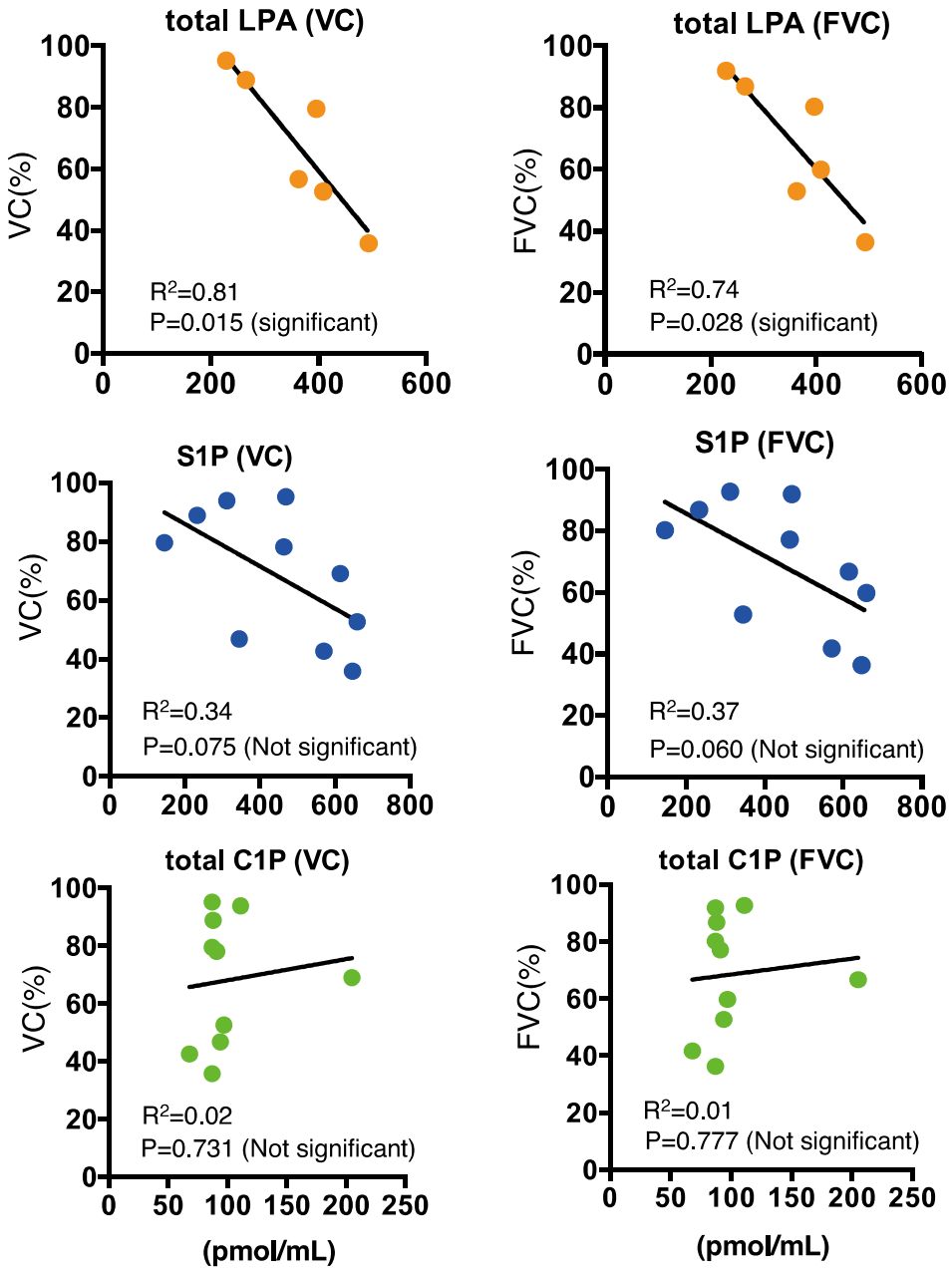

Fig 4. Correlation between plasma lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P) or ceramide 1-phosphate (C1P) levels and vital capacity (VC) or forced vital capacity (FVC) of patients with idiopathic pulmonary fibrosis (IPF). Values of VC or FVC versus concentration of LPA ($n = 6$), S1P ($n = 10$), or C1P ($n = 10$) are shown. The correlation coefficient was obtained by simple linear regression analysis. A p-value of <0.05 was considered statistically significant.

expression is upregulated in the lungs of IPF patients, and that S1P levels in the BALF and serum of IPF patients are significantly higher than those in control subjects (13). We found that IPF patients had significantly higher plasma S1P levels than those in healthy subjects. This result is consistent with those of previous reports (13). Although it is not statistically significant, the relation between S1P level and VC shows a similar trend to that observed in LPA.

In summary, we determined lipid mediators in plasma of IPF patients. We found that S1P levels were significantly higher in IPF patients than those in healthy subjects. There is a significant negative correlation between VC and plasma LPA levels. Although it is not statistically significant, a similar trend was also observed in S1P. These results indicated that plasma LPA and S1P may be involved in the progress and deterioration of pulmonary function. This study was conducted with a limited number of subjects. A larger number of IPF patients with varying severities are needed to verify this conclusion. It is also important to examine plasma concentration of these lipid mediators of non-IPF interstitial pneumonia, such as hypersensitivity interstitial pneumonia and collagen disease-related interstitial pneumonia to know the specificity of the phenomenon observed here.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests.

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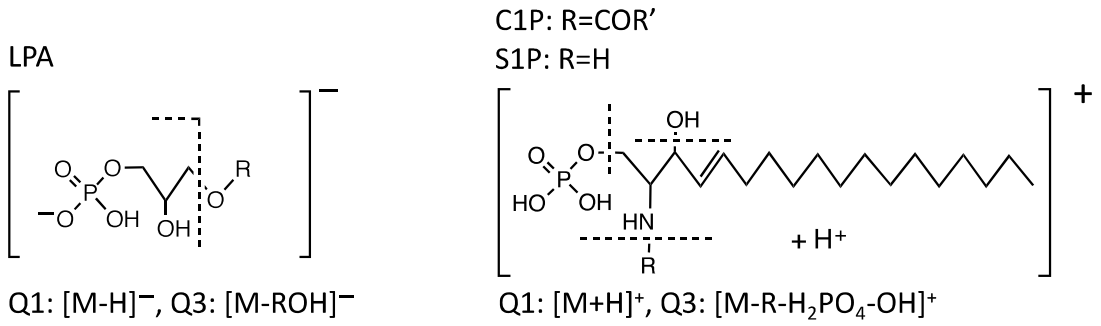
Supplementary Table 1. Details of subjects

Patient No.	[a]M/F	Age	[b]DX	[d]SMK	[h]VC %	[i]FVC %	[j]DLco/VA %	[k]KL-6 U/mL	[l]SP-D ng/mL	[m]LDH U/L	[n]tLPA pmol/mL	[o]tC1P pmol/mL	[p]S1P pmol/mL
IPF													
1	M	68	[c]IPF	[e]S	52.6	59.8	-	1031	636	287	409	97	659
2	M	69	[c]IPF	[f]N	95.2	91.9	98.4	1531	193	242	229	87	469
3	F	80	[c]IPF	[f]N	69.0	66.7	94.6	705	319	182	-	205	613
4	M	62	[c]IPF	[e]S	42.6	41.7	-	835	263	286	-	68	571
5	F	80	[c]IPF	[f]N	-	-	-	781	121	245	-	101	667
6	F	74	[c]IPF	[f]N	35.8	36.3	-	923	421	240	493	87	647
7	F	69	[c]IPF	[f]N	56.8	52.8	-	803	196	258	363	94	345
8	M	78	[c]IPF	[g]NS	88.9	86.8	70.3	1039	179	195	265	88	233
9	M	76	[c]IPF	[g]NS	79.5	80.2	-	513	165	198	396	87	146
10	F	71	[c]IPF	[f]N	78.1	77.1	112.1	1572	267	263	-	91	464
11	M	75	[c]IPF	[g]NS	93.9	92.7	91.3	663	237	224	-	111	312
Healthy control													
12	M	54	-	[f]N	-	-	-	-	-	-	282	105	188
13	M	54	-	[f]N	-	-	-	-	-	-	433	106	296
14	M	29	-	[f]N	-	-	-	-	-	-	357	263	180
15	M	65	-	[f]N	-	-	-	-	-	-	276	92	219
16	M	79	-	[f]N	-	-	-	-	-	-	392	108	364
17	F	74	-	[f]N	-	-	-	-	-	-	356	65	135
18	F	62	-	[f]N	-	-	-	-	-	-	241	75	239

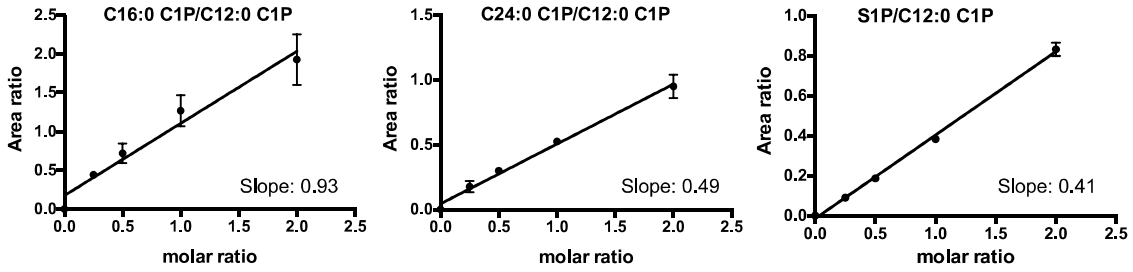
[a]M/F, male/female ; [b]DX, diagnosis ; [c]IPF, idiopathic pulmonary fibrosis ; [d]SMK, smoking history ; [e]S, smoker who consumed one or more cigarettes per day ; [f]N, non-smoker ; [g]NS, non-smoker with past smoking history ; [h]VC, vital capacity ; [i]FVC, forced vital capacity ; [j]DLco/VA, diffusing capacity for carbon monoxide/alveolar volume, [k]KL-6, Krebs von den Lungen-6 ; [l]SP-D, surfactant protein-D ; [m]LDH, lactate dehydrogenase ; [n]tLPA, total lysophosphatidic acid (LPA) ; [o]tC1P, total ceramide 1-phosphate (C1P) ; [p]S1P, sphingosine 1-phosphate.

Supplementary Table 2. Analytical details for determination of S1P, C1P, and LPA in LC-MS/MS

Molecular species	Detection mode	Q1 *m/z*	Q3 *m/z*	Internal standard	Correction factor
S1P					
*d*18:1	positive	380.4	264.4	C1P (*d*18:1/12:0)	2.5
C1P					
*d*18:1/16:0	positive	618.5	264.4	C1P (*d*18:1/12:0)	1.0
*d*18:1/24:1	positive	728.6	264.4	C1P (*d*18:1/12:0)	2.0
*d*18:1/24:0	positive	730.6	264.4	C1P (*d*18:1/12:0)	2.0
LPA					
16:0	negative	409.2	153.0	LPA (17:0)	1.0
16:1	negative	407.2	153.0	LPA (17:0)	1.0
18:0	negative	437.2	153.0	LPA (17:0)	1.0
18:1	negative	435.2	153.0	LPA (17:0)	1.0
18:2	negative	433.2	153.0	LPA (17:0)	1.0
20:3	negative	459.3	153.0	LPA (17:0)	1.0
20:4	negative	457.2	153.0	LPA (17:0)	1.0
20:5	negative	455.2	153.0	LPA (17:0)	1.0
22:6	negative	481.2	153.0	LPA (17:0)	1.0



Supplementary Fig 1. Structures of molecular ions (Q1) and fragment ions (Q3) used for determination of LPA, S1P, and C1P in LC-MS/MS.



Supplementary Fig 2. Standard curves prepared for determination of C1P and S1P using C12:0 C1P as an internal standard.