Research Article
Experimental and Translational Hepatology

JOURNAL
OF HEPATOLOGY

Hepatopulmonary syndrome is associated with low sphingosine-1-phosphate levels and can be ameliorated by the functional agonist fingolimod

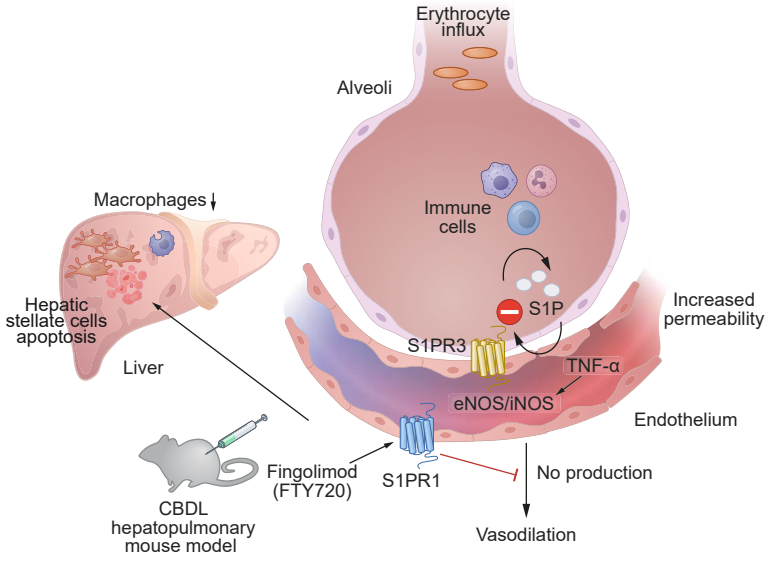
Authors

Sukriti Baweja, Anupama Kumari, Preeti Negi, …, Chhagan Bihari, Rakhi Maiwall, Shiv Kumar Sarin

Correspondence

sukriti@ilbs.in, sukritibiochem@gmail.com (S. Baweja), shivsarin@gmail.com, sksarin@ilbs.in (S.K. Sarin).

Graphical abstract



Highlights

- Hepatopulmonary syndrome is associated with low levels of sphingosine-1-phosphate (S1P).
- Fingolimod (a functional agonist of S1P) improves pulmonary vascular tone, oxygenation, and survival in an experimental model.
- Fingolimod improves hepatocyte proliferation and portal pressure and decelerates hepatic fibrosis in a CBDL mouse model.

Impact and implications

A low level of plasma sphingosine-1-phosphate (S1P) is associated with severe pulmonary vascular shunting, and hence, it can serve as a marker of disease severity in patients with hepatopulmonary syndrome (HPS). Fingolimod, a functional agonist of S1P, reduces hepatic inflammation, improves vascular tone, and thus retards the progression of fibrosis in a preclinical animal model of HPS. Fingolimod is being proposed as a potential novel therapy for management of patients with HPS.

https://doi.org/10.1016/j.jhep.2023.03.018
 J. Hepatol. 2023, 79, 167–180

Research Article
Experimental and Translational Hepatology

JOURNAL
OF HEPATOLOGY

Hepatopulmonary syndrome is associated with low sphingosine-1-phosphate levels and can be ameliorated by the functional agonist fingolimod

Sukriti Baweja[1,*], Anupama Kumari[1], Preeti Negi[1], Arvind Tomar[2], Dinesh Mani Tripathi[1], Akash Kumar Mourya[1], Aayushi Rastogi[3], P. Debishree Subudhi[1], Swati Thangariyal[1], Guresh Kumar[1], Jitendra Kumar[1], G. Srinivasa Reddy[4], Arun Kumar Sood[5], Chitranshu Vashistha[4], Vivek Sarohi[6], Chhagan Bihari[7], Rakhi Maiwall[4], Shiv Kumar Sarin[1,4,*]

Journal of Hepatology **2023**. vol. 79 | 167–180

Background & Aims: Hepatopulmonary syndrome (HPS) is characterised by a defect in arterial oxygenation induced by pulmonary vascular dilatation in patients with liver disease. Fingolimod, a sphingosine-1-phosphate (S1P) receptor modulator, suppresses vasodilation by reducing nitric oxide (NO) production. We investigated the role of S1P in patients with HPS and the role of fingolimod as a therapeutic option in an experimental model of HPS.

Methods: Patients with cirrhosis with HPS (n = 44) and without HPS (n = 89) and 25 healthy controls were studied. Plasma levels of S1P, NO, and markers of systemic inflammation were studied. In a murine model of common bile duct ligation (CBDL), variations in pulmonary vasculature, arterial oxygenation, liver fibrosis, and inflammation were estimated before and after administration of S1P and fingolimod.

Results: Log of plasma S1P levels was significantly lower in patients with HPS than in those without HPS (3.1 ± 1.4 *vs*. 4.6 ± 0.2; p <0.001) and more so in severe intrapulmonary shunting than in mild and moderate intrapulmonary shunting (p <0.001). Plasma tumour necrosis factor-α (76.5 [30.3–91.6] *vs*. 52.9 [25.2–82.8]; p = 0.02) and NO (152.9 ± 41.2 *vs*. 79.2 ± 29.2; p = 0.001) levels were higher in patients with HPS than in those without HPS. An increase in Th17 (p <0.001) and T regulatory cells (p <0.001) was observed; the latter inversely correlated with plasma S1P levels. In the CBDL HPS model, fingolimod restored pulmonary vascular injury by increasing the arterial blood gas exchange and reducing systemic and pulmonary inflammation, resulting in improved survival (p = 0.02). Compared with vehicle treatment, fingolimod reduced portal pressure (p <0.05) and hepatic fibrosis and improved hepatocyte proliferation. It also induced apoptotic death in hepatic stellate cells and reduced collagen formation.

Conclusions: Plasma S1P levels are low in patients with HPS and even more so in severe cases. Fingolimod, by improving pulmonary vascular tone and oxygenation, improves survival in a murine CBDL HPS model.

Introduction

Hepatopulmonary syndrome (HPS) is characterised by a defect in arterial oxygenation induced by pulmonary vascular dilatation in the setting of liver disease.[1] In clinical practice, persistent dyspnoea and the presence of hypoxaemia indicate HPS.[2] In a recent study of the 42,749 individuals, HPS was detected in 194 (0.45%) patients, of which 182 had cirrhosis. Among them, 143 (78.5%) patients underwent contrast-enhanced transthoracic echocardiography, and 98 (54%) had delayed shunting. Forty-one (22.5%) of these patients with cirrhosis had confirmed HPS.[3] Most patients have underlying intrapulmonary vasodilatation, which worsens over time with progressive hypoxaemia.[4] The presence of HPS increases the frequency and severity of complications related to portal hypertension and thus mortality. Moreover, successful transplantation is often limited by progressive hypoxaemia.[5]

The hallmark of HPS is microvascular dilatation in the pulmonary arterial circulation, and such dilatations, especially in alveolar regions, contribute to hypoxaemia.[6] The vasodilation is assumed to result from excessive vascular production of vasodilators, particularly nitric oxide (NO).[7] The exact mechanism of increased endogenous NO production and its relationship to the presence of portal hypertension, hyperdynamic circulation, and degree of liver injury remain uncertain. Overproduction of tumour necrosis factor alpha (TNF-α), as a result of endotoxin stimulation of Kupffer cells is one of the mechanisms leading to

Keywords: Advanced cirrhosis; Liver transplant; Orthodeoxia; Dyspnoea; Liver cirrhosis; Oxygen therapy; Hypoxia; Pulmonary vascular disorders.
Received 5 August 2021; received in revised form 28 February 2023; accepted 3 March 2023; available online 28 March 2023
* Corresponding authors. Addresses: Department of Molecular and Cellular Medicine, Institute of Liver and Biliary Sciences, New Delhi, 110070, India. +91-11-46300000; extension 24113 and fax number 46300010. (S. Baweja); Department of Hepatology, Institute of Liver and Biliary Sciences, New Delhi, 110070, India. +91-11-46300000; extension 24113 and fax number 46300010. (S.K. Sarin).
E-mail addresses: sukriti@ilbs.in, sukritibiochem@gmail.com (S. Baweja), shivsarin@gmail.com, sksarin@ilbs.in (S.K. Sarin).
https://doi.org/10.1016/j.jhep.2023.03.018

HPS development. Endothelin-1 (ET-1) and TNF-α interaction, occurring in the lung vasculature, has been shown in an experimental model to contribute to the development of experimental HPS.[8] Pulmonary microvascular endothelial changes appear to be induced by increased endothelial NO synthase (eNOS)-derived NO production as well as by enhanced expression of inducible NO synthase (iNOS) activity in intravascular macrophages.[9]

At present, there are no effective medical therapies for HPS. In a small subset of patients, pentoxifylline has been found to be effective,[10] possibly owing to its anti-TNF actions.[11] Larger studies are required to confirm the therapeutic potential of pentoxifylline. In another study from our group, we found that the combination of pentoxifylline and rifaximin showed additional benefits over pentoxifylline alone.[12] However, larger studies are needed to provide an effective therapy for HPS. Supplementary oxygen therapy does improve oxygenation in patients with HPS. However, the pulmonary capillaries are grossly dilated, and oxygen molecules from the adjacent alveoli are unable to permeate sufficiently to the centre of the dilated vessel. This diffusion defect is able to be only partially overcome by the use of supplementary oxygen.[13] In addition, results from uncontrolled trials and anecdotal evidence indicate that treatment of HPS with almitrine, antibiotics, beta-blockers, cyclooxygenase inhibitors, systemic glucocorticoids and cyclophosphamide, inhaled NO, NO inhibitors, and somatostatin is not effective.[14,15] The transjugular intrahepatic portosystemic shunt creation in patients with HPS showed only a transient relief from clinical symptoms. However, this was not maintained in half of the patients after 3 months, indicating a limited role of transjugular intrahepatic portosystemic shunt as a bridge to transplantation in patients with HPS.[16] Liver transplantation remains the only effective treatment for patients with HPS till date.

Sphingosine-1-phosphate (S1P) is a naturally occurring bioactive lipid with various biological functions.[17] During normotension conditions, through its receptor, Sphingosine-1-phosphate receptor 1 (S1PR1), it mediates the flow, decreases the vascular tone, and induces the vasoconstriction. During hypertensive conditions, it works through S1PR3 and increases the vascular tone. Its functional agonist, fingolimod, is known to reduce NO levels and vasodilation.[18]

Fingolimod is also an immunomodulator that sequesters lymphocytes in lymph nodes, preventing them from contributing to an autoimmune reaction.[19] It is phosphorylated intracellularly to fingolimod phosphate, which binds to the S1P receptor, and reduces the recirculation of lymphocytes from lymph nodes to blood and peripheral tissue.[20] Presently, it is used as a prophylactic drug in acute rejection after solid organ transplantation and for the treatment of multiple sclerosis.[21] Antifibrotic effects of fingolimod have been reported in renal, cardiac, and muscular tissues.[22]

We hypothesise that a low level of S1P is associated with the development of HPS in cirrhosis. We further evaluated whether supplementation with fingolimod, an S1P agonist, can improve hypoxaemia and vasoconstriction. We undertook the present study to investigate the role of S1P in HPS and its association with systemic inflammation in patients with cirrhosis. As S1P has a short half-life, fingolimod might represent an attractive therapy for HPS.

Patients and methods

Patients

Patients with cirrhosis of the liver seen at the Institute of Liver and Biliary Sciences (ILBS), New Delhi, India, were screened for the presence of HPS and were included in the study. The study was approved by the institutional ethics committee (F.25/5/107/ILBS/AC/2016/11252/299). Patients aged between 18 and 64 yr with HPS were enrolled after taking written informed consent. HPS was diagnosed by alveolar–arterial oxygen gradient >15 mmHg (or >20 mmHg in patients >64 yr of age) and intrapulmonary vasodilatation as confirmed by saline contrast echocardiography. Patients were further classified based on arterial blood gas analysis and were graded as mild (partial pressure of oxygen [PaO_2], 80–90 mmHg), moderate (PaO_2, 60 to <80 mmHg), severe (PaO_2, 50 to <60 mmHg), and very severe (PaO_2, <50 mmHg).[15] Patients with significant intrinsic cardiopulmonary disease, advanced hepatic encephalopathy, inadequate echocardiogram, antibiotic use within the past 1 month, any current use of exogenous nitrates, active infection, or presence of hepatocellular carcinoma or any other malignancy were excluded from the study. The laboratory staff performing the experiments were blinded to the clinical details. Patients were managed according to the standard of care. The patient groups were compared with a group of age- and sex-matched healthy controls (n = 25).

Peripheral blood

Whole blood was subjected to red blood cell lysis followed by immune phenotyping, to analyse the innate and adaptive immune cells. RNA was isolated, and then real-time PCR was used for gene expression analysis of candidate genes. Plasma was separated and stored at -80 °C until further use for cytokine ELISA for S1P, NO, IL-1β, and TNF-α. This is further detailed in the Supplementary methods.

Animal model of common bile duct ligation

The animal study was approved by the Institutional Animal Ethics Committee of the ILBS (IAEC/ILBS/17/01). C57BL/6 male mice (age 10–12 wk) were procured from the Center of Comparative Medicine, ILBS. All the animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals Eighth Edition* (2011) published by the National Research Council of the National Academies and the Committee for the Purpose of Control and Supervision of Experiments on Animals. Details of surgical procedure and scheme of experimentation is found in the Supplementary methods.

Statistical analysis

The baseline clinical parameters of the patients and animals are presented as mean ± SD or median (IQR), as appropriate. The normally distributed data were analysed using Student's *t* test and one-way ANOVA followed by *post hoc* comparisons by the Bonferroni method. The skewed data were analysed using the Kruskal–Wallis test followed by multiple comparisons by adjusting the probability. Spearman's correlation was calculated for correlation analysis. The log transformation for S1P,

TNF-α, and IL-1β was also applied. The Kaplan–Meier analysis was performed for survival analysis. For finding the threshold value, receiver operating characteristic (ROC) curve was used. Statistical analyses were performed using SPSS for Windows version 22 (IBM SPSS, Armonk, NY, USA). The representative graphs were prepared using GraphPad Prism version 8.0 (GraphPad Software, Insight partners, CA, USA).

Results

Patient characteristics

We recruited 297 patients with cirrhosis. These patients were subjected to arterial blood gas analysis. Patients with PaO_2 <90 mmHg (n = 57, 19.6%) and with intrapulmonary shunting on saline contrast echocardiography (n = 44, 14.8%) were included as HPS in the study, and the rest as no HPS. Patients with cirrhosis (n = 89), with PaO_2 >90 mmHg and no evidence of HPS and matched for age, sex, and model of end-stage liver disease (MELD) score with the patients with HPS, were included as disease controls. The clinical parameters between the patients with HPS (group B) and those without HPS (group A) were comparable (Table 1). The heart rate and mean arterial pressure, the Child–Turcotte–Pugh and MELD scores were also comparable. Of the patients, 80% had received diuretics and 60% were on beta-blockers. The aetiology of cirrhosis was dominantly alcohol in both the groups, followed by non-alcoholic fatty liver disease and viral hepatitis (Table 1).

Low levels of S1P predict severe shunting in patients with HPS

At baseline, plasma S1P levels of study patients and healthy controls were measured. We observed significantly reduced S1P concentrations in patients with cirrhosis compared with healthy controls (p <0.001). S1P levels were lower in patients with HPS than in those without HPS (p <0.001) (Fig. 1B).

We also compared the S1P levels in patients with mild, moderate, and severe HPS. We found significantly lower levels of S1P in patients with severe shunting than in those with mild shunting (p <0.001) (Fig. 1C). The PaO_2 levels were lower in patients with HPS than in those without HPS (p = 0.001) (Fig. 1A). The S1P levels directly correlated with PaO_2 levels (r = 0.807, p <0.001), as shown in Table S1. A significant direct correlation of S1P levels with haemoglobin (r = 0.366, p <0.01) and platelets (r = 0.243, p = 0.005) and an inverse correlation with MELD score (r = -0.303, p = 0.003) were seen (Table S1).

The logistic regression analysis showed that the per-unit increase in S1P decreased the risk of HPS by 5% (odds ratio 0.95, 95% CI 0.936–0.967; p = 0.001). The ROC analysis showed an AUC of 0.82 (specificity, 71.4%; sensitivity, 71.6%; accuracy, 71.5%; p = 0.001), a positive predictive value of 54.5%, and a negative predictive value of 84.0% (Fig. 1D).

The ROC analysis of mild *vs*. severe HPS using S1P showed an AUC of 0.78 (specificity, 78.6%; sensitivity, 79.1%; accuracy, 79.3%; p = 0.07), a positive predictive value of 80.1%, and a negative predictive value of 78.6% (Fig. 1E). The overall mortality was significantly higher in patients with HPS than in those without HPS (p <0.001) (Fig. 1F). Based on the area under the ROC of S1P, a cut-off value of 99 ng/ml or more showed 80% sensitivity and 73% specificity for 30-day survival (Fig. 1G). Decreased S1P correlated with higher mortality in patients with cirrhosis.

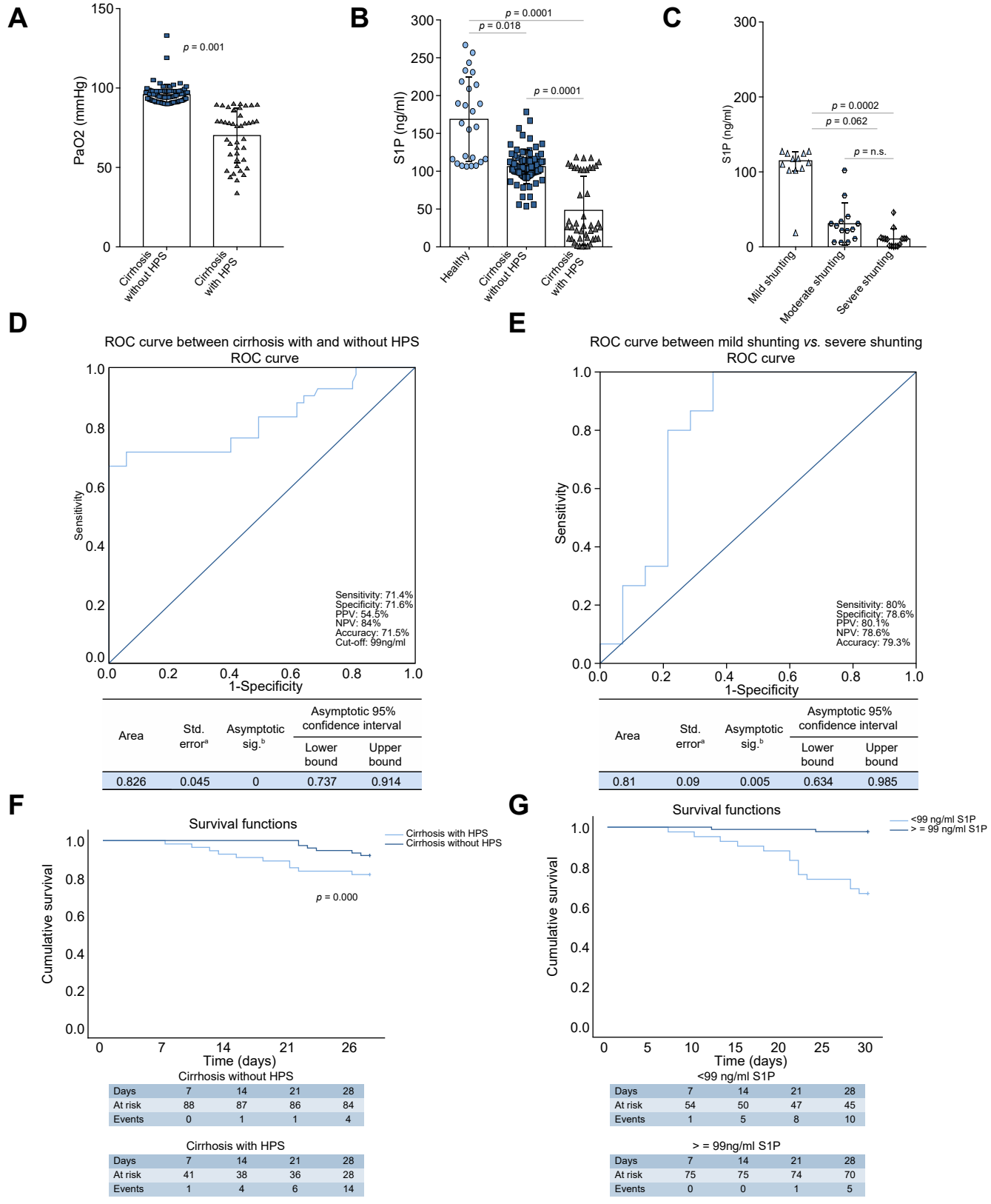
Vasodilators in systemic circulation in patients with HPS

In HPS, the vasodilation is assumed to result from excessive vascular production of vasodilators, particularly NO and pro-inflammatory cytokines such as TNF-α and ET-1. These are implicated in induction of genes via iNOS and eNOS, which in turn are responsible for intrapulmonary vasodilation (Fig. 2A). The plasma NO levels were significantly higher in patients with cirrhosis than in controls, and in patients with HPS than in

Table 1. Baseline characteristics of cirrhosis patients with cirrhosis with and without HPS. Data are presented as mean ± SD or median (IQR) depending on the nature of variables (normally or non-normally distributed).

Variable	Cirrhosis without HPS (group A; n = 89)	Cirrhosis with HPS (group B; n = 44)	p value
Age (yr)	49 ± 10.9	50 ± 9	0.74
Sex (male), n (%)	80 (90.9)	37 (88)	0.64
Haemoglobin (g/dl)	10.6 ± 2.1	9.62 ± 1.9	0.76
RBC counts (10^9/L)	3.2(1.9–5.3)	2.81 (1.74–4.0)	0.84
Platelets (10^9/L)	90 (50–118)	77 (52–101.7)	0.13
Total leucocyte count (10^3 cells/ml)	5.0 (3.4–6.4)	5.8 (4.2–8.5)	0.35
Serum sodium (mEq/L)	134.5 ± 5.6	133.02 ± 5.5	0.17
Serum potassium (mEq/L)	4.1 ± 0.9	3.88 ± 0.4	0.33
Aspartate aminotransferase (IU/ml)	60 (39.5–76.5)	58 (39–82)	0.24
Alanine aminotransferase (IU/ml)	37 (23.1–49.5)	27.2 (18–40)	0.42
Presented with ascites, n (%)			
Grade I	60 (84.5)	26 (72.2)	0.50
Grade II	11 (15.4)	10 (27.7)	
Presented with hepatic encephalopathy, n (%)	4 (4.5)	2 (4.7)	0.12
Presented with bleed, n (%)	1(1.1)	3 (7.1)	0.81
Oesophageal varices, n (%)	73 (82.2)	40 (90.9)	0.92
Gastric varices, n (%)	4 (4.4)	8 (18.1)	0.13
Hepatorenal syndrome, n (%)	2 (2.3)	4 (9.5)	0.35
Aetiology: alcohol, n (%)	54 (61.4)	30 (71)	0.75
Viral hepatitis, n (%)	5 (5.6)	3 (7)	0.82
Non-alcoholic steatohepatitis, n (%)	22 (25)	8 (21)	0.08
Others, n (%)	7 (8)	1 (2.3)	0.38
MELD score	16.2 ± 3.8	20.2 ± 4.3	0.01

Values of p were given for the comparison between patients with cirrhosis with and without HPS, and p <0.05 is considered significant. HPS, hepatopulmonary syndrome; MELD, model for end-stage liver disease; RBC, red blood cell.



Area	Std. error[a]	Asymptotic sig.[b]	Asymptotic 95% confidence interval	
			Lower bound	Upper bound
0.826	0.045	0	0.737	0.914

Area	Std. error[a]	Asymptotic sig.[b]	Asymptotic 95% confidence interval	
			Lower bound	Upper bound
0.81	0.09	0.005	0.634	0.985

Cirrhosis without HPS

Days	7	14	21	28
At risk	88	87	86	84
Events	0	1	1	4

Cirrhosis with HPS

Days	7	14	21	28
At risk	41	38	36	28
Events	1	4	6	14

<99 ng/ml S1P

Days	7	14	21	28
At risk	54	50	47	45
Events	1	5	8	10

> = 99ng/ml S1P

Days	7	14	21	28
At risk	75	75	74	70
Events	0	0	1	5

Fig. 1. Low levels of S1P associated with HPS. (A) The scatterplot depicts the PaO_2 levels (mmHg) in patients with cirrhosis with and without HPS (Student's *t* test; $p = 0.001$). (B) The scatterplot depicts the plasma S1P levels (ng/ml) in healthy controls and patients with cirrhosis without HPS and with HPS. (C) S1P levels in patients with HPS as subgrouped into mild, moderate, and severe shunting (mild *vs*. severe; $p < 0.01$). (D) For finding the threshold value, the ROC curve was used for S1P between patients with cirrhosis with and without HPS. (E) The ROC curve within the patients with HPS of S1P, compared with those with mild–moderate HPS of S1P. (F) Kaplan–Meier curve analysis elucidated the overall mortality in patients with HPS compared with those without HPS ($p = 0.00$). (G) Based on the AUROC of S1P, a cut-off value of 99 ng/ml or more showed 80% sensitivity and 73% specificity for survival and an increase in 30-day mortality in patients with S1P <99 ng/ml. AUROC, area under the ROC curve; HPS, hepatopulmonary syndrome; NPV, negative predictive value; PaO_2, partial pressure of oxygen; PPV, positive predictive value; ROC, receiver operating characteristic; S1P, sphingosine-1-phosphate.

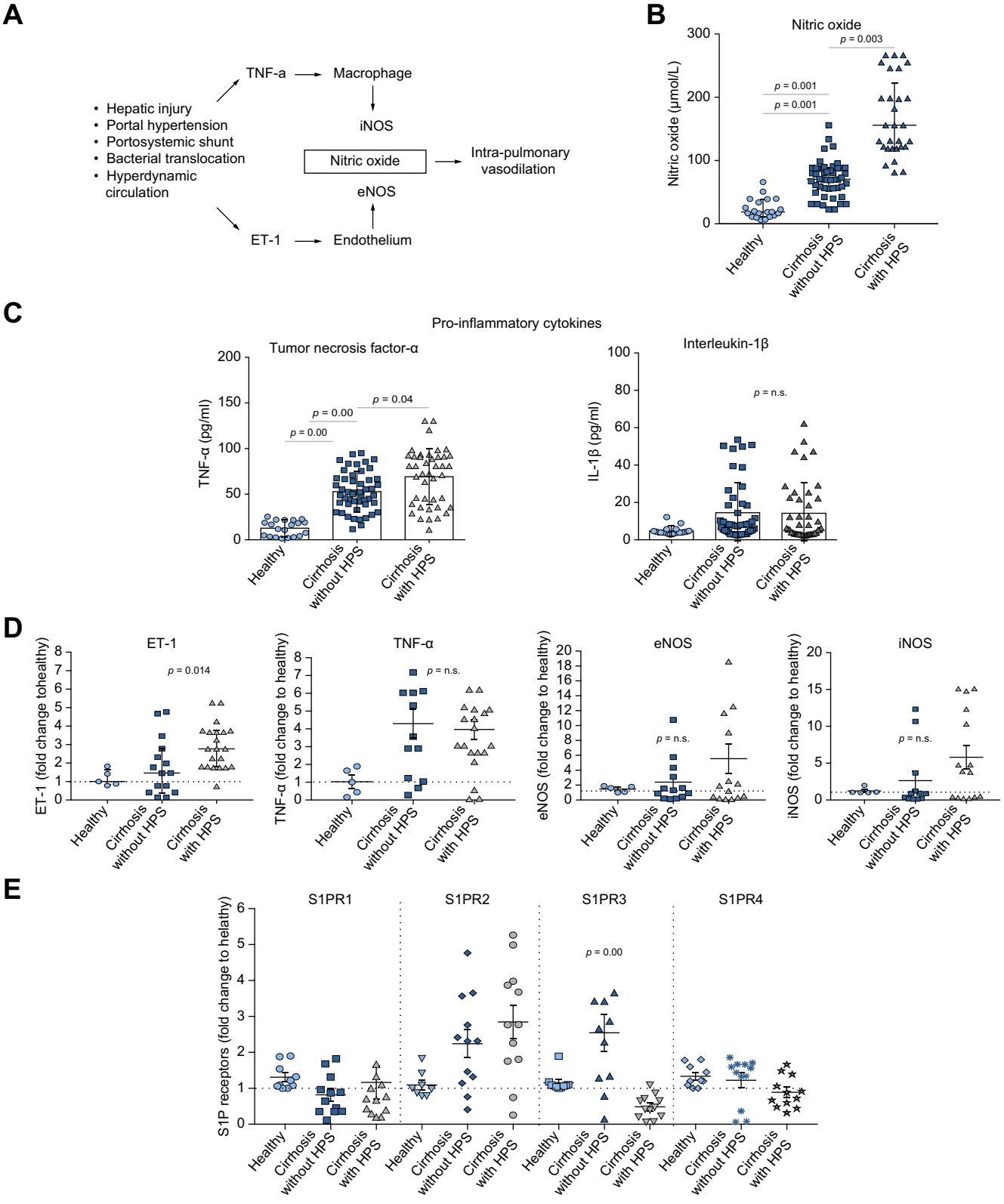
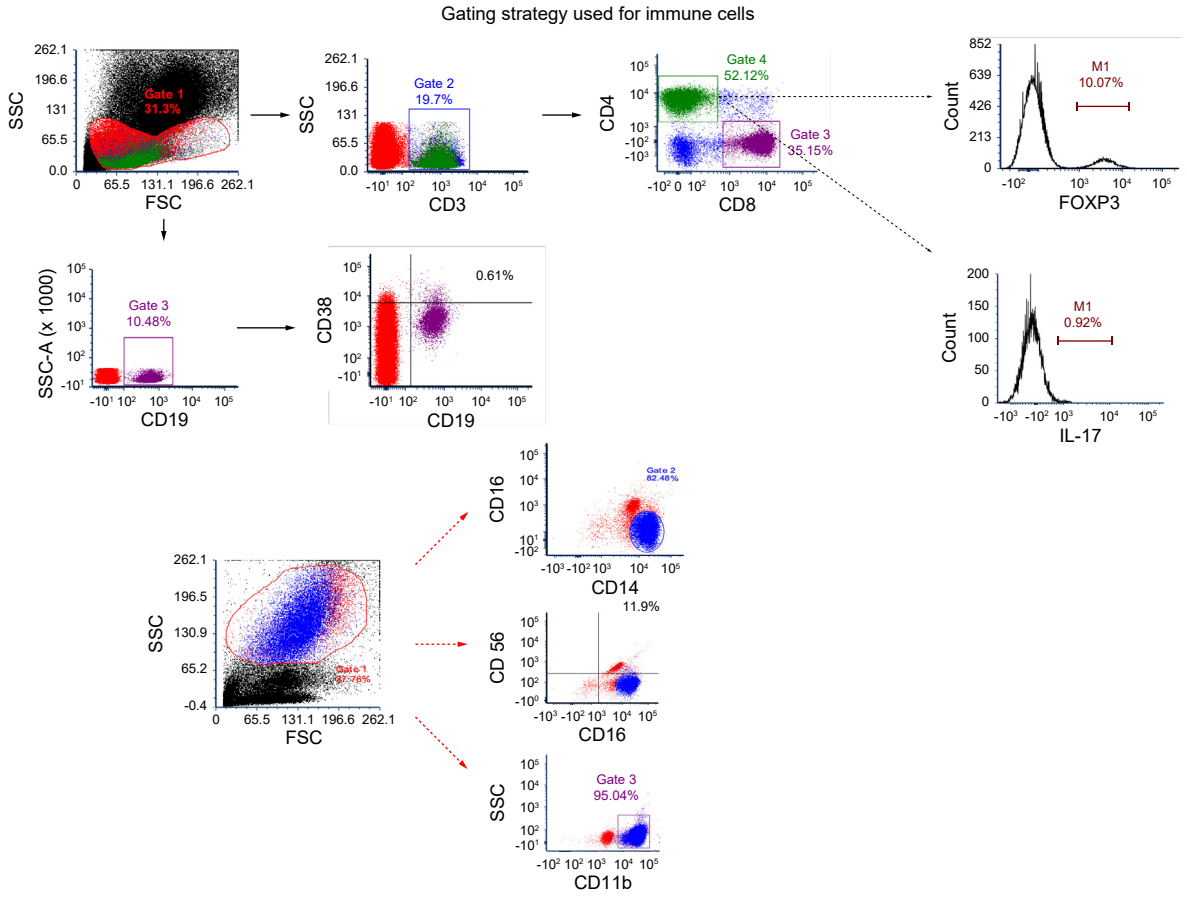


Fig. 2. Vasodilators in systemic circulation in patients with HPS. (A) The flowchart shows the mechanism known for intrapulmonary vasodilation. (B) The scatterplot depicts the plasma NO levels (μmol/L) as measured using human ELISA, which were significantly low in healthy controls than in patients with cirrhosis ($p = 0.001$) in systemic circulation. Patients with HPS had significantly higher levels of NO in circulation than those without HPS ($p = 0.003$) (Kruskal–Wallis test with correction). (C) Peripheral blood plasma cytokine TNF-α level was significantly higher in patients with cirrhosis with or without HPS than in healthy controls ($p = 0.001$) and further increased in patients with HPS compared with those without HPS ($p = 0.04$). (Kurskal–Wallis test with correction). (D) The whole blood mRNA levels of candidate genes regulation associated with NO production was measured using quantitative real-time PCR. The scatterplots represent the fold change values normalised to healthy controls (as calculated by using 2-power to delta CT values). Significant upregulation was found between patients with HPS and those without HPS in ET-1, whereas no significance was achieved in mRNA gene expression in whole blood for TNF-α, eNOS, and iNOS levels. (E) Among all the receptors, that is, S1PR1 to S1PR4, only S1PR3 was significantly downregulated in patients with HPS compared with those without HPS (one-way ANOVA). eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; HPS, hepatopulmonary syndrome; iNOS, inducible nitric oxide synthase; NO, nitric oxide; S1PR1 to S1PR4, S1P receptor 1 to S1P receptor 4; TNF-α, tumour necrosis factor-alpha.

A



B

Frequency of immune cells in HPS patients (%)

Immune Cells	Healthy controls Group 1 median (IQR)	Cirrhosis without HPS Group 2 median (IQR)	Cirrhosis with HPS Group 3 median (IQR)	*p* value Group 1 *vs.* 2	*p* value Group 1 *vs.* 3	*p* value Group 2 *vs.* 3
$CD3^+CD4^+$ (Th cells)	50.6 (47.6-56.1)	15.5 (5.5-23.9)	7.2 (3.7-20.8)	0.000	0.000	n.s.
$CD3^+CD8^+$ (Tc cells)	18.2 (14.2-19.8)	4.4 (1.0-10.3)	3.0 (0.7-6.3)	0.000	0.000	n.s.
$CD3^+CD4^+$ FoxP3 (T regs)	2.3 (2.0-2.8)	1.0 (0.2-1.3)	4.0 (1.7-7.7)	0.000	n.s.	0.000
$CD3^+CD127^+IL\text{-}17^+$ (Th-17 cells)	1.0 (0.7-1.3)	0.05 (.01-0.7)	3.2 (0.8-6.2)	0.001	n.s.	0.000
$CD3^-CD19^+$ (B cells)	10.6 (8.3-16.8)	6.7 (1.8-8.8)	3.9 (1.4-8.0)	0.003	0.001	n.s.
$CD14^+CD16^-$ (Monocytes)	12.8 (12.5-13.0)	2.0 (1.2-2.9)	2.9 (1.2-5.7)	0.000	0.000	n.s.
$CD11b^+CD16^+$ (Neutrophils)	49.3 (47.1-51.3)	56.6 (51.3-64.4)	57.0 (50.7-65.9)	0.006	0.008	n.s.
$CD16^+CD56^+$ (NK cells)	13.3 (12.5-16.5)	1.9 (1.2-2.9)	2.5 (0.9-4.4)	0.000	0.000	n.s.

C

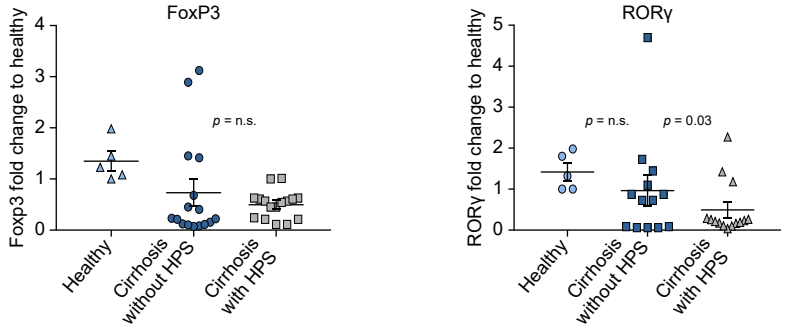


Fig. 3. Elevated population of Treg and Th17 cells in patients with HPS. (A) The flow cytometry was used to determine the frequency of various immune cells. The dot plots show the gating strategy used to enumerate different immune cells in peripheral blood. (B) The table shows percentage frequency of various immune cells in healthy patients and those with cirrhosis with HPS and without HPS (depicted in median [IQR], using the Kruskal–Wallis test followed by multiple comparisons by adjusting the probability). (C) Performed mRNA gene expression in whole blood. Data are presented as fold change with reference to healthy controls for transcription factors for Treg cells (FoxP3) in patients with and without HPS. For Th17 transcription factor-associated (RoRg) mRNA levels were lower in patients with HPS than in those without HPS (one-way ANOVA). HPS, hepatopulmonary syndrome; NK, natural killer; Treg, T regulatory. (This figure appears in color on the web.)

those without HPS (p = 0.003) (Fig. 2B). Serum TNF-α levels were higher in patients with cirrhosis with and without HPS than in controls (p <0.001) (Fig. 2C). We found an inverse correlation of S1P with TNF-α (r = -0.356, p <0.001) and IL-1β (r = -0.371, p <0.001) (Table S1). In the whole blood, an upregulation in the mRNA levels of ET-1 gene was seen in patients with HPS (p = 0.014) (Fig. 2D).

S1P also regulates the NO generation with internalisation of its different receptors. We found that among various receptors, namely, S1PR1 to S1PR4, only S1PR3 mRNA was down-regulated in patients with HPS (p <0.001) (Fig. 2E).

Increased population of Treg and Th17 cells in patients with HPS

S1P is known for stimulating lymphocyte trafficking. To understand the correlation between low levels of S1P with inflammation and immune cells, we performed the multicolour flow cytometric immune phenotyping in the whole blood of patients and controls. The gating strategy used for whole blood analysis of immune cells is shown in Fig. 3A. We found a higher number of T regulatory (Treg) cells and IL-17-producing Th17 cells in patients with HPS (p <0.001) (Fig. 3B). An inverse correlation between S1P levels and Treg cell populations was noted in patients with cirrhosis (r >0.3, p = 0.00).

Reversal of hypoxaemia and intrapulmonary shunting in the CBDL HPS mouse model

The therapeutic efficacy of fingolimod was evaluated in a murine model of common bile duct ligation (CBDL)-induced HPS. The scheme of CBDL mouse model development and administration of i.p. S1P (1 μM/kg/body weight) and fingolimod (1 mg/kg/body weight) is shown in Fig. 4A. The doses were

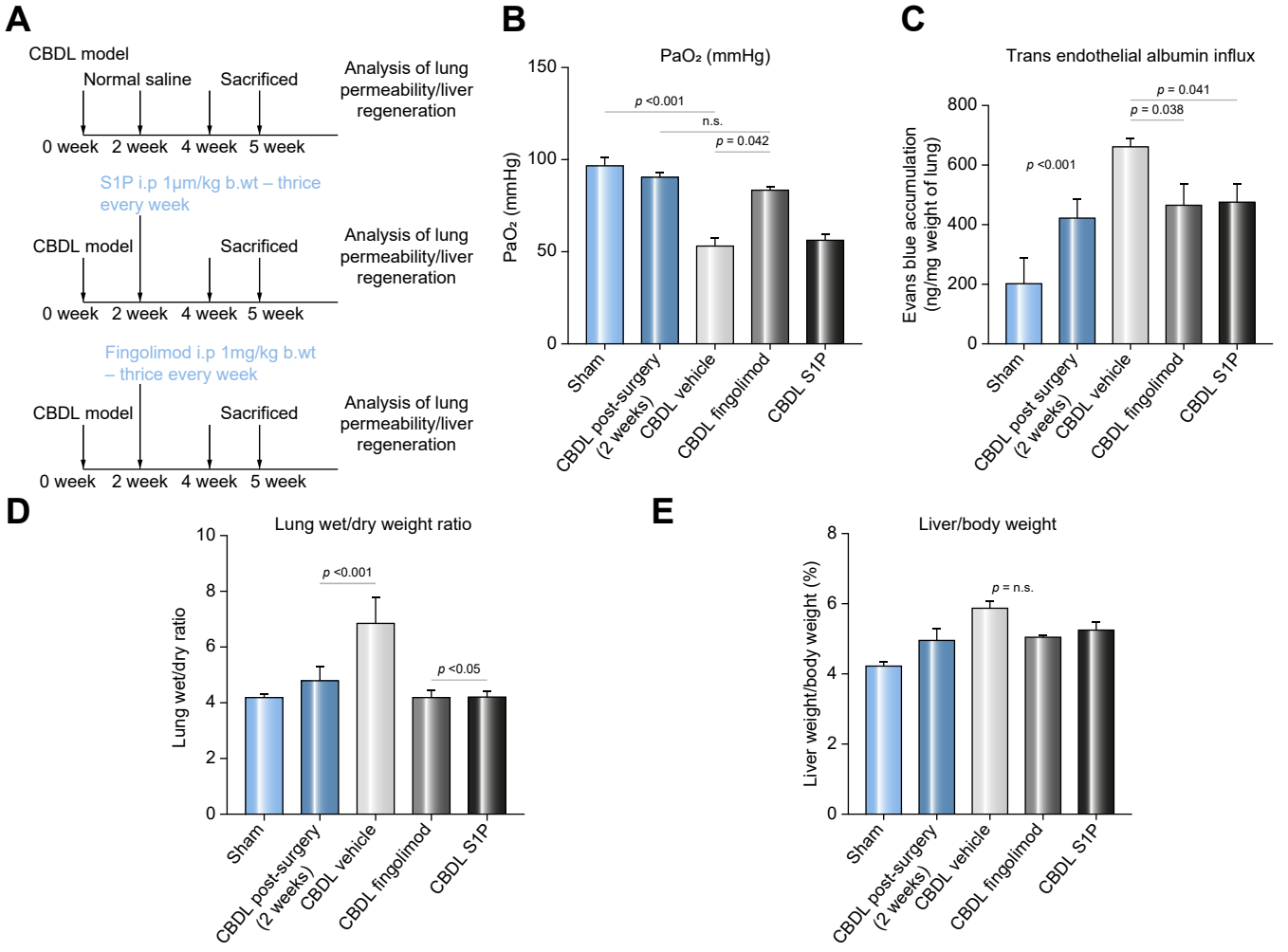


Fig. 4. Reversal of hypoxaemia and intrapulmonary shunting in the CBDL HPS mouse model. (A) The scheme of CBDL mouse model development and administration of i.p. S1P (1 μM/kg/body weight) every alternate day started 2 wk of post-surgery , and fingolimod (1 mg/kg/body weight) was administered twice a week 2 wk of post-surgery in CBDL mouse model. (B) At 2 wk post surgery, the CBDL mice suffered from significant hypoxaemia (as demonstrated by bar diagrams based on arterial blood gas analysis), which significantly improved after fingolimod treatment. (C) The presence of intrapulmonary shunting was evaluated using Evans blue dye by albumin accumulation in the lungs. The bar graph shows the transendothelial albumin influx in ng/mg weight of the lung: sham-operated *vs*. CBDL post-surgery 2 wk without any intervention *vs*. CBDL vehicle *vs*. CBDL fingolimod *vs*. CBDL S1P (one-way ANOVA). (D) The lung oedema was estimated by lung wet/dry ratio: sham-operated *vs*. CBDL post-surgery 2 wk without any intervention *vs*. CBDL vehicle *vs*. CBDL fingolimod *vs*. CBDL S1P. (E) The CBDL mouse model resulted in increased liver weight starting from wk 1 and continuing until wk 4 and decreased body weight at wk 4 post surgery, compared with the liver weight of sham-operated mice, but did not achieve significance (one-way ANOVA). CBDL, common bile duct ligation; HPS, hepatopulmonary syndrome; PaO_2, partial pressure of oxygen; S1P, sphingosine-1-phosphate.

determined after initial experiments, where three different doses for S1P (0.2, 0.5, and 1 μM/kg) and fingolimod (200 ng, 400 ng, and 1 mg/kg) were investigated. The highest dose of S1P and fingolimod gave high mortality, whereas the lowest doses for both did not show efficacy; thus, the medium dose was accepted (Fig. 4A).

We studied the acute effects of fingolimod administration after 24 h but did not find any effect on hypoxaemia and portal pressure (Fig. S1).

At 2 wk post surgery, the CBDL mice suffered from significant hypoxaemia compared with sham-operated animals (p <0.001), and the PaO_2 levels reduced further at Wk 4 in vehicle control mice compared with those in the sham-operated animals (p = 0.001). In the fingolimod-treated mice, the PaO_2 levels did not decline any further and had improved oxygenation compared with those in the vehicle-treated CBDL animals (p = 0.042) (Fig. 4B).

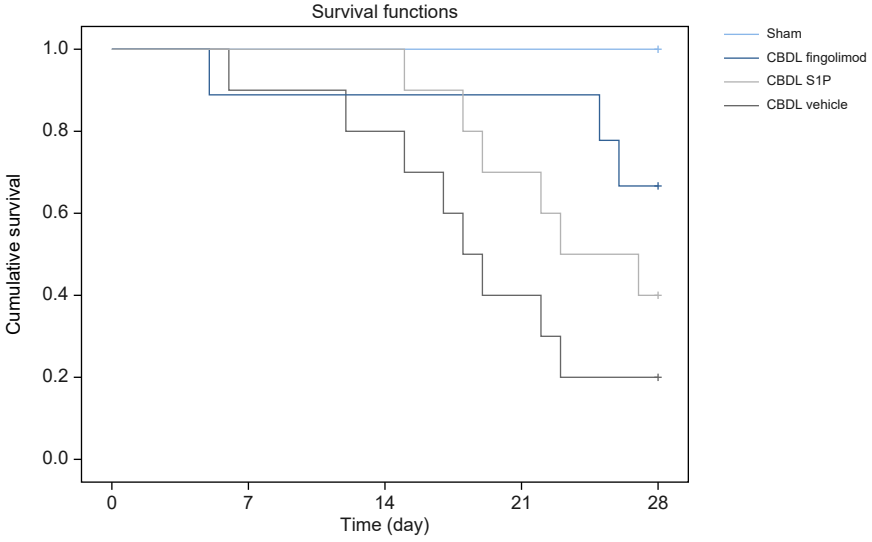
Intrapulmonary shunting was evaluated using Evans blue dye by albumin accumulation in the lungs. The transendothelial albumin influx was significantly higher in vehicle control mice than in the sham-operated animals (p <0.001), at both Wk 2 and 4 post surgery (Fig. 4C). In both the fingolimod- and S1P-treated groups, the Evans blue dye accumulation decreased compared with that in the vehicle-treated animals (p = 0.041 and p = 0.038, respectively). In addition, the lung wet/dry ratio was higher after 2 wk in CBDL animals than in sham-operated animals (p <0.001). The lung oedema improved in fingolimod- and S1P-treated mice compared with that in CBDL vehicle mice at 4 wk (p <0.05) (Fig. 4D). CBDL animals showed a non-significant increase in liver weight until wk 4 and then a decrease post surgery compared with that of sham-operated mice (Fig. 4E).

Fingolimod improves survival in the CBDL mouse model

Four weeks after CBDL, the vehicle control mice, compared with the sham-operated animals, had higher mortality (p <0.001) (Fig. 5). Fingolimod-treated mice had reduced 28-day mortality compared with that of the vehicle-treated group (p = 0.023) (Fig. 5). A reduction in mortality was also found in mice with S1P administration compared with the vehicle control animals, although the difference was not significant.

Pulmonary inflammation in bronchoalveolar lavage

In the lung tissues, we observed necrosis and disruption of normal alveoli in CBDL vehicle, which were comparable after fingolimod and S1P treatment (Fig. 6A). We investigated the bronchoalveolar lavage (BAL) fluid in all animal treatment groups. We observed macrophage infiltration and activation of macrophages in CBDL mice, but macrophage infiltration was significantly reduced in fingolimod-treated mice (84 ± 26 *vs*. 12 ± 5 per area; p = 0.002). Furthermore, in S1P-treated mice, in



Group		Pairwise comparisons							
		Sham		CBDL vehicle		CBDL fingolimod		CBDL S1P	
		Chi-square	*p* value	Chi-square	*p* value	Chi-square	*p* value	Chi-square	*p* value
Log-rank (Mantel-Cox)	Sham			10.813	0.001	3.024	0.082	6.657	0.01
	CBDL	10.813	0.001			5.171	0.023	1.938	0.164
	CBDL + fingolimod	3.024	0.082	5.171	0.023			1.362	0.243
	CBDL + S1P	6.657	0.01	1.938	0.164	1.362	0.243		

Fig. 5. Fingolimod improves survival in the CBDL mice model. Pairwise comparisons using the log-rank test was performed for multiple comparisons. The Kaplan–Meir plot shows that sham-operated animals had higher mortality than CBDL vehicle mice. On administration of fingolimod, a significant decrease in mortality was observed at 28 days compared with that of vehicle treated group. A reduction in mortality was also found in S1P-administered animals compared with that of vehicle treated animals, although the difference was not significant. CBDL, common bile duct ligation; S1P, sphingosine-1-phosphate.

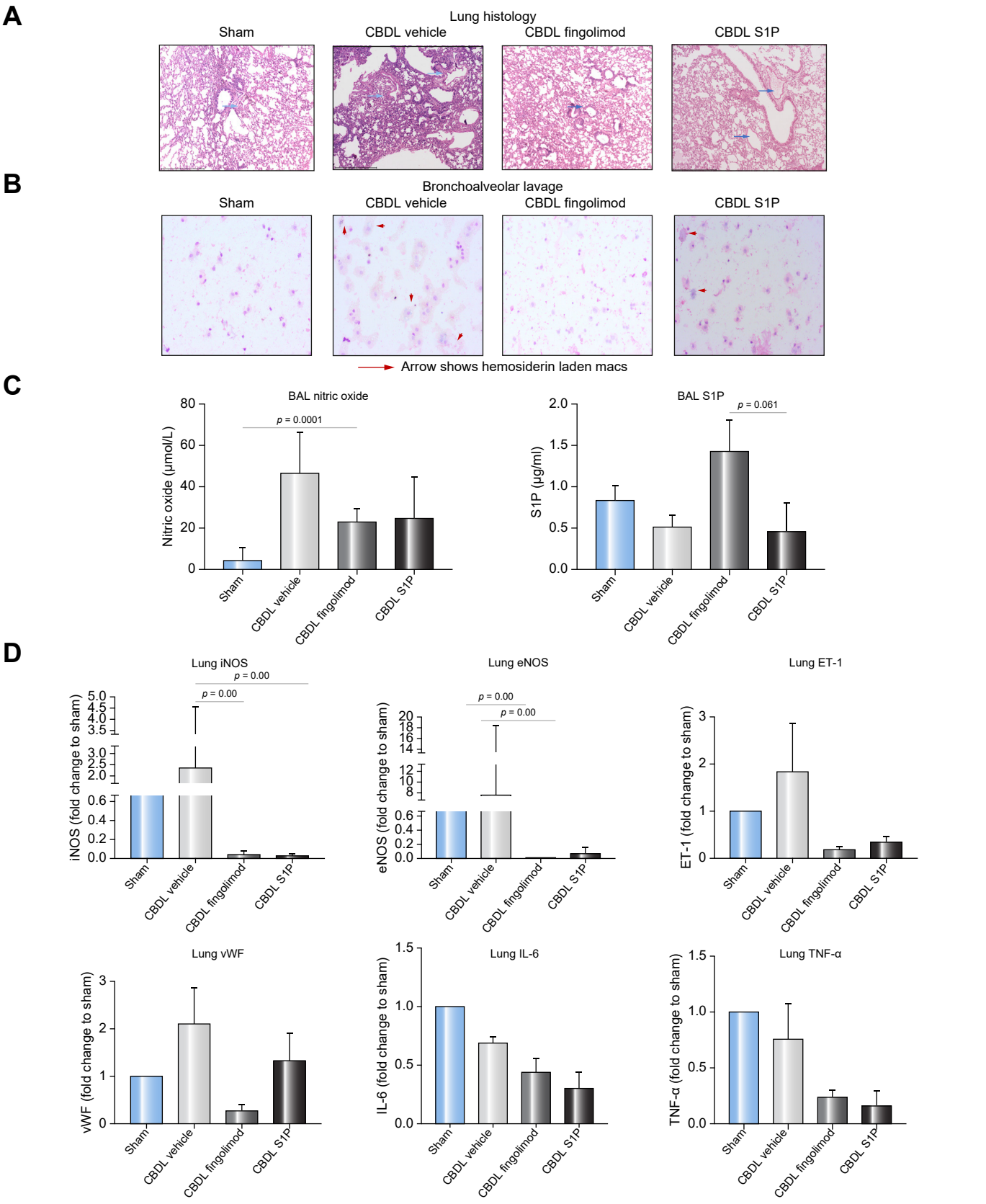


Fig. 6. Pulmonary Inflammation in BAL. (A) The lung histology shows necrosis and disruption of normal vasculature in the CBDL vehicle-treated group, and these improved after fingolimod treatment.(B) In addition, BAL fluid H&E staining along with haemosiderin stain was performed. The representative micrographs show macrophage infiltration and activated macrophages as positive for haemosiderin laden stains. (C) The bar graphs show the nitric oxide and S1P levels in BAL fluid. (D) The bar graphs shows the mRNA levels of candidate genes associated with nitric oxide synthesis and inflammation in lungs. Upon observing the gene regulation in the lung tissue, we found a significant reduction of >10-fold in eNOS, >5-fold in iNOS, and >0.5-fold in CBDL fingolimod- and S1P-treated mice compared with those in CBDL vehicle-treated mice (fold change to sham-operated mice); all the mRNA level fold change values were calculated after normalising to the sham-operated mice. One-way ANOVA was used. The scale bars for H&E staining is 200 μm; the arrows shows the positive staining. BAL, bronchoalveolar lavage; CBDL, common bile duct ligation; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; iNOS, inducible nitric oxide synthase; S1P, sphingosine-1-phosphate; TNF-α, tumour necrosis factor-alpha. (This figure appears in color on the web.)

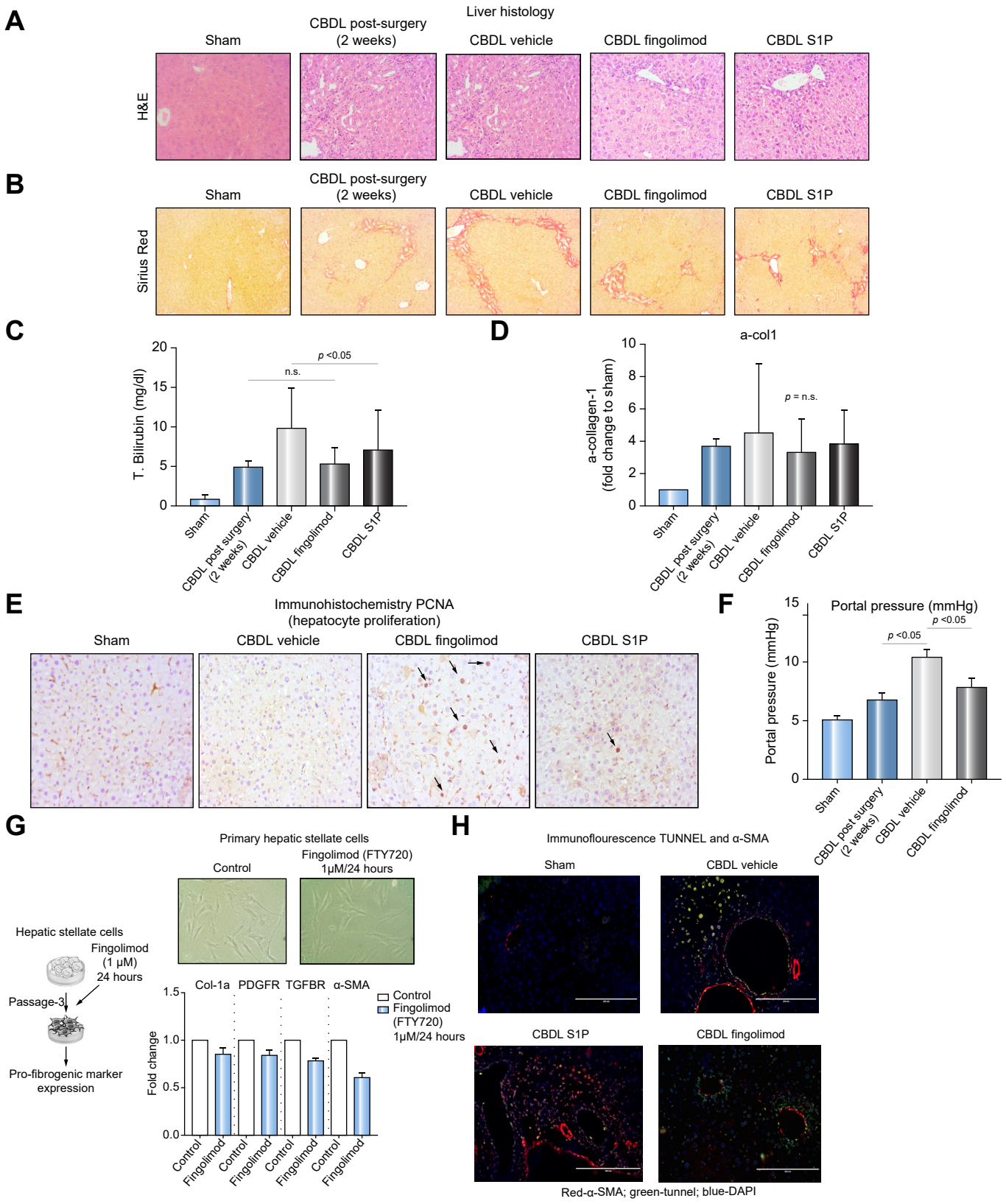


Fig. 7. Effect of S1P and fingolimod treatment on hepatic fibrosis. (A) Liver histology is visualised using H&E staining. In the CBDL mouse model, at 2 wk post surgery, liver histology showed bile duct proliferation and inflammation, whereas no morphological changes in sham-operated mice were observed. After administration of fingolimod, no further increase in inflammation, cholestasis, and liver fibrosis scores was observed compared with scores in 2-wk post-surgery CBDL mice. (B) Liver fibrosis as analysed using Sirius Red staining. Fibrosis as visualised by Sirius Red staining started by Wk 2 in post-surgery CBDL mice. On administration of fingolimod, a reduction is noticed in inflammation with reduction in inflammatory cells, cholestasis, and liver fibrosis compared with those on vehicle treatment. (C) The

comparison with vehicle-treated mice, the infiltration was reduced (84 ± 26 *vs*. 24 ± 18 per area; $p = 0.032$) (Fig. 6B).

Fingolimod- and S1P-treated mice had significantly reduced BAL-NO levels compared with those of the vehicle control group ($p < 0.001$) (Fig. 6C). To understand the compartmentalisation of S1P, we estimated S1P in the plasma of the hepatic vein, the heart, and pulmonary venous blood. The S1P levels in different organs were comparable (Table S2).

Upon observing the gene regulation in the lung tissue, we found a significant reduction of >10-fold in eNOS, >5-fold in iNOS, and >0.5-fold in ET-1 levels in fingolimod- and S1P-treated mice (fold change to sham-operated mice; $p < 0.001$) (Fig. 6D).

Effect of S1P and fingolimod on hepatic fibrosis

In the CBDL experimental mouse model, liver histology showed bile duct proliferation and inflammation, whereas no morphological changes in sham-operated animals were observed. In the CBDL mouse model, liver fibrosis was observed by Wk 2, which progressively increased by Wk 4 (Fig. 7A and B). After administration of fingolimod, we found no further increase in inflammation, cholestasis, and liver fibrosis scores compared with scores of 2-wk post-surgery CBDL mice (Fig. 7A). A significant difference was seen in Sirius Red staining and inflammation after fingolimod treatment, compared with those in CBDL vehicle controls and S1P-treated mice.

Serum bilirubin levels increased by 4 wk post surgery in the CBDL vehicle group compared with those in the sham-operated group ($p = 0.001$), but there was no difference in the fingolimod-treated group and the 2-wk post-surgery vehicle CBDL group (Fig. 7C). The total liver pro-collagen-1 alpha gene expression was comparable between groups (Fig. 7D).

We also measured Mdr2 (Abcb4) gene expression and found that fingolimod-treated animals had improved expression of mdr2 compared with that of controls or S1P-treated CBDL animals, indicating the beneficial effects of fingolimod on improving cholestasis (Fig. S2).

Next, the hepatocyte proliferation was quantitatively measured using proliferating cell nuclear antigen staining. Hepatocytes were found to proliferate more after fingolimod treatment (8 ± 5 per area) than after vehicle treatment (0 per area; $p < 0.001$) or S1P treatment (2 ± 1 per area; $p < 0.001$) (Fig. 7E).

In the 2-wk post-CBDL mice, the portal pressure significantly increased (6.7 ± 0.4 mmHg) compared with that in the sham-operated animals (5.0 ± 0.2 mmHg; $p < 0.05$). We found 25% reduction in portal pressure (7.8 ± 0.6 mmHg) in fingolimod-treated mice at 4 wk compared with vehicle-treated mice (10.4 ± 0.5 mmHg; $p < 0.05$). The reduction in portal pressure was partially accompanied by reduction in fibrosis, as evidenced by reduced collagen proportionate area in fingolimod-treated animals (Fig. 7F).

Fingolimod treatment caused a significant decrease in ductular proliferation, accompanied by reduced mononuclear cell recruitment and low mRNA levels of inflammatory cytokines. This suggests that reduction in hepatic fibrosis may lead to improved pulmonary vasodilation. To investigate it further, primary hepatic stellate cells were isolated from wild-type mice and cultured. On treatment with fingolimod *in vitro*, we found a decrease in expression of pro-fibrogenic genes such as transforming growth factor beta, alpha-smooth muscle actin, and pro-collagen-1 alpha (Fig. 7G).

To understand the mechanism of action of fingolimod, we performed the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay on hepatic stellate cells. A significantly higher expression of TUNEL and alpha-smooth muscle actin colocalisation in liver tissues of CBDL mice treated with fingolimod was observed than in vehicle control and S1P-treated animals ($p = 0.001$). Immunohistochemistry showed lower levels of pro-collagen-1 alpha but comparable levels of TIMP1 and MMP9 expression in fingolimod-treated mice than in vehicle controls ($p = 0.03$) (Fig. 7H). This indicates that fingolimod induces hepatic stellate cell deactivation or death, resulting in reduced collagen formation and fibrosis.

As proof of concept, proteomic analysis was done on whole liver tissues of CBDL mice and analysed using the PLS-DA plot. The partial least squares-discriminant analysis (PLS-DA) plot segregated fingolimod-treated animals from sham-operated and vehicle-treated ones with distinct expression of various proteins (Fig. S3A and B). The fingolimod-treated animals had lower collagen-1 alpha and collagen-1 alpha 2 sub-forms. An upregulation in leutrienes, kyuneurine, and asialoglycoprotein receptor II was seen, suggesting that after fingolimod treatment, metabolic changes occur, which might aid in attenuating fibrosis progression (Fig. S3C). This was also supported by the significantly lower expression of macrophage inflammatory protein-1 alpha and monocyte chemotactic protein-1 with lower infiltration of macrophages in the liver (Fig. S3D).

Discussion

The results of this novel study show that patients with cirrhosis with HPS have significantly lower levels of S1P than those without HPS. High plasma TNF-α, NO levels, and Th17 and

serum bilirubin levels are represented as bar graphs. A significant increase is seen in the CBDL vehicle group compared with the sham-operated mice, and no further elevation in CBDL fingolimod-treated mice compared with CBDL vehicle-treated mice ($p = 0.05$) was observed. (D) The bar diagram shows a fold change difference in mRNA expression of pro-fibrogenic gene pro-col-1α from liver tissues of different CBDL groups. (E) The hepatocyte proliferation was quantitatively measured using PCNA staining. Hepatocytes were found to proliferate more after fingolimod treatment than without vehicle treatment or after S1P treatment. (F) Haemodynamic assessment measured the portal pressure in the experimental CBDL mouse model. The bar diagram shows the portal pressure (mmHg). In 2-wk post-CBDL mice, the portal pressure significantly increased in comparison with that in the sham-operated animals. The portal pressure decreased in fingolimod-treated mice in comparison with that in vehicle treated mice. (G) The primary hepatic stellate cells isolated from wild-type mice and cultured until passage 3 before being converted to myofibroblasts. On treatment with fingolimod for 24 h *in vitro*, the mRNA expression was estimated using real-time PCR for pro-fibrogenic genes such as TGF-β, α-SMA, pro-col-1α, and PDGFR. The bar shows the fold change normalised to vehicle-treated controls. One-way ANOVA was used. The scale bars for H&E staining is 200 μm; the arrows shows the positive staining. (H) The immunofluorescence was done for TUNEL and for α-SMA (marker for HSCs) to estimate the apoptosis of HSCs. The confocal microscopy was performed to capture the images (TUNEL, 488 nm; α-SMA, 594 nm). α-SMA, alpha smooth muscle actin; CBDL, common bile duct ligation; col-1α, collagen-1 alpha; HSC, hepatic stellate cell; PCNA, proliferating cell nuclear antigen; PDGFR, platelet-derived growth factor receptor; S1P, sphingosine-1-phosphate; TGF-β, transforming growth factor beta; TGFBR, transforming growth factor beta receptor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling. (This figure appears in color on the web.)

Treg cell populations in systemic circulation indicate a hyper-inflammatory state in patients with HPS. In the murine CBDL model of HPS, administration of fingolimod, a structural and functional analogue of S1P, ameliorated the vascular injury and restored the vascular functions by increasing the arterial blood gas exchange. Fingliomod reduced systemic inflammation and portal pressures, and attenuated hepatic fibrogenesis, resulting in improved animal survival.

Patients with cirrhosis have multiple concurrent pathophysiological events, including hepatocellular and endothelial injury, release of toxic metabolites, systemic inflammation, immunologic disbalance, and progressive deterioration in liver functions.[23] Bacterial infections in patients with cirrhosis can trigger acute deterioration and are linked with low S1P levels and high mortality.[24] Our data supplement these observations as we also found lower S1P levels in patients with HPS than without HPS.

In the circulatory system, red blood cells constitute 95% of total blood cells and are the main source of S1P, followed by endothelial cells.[25] Although we found lower haemoglobin concentration in patients with HPS despite hypoxaemia, the red blood cell counts were comparable with those in patients without HPS. The liver plays a crucial role in maintaining the S1P gradient in the blood as hepatocytes express and secrete majority of S1P carriers in the blood, namely, apolipoprotein M (65%) and albumin (30%) respectively. Apolipoprotein M binds S1P with high affinity in a hydrophobic binding pocket.[26] In severe systemic inflammation, as is found in HPS, maintenance of vascular barrier function is crucial to prevent complications, such as haemorrhage, tissue ischaemia, and oedema. This observation has a major bearing on the natural history of patients with HPS. Furthermore, S1P plays a major role in maintaining endothelial functions.[27] Our observations of a marked reduction in S1P levels in patients with cirrhosis with HPS reflect defects in vascular function including tone. Low S1P levels in patients with cirrhosis could partly be attributable to deranged functions of a recently identified S1P exporter, Mfsd2b, which is highly expressed in erythrocytes and platelets. It exports and contributes approximately up to 50% of plasma S1P.[28] It would be interesting to study the status of this exporter further.

S1P has also been identified as a mediator of lymphocyte egress from lymphoid tissues to blood and is modulated by S1P/S1PR1 interactions.[29] We observed low population of Treg cells and high Th17 T-lymphocyte subsets, which were directly proportional to low levels of S1P in plasma. These observations, taken together, link a protective role of S1P in the prevention of progression of HPS and allow the introduction of potential therapies for these patients. It would seem logical to raise plasma S1P levels therapeutically. However, the use of S1P poses challenges as endogenous S1P has a very short half-life.[30] It should also be taken into account that S1P has pleiotropic effects that mainly depend on the S1P receptor expression pattern on targeted cell types.[31] Therefore, activating or deactivating specific S1P receptors may be a better approach.

A structural analogue of S1P, fingolimod, is a potent functional antagonist of the S1P subtype 1 receptor and has been approved to treat patients with relapsing multiple sclerosis since 2010.[21] Treatment with this novel immunomodulator seems to reduce endothelial permeability in systemic inflammation. It has also been shown to modulate S1P-related lymphocyte egress into the blood.[27] These preclinical observations encourage investigative use of this agent in endothelial barrier enhancement and immunomodulation. In the present study, we observed that fingolimod was a potent agent to reduce systemic and pulmonary inflammation, enhance hepatocyte proliferation, and reduce portal pressure with improvement in arterial blood gases. The S1P administration itself also showed similar trends, but owing to the short half-life, the results were inferior to those of administration of fingolimod. Specific hepatoprotective effects of fingolimod have been described earlier in rodent ischaemia reperfusion models in both normal and cirrhosis animals.[32] Fingolimod can act on multiple target.[33] It reduces macrophage accumulation in liver by reducing monocyte chemoattractants, namely, monocyte chemotactic protein 1 and macrophage inflammatory protein-1 alpha.[33] It also downregulates matrix metalloprotease 2 and 9 in the glioblastoma cell line.[34] These biological properties make fingolimod an attractive therapeutic option.

However, drug-induced liver injury has been reported in patients with multiple sclerosis treated with Fingolimod.[35] These adverse events include increase in liver enzymes up to three times the upper limit of normal in 8% of patients.[36] There might be additional concerns in patients with cirrhosis owing to possible reduction of NO production in the intrahepatic circulation, which can affect portal pressure gradients which needs further investigations.

In the experimental CBDL model, we demonstrated that fingolimod treatment significantly decreased ductular proliferation and mononuclear cell recruitment and lowered mRNA levels of inflammatory cytokines. The increase in portal pressure from Wk 2 to 4 was probably a result of persistently raised intrahepatic resistance and progressive fibrosis. It is noteworthy that chronic administration of fingolimod led to a significant amelioration in portal pressure. Furthermore, our data clearly demonstrate that liver fibrosis progression was attenuated by fingolimod via apoptosis of hepatic stellate cells with reduced production of collagen. Hence, we propose that fingolimod is a potent drug, which has multiple targets through which it reduces inflammation, vascular injury, fibrotic activity, and portal pressure. The improvement in liver fibrosis also helped increase arterial blood gas exchange and reduced systemic and pulmonary inflammation, and mortality.

HPS is not a very common complication of cirrhosis and is often underdiagnosed. Our study was carried out in 44 well-documented patients and provided useful clinical cut-offs. It would be worthwhile to measure S1P levels in the pulmonary tissue or in BAL in patients with cirrhosis. We believe that our observations stand in line with current knowledge of the role of S1P in severe inflammatory conditions and hepatic diseases, and we demonstrate it to be a key mediator in the pathogenesis and progression of HPS in patients with cirrhosis. Measurement of S1P levels can help stratify patients requiring priority treatment including liver transplantation.

Our data justify further elucidation of the diagnostic and therapeutic role of S1P and its functional analogue, fingolimod, in patients with HPS.

Affiliations

[1]Department of Molecular and Cellular Medicine, Institute of Liver and Biliary Sciences, New Delhi, India; [2]Department of Pulmonary Medicine, Institute of Liver and Biliary Sciences, New Delhi, India; [3]Department of Epidemiology, Institute of Liver and Biliary Sciences, New Delhi, India; [4]Department of Hepatology, Institute of Liver and Biliary Sciences, New Delhi, India; [5]Department of Cardiology, Institute of Liver and Biliary Sciences, New Delhi, India; [6]Indian Institute of Technology, Mandi, Suran, India; [7]Department of Pathology, Institute of Liver and Biliary Sciences, New Delhi, India

Abbreviations

BAL, bronchoalveolar lavage; CBDL, common bile duct ligation; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; HPS, hepatopulmonary syndrome; ILBS, Institute of Liver and Biliary Sciences; iNOS, inducible nitric oxide synthase; MELD, model for end-stage liver disease; NO, nitric oxide; PaO_2, partial pressure of oxygen; ROC, receiver operating characteristic; S1P, sphingosine-1-phosphate; S1PR1 to S1PR4, S1P receptor 1 to S1P receptor 4; TNF-α, tumour necrosis factor-alpha; Treg, T regulatory; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

Financial support

The work is funded by Science and Engineering Research Board (SERB) Early Career Grant no. ECR/2016/001687.

Conflicts of interest

All authors have declared no conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Contributed to hypothesis formation, experimental design, intellectual support, supervision of the study, and manuscript writing: SB, SKS. Performed the experiments and data collection: SB, DMT, SG, PDS, ST, AK, PN, JK. Did the patient selection and patient sample collection: AT, CV, AKS, RM. Contributed to statistical and data analysis: GK, AR. Performed pathological analysis: CB. Performed the proteomics and its analysis: VS. Have approved this manuscript: all authors.

Data availability statement

The data that support the findings of this study are available from the corresponding author.

Acknowledgment

We wish to thank Dr. Basak Trayambak from IIT Mandi for his extensive help in analysis of liver proteomics data of CBDL experimental mouse model.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2023.03.018.

References

[1] Rodríguez-Roisin R, Krowka MJ. Hepatopulmonary syndrome—a liver-induced lung vascular disorder. N Engl J Med 2008;358:2378–2387.

[2] Fallon MB, Abrams GA. Pulmonary dysfunction in chronic liver disease. Hepatology 2000;32:859–865.

[3] Bommena S, Gerkin RD, Agarwal S, Raevens S, Glassberg MK, Fallon MB. Diagnosis of hepatopulmonary syndrome in a large integrated health system. Clin Gastroenterol Hepatol 2021;19:2370–2378.

[4] Krowka MJ. Hepatopulmonary syndrome and portopulmonary hypertension: implications for liver transplantation. Clin Chest Med 2005;26:587–597.

[5] Arguedas MR, Abrams GA, Krowka MJ, Fallon MB. Prospective evaluation of outcomes and predictors of mortality in patients with hepatopulmonary syndrome undergoing liver transplantation. Hepatology 2003;37:192–197.

[6] Luo B, Abrams GA, Fallon MB. Endothelin-1 in the rat bile duct ligation model of hepatopulmonary syndrome: correlation with pulmonary dysfunction. J Hepatol 1998;29:571–578.

[7] Rolla G, Brussino L, Colagrande P, Dutto L, Polizzi S, Scappaticci E, et al. Exhaled nitric oxide and oxygenation abnormalities in hepatic cirrhosis. Hepatology 1997;26:842–847.

[8] Fallon MB, Abrams GA, Luo B, Hou Z, Dai J, Ku DD. The role of endothelial nitric oxide synthase in the pathogenesis of a rat model of hepatopulmonary syndrome. Gastroenterology 1997;113:606–614.

[9] Luo B, Liu L, Tang L, Zhang J, Ling Y, Fallon MB. ET-1 and TNF-α in HPS: analysis in prehepatic portal hypertension and biliary and nonbiliary cirrhosis in rats. Am J Physiol Gastrointest Liver Physiol 2004;286:G294–G303.

[10] Gupta LB, Kumar A, Jaiswal AK, Yusuf J, Mehta V, Tyagi S, et al. Pentoxifylline therapy for hepatopulmonary syndrome: a pilot study. Arch Intern Med 2008;168:1820–1823.

[11] Martino RB, Coelho AMM, Kubrusly MS, Leitão R, Sampietre SN, Machado MCC, et al. Pentoxifylline improves liver regeneration through down-regulation of TNF-α synthesis and TGF-β1 gene expression. World J Gastrointest Surg 2012;4:146–151.

[12] Poster session 1: encephalopathy and other complications. Hepatology 2014;60:375A–401A.

[13] Ho V. Current concepts in the management of hepatopulmonary syndrome. Vasc Health Risk Manag 2008;4:1035–1041.

[14] European Association for the Study of the Liver. EASL Clinical Practice Guidelines for the management of patients with decompensated cirrhosis. J Hepatol 2018;69:406–460.

[15] Rodríguez-Roisin R, Krowka MJ, Hervé P, Fallon MB. ERS task force pulmonary-hepatic vascular disorders (PHD) scientific committee. Pulmonary-hepatic vascular disorders (PHD). Eur Respir J 2004 Nov;24(5):861–880.

[16] Benítez C, Arrese M, Jorquera J, Godoy I, Contreras A, Loyola S, et al. Successful treatment of severe hepatopulmonary syndrome with a sequential use of TIPS placement and liver transplantation. Ann Hepatol 2009 Mar;8(1):71–74.

[17] Igarashi J, Michel T. Sphingosine-1-phosphate and modulation of vascular tone. Cardiovasc Res 2009;82:212–220.

[18] Cantalupo A, Gargiulo A, Dautaj E, Liu C, Zhang Y, Hla T, et al. S1PR1 (sphingosine-1-phosphate receptor 1) signaling regulates blood flow and pressure. Hypertension 2017;70:426–434.

[19] Aktas O, Küry P, Kieseier B, Hartung HP. Fingolimod is a potential novel therapy for multiple sclerosis. Nat Rev Neurol 2010;6:373–382.

[20] Chun J, Hartung HP. Mechanism of action of oral fingolimod (FTY720) in multiple sclerosis. Clin Neuropharmacol 2010;33:91–101.

[21] Kappos L, Radue EW, O'Connor P, Polman C, Hohlfeld R, Calabresi P, et al. A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. N Engl J Med 2010;362:387–401.

[22] Ahmed N, Farooq J, Sadiq S, Meo SA, Jan A, Cheema FH, et al. Fingolimod (FTY720) preserves high energy phosphates and improves cardiac function in heterotopic heart transplantation model. Int J Mol Sci 2020;21:6548.

[23] Albillos A, Lario M, Álvarez-Mon M. Cirrhosis-associated immune dysfunction: distinctive features and clinical relevance. J Hepatol 2014;61:1385–1396.

[24] Mücke VT, Maria Schwarzkopf K, Thomas D, Mücke MM, Rüschenbaum S, Trebicka J, et al. Serum sphingosine-1-phosphate is decreased in patients with acute-on-chronic liver failure and predicts early mortality. Hepatol Commun 2020;4:1477–1486.

[25] Książek M, Chacińska M, Chabowski A, Baranowski M. Sources, metabolism, and regulation of circulating sphingosine-1-phosphate. J Lipid Res 2015 Jul;56:1271–1281.

[26] Hajny S, Christoffersen C. A novel perspective on the ApoM–S1P axis, highlighting the metabolism of ApoM and its role in liver fibrosis and neuroinflammation. Int J Mol Sci 2017;18:E1636.

[27] Sukriti S, Tauseef M, Yazbeck P, Mehta D. Mechanisms regulating endothelial permeability. Pulm Circ 2014 Dec;4:535–551.

[28] Nguyen TQ, Vu TM, Tukijan F, Muralidharan S, Foo JC, Li Chin JF, et al. Erythrocytes efficiently utilize exogenous sphingosines for S1P synthesis and export via Mfsd2b. J Biol Chem 2021;296:100201.

[29] Tiper IV, East JE, Subrahmanyam PB, Webb TJ. Sphingosine 1-phosphate signaling impacts lymphocyte migration, inflammation and infection. Pathog Dis 2016;74:ftw063.

[30] Diab KJ, Adamowicz JJ, Kamocki K, Rush NI, Garrison J, Gu Y, et al. Stimulation of sphingosine 1-phosphate signaling as an alveolar cell survival strategy in emphysema. Am J Respir Crit Care Med 2010;181:344–352.

[31] Roggeri A, Schepers M, Tiane A, Rombaut B, van Veggel L, Hellings N, et al. Sphingosine-1-phosphate receptor modulators and oligodendroglial cells: beyond immunomodulation. Int J Mol Sci 2020;21:7537.

[32] Wei Y, Yemisci M, Kim HH, Yung LM, Shin HK, Hwang SK, et al. Fingolimod provides long-term protection in rodent models of cerebral ischemia. Ann Neurol 2011;69:119–129.
[33] Mauer AS, Hirsova P, Maiers JL, Shah VH, Malhi H. Inhibition of sphingosine 1-phosphate signaling ameliorates murine nonalcoholic steatohepatitis. Am J Physiol Gastrointest Liver Physiol 2017;312:G300–G313.
[34] Zhang L, Wang H, Zhu J, Ding K, Xu J. FTY720 reduces migration and invasion of human glioblastoma cell lines via inhibiting the PI3K/AKT/mTOR/p70S6K signaling pathway. Tumour Biol 2014;35:10707–10714.
[35] Antonazzo IC, Poluzzi E, Forcesi E, Riise T, Bjornevik K, Baldin E, et al. Liver injury with drugs used for multiple sclerosis: a contemporary analysis of the FDA Adverse Event Reporting System. Mult Scler 2019;25:1633–1640.
[36] Joni SS, Cheshmavar M, Shoureshi P, Zamani Z, Taoosi N, Akbari M, et al. Effects of fingolimod treatments on alanine transaminase and aspartate transaminase levels in patients with multiple sclerosis. Int J Physiol Pathophysiol Pharmacol 2020;12(3):88–94.