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

Running title:

Hepatopulmonary Syndrome and Outcomes

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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

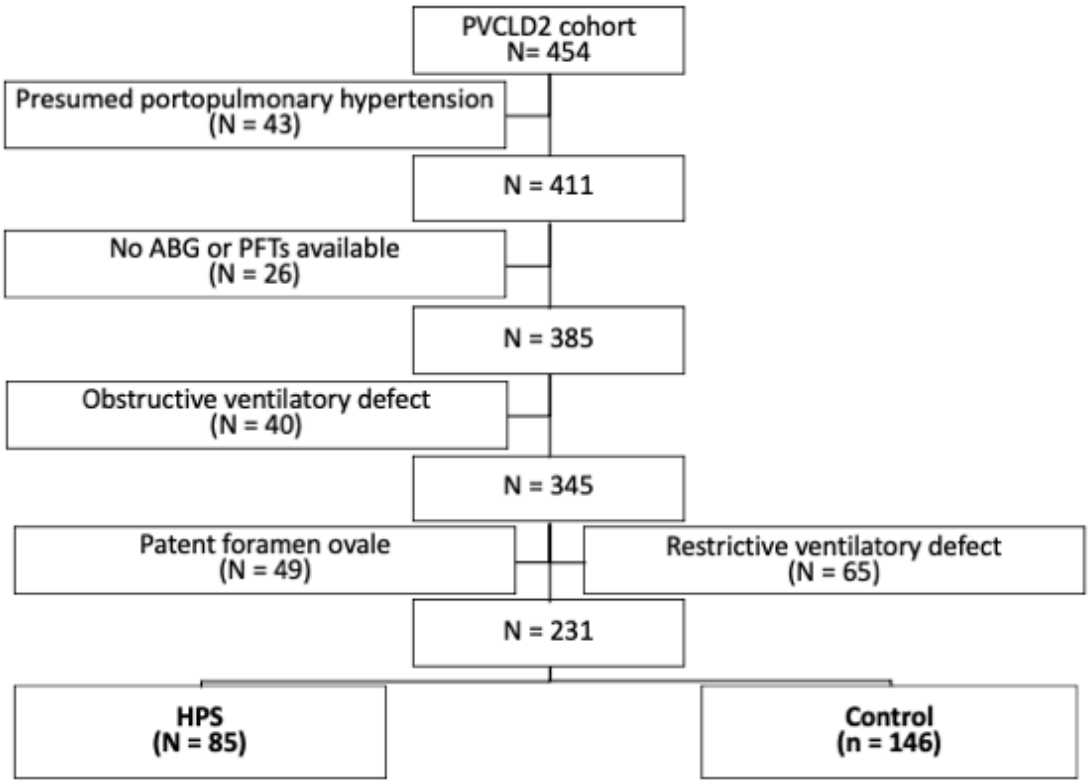


Figure E2: Time to liver transplantation

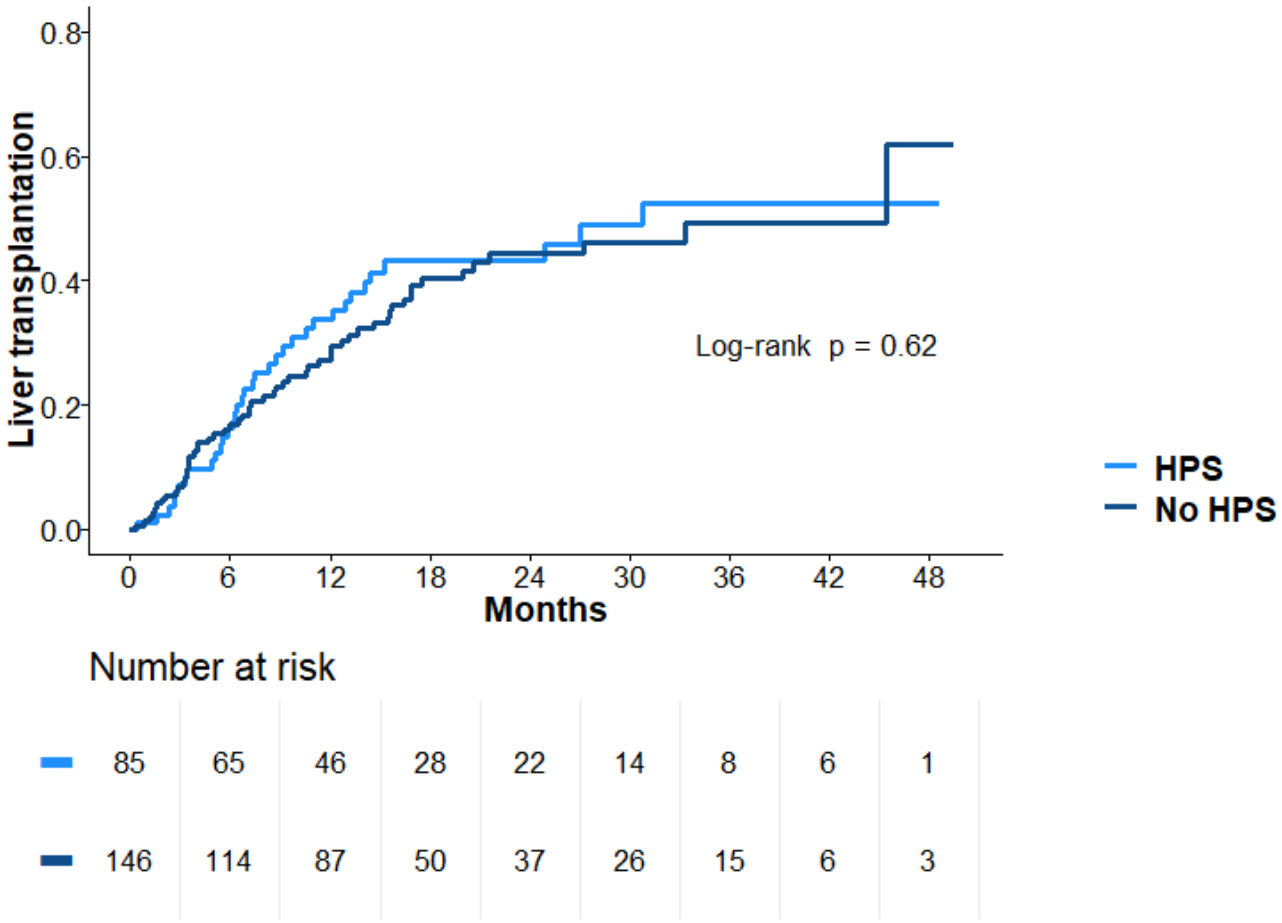


Figure E3: Mean cumulative function plot of hospitalizations from date of evaluation

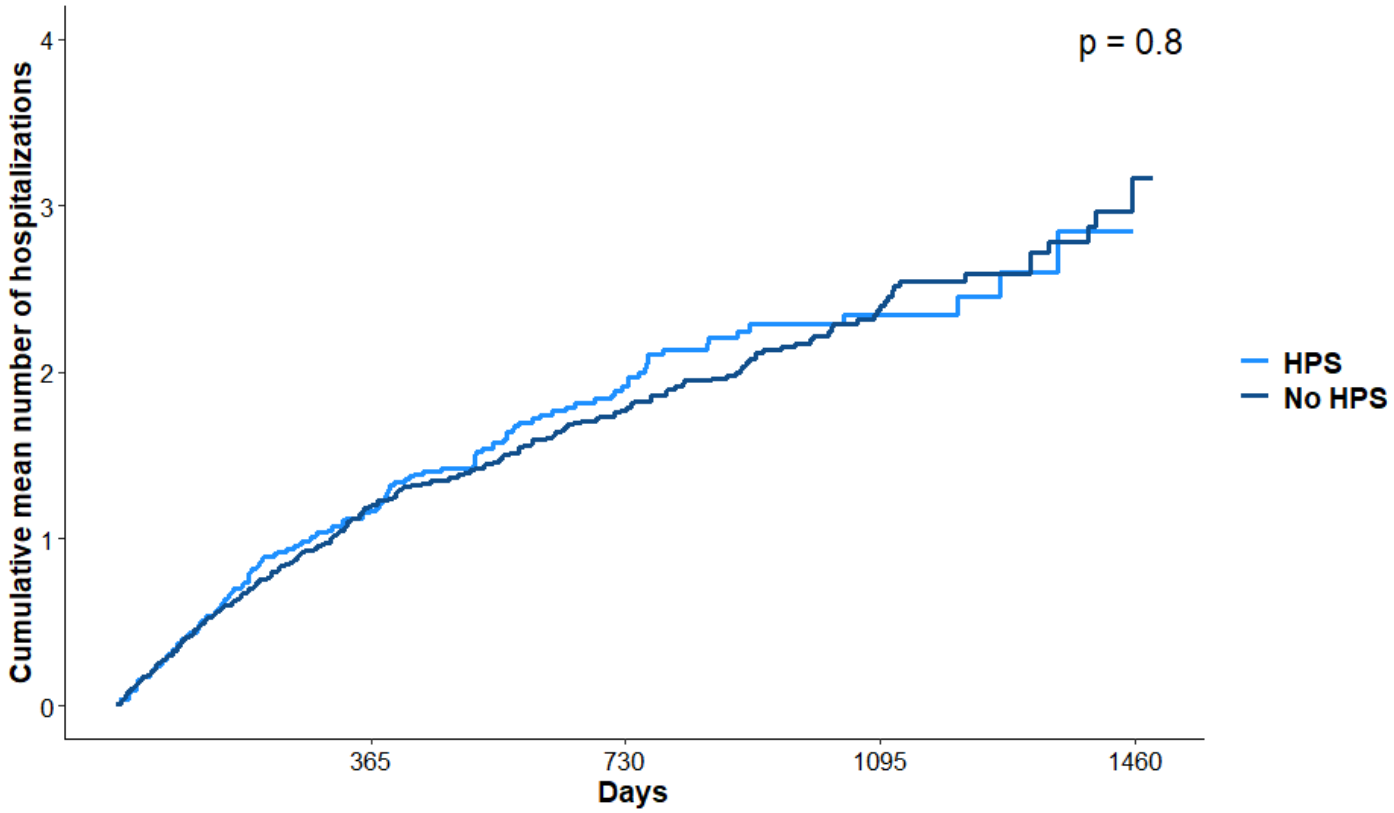


Figure E4: Hospitalizations in HPS and non-HPS groups

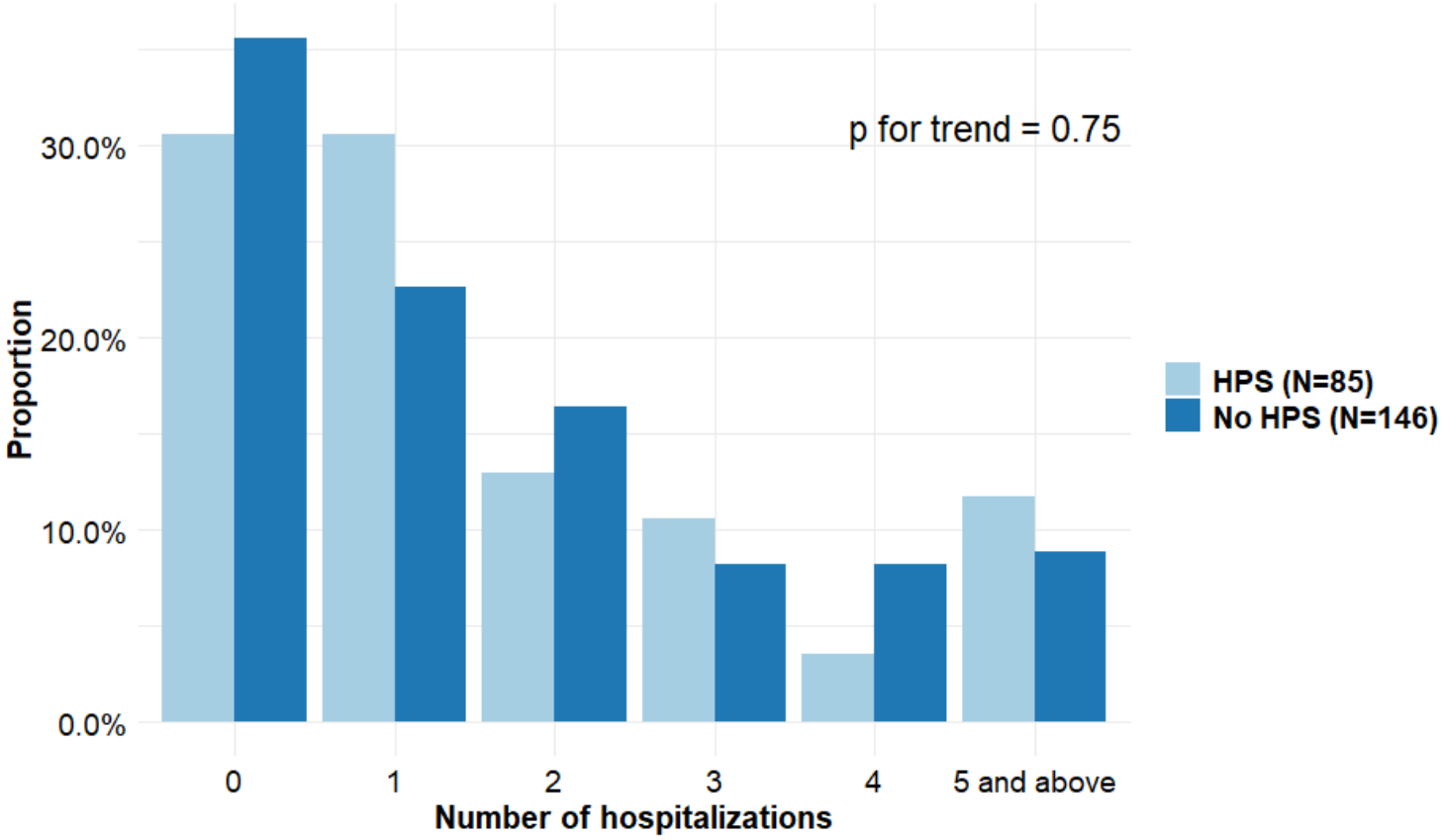
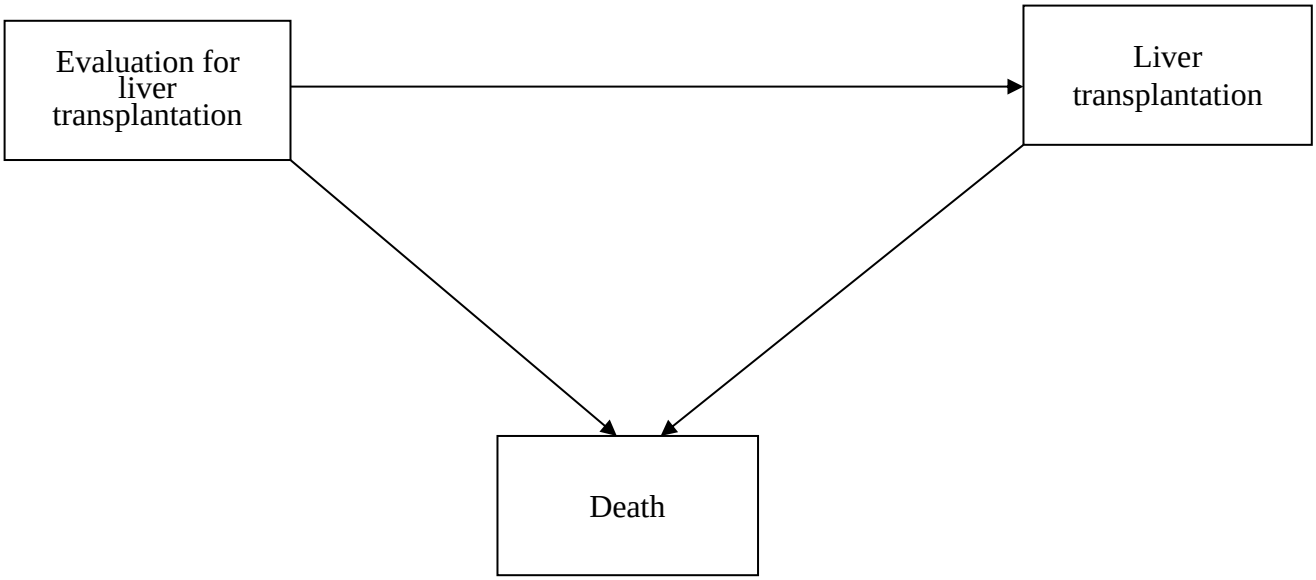


Figure E5: Schema for liver transplantation and death multistate model



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