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Impact of Hepatopulmonary Syndrome in Liver Transplantation Candidates and the Role of Angiogenesis

Steven M. Kawut, MD, MS,[1,2] Michael J. Krowka, MD,[4] Kimberly A. Forde, MD, PhD,[1,2] Nadine Al-Naamani, MD, MS,[1] Karen L. Krok, MD,[5] Mamta Patel, RN, BSN,[1,2] Carlo R. Bartoli, MD, PhD,[3,6] Margaret Doyle, PhD,[7] Jude Moutchia, MS,[2] Grace Lin, MD,[4] Jae K. Oh, MD,[4] Carl D. Mottram, RRT,[4] Paul D. Scanlon, MD,[4] and Michael B. Fallon, MD[8]

for the Pulmonary Vascular Complications of Liver Disease Study Group

[1]Department of Medicine and the [2]Center for Clinical Epidemiology and Biostatistics, Department of Biostatistics, Epidemiology, and Informatics, and the [3]Division of Cardiovascular Surgery, Department of Surgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

[4]Department of Medicine, Mayo Clinic, Rochester, MN, USA

[5]Department of Medicine, Penn State Milton S. Hershey Medical Center, Hershey, PA, USA

[6]Division of Cardiothoracic Surgery, Department of Medicine, Geisinger Medical Center, Danville, PA, USA

[7]Department of Pathology, University of Vermont, Burlington, VT, USA

[8]Department of Medicine, University of Arizona, Phoenix, AZ, USA

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Correspondence to:

Steven M. Kawut, MD, MS

727 Blockley Hall

423 Guardian Dr.

Philadelphia, PA 19104

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Data and analytic methods may be shared on a limited case-by-case basis.

Take Home Message:
The presence of hepatopulmonary syndrome with even mild oxygenation abnormalities was associated with shorter survival in candidates evaluated for liver transplantation and was characterized by higher levels of pro-angiogenic biomarkers.

Abstract

Hepatopulmonary syndrome affects 10-30% of patients with cirrhosis and portal hypertension. We evaluated the serum angiogenic profile of hepatopulmonary syndrome and assessed the clinical impact of hepatopulmonary syndrome in patients evaluated for liver transplantation.

The Pulmonary Vascular Complications of Liver Disease 2 study was a multicentre, prospective cohort study of adults undergoing their first liver transplantation evaluation. Hepatopulmonary syndrome was defined as an alveolar-arterial oxygen gradient ≥ 15 mm Hg (≥ 20 mm Hg if age > 64 years), positive contrast-enhanced transthoracic echocardiography, and absence of lung disease.

We included 85 patients with hepatopulmonary syndrome and 146 patients without hepatopulmonary syndrome. Patients with hepatopulmonary syndrome had more complications of portal hypertension and slightly higher Model for End-stage Liver Disease-Na score compared to those without hepatopulmonary syndrome (median [interquartile range] 15 [12, 19] vs. 14 [10, 17], p = 0.006). Hepatopulmonary syndrome patients had significantly lower six minute walk distance and worse functional class. Hepatopulmonary syndrome patients had higher circulating angiopoietin-2, Tie2, tenascin-C, c-kit, VCAM-1, and von Willebrand factor levels, and lower E-selectin levels. Patients with hepatopulmonary syndrome had an increased risk of death (hazard ratio 1.80 [1.03 – 3.16], p = 0.04) which persisted despite adjustment for covariates (hazard ratio 1.79 [1.02 – 3.15], p = 0.04). This association did not vary based on levels of oxygenation reflecting the severity of hepatopulmonary syndrome.

Hepatopulmonary syndrome was associated with a profile of abnormal systemic angiogenesis, worse exercise and functional capacity, and an overall increased risk of death.

Key Words: Pulmonary circulation; Liver diseases; Hypoxemia; Portal hypertension

INTRODUCTION

The hepatopulmonary syndrome (HPS) occurs when intrapulmonary vascular dilation and pulmonary arteriovenous malformations lead to abnormal systemic oxygenation in the setting of liver disease or portal hypertension[1]. This syndrome has been found in 10-30% of patients with cirrhosis being evaluated for liver transplantation.[2-4] We and others have shown that HPS is associated with a doubling in the risk of death even after accounting for liver transplantation, which is curative.[3, 5, 6]

The mechanism of HPS is currently unknown. Prior studies of patients and the experimental model of HPS have suggested that dysregulated angiogenesis plays an important role in this manifestation of advanced liver disease. Increased circulating levels of hematopoietic progenitor cells/monocytes were found in the common bile duct ligation rat model.[7-9] Genetic variants of the gene which codes for von Willebrand factor (vWF) and higher vWF levels as well as the precursor of endostatin were associated with HPS in a prior study.[10] Anti-angiogenic interventions (such as endostatin and angiostatin expression and sorafenib) improved gas exchange and shunting in the HPS experimental model.[11] While a small Phase II randomized double-blind placebo-controlled trial of sorafenib showed a reduction in vascular endothelial growth factor (VEGF) receptor-2, sorafenib did not impact on gas exchange, exercise capacity, or quality of life in patients with HPS.[12]

Some have questioned the importance of the impact of HPS, especially when presenting subclinically without significant hypoxemia. We previously published a prospective multicentre cohort of liver transplantation candidates in which most HPS was characterized by mild reductions in partial pressure of oxygen in arterial blood (PaO_2) and oxygen saturation in arterial blood and infrequent severe hypoxemia.[3] Even so, HPS had a clinically significant negative impact on functional status, health-related quality of life, and survival. However, there are no prospective,

multicentre human studies of the angiogenic milieu of HPS and the impact of HPS on other clinical end points with systematic evaluation of heart and lung function and adjustment for confounders.

Therefore, we evaluated the clinical characteristics, angiogenic biomarker profile, exercise capacity, and risk of hospitalization and mortality in patients with HPS compared to those without HPS in patients with advanced liver disease being evaluated for liver transplantation.

MATERIALS AND METHODS

Study Design and Study Sample

The Pulmonary Vascular Complications of Liver Disease (PVCLD2) study enrolled a cohort of 454 patients evaluated for liver transplantation at centres in the United States between 2013 and 2017 (Supplemental Figure E1). The only inclusion criterion was the presence of portal hypertension with or without intrinsic liver disease. We excluded patients with active infection, recent (< two weeks) gastrointestinal bleeding, or who had undergone prior liver or lung transplantation. The study was approved by the Institutional Review Board of each centre.

The study sample for this analysis was drawn from enrolled patients undergoing their first liver transplantation evaluation at the University of Pennsylvania, Mayo Clinic, and the University of Texas at Houston (Supplemental Figure E1). We excluded patients thought to have portopulmonary hypertension.

HPS was defined as: 1) alveolar-arterial oxygen gradient ($AaPO_2 \geq 15$ mm Hg (or $AaPO_2 \geq$ 20 mm Hg if age > 64 years); 2) late passage of contrast on contrast-enhanced transthoracic echocardiography ("positive contrast-enhanced transthoracic echocardiography); and 3) absence of a significant obstructive and restrictive ventilatory defect on spirometry; and 4) absence of intracardiac shunting.[1, 3]

The main analysis excluded patients with obstructive or restrictive ventilatory defects (defined below), missing testing, or those with intracardiac shunting from the control group. A sensitivity analysis for outcomes included all other patients undergoing their first liver transplantation evaluation without portopulmonary hypertension who did not meet diagnostic criteria for HPS.

Data Collection and Variables

Informed consent was obtained from eligible patients, who were then scheduled for research assessment, which included a history, anthropometrics, physical examination, phlebotomy, pulse oximetry, arterial blood gas sampling, spirometry, six minute walk testing, and contrast echocardiography. All study visits and study procedures were conducted in the outpatient setting. Patients were asked to avoid smoking before the research assessment.

Phlebotomy was performed after overnight fasting except water. Serum and plasma were banked at -80 C, while samples for flow cytometry were processed within 24 hours. All samples were shipped and assays performed at the University of Vermont Laboratory for Clinical Biochemistry Research except for vWF multimer studies which were performed at the University of Pennsylvania.

Clinical data were collected from formal interviews on the date of study procedures and from the medical record. Pulse oximetry was performed using a standard professional grade oximeter after the study participant maintained an upright seated posture for five minutes and then was repositioned supine for five minutes. Patients underwent a physical examination. Clinical laboratory results obtained closest to the date of the study visit were recorded. The Model for End-Stage Liver Disease (MELD-Na) score was calculated using the following formulae: MELD=10 * ((0.957 * ln(Creatinine)) + (0.378 * ln(Bilirubin)) + (1.12 * ln(International normalized ratio))) + 6.43 and MELD-Na=MELD + (0.32 *(137 - Na)) - (0.033 * MELD * (137 - Na).[13, 14]

Radial artery blood gas sampling was performed on ambient air in a seated position after 10 minutes of rest. The samples were processed in a blood gas analyzer after a one-point calibration. The $AaPO_2$ was calculated using the following formula: $AaPO_2=[(FiO2*[P_{atm} - P_{H20}])-(PaCO_2 /R)]] - PaO_2$ where R was assumed to be 0.8 and P_{atm} was the barometric pressure measured on the date (and in the city) of the study visit.[15]

Pre-bronchodilator spirometry was performed according to American Thoracic Society and European Respiratory Society recommendations.[16] Obstructive ventilatory defect was defined as forced expiratory volume in 1 second (FEV1)/forced vital capacity (FVC) < 0.70 with FEV1 < 80% predicted, and restrictive ventilatory defect was defined as FVC < 70% predicted. A minimum of three efforts with no acceptability errors and at least 2 with repeatability per standards (FVC within 150 mL of largest, FEV1 within 150 mL of largest, and peak flow within 15% of largest) were required. Testing was continued until the above criteria were met, a total of 8 tests were performed, or the patient was unable to continue testing. Sex, age and race specific equations were used to determine percent predicted based on spirometric reference values derived from the National Health and Nutrition Evaluation Survey III.[17] The six minute walk test was performed according to American Thoracic Society Guidelines.[18]

Contrast-enhanced transthoracic echocardiography was performed by the injection of agitated saline via a peripheral vein during transthoracic echocardiographic imaging. The apical four-chamber view was the preferred window for image acquisition, although the parasternal long axis, modified or para-apical four-chamber view, or subcostal views were utilized if the four-chamber view was suboptimal or unavailable. At least 10 continuous cardiac cycles were captured, beginning immediately prior to contrast injection to allow accurate assessment of cardiac cycles to determine delay from injection of agitated saline until visualization of contrast entering the left heart. Identification of microbubbles in either the left atrium or left ventricle after ≥ 3 cardiac cycles was

considered to indicate the presence of intrapulmonary vascular dilatation.[19] Patients with evidence of immediate (< 3 cycles) opacification of the left atrium or left ventricle were presumed to have an intra-cardiac shunt. A Doppler flow signal across the atrial septum was presumed to indicate a patent foramen ovale, also considered to be an intra-cardiac shunt. Post-Valsalva images were not utilized for study purposes. The Echocardiography Core Laboratory at the Mayo Clinic evaluated all contrast echocardiograms performed at individual study sites and echocardiographers interpreted the studies offline while blinded to all clinical information.

World Health Organization Functional Class

Assessment of symptoms and World Health Organization functional class was performed at baseline. The World Health Organization functional classification is modified from the New York Heart Association functional classification, with Class I being defined by no symptoms, Class II as symptoms with more than usual activity, Class III as symptoms with less than usual activity, and Class IV with symptoms at rest.

Patients were contacted by the research team every six months until 2017. Dates of hospitalization, liver transplantation, and death were obtained from the patients, medical record, and the subjects' physicians. Patients who were alive at the end of follow-up were censored at May 2017.

Laboratory Assays

All assays were performed in bulk at the conclusion of the study except for flow cytometry. We used the MILLIPLEX Human Angiogenesis Panel 2 (#HANG2MAG-12K) which is a bead-based Luminex multiplex assay to measure plasma angiostatin, soluble c-Kit, soluble E-selectin, soluble epithelial growth factor receptor, tenascin-C, soluble Tie-2, soluble VEGF receptors-1, -2, and -3, platelet derived growth factor AB/BB, and platelet endothelial cell adhesion molecule-1

(MilliporeSigma, Burlington, MA). We used the MILLIPLEX Human Cytokine/Chemokine Magnetic Bead Panel - Immunology Multiplex Assay (#HCYTOMAG-60K) to measure plasma fractalkine and VEGF-A (MilliporeSigma, Burlington, MA). We measured plasma angiopoietin-2 using the Meso Scale Discovery Human Angiopoietin-2 Kit (#K151KCD, Mesa Scale Diagnostics, Rockville MD). We measured plasma vascular cell adhesion molecule -1 (#DVC00) and plasma endostatin (#DNST0) using ELISAs (R&D Systems, Minneapolis, MN). vWF antigen was assessed using an immunoturbidimetric method from Stago (Cat #00518).

Details of vWF multimer studies and flow cytometry are provided in the Supplement. As flow cytometry occurred in "real time" throughout the cohort, we used the standard flow cytometric classifications at that time of cohort initiation, which have generally remained similar.[20] We focused on hematopoietic progenitor cells defined by CD34+, CD34+CD133+ and CD34+CD133+KDR+, which were CD45dim (expressed as % of peripheral blood mononuclear cells) and intermediate class (M2) monocytes and TIE2-expressing M2 monocytes (CD14+CD16+ and CD14+CD16+TIE2+), both expressed as percent of CD14+ cells.

Statistical Analysis

Continuous data were summarized using mean ± standard deviation or median [interquartile range], as appropriate. Categorical variables were summarized with n (%). We compared HPS to non-HPS patients using unpaired Student's t-tests, Wilcoxon rank sum tests, chi-squared tests, and Fisher's exact tests, as appropriate. We used bivariate and multivariate linear regression to analyse the association between HPS status and the six minute walk distance and biomarkers of angiogenesis.

We analysed the association of HPS with the risk of hospitalization using relevant models for recurrent event analysis including gamma frailty, Andersen-Gill, Prentice-Williams-Peterson gap-time and total-time, and multistate models.[21] Survival was assessed using the Kaplan-Meier estimator and Cox proportional hazards models and expressed the results with a hazard ratio (HR) in bivariate and multivariate analyses. We included age and MELD-Na as covariates in the multivariable models. We analysed overall survival (including time before and after liver transplantation) as the primary analysis, since ultimately that is what is of clinical importance to patients and clinicians. We performed sensitivity analyses with censoring at liver transplantation, considering liver transplantation a competing risk for death,[22] and adjusting for liver transplantation as a time-varying covariate. We also performed multistate modeling.[23] We calculated E-values for the main survival analyses.[24] We used laboratory parameters that were correlated with MELD-Na and a random forest imputation algorithm to impute missing MELD-Na scores (2%) for the adjusted analyses.[25] Due to the independent hypotheses investigated, there was no correction for multiple comparisons. All analyses used R version 3.6.1.[26]

RESULTS

Four hundred and fifty-four patients were enrolled in the cohort (Supplement Figure E1). Forty-three were excluded for presumed portopulmonary hypertension, leaving 411. Of these, 26 were excluded for lack of arterial blood gas or pulmonary function testing and 40 were excluded for obstructive (and 65 for restrictive) ventilatory defects, leaving 280. Forty-nine patients had evidence of patent foramina ovalia. Of the remaining 231, 85 (37%, 95%CI 31% – 43%) met criteria for HPS. Of the full cohort without a possible diagnosis of portopulmonary hypertension (N = 411), at least 21% (95%CI 17% – 25%) had HPS. There were no substantive differences between the final study

sample (N = 231) and those new patients without possible portopulmonary hypertension who were excluded (N= 180) (Supplement Table E1).

There were 85 patients with HPS and 146 without HPS (Table 1). Patients with HPS had slightly younger age and were somewhat more likely to be female. HPS patients were more likely to be non-Hispanic white than non-HPS patients, but had similar educational attainment and household income. A high proportion of patients in both groups had liver disease attributable to alcohol use and/or hepatitis C infection. The median MELD-Na score was one point higher in patients with HPS compared to those without HPS (15 [12, 19] vs 14 [10, 17], p = 0.006). Patients with HPS were more likely to have a history of ascites, varices, and encephalopathy but were less likely to have a history of hepatocellular carcinoma. Smoking and alcohol use were similar between the groups.

Dyspnoea was more common and functional class was significantly worse in patients with HPS (Table 2); cyanosis and jaundice were more common in HPS. Other physical examination findings such as clubbing and asterixis appeared more common in patients with HPS although ascites, spider angiomata, and degree of encephalopathy were not.

Patients with HPS had a mean oxygen saturation of 96% by pulse oximetry while sitting which was on average only 2% lower than the oxygen saturation of liver disease patients without HPS (Table 2). While orthodeoxia (decrease in oxygen saturation by pulse oximetry ≥ 3 % from supine to seated position) was significantly more common in HPS, only 12% of patients with HPS demonstrated this. Lung function between the groups was similar and PaO_2 and $AaPO_2$ were significantly different between the groups (Table 3). Only seven (8%) HPS patients had PaO_2 < 60 mm Hg or oxygen saturation by pulse oximetry < 90% on ambient air. Abdominal imaging demonstrated ascites in 50 (60.2%) of patients with HPS and 67 (46.5%) of patients without HPS (p = 0.05). Six patients in each group had portal vein thrombosis (p = 0.36).

Patients with HPS had 29 m [95% CI, 3 to 56] shorter six minute walk distance compared to liver disease controls with adjustment for age, sex, and MELD-Na ($p = 0.04$, $N = 197$) (Figure 1). There were no significant differences in oxygen saturation, heart rate, or Borg score at the end of the walk.

We performed a panel of blood biomarkers of angiogenesis with adjustment for age and MELD-Na score (Figure 2, Supplement Table E2). Several pro-angiogenic biomarkers were significantly higher in patients with HPS compared to liver disease controls, including angiopoietin 2, c-kit, vascular cell adhesion molecule 1, and tenacin-C. Tie-2 and platelet-derived growth factor may also have been higher in HPS patients compared to liver disease controls. Endostatin and angiostatin (both anti-angiogenic molecules) tended to be lower in HPS. We did not find differences in VEGF-1 or VEGF receptors or other protein or flow cytometry biomarkers (Supplement Table E2). There was no association between angiogenesis biomarkers and PaO_2 or $AaPO_2$ after adjustment for MELD-Na in patients with HPS (data not shown).

vWF antigen levels were significantly higher in patients with HPS compared to liver disease controls (Figure 3, Supplement Table E2). Based on this finding, we performed analyses of circulating vWF multimer size in 40 randomly selected patients with HPS and 60 liver disease controls. HPS patients had significantly higher circulating levels of low-molecular weight vWF multimers and vWF degradation fragments compared to liver disease controls after adjustment for age and MELD-Na. These findings were accompanied by significantly elevated levels of vWF clotting function in HPS (vWF:collagen binding). Levels of vWF antigen were strongly associated with angiopoietin 2 levels ($r = 0.50$, $p < 0.001$)

The median follow-up time in the cohort was 2 years (interquartile range, 1.2 – 2.8 years), and there were 461.1 person-years of follow-up. Ninety-two patients underwent liver transplantation (~40% in each group), and there was no difference in the time to liver transplantation (Supplement

Figure E2). Thirteen percent of patients were not censored as alive or died by the end of follow-up date.

There were 421 hospitalizations (89 per 100 person-years); the median number of hospitalizations per patient was 1 (interquartile range, 0 – 3). Liver transplantation was not considered as a hospitalization. Supplemental Figure E3 shows that the mean cumulative function plots of hospitalizations were similar for patients with HPS and liver disease controls, and Supplement Figure E4 shows the number of hospitalizations in the groups. HPS was not associated with the risk of recurrent hospitalization with adjustment for age, sex, and MELD-Na using the gamma frailty model (hazard ratio (HR) 1.06, 95%CI 0.77 – 1.46, p = 0.70). Sensitivity analyses with adjustment for transplantation, examining transplantation-free hospitalization, and using Andersen-Gill, Prentice-Williams-Peterson gap-time and total-time, and multistate models still showed no difference in hospitalization (Data not shown).

Patients with HPS had a worse survival than liver disease controls (Figure 4, log rank test p = 0.04). Twenty-four (28%) HPS patients and 25 (17%) liver disease controls died during follow-up. Patients with HPS had a lower probability of being alive than liver disease controls at one year (87% vs 92%), two years (73% vs. 83%), and three years (63% vs 81%). Causes of death are shown in Supplement Table E3. Cox proportional hazards models showed that patients with HPS had an 80% increase in the risk of death in bivariate (HR 1.80, 95%CI 1.03-3.16, p = 0.04) and multivariate analyses (HR 1.79, 95%CI 1.02 – 3.15, p = 0.04) adjusted for age and MELD-Na (Table 3). A model with adjustment for liver transplantation as a time-varying covariate showed similar results (Table 3). When transplantation was considered as a competing risk using the Fine-Gray model, the subdistributional HR of HPS vs liver disease controls for death was 1.91 (95%CI, 1.06 – 3.45, p = 0.03) after adjustment for age and MELD-Na (Figure 5). A multistate model (Supplement Figure E5) suggested that HPS was associated with an increased risk of death in patients without liver

transplantation (HR = 1.84, 95%CI 0.99 – 3.44, p = 0.05) but not with the chances of receiving liver transplantation (HR = 1.03, 95%CI 0.67 – 1.58, p = 0.89) or the risk of death after liver transplantation (HR 0.99, 95%CI 0.24 – 4.15, p = 0.99). The association of HPS with overall survival was partially attenuated after adjustment for angiopoietin 2 (28% attenuated, HPS vs no HPS HR = 1.30, 95%CI 0.70 – 2.41, p = 0.40) or vWF levels (21% attenuated, HPS vs no HPS HR = 1.42, 95%CI 0.77-2.61, p = 0.30). This suggests that these biomarkers are in the causal pathway or are confounders of the association of HPS with outcomes. Other biomarkers did not have qualitatively important impact on the effect estimate. After excluding patients with hepatocellular carcinoma, findings were generally consistent with the main results albeit non-significant in some cases with the smaller sample size and lower power.

The differences in overall risk of death for HPS vs. liver disease controls did not differ based on PaO_2 (p for interaction = 0.30) or $AaPO_2$ (p for interaction = 0.40) suggesting that the relationship between HPS and worse outcomes was not dependent on the severity of HPS. We also compared the survival of patients with HPS with all others in the prospective cohort without portopulmonary hypertension, including those with restrictive or obstructive lung diseases, patent foramina ovalia, or missing data who were excluded from the primary analyses. Many of these excluded patients likely had HPS coexisting with their underlying exclusion. For example, of the 105 patients excluded for restrictive or obstructive lung disease, 49 (47%) had a positive contrast-enhanced transthoracic echo and 38 (36%) had abnormal $AaPO_2$ and a positive contrast-enhanced transthoracic echo. Even with including these patients in the "control" group, HPS still appeared to be associated with a higher risk of death (HR 1.56, 95%CI 0.97 – 2.50, p = 0.07) with a weaker effect estimate, as expected with the likely presence of undiagnosable HPS in the "control" group.

DISCUSSION

We have shown that HPS is a common complication in patients who are referred for evaluation for liver transplantation. In contrast to some prior studies, patients with HPS had more severe liver disease and complications of portal hypertension. Despite only mild abnormalities in oxygenation of the blood (with only 8% being clinically hypoxemic), HPS patients had more respiratory symptoms, worse functional class, and lower six minute walk distance after adjustment for severity of liver disease, which are novel findings. HPS patients were characterized by a profile of dysregulated angiogenic peptides compared to liver disease controls even after accounting for differences in the severity of liver disease, which has not been demonstrated previously. Patients with HPS had a risk of hospitalization which was similar to that of liver disease controls, however HPS patients had a significantly increased risk of death overall regardless of the degree of abnormal oxygenation. Some of this increased risk was accounted for by higher levels of angiopoietin 2 and vWF in patients with HPS, representing the first possible biologic mechanisms for how HPS impacts on outcome in patients. This study used sophisticated research-grade prospective heart and lung phenotyping and adjusted for confounders in multivariate analyses (notably severity of liver disease), distinguishing our results from those of other studies.

We found that 37% of our patients without other potential causes of oxygen abnormalities and 21% or more of the entire cohort had HPS based on established diagnostic criteria.[3] A prior study (which recruited candidates for liver transplantation 10 years before the current study) did not show differences in severity of liver disease between patients with HPS and those without HPS. This may be due to the evolving characteristics of patients being evaluated for liver transplantation over time or spectrum bias. For example, almost one quarter of patients in the current sample had non-alcoholic fatty liver disease compared to only 11% of the prior study, and approximately one-third

of the current sample had hepatocellular carcinoma compared to only 9% of the prior study. These differences may reflect not only changes in the general population (e.g., increased obesity) and aetiologies of advanced liver disease but also newer exception point policies which prioritize certain patients for transplantation (e.g., with hepatocellular carcinoma) in the US, thereby influencing the composition of referrals for evaluation. The higher severity of liver disease in patients with HPS in the current study (reflected by higher MELD-Na score and more complications of portal hypertension) could either be a cause or consequence of HPS and has been seen in other recent cohorts.[2, 4, 27] One study of patients listed for liver transplantation actually showed lower laboratory MELD scores in those with HPS listed for liver transplantation (although this study's focus on HPS with exception scores could introduce selection bias).[28] More severe liver disease could lead to HPS as one of the sequelae or the presence of HPS could causally contribute to more liver disease complications; angiogenesis or other pathobiological processes could cause both worse liver disease and HPS.

There were small differences in oxygen saturation in HPS and non-HPS patients, and only 8% of HPS patients were hypoxemic.[3] Even so, patients with HPS had both clinically and statistically significantly shorter distance walked in six minutes compared to other patients with liver disease. This difference in exercise capacity substantiates the greater symptoms reported by patients with HPS and the worse functional class assigned by clinicians.[29] Worse liver disease did not explain the difference in exercise capacity, since this was independent of MELD-Na. Other systemic processes may cause worse symptoms and exercise capacity in HPS.

Studies in experimental models of HPS have demonstrated increased lung angiogenesis.[11] We showed higher levels of several important circulating biomarkers of angiogenesis, including angiopoietin 2, c-kit, and possibly Tie-2, in HPS even after adjustment for the differences in severity of liver disease. Higher angiopoietin 2 (which signals via Tie-2) has been linked to increased

pathologic angiogenesis in several studies of patients with liver disease.[30-32] Tenascin-C is an extracellular matrix protein which has been linked to vascular remodelling in the lung. Both angiostatin and endostatin tended to be lower in HPS, which parallels their roles as anti-angiogenic molecules which prevented HPS in the experimental model.[11] Vascular cell adhesion molecule-1 was higher in HPS as in one prior study, suggesting that leukocyte adhesion to the endothelium may play a role.[33] We have previously demonstrated lower bone morphogenetic protein 9 and 10 levels (linked to increased angiogenesis) in a small sample of HPS patients from this cohort.[34]

HPS patients had significantly higher levels of vWF antigen, low-molecular-weight vWF multimers, and vWF degradation fragments. Levels of vWF multimers and/or vWF degradation fragments may alter angiopoietin signalling, a potent destabilizer of blood vessels;[35-37] vWF levels were significantly associated with angiopoietin-2 levels in our study.[35-39] Indeed, abnormalities in vWF metabolism are associated with angiodysplasia and arteriovascular malformations in multiple diseases.[40-47] In infants with single ventricle anatomy and a superior-cavopulmonary (Glenn) circulation, abnormalities in vWF and angiopoietin 2 play a role in the development of pulmonary angiodysplasia and arteriovascular malformations also without increased VEGF.[40-48] High shear stress from continuous-flow left ventricular assist devices increases enzymatic degradation of large vWF multimers into vWF degradation fragments,[38] which may alter circulating levels of angiopoietin 2[49] causing mucosal arteriovascular malformations.[38, 39] Patients with HPS may have more commonly had varices which may relate to these biomarker findings.

There was no association between the presence of HPS and the risk of hospitalization. Even so, we found a significant association between HPS and a higher overall risk of death with similar findings when adjusting for liver transplantation, analysing transplantation-free survival, and with liver transplantation as a competing risk. The major population accounting for this finding appeared to be patients who did not receive liver transplantation; HPS does not generally pose a significant

increase in risk after liver transplantation in recent studies.[2, 27, 28, 50] When comparing patients with HPS to the rest of the liver transplantation candidates in our cohort (including patients with restrictive and obstructive lung diseases, patent foramina ovalia, etc.), there was still a 56% increase in risk of death overall (95%CI, -3% - 250%, a conservative estimate considering that the comparison group likely included up to one-third patients with HPS (undiagnosable due to the other comorbid conditions).

Prior studies of survival have been single centre, without multivariate analyses, and without careful phenotyping of the presence of HPS (or the exclusion of patients with other reasons for abnormal oxygenation.)[4-6, 51] Our prior prospective multicentre cohort study showed that HPS was independently associated with an increased risk of death even after adjusting for MELD score and liver transplantation.[3] More recently, two single centre cohort studies showed that HPS was associated with an increased risk of death, but not after multivariate adjustment.[4, 27] In a prior study in patients listed for liver transplantation in United Network for Organ Sharing, we found that patients with HPS actually had better overall survival than patients without HPS, which was attributable to HPS patients receiving higher priority for liver transplantation from MELD exception.[28]

The association of HPS with worse outcomes in liver disease has vexed some due to the very mild subclinical abnormalities in oxygenation in most HPS patients. However, we have now replicated this finding in two distinct multicentre prospective cohort studies with similar effect estimates. The role of angiogenesis (which causes other well-known sequelae of portal hypertension) may address the question of how HPS impacts on survival, although a recent clinical trial of sorafenib to target angiogenesis in patient with HPS was null.[12]

The only therapy which reverses HPS is liver transplantation.[1, 27] The association of HPS with worse survival than other liver disease patients particularly in those who do not receive liver transplantation underscores the need to develop effective medical therapies. These findings may further justify screening for HPS using arterial blood gases and contrast-enhanced transthoracic echocardiography in all candidates for liver transplantation[52] and argue for the consideration of the presence of carefully-phenotyped HPS (irrespective of the PaO_2) in the prioritization for liver transplantation.

There are several limitations to this study. First, we only included patients being considered for liver transplantation in the United States which is a selected population. Differences in the makeup of the liver transplantation populations and allocation policies in other countries warrant similar studies of HPS in those populations. Second, we excluded some patients from the main analyses in order to create "clean" phenotypes. However, survival analyses including all patients still suggested an increased risk of death for patients with HPS. Third, it is possible that unmeasured or imprecisely measured variables could have confounded the findings, quantified by the E-values. Hypothesis-generating analyses suggested that angiopoietin 2, vWF, and other angiogenic biomarkers could explain how HPS reduces survival. We did not correct for multiple comparisons due to the multiple hypotheses investigated, therefore Type 1 error is possible. Finally, although our study was multicentre and conducted over several years, the sample size is relatively small. Still, this cohort remains one of the largest (and only) prospective multicentre study of HPS with extensive protocolized lung and heart phenotyping to our knowledge.

In summary, HPS is common in patients being evaluated for liver transplantation and is associated with worse functional status, exercise capacity, and overall survival. Understanding the mechanisms of the adverse effects of HPS and developing effective medical therapies merit high priority to improve the outcomes of patients with advanced liver disease.

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Figure Legends

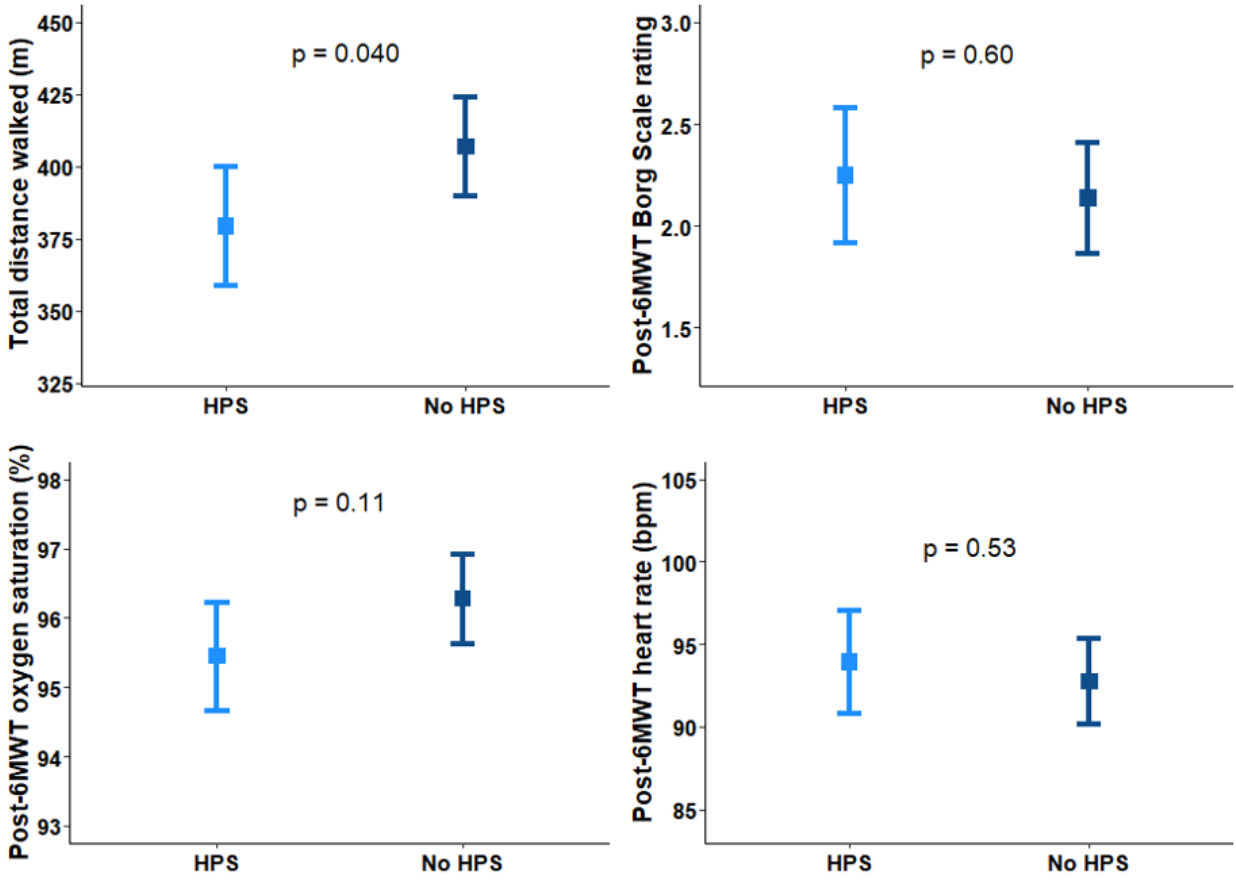


Figure 1: Least square means and 95% confidence intervals of six minute walk test parameters. All values are adjusted for age, gender, MELD-Na, and baseline values (other than distance).

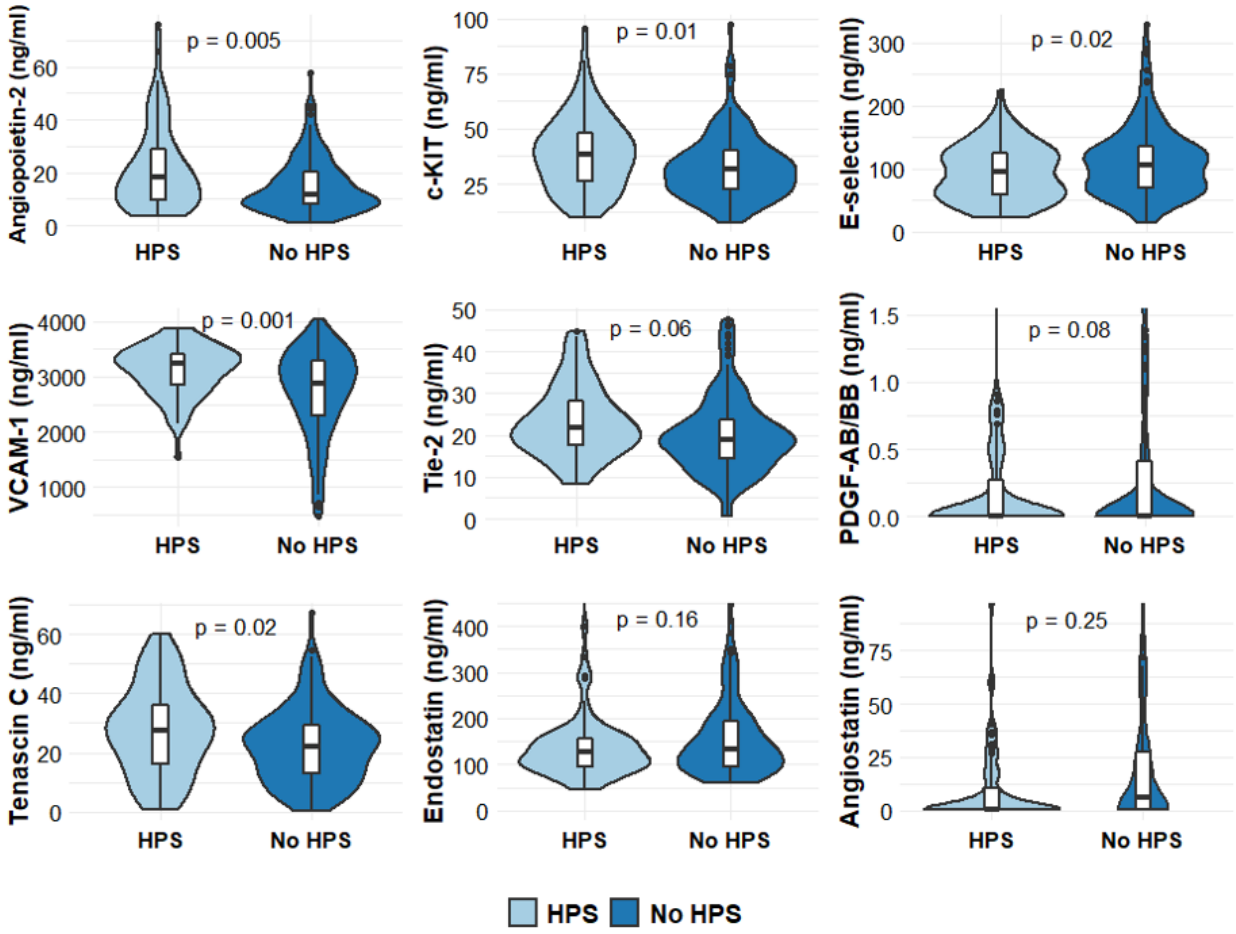


Figure 2: Violin plots of selected angiogenesis biomarkers. Boxes are interquartile ranges with median. Whiskers are observations within 1.5*interquartile range. Plots of platelet derived growth factor (PDGF)-AB/BB, endostatin, and angiostatin only show data that are within 1.5*interquartile range. p values are from multivariable linear regression models adjusted for age and MELD-Na score. C-kit: tyrosine protein kinase Kit, VCAM-1: vascular cell adhesion molecule 1, Tie-2: TEK tyrosine kinase, endothelial

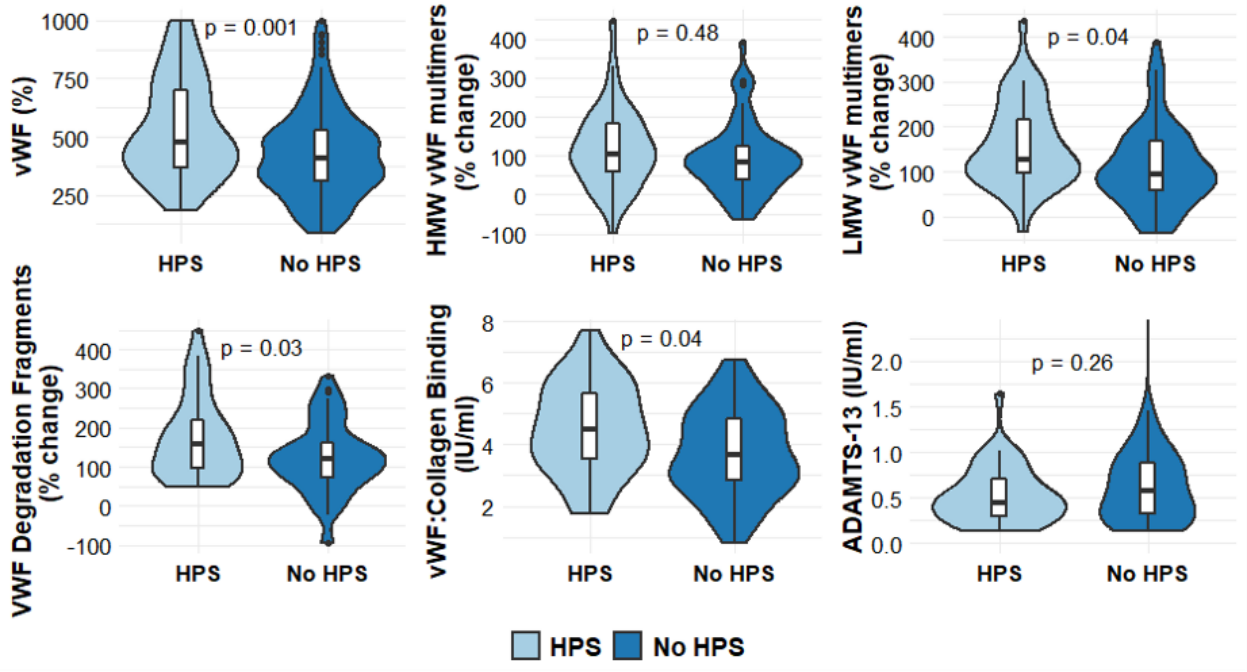


Figure 3: Violin plots of von Willebrand factor biomarkers. Boxes are interquartile ranges with median. Whiskers are observations within 1.5*interquartile range. p values are from multivariable linear regression models adjusted for age and MELD-Na score. HMW: high molecular weight, LMW: low molecular weight, vWF: von Willebrand factor

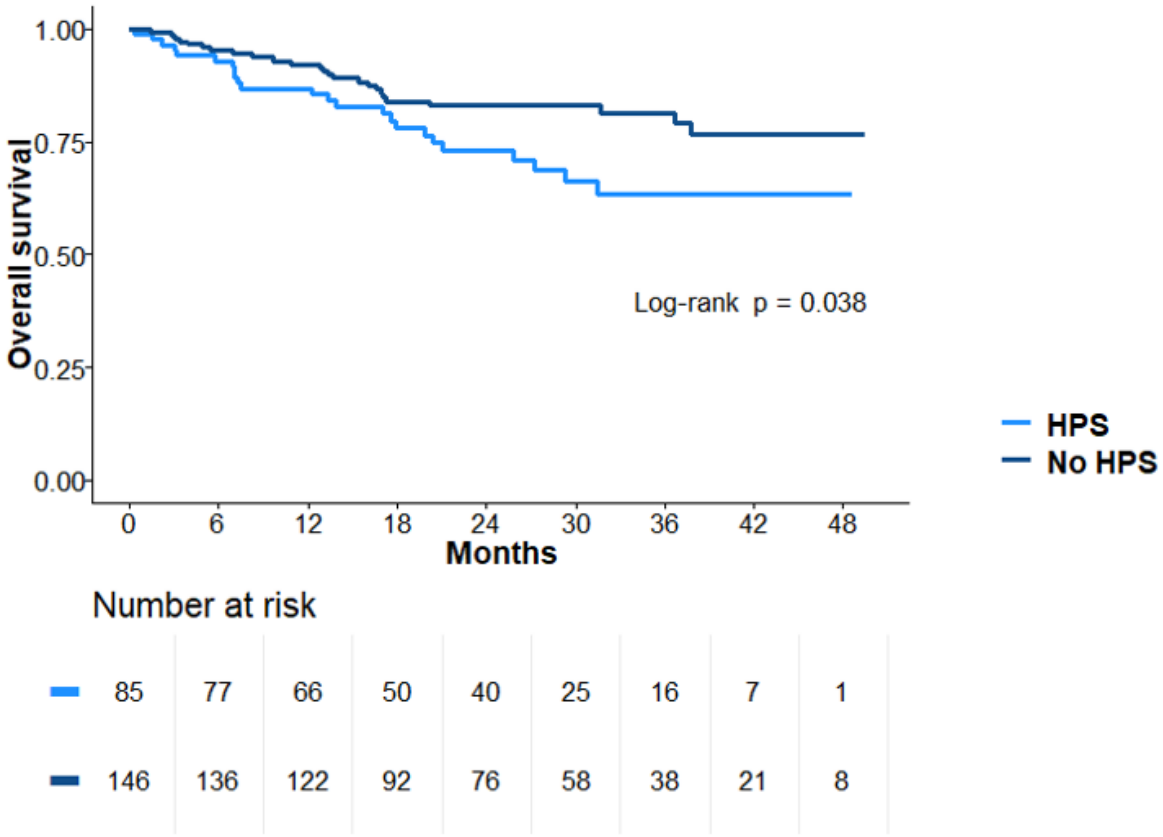


Figure 4: Kaplan-Meier curves comparing patients with HPS and liver disease controls

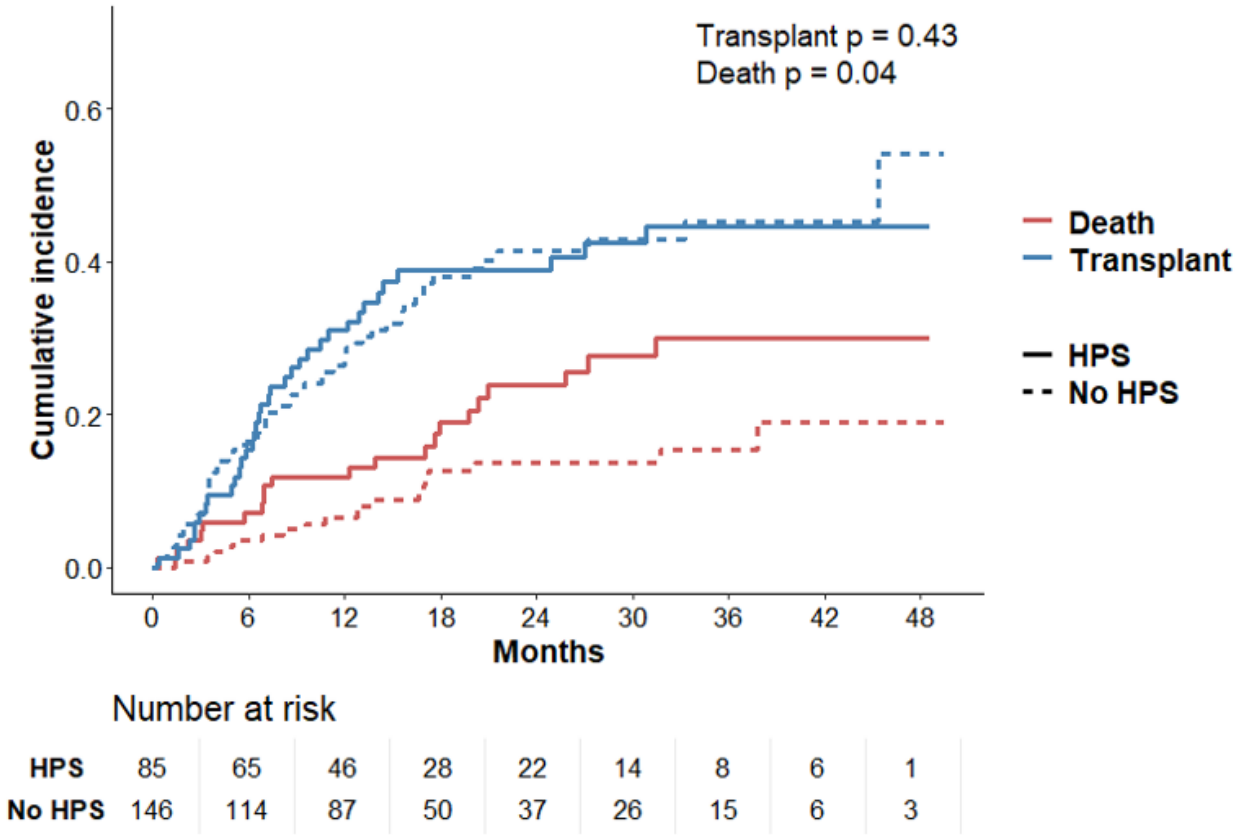


Figure 5: Predicted cumulative incidences of death and liver transplantation for patients with HPS and liver disease controls

Table 1. Demographics, liver disease characteristics, and past medical history

Variable	N	HPS (N=85)	No HPS (N=146)	p value*
Age (years), mean ± SD	231	55.2 ± 9.4	57.6 ± 8.9	0.06
Female gender, n (%)	231	33 (38.8)	40 (27.4)	0.07
Race/Ethnicity, n (%)	231			0.02
Non-Hispanic white		70 (82.4)	95 (65.1)	
Hispanic white		12 (14.1)	29 (19.9)	
Non-Hispanic black		2 (2.4)	16 (11.0)	
Other		1 (1.2)	6 (4.1)	
Born in the United States/Puerto Rico, n (%)	230	74 (88.1)	135 (92.5)	0.27
Language spoken in the household, n (%)	230			1.0
English		78 (92.9)	133 (91.1)	
Spanish		1 (1.2)	3 (2.1)	
Other		5 (6.0)	10 (6.8)	
Education, n (%)	230			0.51
No schooling or Grades 1-11		11 (13.1)	24 (16.4)	
High school or GED		26 (31.0)	42 (28.8)	
Some college education or Technical/Vocational certificate		18 (21.4)	25 (17.1)	
Associate or Bachelor's degree		26 (31.0)	42 (28.8)	
Professional or Graduate degree		3 (3.6)	13 (8.9)	
Family income for past 12 months, n (%)	230			0.33
$19,999 and below		18 (21.4)	39 (26.7)	
$20,000 – $49,999		22 (26.2)	31 (21.2)	
$50,000 – $99,999		17 (20.2)	28 (19.2)	
$100,000 and above		14 (16.7)	35 (24.0)	
Unknown		13 (15.5)	13 (8.9)	
Aetiology of liver disease, n (%)				
Alcohol	231	34 (40.0)	48 (32.9)	0.28
Hepatitis C infection	231	37 (43.5)	62 (42.5)	0.88
Autoimmune hepatitis	231	4 (4.7)	6 (4.1)	1.0
Non-alcoholic fatty liver disease	231	20 (23.5)	33 (22.6)	0.87
Hepatitis B infection	231	1 (1.2)	6 (4.1)	0.43
Primary sclerosing cholangitis	231	4 (4.7)	8 (5.5)	1.0
Primary biliary cholangitis	231	9 (10.6)	6 (4.1)	0.05
Cryptogenic cirrhosis	231	3 (3.5)	11 (7.5)	0.22
Other	231	6 (7.1)	5 (3.4)	0.22

MELD-Na score, median [IQR]	227	15.0 [12.0, 19.0]	14.0 [10.0, 17.0]	0.006
History of liver disease complications, n (%)				
Ascites	231	64 (75.3)	91 (62.3)	0.04
Varices	231	64 (75.3)	93 (63.7)	0.07
Variceal bleeding	231	29 (34.1)	42 (28.8)	0.40
Encephalopathy	231	55 (64.7)	73 (50.0)	0.03
Multiple paracenteses	231	31 (36.5)	42 (28.8)	0.23
Spontaneous bacterial peritonitis	231	4 (4.7)	8 (5.5)	1.0
Hepatocellular carcinoma	231	22 (25.9)	58 (39.7)	0.03
Hepatic hydrothorax	231	10 (11.8)	13 (8.9)	0.48
Transjugular intrahepatic porto-systemic shunt	231	11 (12.9)	8 (5.5)	0.05
Past medical history, n (%)				
Chronic obstructive pulmonary disease	231	5 (5.9)	7 (4.8)	0.76
Chronic bronchitis	231	7 (8.2)	7 (4.8)	0.30
Asthma	231	12 (14.1)	7 (4.8)	0.01
Venous thromboembolism	231	5 (5.9)	5 (3.4)	0.50
Diabetes mellitus	231	22 (25.9)	63 (43.2)	0.009
Hypertension	231	28 (32.9)	82 (56.2)	0.001
Hypercholesterolemia	231	16 (18.8)	28 (19.2)	0.95
Congestive heart failure	231	3 (3.5)	8 (5.5)	0.75
Smoked at least 100 cigarettes in lifetime, n (%)	230	53 (63.1)	82 (56.2)	0.30
Pack-years for ever-smokers, median [IQR]	106	22 [6, 35]	11 [4, 27]	0.12
Smoked in the last 30 days, n (%)	231	13 (15.3)	16 (11.0)	0.34
Consumed alcohol, n (%)	230	76 (90.5)	138 (94.5)	0.25
Duration of alcohol consumption (years), median [IQR]	214	30 [20, 37]	32 [22, 40]	0.12
Current alcohol use, n (%)	231	5 (5.9)	12 (8.2)	0.51
Medications, n (%)				
B-blockers	230	44 (51.8)	72 (49.7)	0.76
Spontaneous bacterial peritonitis prophylaxis/Antibiotics	230	42 (49.4)	61 (42.1)	0.28
Bile acid resins	230	14 (16.5)	13 (9.0)	0.09
Midodrine	230	0 (0.0)	2 (1.4)	0.53

*Pearson's Chi-squared test; Fisher's exact test; two sample t-test; Wilcoxon rank sum test

GED: General Educational Development

Table 2. Symptoms, signs, physical findings and laboratory evaluation

Variable	N	HPS (N=85)	No HPS (N=146)	p value*
Symptoms, n (%)				
Dyspnoea	231	34 (40.0)	33 (22.6)	0.005
Chest Pain	231	8 (9.4)	7 (4.8)	0.17
Orthopnoea	230	1 (1.2)	4 (2.7)	0.65
Palpitations	231	5 (5.9)	8 (5.5)	1.0
Syncope	231	2 (2.4)	2 (1.4)	0.63
Platypnoea	230	2 (2.4)	2 (1.4)	0.62
WHO functional class, n (%)	231			<0.001
I		16 (18.8)	64 (43.8)	
II		47 (55.3)	57 (39.0)	
III		22 (25.9)	25 (17.1)	
IV		0 (0.0)	0 (0.0)	
Signs, n (%)				
Cyanosis	231	7 (8.2)	1 (0.7)	0.004
Jaundice	231	43 (50.6)	34 (23.3)	<0.001
Lower extremity oedema	231	47 (55.3)	65 (44.5)	0.11
Clubbing	231	11 (12.9)	6 (4.1)	0.01
Spider angiomata	231	3 (3.5)	6 (4.1)	1.0
Asterixis	230	36 (42.4)	43 (29.7)	0.05
Ascites	231			0.47
Absent		45 (52.9)	89 (61.0)	
Mild-Moderate		32 (37.6)	47 (32.2)	
Severe		8 (9.4)	10 (6.8)	
Encephalopathy	231			0.27
Absent		67 (78.8)	127 (87.0)	
Mild (I-II)		17 (20.0)	18 (12.3)	
Severe (III-VI)		1 (1.2)	1 (0.7)	
Physical examination, mean ± SD				
Body mass index (kg/m^2)	231	31 ± 7	30 ± 7	0.22
Waist-hip ratio	218	1.0 ± 0.1	1.0 ± 0.1	0.67
Pulse (beats per minute)	231	74 ± 14	72 ± 13	0.26
Respiratory rate (breaths per minute)	230	15 ± 3	16 ± 3	0.17

Systolic blood pressure (mm Hg)	231	121 ± 16	124 ± 18	0.26
Diastolic blood pressure (mm Hg)	231	66 ± 9	70 ± 11	0.006
Oxygen saturation (%)	231	96 ± 4	98 ± 2	<0.001
Orthodeoxia[†], n (%)	227	10 (12)	7 (5)	0.04
Laboratory results, median [IQR]				
Blood urea nitrogen (mg/dl)	209	14 [10, 20]	15 [11, 21]	0.19
Creatinine (mg/dl)	230	0.9 [0.8, 1.1]	1.0 [0.8, 1.2]	0.06
Haemoglobin (g/dl)	231	11.8 [10.4, 13.7]	12.4 [10.9, 13.6]	0.52
Platelet count (10^9/l)	229	86 [62, 109]	92 [66, 136]	0.10
International normalized ratio	228	1.4 [1.2, 1.6]	1.3 [1.1, 1.5]	0.002
Alanine aminotransferase (U/l)	230	38 [28, 64]	46 [27, 72]	0.33
Aspartate aminotransferase (U/l)	230	64 [41, 98]	58 [36, 92]	0.17
Total bilirubin (mg/dl)	230	2.4 [1.5, 3.7]	1.5 [0.8, 2.8]	<0.001
Direct bilirubin (mg/dl)	226	0.9 [0.6, 1.6]	0.6 [0.2, 1.1]	<0.001
Alkaline phosphatase (U/l)	230	149 [93, 220]	148 [112, 194]	0.98
Total protein (g/dl)	230	6.8 [6.4, 7.3]	7.2 [6.6, 7.6]	0.004
Albumin (g/dl)	230	3.0 [2.6, 3.4]	3.2 [2.8, 3.7]	0.01
Pulmonary function testing, mean ± SD				
Forced vital capacity (% predicted)	231	88 ± 10	91 ± 12	0.15
Forced expiratory volume in 1 s (% predicted)	231	89 ± 11	89 ± 12	0.68
Forced expiratory volume in 1 s/Forced vital capacity	231	0.77 ± 0.05	0.78 ± 0.06	0.26
Arterial blood gas, mean ± SD				
pH	231	7.45 ± 0.04	7.44 ± 0.04	0.17
$PaCO_2$ (mmHg)	231	33 ± 5	35 ± 5	0.001
PaO_2 (mmHg)	231	78 ± 13	92 ± 14	< 0.001
Alveolar-arterial oxygen gradient (mmHg), median [IQR]	231	26 [20, 37]	12 [7, 19]	< 0.001

[*]Pearson's Chi-squared test, Fisher's exact test, two sample t-test, Wilcoxon rank sum test, as appropriate.
$PaCO_2$: Partial pressure of carbon dioxide in arterial blood
PaO_2: Partial pressure of oxygen in arterial blood
[†]Increase of 3% or more in oxygen saturation from pulse oximetry from sitting to supine position

Table 3. Models for the risk of death

Models	HR (95% CI)	p value	aHR (95% CI)	p value*	E–value (for the limit of the CI)
Overall survival	1.80 (1.03, 3.16)	0.04	1.79 (1.02, 3.15)	0.04	2.35 (1.13)
Overall survival with transplant as a time-varying covariate	1.78 (1.02, 3.13)	0.04	1.71 (0.97, 3.00)	0.06	2.25 (1.00)
Transplant-free Survival	1.99 (1.07, 3.71)	0.03	1.84 (0.99, 3.44)	0.05	2.42 (1.00)
Survival with transplant as competing risk (Fine-Gray model)†	1.91 (1.06, 3.45)	0.03	1.87 (1.04, 3.35)	0.04	2.45 (1.20)
Multistate model††					
Transition from evaluation to liver transplant	1.11 (0.73, 1.69)	0.62	1.03 (0.67, 1.58)	0.89	
Transition from evaluation to death without liver transplant	1.99 (1.07, 3.71)	0.03	1.84 (0.99, 3.44)	0.05	2.42 (1.00)
Transition from liver transplant to death	0.94 (0.23, 3.80)	0.93	0.99 (0.24, 4.15)	0.99	

*Adjusted for age and MELD-Na score

†Subdistributional hazard ratio for mortality

†† Schema for multistate model is shown in Figure S3

Impact of Hepatopulmonary Syndrome in Liver Transplantation Candidates and the Role of Angiogenesis

Steven M. Kawut, MD, MS,[1,2] Michael J. Krowka, MD,[4] Kimberly A. Forde, MD PhD,[1,2] Nadine Al-Naamani, MD, MS,[1] Karen L. Krok, MD,[5] Mamta Patel, RN, BSN,[1,2] Carlo R. Bartoli, MD, PhD,[3,6] Margaret Doyle, PhD,[7] Jude Moutchia, MS,[2] Grace Lin, MD,[4] Jae K. Oh, MD,[4] Carl D. Mottram, RRT,[4] Paul D. Scanlon, MD,[4] and Michael B. Fallon, MD[8]

for the Pulmonary Vascular Complications of Liver Disease Study Group

Online Data Supplement

Supplemental Methods

vWF Assays

Gel Electrophoresis and Immunoblotting to Quantify vWF Multimers and Fragments

High-molecular-weight plasma vWF multimers and vWF degradation fragments were resolved by standard vertical gel electrophoresis and immunoblotting techniques as previously described in detail.[1-4] To resolve multimers, plasma was diluted in NuPAGE LDS Sample Buffer (Invitrogen, Carlsbad, CA), heated, and loaded into vertical 1% agarose-SDS gels. Electrophoresis was performed in Tris-Acetate SDS running buffer (Invitrogen) in an XCell SureLock Mini-Cell Electrophoresis System (Invitrogen).

Proteins were transferred to PVDF using the iBlot dry transfer device (Invitrogen). Membranes were blocked and incubated with rabbit anti-human vWF primary antibody (1:500, Dako, Carpinteria, CA) overnight. Membranes were incubated with goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (1:3,000, Cell Signaling, Danvers, MA), developed with Luminata Forte Western Blot HRP Substrate (Millipore, Billerica, MA), and imaged with an ImageQuant LAS 4000 (GE Healthcare, Piscataway, NJ).

To resolve VWF degradation fragments, plasma was diluted in NuPAGE LDS Sample Buffer (Invitrogen), heated, and loaded into vertical NuPAGE 3-8% Tris-Acetate Polyacrylamide gels (Invitrogen). Electrophoresis was performed in Tris-Acetate SDS running buffer (Invitrogen). Protein was transferred, blocked, probed for vWF, and imaged as described above. As a loading control, each membrane was probed for human plasma albumin with a goat anti-human albumin HRP-conjugated antibody (1:10,000 Abcam, Cambridge, MA).

Quantification of Plasma vWF Multimers and vWF Degradation Fragments

Study patient plasma samples were blotted in adjacent lanes to a pooled control sample from healthy volunteer blood donors (n=20). High-molecular-weight vWF multimers were quantified as percent difference in total length of the vWF multimer profile versus the pooled control as described in detail.[5] The density of low-molecular-weight vWF multimers and vWF degradation fragments was quantified as the mean difference in density of all multimers or all vWF degradation fragments in HPS and non-HPS versus the pooled control. ImageQuantTL (GE Healthcare) and ImageJ (National Institutes of Health) were used to generate and analyse densitometric plots, respectively.

vWF:Collagen Binding Activity

VWF:collagen binding activity was determined with via ELISA (Technozym, vWF:CBA ELISA, Technochlone, Vienna Austria). Plasma samples were incubated with wells coated with human collagen III. An HRP-conjugated polyclonal anti-vWF antibody was co-incubated in solution and bound the vWF-collagen III complex. Tetramethylbenzidine reagent was added to elicit a colorimetric reaction that was quenched with a stopping solution. A standard curve was constructed from reference plasma samples with known vWF concentrations. vWF:collagen binding activity was determined by interpolation of the colorimetric intensity values from the standard curve.

Quantification of Plasma ADAMTS-13

Plasma ADAMTS-13, the vWF-specific protease, was measured with a quantitative solid-phase sandwich-based ELISA (R&D Systems, Minneapolis, MN). Briefly, plasma samples were added to wells coated with a monoclonal anti-human ADAMTS-13 antibody. Wells were incubated with HRP-conjugated polyclonal anti-human ADAMTS-13. Tetramethylbenzidine was added to

elicit a colorimetric reaction that was quantified with spectrophotometry by a microplate reader (μQuant, Bio-Tek Instruments, Highland Park, VT). Data were analysed with Gen5, version 2.05 (Bio-Tek). Plasma ADAMTS-13 values were interpolated from a standard curve.

Flow Cytometry

Heparinized whole blood was shipped overnight to the University of Vermont Laboratory for Clinical Biochemistry Research using temperature controlled (15-30^{o}C) shipping containers. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation and cells were washed to remove platelet contamination. PBMCs (10^{6} cells) were labelled with PeCy5.5 anti CD45 (Invitrogen Cat#MHCD4518), FITC anti-CD34 (BD Cat# 555821), PE anti-KDR (VEGFR2) (R&D Cat# FAG357P) and APC anti-CD133 (Miltenyi Cat# 130-90-826), or with appropriate isotype controls. Contaminating RBCs were lysed and the cells fixed with 1% paraformaldehyde. Samples were analysed on a MacsQuant 10 (Miltenyi Biotech) using the MacsQuantify software. Single colour controls were used for machine compensation and negative gates were set with isotype controls. We focused on hematopoietic progenitor cells defined by CD34+, CD34+CD133+ and CD34+CD133+KDR+, which were CD45dim (expressed as % of peripheral blood mononuclear cells) and intermediate class (M2) monocytes and TIE2-expressing M2 monocytes (CD14+CD16+ and CD14+CD16+TIE2+), both expressed as percent of CD14+ cells.

Table E1: Comparison between study sample and excluded subjects

Variable	N	Non-missing (N=231)	Missing (N=180)	Standardized difference
Age (years), mean ± SD	411	56.7 ± 9.1	56.1 ± 9.4	0.06
Female gender, n (%)	411	73 (31.6)	72 (40.0)	0.18
Race/Ethnicity, n (%)	411			0.14
Non-Hispanic white		165 (71.4)	133 (73.9)	
Hispanic white		41 (17.7)	24 (13.3)	
Non-Hispanic black		18 (7.8)	18 (10.0)	
Other		7 (3.0)	5 (2.8)	
Body mass index (kg/m^2)	407	31 ± 7	30 ± 7	0.04
Education, n (%)	410			0.38
No schooling or Grades 1-11		35 (15.2)	12 (6.7)	
High school or GED		68 (29.6)	59 (32.8)	
Some college education or Technical/Vocational certificate		43 (18.7)	54 (30.0)	
Associate or Bachelor's degree		68 (29.6)	41 (22.8)	
Professional or Graduate degree		16 (7.0)	14 (7.8)	
Etiology of liver disease, n (%)				
Alcohol	411	82 (35.5)	80 (44.4)	0.18
Hepatitis C infection	411	99 (42.9)	62 (34.4)	0.17
Autoimmune hepatitis	411	10 (4.3)	6 (3.3)	0.05
Non-alcoholic fatty liver disease	411	53 (22.9)	41 (22.8)	0.0
Hepatitis B infection	411	7 (3.0)	1 (0.6)	0.19
Primary sclerosing cholangitis	411	12 (5.2)	6 (3.3)	0.09
Primary biliary cirrhosis	411	15 (6.5)	11 (6.1)	0.02
Cryptogenic cirrhosis	411	14 (6.1)	7 (3.9)	0.1
Other	411	11 (4.8)	12 (6.7)	0.08
MELD-Na score, median [IQR]	411	14.0 [10.5, 18.0]	13.5 [10.0, 18.2]	0.06
History of liver disease complications, n (%)				
Ascites	411	155 (67.1)	131 (72.8)	0.12
Varices	411	157 (68.0)	123 (68.3)	0.01
Variceal bleeding	411	71 (30.7)	62 (34.4)	0.08
Encephalopathy	411	128 (55.4)	111 (61.7)	0.13
Multiple paracenteses	411	73 (31.6)	68 (37.8)	0.13
Spontaneous bacterial peritonitis (SBP)	411	12 (5.2)	14 (7.8)	0.1
Hepatocellular carcinoma	411	80 (34.6)	47 (26.1)	0.19

Hepatic hydrothorax	411	23 (10.0)	29 (16.1)	0.18
Transjugular intrahepatic porto-systemic shunt	411	19 (8.2)	15 (8.3)	0.0
Smoked at least 100 cigarettes in lifetime, n (%)	410	135 (58.7)	115 (63.9)	0.11
Pack-years for ever-smokers, median [IQR]	182	13 [4, 30]	15 [7, 35]	0.17
Smoked in the last 30 days, n (%)	411	29 (12.6)	38 (21.1)	0.23
Consumed alcohol, n (%)	410	214 (93.0)	165 (91.7)	0.05

Standardized Difference- Difference in means, ranks, or proportions divided by the standard deviation. Standardized differences > 0.20 may suggest imbalance.

Table E2: Angiogenesis biomarkers

Variable	N	HPS (N=85)	No HPS (N=146)	β (95% CI)	p value	aβ (95% CI)*	p value
VEGF (ng/ml)	222	1.0 ± 2.1	0.8 ± 1.8	0.17 (-0.35, 0.7)	0.51	0.19 (-0.34, 0.72)	0.48
VEGFR-1 (ng/ml)	222	0.4 ± 0.5	0.4 ± 0.8	-0.06 (-0.26, 0.14)	0.55	-0.04 (-0.24, 0.17)	0.73
VEGFR-2 (ng/ml)	222	9.6 ± 3.7	11.1 ± 10.8	-1.45 (-3.92, 1.03)	0.25	-1.22 (-3.74, 1.3)	0.34
VEGFR-3 (ng/ml)	222	6.0 ± 9.6	9.0 ± 42.9	-3.06 (-12.7, 6.58)	0.53	-1.73 (-11.52, 8.06)	0.73
Fractalkine (ng/ml)	222	0.6 ± 1.1	0.5 ± 0.7	0.12 (-0.12, 0.36)	0.34	0.11 (-0.13, 0.36)	0.37
Angiostatin (ng/ml)	222	9.3 ± 17.0	24.8 ± 85.7	-15.53 (-34.76, 3.69)	0.11	-11.29 (-30.62, 8.05)	0.25
Endostatin (ng/ml)	222	145.7 ± 89.5	160.0 ± 89.5	-14.29 (-39.01, 10.44)	0.26	-17.78 (-42.81, 7.25)	0.16
Tie-2 (ng/ml)	222	23.3 ± 8.5	20.1 ± 9.2	3.2 (0.73, 5.67)	0.01	2.26 (-0.12, 4.64)	0.06
c-KIT (ng/ml)	222	39.1 ± 16.9	32.6 ± 14.8	6.47 (2.17, 10.76)	0.01	5.55 (1.22, 9.88)	0.01
EGFR (ng/ml)	222	1.7 ± 1.1	1.9 ± 3.0	-0.25 (-0.92, 0.43)	0.47	-0.13 (-0.81, 0.56)	0.71
E-selectin (ng/ml)	222	94.4 ± 44.2	111.3 ± 55.7	-16.91 (-31.26, -2.57)	0.02	-16.78 (-31.18, -2.39)	0.02
PCAM-1 (ng/ml)	222	9.7 ± 3.7	9.2 ± 5.5	0.51 (-0.86, 1.87)	0.47	0.23 (-1.14, 1.6)	0.74
Tenascin C (ng/ml)	222	27.6 ± 15.0	22.3 ± 12.5	5.3 (1.59, 9.01)	0.01	4.23 (0.58, 7.89)	0.02
PDGF-AB/BB (ng/ml)	222	0.2 ± 0.4	0.6 ± 1.5	-0.33 (-0.68, 0.02)	0.07	-0.31 (-0.67, 0.04)	0.08
Angiopoietin-2 (ng/ml)	222	21.5 ± 15.5	15.1 ± 10.5	6.39 (2.95, 9.84)	<0.001	4.56 (1.43, 7.7)	0.005
VCAM-1 (ng/ml)	222	3,118 ± 452	2,731 ± 774	387.46 (200.29, 574.63)	<0.001	289.1 (124.58, 453.63)	0.001
vWF antigen (%)	222	537 ± 213	430 ± 189	106.56 (51.87, 161.25)	<0.001	82.88 (32.75, 133.01)	0.001
High-molecular-weight vWF multimers (% change)	100	119 ± 103	95 ± 92	24 (-14.96, 62.96)	0.22	13.96 (-24.9, 52.83)	0.48
Low-molecular-weight vWF multimers (% change)	100	161 ± 94	112 ± 90	49.62 (12.37, 86.86)	0.01	37.93 (1.31, 74.54)	0.04
vWF Degradation Fragments (% change)	100	174 ± 104	121 ± 90	53.39 (14.62, 92.15)	0.007	41.06 (3.65, 78.47)	0.03
vWF:Collagen Binding (IU/ml)	100	4.5 ± 1.5	3.7 ± 1.4	0.8 (0.21, 1.38)	0.008	0.6 (0.03, 1.18)	0.04
ADAMTS-13 (IU/ml)	100	0.5 ± 0.3	0.7 ± 0.6	-0.15 (-0.37, 0.07)	0.18	-0.13 (-0.35, 0.1)	0.26
Flow cytometry							
CD34+CD45dim, % of PBMCs	194	0.5 ± 0.4	0.5 ± 0.4	0.04 (-0.07, 0.15)	0.48	0.05 (-0.06, 0.17)	0.38
CD133+CD45dim, % of PBMCs	194	4.1 ± 2.6	4.5 ± 3.2	-0.43 (-1.3, 0.45)	0.34	-0.56 (-1.45, 0.33)	0.21
CD34+CD133+CD45dim, % of PBMCs	194	20.9 ± 18.6	20.1 ± 16.2	0.78 (-4.26, 5.81)	0.76	1.28 (-3.82, 6.38)	0.62

CD34+CD133+KDR+CD4dim, % of PBMCs	194	2.2 ± 2.7	2.3 ± 3.4	-0.11 (-1.03, 0.81)	0.81	-0.01 (-0.94, 0.92)	0.98
CD14++CD16+, % of CD14+ cells PBMCs	198	13.5 ± 8.6	12.4 ± 9.1	1.02 (-1.59, 3.64)	0.44	1.24 (-1.41, 3.88)	0.36
Tie2-expressing CD14++CD16+, % of CD14+ cells	198	1.5 ± 1.1	1.7 ± 1.5	-0.25 (-0.64, 0.14)	0.21	-0.29 (-0.69, 0.11)	0.15

*Beta coefficient (mean difference) adjusted for age and MELD-Na

vWF: von Willebrand factor, PBMCs: Peripheral blood mononuclear cells

Table E3: Causes of death

Patient	Days from evaluation	Status	Cause of death	Other causes of death
1	67	HPS	Brain hematoma	
2	537	HPS	Sepsis/Septic shock	
3	89	HPS	Respiratory failure	
4	510	HPS	Hepatocellular carcinoma	
5	226	HPS	Unknown	
6	368	HPS	Unknown	
7	775	HPS	Cholangiocarcinoma	Acute kidney injury
8	209	HPS	Unknown	
9	10	HPS	Sepsis/Septic shock	
10	817	HPS	Unknown	
11	879	HPS	Sudden cardiac arrest	
12	45	HPS	Disseminated intravascular coagulation	Haemorrhagic and septic shock
13	210	HPS	Liver failure	
14	93	HPS	Respiratory failure	
15	527	HPS	Brain embolism	
16	418	HPS	Liver failure	
17	399	HPS	Liver transplantation rejection	
18	612	HPS	Respiratory failure	
19	631	HPS	Liver disease	
20	942	HPS	Liver disease	
21	206	HPS	Unknown	
22	170	HPS	Multisystem organ failure	
23	593	HPS	Liver disease	
24	220	HPS	Hepatic and uremic encephalopathy	
25	605	No HPS	Unknown	
26	117	No HPS	Sepsis/Septic shock	
27	499	No HPS	Liver disease	
28	398	No HPS	Unknown	
29	245	No HPS	Acute renal failure	
30	385	No HPS	Sudden cardiac arrest	
31	1134	No HPS	*C. difficile* colitis	Sepsis/Septic shock, Multiorgan system failure
32	162	No HPS	Unknown	
33	147	No HPS	Liver failure	
34	950	No HPS	Sudden cardiac arrest during liver transplantation surgery	
35	481	No HPS	Sepsis/Septic shock	
36	412	No HPS	Unknown	
37	42	No HPS	Haemorrhagic ascites	

38	508	No HPS	Multisystem organ failure	
39	462	No HPS	Motor vehicle accident	
40	103	No HPS	Sepsis/Septic shock	
41	511	No HPS	Unknown	
42	515	No HPS	Unknown	
43	206	No HPS	Unknown	
44	83	No HPS	Sepsis/Septic shock	
45	1100	No HPS	Acute alcoholic hepatitis	
46	325	No HPS	Liver failure	
47	90	No HPS	Liver failure	
48	390	No HPS	Infection	Liver disease
49	288	No HPS	Unknown	

Table E4: Survival analyses including patients with missing data for HPS phenotyping as "no HPS"

Models	HR (95% CI)	p value	aHR* (95% CI)	p value	E–value (lower band of 95% CI)
Overall survival	1.51 (0.95, 2.42)	0.09	1.56 (0.97, 2.50)	0.07	2.06 (1.00)
Overall survival with transplantation as a time-varying covariate	1.49 (0.93, 2.39)	0.09	1.47 (0.91, 2.35)	0.11	1.94 (1.00)
Transplantation-free Survival	1.51 (0.91, 2.49)	0.11	1.41 (0.85, 2.34)	0.2	1.85 (1.00)
Survival with transplantation as competing risk (Fine-Gray model)†	1.44 (0.90, 2.31)	0.13	1.43 (0.90, 2.29)	0.13	1.87 (1.00)
Multistate model††					
Transition from waitlist to liver transplantation	1.19 (0.82, 1.73)	0.37	1.33 (0.77, 1.64)	0.54	
Transition from waitlist to death without liver transplantation	1.51 (0.91, 2.49)	0.11	1.42 (0.85, 2.32)	0.18	1.87 (1.00)
Transition from liver transplantation to death	1.44 (0.38, 5.41)	0.59	1.33 (0.34, 5.19)	0.68	

*Adjusted for age and MELD-Na score

†Subdistributional hazards ratio

††Schema for the multistate model is shown in Figure E3

Figure E1: Selection of study sample

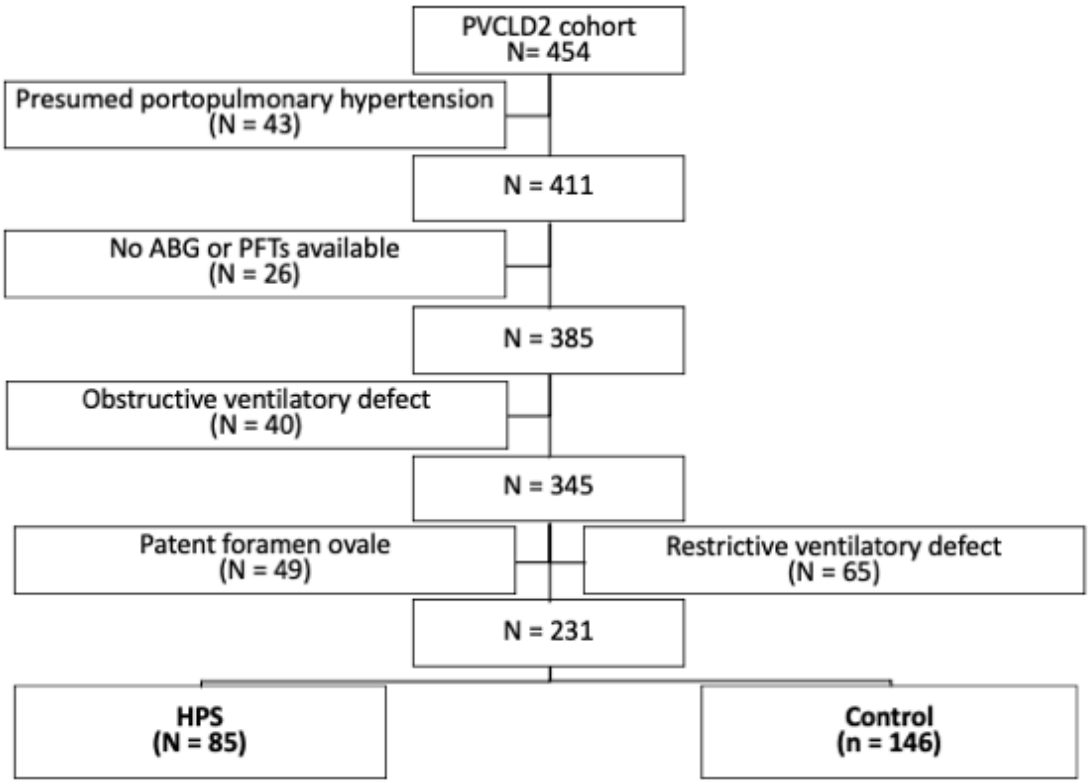


Figure E2: Time to liver transplantation

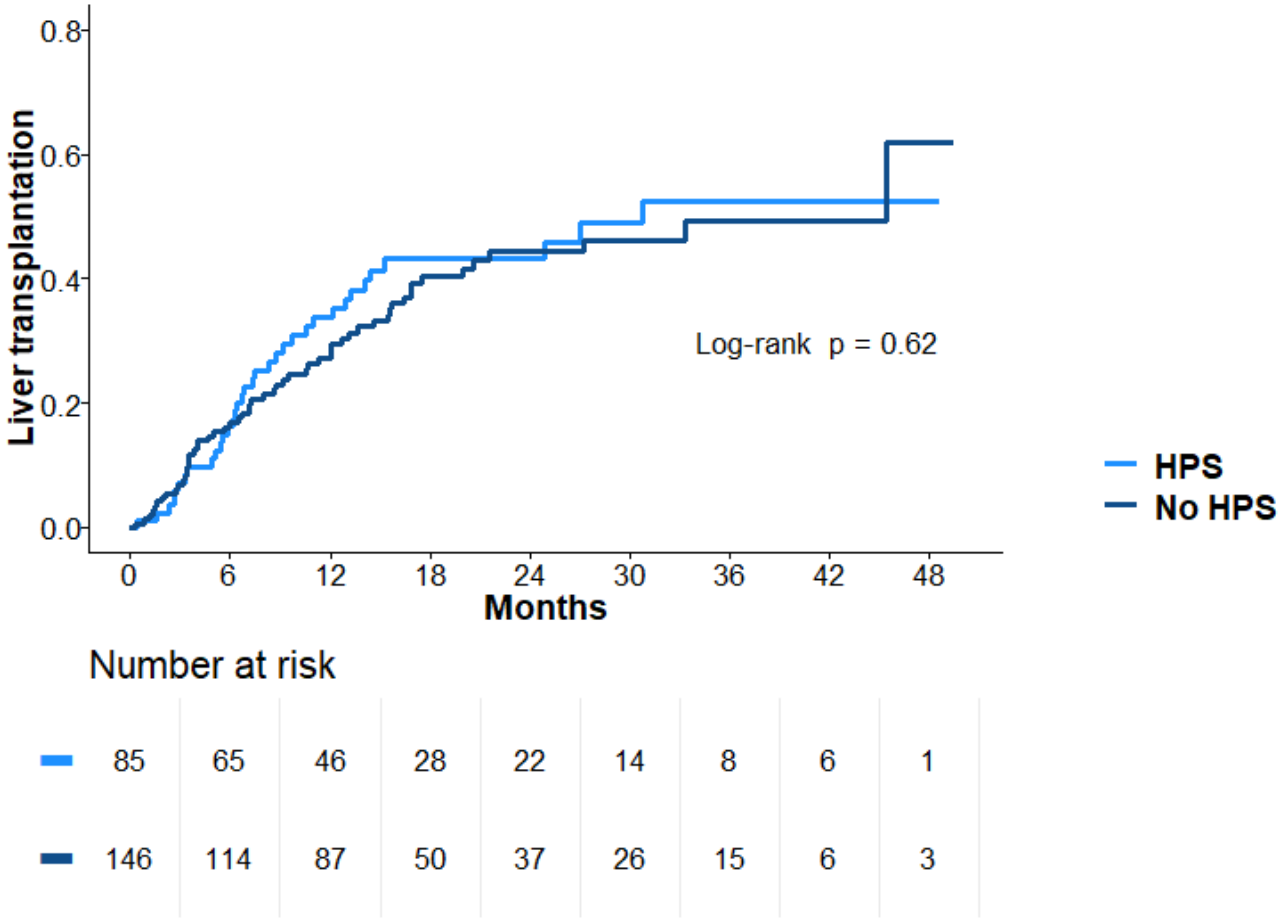


Figure E3: Mean cumulative function plot of hospitalizations from date of evaluation

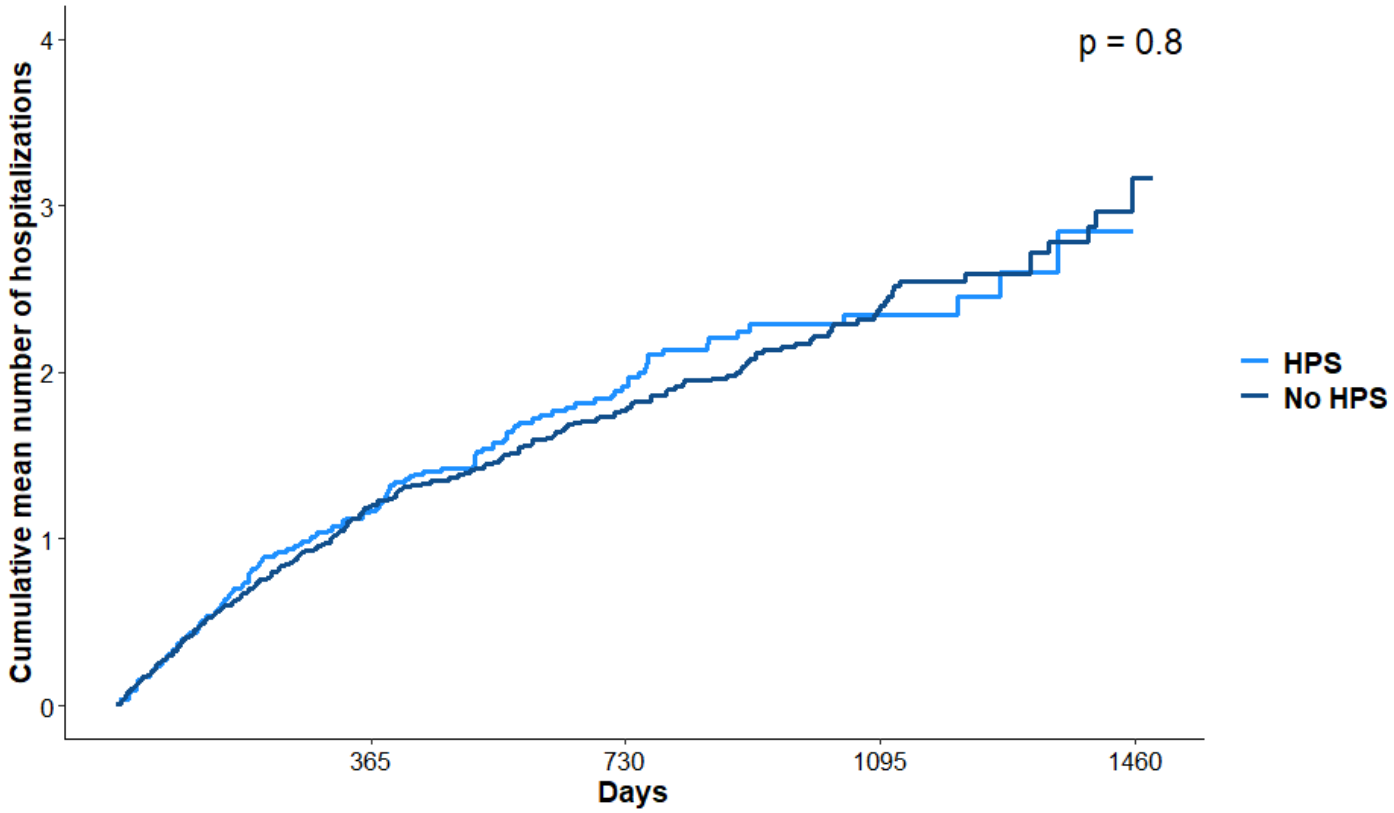


Figure E4: Hospitalizations in HPS and non-HPS groups

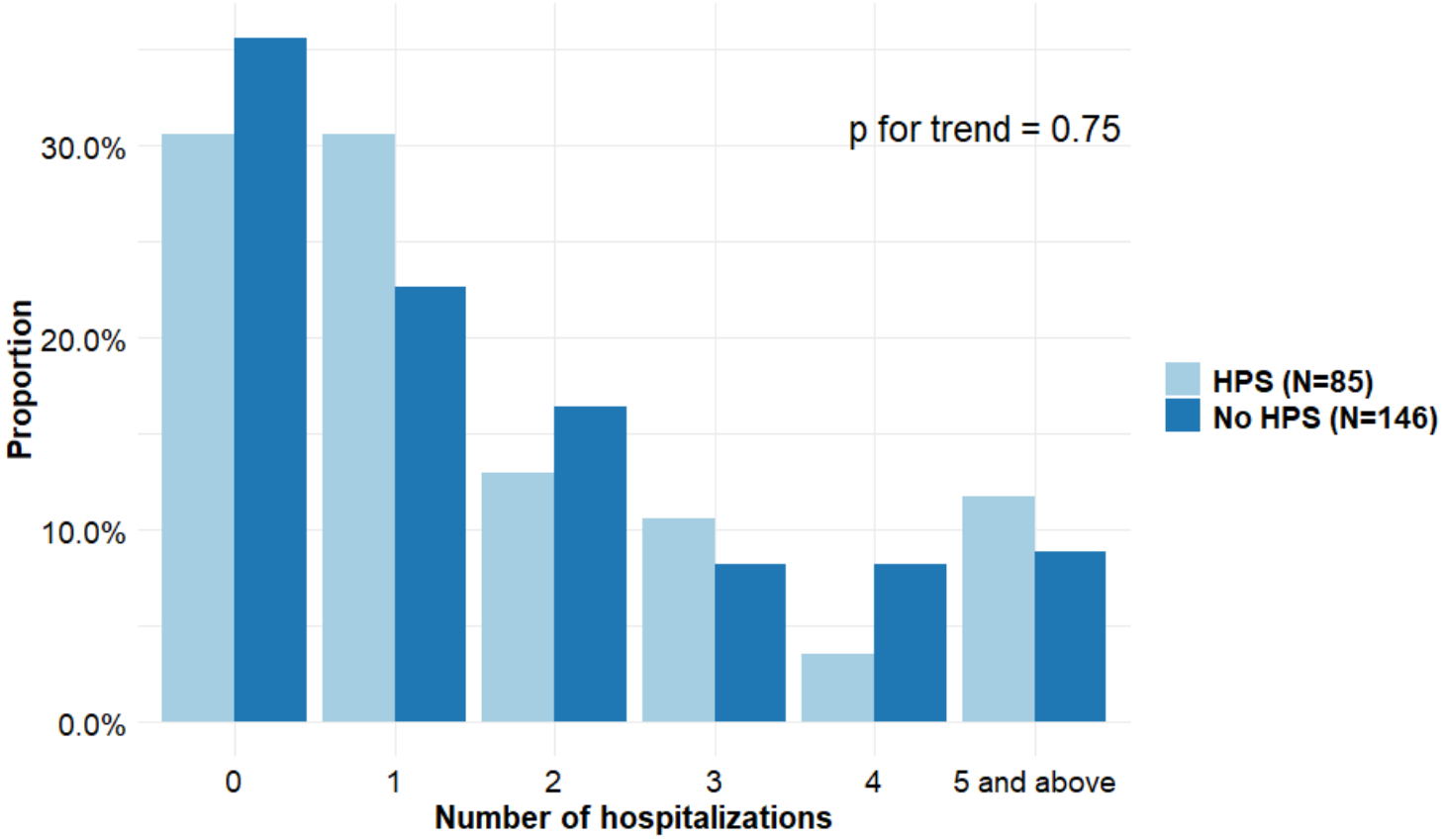
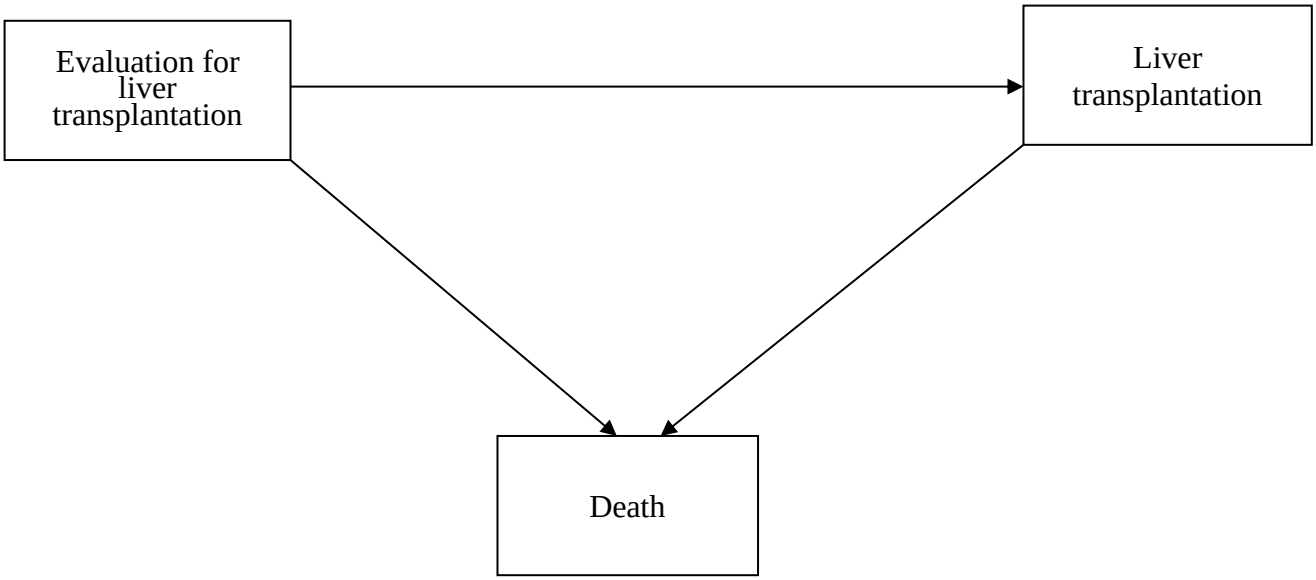


Figure E5: Schema for liver transplantation and death multistate model



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