

# Macrophage migration inhibitory factor as a novel biomarker of portopulmonary hypertension

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**Abstract:** Portopulmonary hypertension (POPH) is a poorly understood complication of liver disease associated with significant morbidity and mortality. We sought to identify novel biomarkers of POPH disease presence and severity. We performed a prospective, multicenter, case-control study involving patients with liver disease undergoing right heart catheterization. POPH cases were defined as a mean pulmonary arterial pressure (mPAP)  $\geq 25$  mmHg and pulmonary vascular resistance (PVR)  $>240$  dynes·s·cm<sup>-5</sup>. Plasma samples were collected from the systemic and pulmonary circulation, and antibody microarray was used to identify biomarkers. Characterization and validation of a candidate cytokine, macrophage migration inhibitory factor (MIF), was performed using enzyme-linked immunosorbent assay. Continuous variables were compared using a Mann-Whitney *U* test and correlated with disease severity using Spearman correlation. MIF levels were elevated in both the systemic and pulmonary circulation in patients with POPH compared with controls (median MIF level [interquartile range] in systemic circulation: 46.68 ng/mL [32.31–76.04] vs. 31.19 ng/mL [26.92–42.17],  $P = 0.009$ ; in pulmonary circulation: 49.59 ng/mL [35.90–108.80] vs. 37.78 [21.78–45.53],  $P = 0.002$ ). In patients with POPH, MIF levels were positively correlated with PVR ( $r = 0.58$ ,  $P = 0.006$ ) and inversely correlated with cardiac output ( $r = -0.57$ ,  $P = 0.007$ ). MIF  $>60$  ng/mL or tricuspid regurgitation gradient  $>50$  mmHg had a 92% sensitivity and specificity for the diagnosis of POPH, with a positive predictive value of 86% and a negative predictive value of 96%. MIF is a promising novel biomarker of POPH disease presence and severity in patients with liver disease and portal hypertension.

**Keywords:** portopulmonary hypertension, macrophage migration inhibitory factor, biomarker, transpulmonary, pulmonary arterial hypertension.

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Portopulmonary hypertension (POPH), a subtype of World Health Organization (WHO) group 1 pulmonary arterial hypertension (PAH) that develops in patients with liver disease and portal hypertension, is the third most common cause of associated PAH and affects 5%–6% of patients referred for liver transplantation.<sup>1–4</sup> POPH is pathologically indistinct from idiopathic PAH, but it is associated with a significantly lower 5-year survival despite a higher cardiac index and lower right atrial pressure at diagnosis.<sup>5,6</sup>

POPH can complicate or preclude liver transplantation because of an elevated risk of perioperative death.<sup>7</sup> Because of this increased risk, patients on the liver transplant list undergo yearly echocardiograms to screen for POPH, and patients with known POPH undergo right heart catheterizations every 3 months to ensure that their hemodynamic characteristics are acceptable for undergoing transplantation.<sup>8,9</sup> As such, identification of noninvasive biomarkers of POPH disease presence, severity, and treatment response is needed to minimize repeated invasive procedures in this group of patients.

The pathogenesis of POPH, characterized by pulmonary vasoconstriction and vascular remodeling, is poorly understood. There

is no known association between the presence of POPH and severity of liver disease or portal hypertension, but both female sex and autoimmune liver disease have been identified as clinical risk factors.<sup>4,10,11</sup> An increased prevalence of spontaneous portosystemic shunts in patients with POPH as well as the development of POPH in patients with congenital portosystemic shunts with normal liver function suggests that vasoactive factors from the splanchnic circulation, rather than the presence of liver failure per se, contribute to disease development.<sup>12,13</sup> These factors may represent novel therapeutic targets and may play an important role in the pathogenesis of other subtypes of PAH.

The purpose of our study was to identify biomarkers of POPH disease presence, severity, and treatment response. We also sought to compare circulating gradients of these biomarkers to determine whether they were derived from the splanchnic or pulmonary circulation. On the basis of the results of exploratory antibody microarray, we identified macrophage migration inhibitory factor (MIF) as a potential biomarker candidate. We chose to focus on MIF for the following reasons: (1) MIF is a pleiotropic proinflammatory cyto-

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kine expressed in the lung, liver, and spleen, key organs that are likely to be involved in the pathogenesis of POPH;<sup>14</sup> (2) MIF is involved in inflammation and endothelial dysfunction in other subtypes of PAH, but its role in POPH has not been previously studied;<sup>15</sup> and (3) high-quality immunoassays were available for measurement of circulating MIF.

## METHODS

### Study design and subject selection

We performed a prospective multicenter case-control study. Patients with liver disease and suspected POPH (based on symptoms and/or echocardiogram) undergoing right heart catheterization as part of their clinical evaluation at 3 academic centers were enrolled between February 2014 and December 2015. Liver disease was defined as a clinical diagnosis of cirrhosis or portal hypertension with imaging or pathology consistent with cirrhosis or portal hypertension. Patients were divided into 2 groups, POPH case patients and liver disease controls, on the basis of pulmonary hemodynamic characteristics. POPH cases were defined as patients with an elevated mean pulmonary arterial pressure (mPAP)  $\geq 25$  mmHg and PVR  $> 240$  dynes  $\cdot$  s  $\cdot$  cm<sup>-5</sup> with a normal pulmonary arterial wedge pressure (PAWP)  $\leq 15$  mmHg and a clinical diagnosis of POPH by an experienced PAH healthcare provider.<sup>8,16</sup> We included patients with incident as well as prevalent disease with prior diagnosis of POPH who were receiving PAH therapy if they had previously met these hemodynamic criteria. Patients with hepatopulmonary syndrome were excluded from the control group. Clinical data, including demographic characteristics, liver disease etiology, liver disease severity, and transthoracic echocardiogram data, including tricuspid regurgitation (TR) gradient, derived from tricuspid regurgitation jet velocity, were collected from review of the medical record. TR gradient was used rather than right ventricular systolic pressure, because there was intercenter variation regarding estimation of right atrial pressure. The study was approved by the institutional review board (IRB; 2013-P-000416), and all patients provided written informed consent.

### Sample collection and assays

At the time of catheterization, plasma samples were collected from the central vein (CV; internal jugular or femoral vein), the right atrium (RA), and the pulmonary arterial wedge (PAW) position. Before each sample collection, 2 mL of blood from the catheter was discarded to ensure that the sample was collected from the intended location. CV samples were collected from the catheter introducer, whereas RA and PAW samples were collected from the distal port of the Swan-Ganz catheter. All samples were collected in EDTA tubes, centrifuged at 3,000 g for 10 minutes, and stored at  $-80^{\circ}\text{C}$ . An exploratory screen using a multiplexed antibody microarray was performed at R&D Systems (Human XL Cytokine Array). Circulating MIF levels were measured using commercial enzyme-linked immunosorbent assay using manufacturer's instructions (R&D Systems). This assay had a sensitivity of 0.068 ng/mL with an intraplate and interplate coefficient of variation of 4.5%–6% and 8.4%–9.7%, respectively.

### Validation and comparison with non-POPH WHO group 1 PAH

To validate our findings and compare MIF levels in patients with POPH with levels in patients with non-POPH WHO group 1 PAH, we also measured circulating MIF levels in a retrospective bank of available superior vena cava plasma samples from a validation cohort of patients with POPH and non-POPH group 1 PAH (idiopathic PAH, HIV-associated PAH, connective tissue disease-associated PAH, and drug/toxin-induced PAH).<sup>17</sup>

### MIF expression in the lung

Archived lung pathology specimens from patients with WHO group 1 or 3 PH (documented mPAP  $\geq 25$  mmHg and a PAWP  $\leq 15$  mmHg) and controls with normal right heart size and function and an estimated right ventricular systolic pressure  $< 35$  mmHg determined by echocardiogram were identified from a search of the pathology laboratory system at a single center. Following hematoxylin and eosin staining, formalin-fixed, paraffin-embedded lung tissue blocks were examined by a thoracic surgical pathologist. Immunohistochemical staining for MIF (polyclonal goat immunoglobulin G [IgG] human MIF antibody, 1:1,000 or 1:1,500 dilution; R&D Systems) was performed using Vector ImmPRESS HRP Anti-Goat IgG Polymer Detection Kit in accordance with the manufacturer's instructions (Burlingame, CA). The pathology study was approved by the IRB with waiver of informed consent.

### Statistical analysis

Data are summarized as medians with interquartile ranges (IQRs) or absolute numbers and percentages. Case patients and controls were compared using a Mann-Whitney *U* test,  $\chi^2$  test, or Fisher exact test,

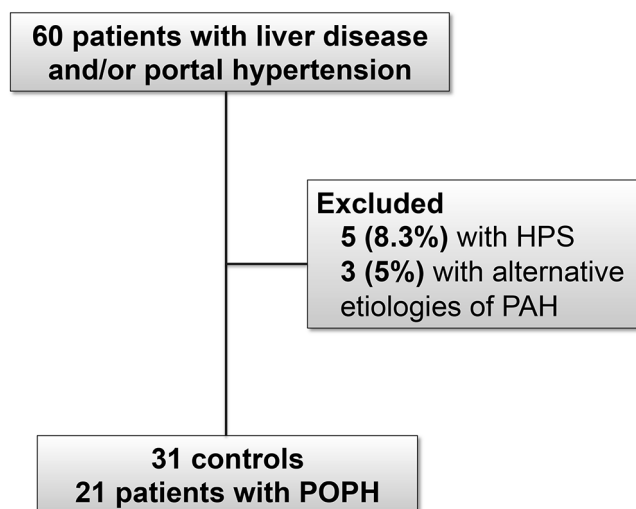


Figure 1. Flow chart of patients included in the study. A total of 8.3% of patients were excluded due to hepatopulmonary syndrome (HPS), and 5% of patients were excluded due to alternative etiologies of pulmonary arterial hypertension (PAH). Thirty-one controls and 21 case patients with portopulmonary hypertension (POPH) were included in the final cohort.

as appropriate. Matched plasma samples within individuals were compared using a Wilcoxon signed-rank test. MIF levels were correlated with continuous variables using Spearman rank correlation. To determine the value of MIF as a potential screening tool for POPH, we generated receiver operating characteristic (ROC) curves for CV MIF

levels and TR gradient by echocardiogram and a combination of the two using a logistic model in controls and incident patients with POPH (i.e., treatment-naive patients with newly diagnosed POPH). We also calculated the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of different MIF and TR

Table 1. Patient characteristics

Characteristic	Controls ( <i>n</i> = 31)	Portopulmonary hypertension ( <i>n</i> = 21)	<i>P</i>
Age, years	58.0 (50.0–65.0)	55.0 (48.0–63.0)	NS
Female sex	9 (29.0)	5 (23.8)	NS
Race/ethnicity			NS
White	25 (80.6)	15 (71.4)	
Hispanic/Latino	4 (12.9)	4 (19)	
Black	2 (6.5)	2 (9.5)	
Other	0	2 (9.5)	
Comorbidities			NS
Hypertension	17 (54.8)	10 (47.6)	
Diabetes mellitus	16 (51.6)	10 (47.6)	
ESRD with hemodialysis	3 (9.7)	1 (4.8)	
HCC	5 (16.1)	4 (19.0)	
Systemic glucocorticoid use	3 (9.7)	1 (5.3) ( <i>n</i> = 19)	
Etiology of liver disease			NS
Alcohol	15 (48.4)	11 (52.4)	
HCV infection	9 (29.0)	8 (38.1)	
HCV infection plus alcohol	1 (3.2)	4 (19.0)	
Nonalcoholic fatty liver disease	4 (12.9)	1 (4.8)	
Autoimmune	0	1 (4.8)	
Other	9 (29.0)	4 (23.8)	
Liver disease severity			
MELD	17 (12–24)	11 (9–14)	0.002
Child class			<0.001
A	2 (6.5)	9 (42.9)	
B	13 (41.9)	11 (52.4)	
C	16 (51.6)	1 (4.8)	
Physical examination			
Body mass index	28.6 (25.6–32.3) ( <i>n</i> = 27)	29.1 (23.7–35.0) ( <i>n</i> = 16)	NS
Oxygen saturation	97 (95.5–99.0) ( <i>n</i> = 29)	96 (95–98) ( <i>n</i> = 18)	NS
Tricuspid regurgitation gradient by echocardiogram, mmHg	37 (35–45) ( <i>n</i> = 26)	53 (42–68) ( <i>n</i> = 20)	0.002
Hemodynamic characteristics			
mPAP, mmHg	24 (19–30)	38 (31–48)	<0.001
PAWP, mmHg	15.5 (11.5–20)	10 (5.5–13.5)	<0.001
PVR, dynes · s · cm <sup>-5</sup>	86.4 (50.4–119.0)	327.7 (267.0–534.9)	<0.001
CO, L/min	7.7 (6.7–10.3)	6.3 (5.3–7.6)	0.001

Note: Patients may have had more than one listed race/ethnicity, diagnosis, or comorbidity. Data are expressed as median (interquartile range) or number (%). CO: cardiac output; ESRD: end-stage renal disease; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; MELD: model for end-stage liver disease; mPAP: mean pulmonary arterial pressure; NS: not significant; PAWP: pulmonary arterial wedge pressure; PVR: pulmonary vascular resistance.

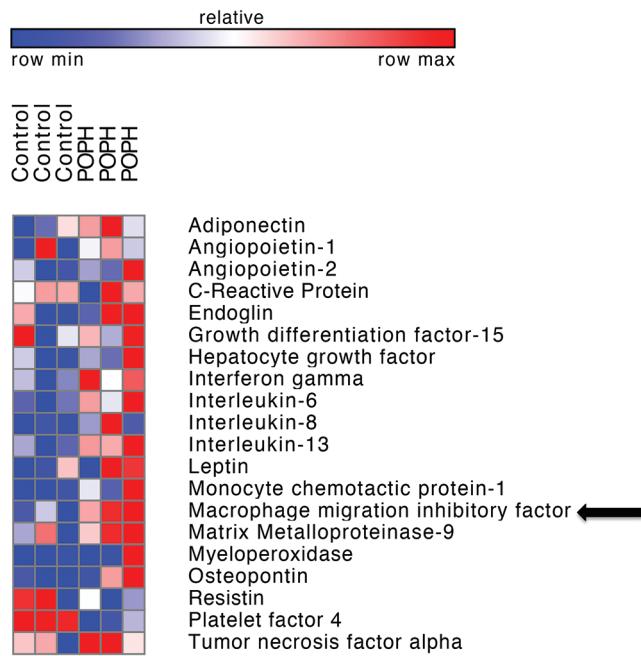


Figure 2. Antibody microarray. Heat map demonstrating relative expression of a subset of biomarkers from screening antibody microarray (Human XL Cytokine Array, R&D Systems) in patients with portopulmonary hypertension (POPH;  $n = 3$ ) versus controls ( $n = 3$ ). Macrophage migration inhibitory factor expression, indicated by an arrow, was 1.6-fold higher in patients with POPH.

gradient thresholds to identify cutoff values with optimal testing characteristics. We chose the CV as our primary site for analysis (rather than RA or PAW), because it would be most reproducibly obtained and similar to peripheral blood samples used for screening. As an exploratory analysis, we also compared patients with high (above the

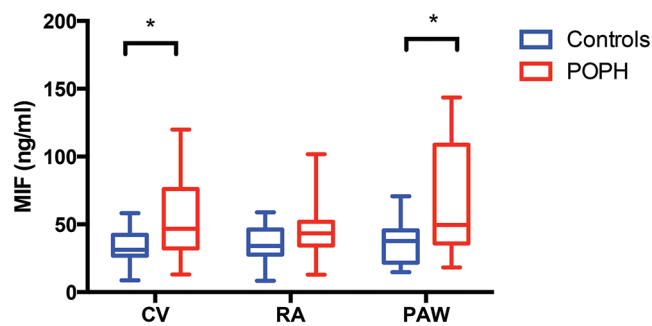


Figure 3. Circulating plasma macrophage migration inhibitory factor (MIF) levels. Median (interquartile range) central vein (CV), right atrium (RA), and pulmonary artery wedge (PAW) macrophage migration inhibitory factor (MIF) levels in patients with portopulmonary hypertension (POPH) versus controls. Both systemic (CV) and pulmonary (PAW) MIF levels were higher in patients with POPH than in controls (CV: 46.68 ng/mL [32.31–76.04] vs. 31.19 ng/mL [26.92–42.17],  $P = 0.009$ ; PAW: 49.59 [35.90–108.80] vs. 37.78 [21.78–45.53],  $P = 0.002$ ). There was also a trend toward higher RA MIF levels in patients with POPH (43.35 ng/mL [34.45–51.99] vs. 34.13 ng/mL [27.81–46.19],  $P = 0.07$ ).

median) and low (below the median) CV and PAW MIF levels to determine whether MIF was predictive of mortality using Cox regression. Vital status was determined from the medical record as any death that occurred between the time of enrollment and the end of the follow-up period (May 20, 2016). Kaplan-Meier survival curves were generated. Patients who were lost to follow-up were censored at the time of their last clinic visit or hospitalization. All tests were 2-sided, and significance was defined as  $P < 0.05$ . Data analysis was performed in GraphPad Prism, version 6.0, and ROC curves were generated in R, version 3.2.2. Heat map was generated using GENE-E software (Broad Institute).

RESULTS

Patient characteristics

Twenty-one patients with POPH and 31 controls with liver disease without pulmonary hypertension were included in the study (Fig. 1). Patients with POPH were not significantly different from controls with respect to age, sex, race/ethnicity, or liver disease etiology (Table 1). Compared with controls, patients with POPH had less severe liver disease as assessed by either Model for End-Stage

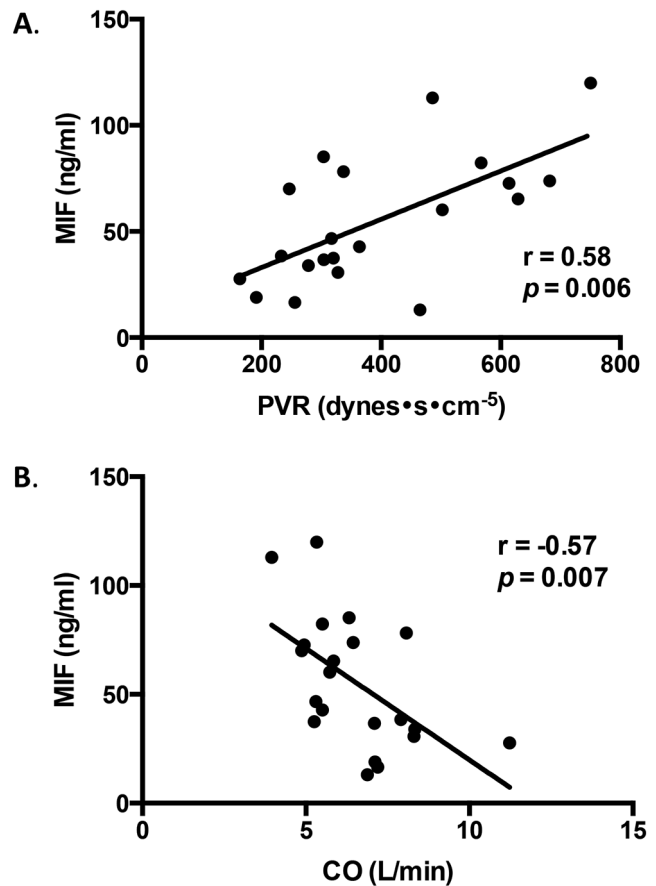


Figure 4. Correlation of macrophage migration inhibitory factor (MIF) levels with pulmonary hemodynamic characteristics. Central venous MIF is significantly correlated with pulmonary vascular resistance ( $r = 0.58$ ,  $P = 0.006$ ; A) and inversely correlated with cardiac output ( $r = -0.57$ ,  $P = 0.007$ ; B).

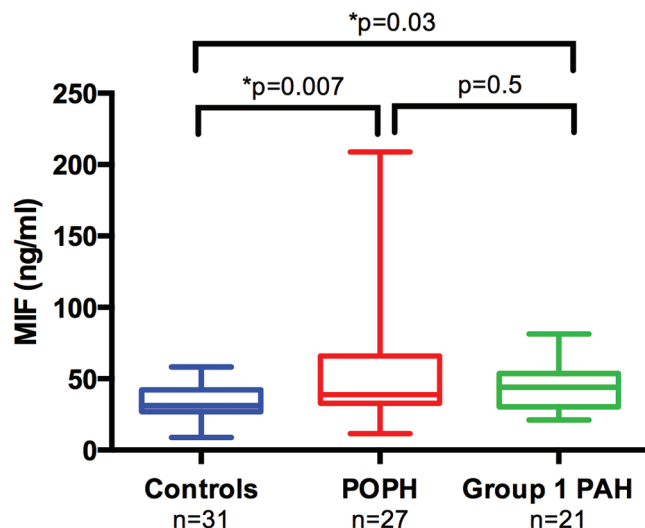


Figure 5. Median (interquartile range) macrophage migration inhibitory factor (MIF) levels in controls, patients with portopulmonary hypertension (POPH), and patients with non-POPH group 1 pulmonary arterial hypertension (PAH). Central vein (CV) MIF levels are higher in an external cohort of patients with POPH (38.85 ng/mL [32.9–66.0];  $n = 27$ ) compared with liver disease controls (31.2 ng/mL [26.9–42.2];  $n = 31$ ) but are not significantly different from patients with non-POPH group 1 PAH (44.1 ng/mL [30.3–53.6];  $n = 21$ ).

Liver Disease (MELD) score ( $P = 0.002$ ) or Child-Pugh class ( $P < 0.001$ ). Patients with POPH had a median mPAP of 38 mmHg (IQR: 31–48 mmHg) and median PVR of 327.7 dynes·s·cm<sup>-5</sup> (IQR: 267.0–534.9 dynes·s·cm<sup>-5</sup>), which were both significantly higher compared with the control group ( $P < 0.001$ ). Patients with POPH

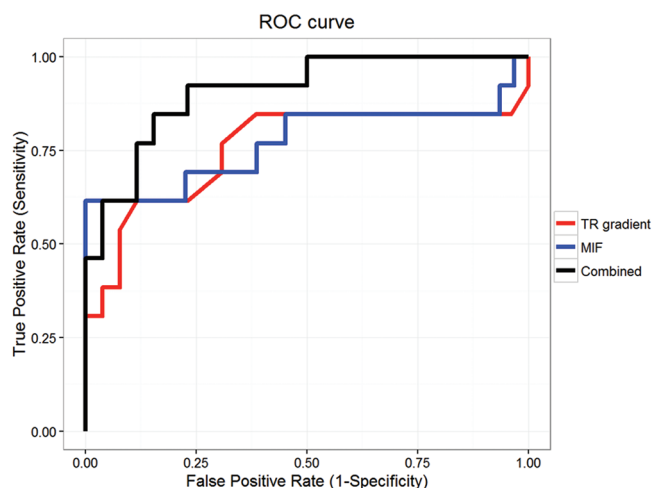


Figure 6. Screening test characteristics. Receiver operating characteristic curves for macrophage migration inhibitory factor (MIF; area under the receiver operating characteristic curve [AUC]: 0.77), tricuspid regurgitation (TR) gradient by transthoracic echocardiogram (AUC: 0.75), and combined MIF plus TR gradient (AUC: 0.91) are shown.

also had a significantly lower cardiac output (CO) and PAWP compared with controls (Table 1).

### Antibody microarray screen

The relative expression of a subset of biomarkers from multiplexed antibody microarray in a limited sample set (3 controls and 3 case patients) is shown in Figure 2. One hundred and eight cytokines were analyzed. Relative cytokine expression was an average 1.18-fold higher in the 3 patients with POPH compared with controls. Nine cytokines were elevated in duplicate testing >1.5-fold in patients with POPH compared with controls (macrophage inflammatory protein-3 beta, hepatocyte growth factor, MIF, platelet-derived growth factor AA, interleukin-17A, monocyte chemoattractant protein-1, myeloperoxidase, leptin, and growth hormone) and no cytokines were reduced >0.5-fold in patients with POPH. For the 3 patients with POPH, compared with 3 controls, duplicate testing of MIF had the lowest  $P$  values without correction for multiple testing (0.01 and 0.009), and MIF was 1.6-fold higher in patients with POPH. The complete raw data set with duplicate values for all cytokines is included in Table S1 (available online). On the basis of microarray results, we focused our efforts on further characterizing MIF as a potential biomarker candidate.

### Circulating MIF levels and correlation with disease severity

Both CV and PAW MIF levels were significantly higher in patients with POPH compared with controls ( $P = 0.009$  and  $P = 0.002$ , respectively), and there was a trend toward higher RA MIF levels in patients with POPH ( $P = 0.07$ ; Fig. 3). PAW MIF levels were significantly higher than RA MIF levels in patients with POPH ( $P = 0.01$ ), whereas controls had similar PAW and RA MIF levels ( $P = 0.5$ ). PAW MIF levels were not significantly different from CV MIF levels in patients with POPH ( $P = 0.27$ ) or controls ( $P = 0.18$ ). The absolute change in MIF across the pulmonary circulation (PAW-RA)

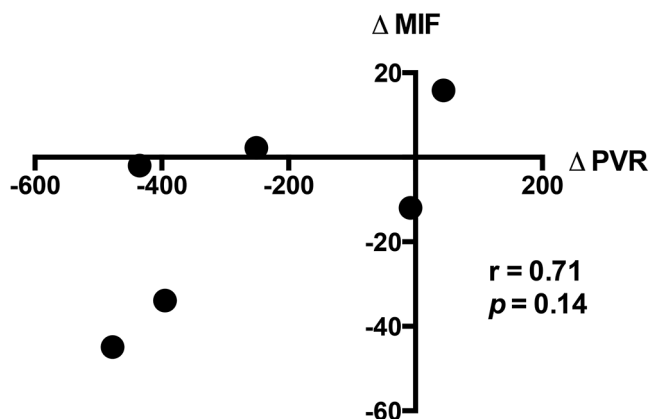


Figure 7. Treatment response, showing the correlation between absolute change in macrophage migration inhibitory factor ( $\Delta$ MIF; ng/mL) and absolute change in pulmonary vascular resistance ( $\Delta$ PVR; dynes·s·cm<sup>-5</sup>) in 6 patients who underwent cardiac catheterization with sample collection before and after initiation of pulmonary arterial hypertension therapy.

Table 2. Treatment response

Patient	Baseline			Posttreatment		
	Hemodynamic characteristics	MIF (ng/mL)	PAH therapy	Hemodynamic characteristics	MIF (ng/mL)	Hemodynamic characteristics
Incident cases before and after initiation of PAH therapy						
1	mPAP: 55 mmHg; PVR: 750 dynes·s·cm <sup>-5</sup> ; CO: 5.3 L/min	120.00	PDE5I and ERA	mPAP: 44 mmHg; PVR: 273 dynes·s·cm <sup>-5</sup> ; CO: 7.0 L/min	75.08	
2	mPAP: 51 mmHg; PVR: 629 dynes·s·cm <sup>-5</sup> ; CO: 5.9 L/min	65.30	PDE5I and INH PGI2	mPAP: 34 mmHg; PVR: 194 dynes·s·cm <sup>-5</sup> ; CO: 8.2 L/min	63.01	
3	mPAP: 40 mmHg; PVR: 364 dynes·s·cm <sup>-5</sup> ; CO: 5.5 L/min	42.80	PDE5I (noncompliant)	mPAP: 42 mmHg; PVR: 408 dynes·s·cm <sup>-5</sup> ; CO: 5.1 L/min	58.60	
4	mPAP: 44 mmHg; PVR: 278 dynes·s·cm <sup>-5</sup> ; CO: 8.3 L/min	33.94	PDE5I	mPAP: 43 mmHg; PVR: 270 dynes·s·cm <sup>-5</sup> ; CO: 7.1 L/min	21.92	
5	mPAP: 49 mmHg; PVR: 614 dynes·s·cm <sup>-5</sup> ; CO: 5.0 L/min	72.70	PDE5I and INH PGI2	mPAP: 32 mmHg; PVR: 219 dynes·s·cm <sup>-5</sup> ; CO: 7.0 L/min	38.84	
6	mPAP: 46 mmHg; PVR: 465 dynes·s·cm <sup>-5</sup> ; CO: 6.9 L/min	13.01	ERA and PDE5I	mPAP: 30 mmHg; PVR: 214 dynes·s·cm <sup>-5</sup> ; CO: 8.6 L/min	15.21	
Prevalent cases before and after up-titration of PAH therapy						
7	mPAP: 31 mmHg; PVR: 317 dynes·s·cm <sup>-5</sup> ; CO: 5.3 L/min on PDE5I and INH PGI2	46.68	PDE5I, ERA, and INH PGI2	mPAP: 34 mmHg; PVR: 171 dynes·s·cm <sup>-5</sup> ; CO: 8.0 L/min	30.46	
8	mPAP: 31 mmHg; PVR: 233 dynes·s·cm <sup>-5</sup> ; CO: 7.9 L/min on PDE5I	38.48	PDE5I and ERA	mPAP: 31 mmHg; PVR: 159 dynes·s·cm <sup>-5</sup> ; CO: 11.1 L/min	56.81	

Note: Pulmonary hemodynamic characteristics and central venous macrophage migration inhibitory factor (MIF) levels before and after initiation or up-titration of pulmonary arterial hypertension therapy. CO: cardiac output; ERA: endothelin receptor antagonist; INH: inhaled; mPAP: mean pulmonary arterial pressure; PAH: pulmonary arterial hypertension; PDE5I: phosphodiesterase 5 inhibitor; PGI2: prostacyclin; PVR: pulmonary vascular resistance.

was higher in patients with POPH compared with controls (median [IQR]: 9.04 ng/mL [1.22–48.40] vs. 2.15 ng/mL [–5.52 to 8.04];  $P = 0.02$ ). In a subgroup analysis, median (IQR) CV MIF in patients with incident POPH (65.30 ng/mL [35.68–80.26];  $n = 13$ ) was not significantly different from that in patients with prevalent POPH who were receiving PAH therapy (37.59 ng/mL [28.46–67.06];  $n = 8$ ;  $P = 0.3$ ). There was no difference in CV MIF levels by sex ( $P = 0.35$ ) or internal jugular versus femoral central venous site of access ( $P = 0.6$ ).

Circulating CV MIF was significantly correlated with PVR ( $r = 0.58$ ,  $P = 0.006$ ), inversely correlated with CO ( $r = -0.57$ ,  $P = 0.007$ ; Fig. 4A, 4B), and was not correlated with mPAP ( $r = 0.29$ ,  $P = 0.2$ ) or PAWP ( $r = 0.01$ ,  $P = 0.95$ ). CV MIF levels were also not correlated with liver disease severity as assessed by the MELD score ( $r = -0.19$ ,  $P = 0.4$ ).

Using the same internal controls, we compared CV MIF levels to those in a retrospective cohort of patients with POPH ( $n = 27$ ) and non-POPH WHO group 1 PAH ( $n = 21$ ). Compared with control subjects, MIF was significantly higher in patients with POPH (median [IQR]: 38.85 ng/mL [32.90–65.97] vs. 31.19 ng/mL [26.92–42.17];  $P = 0.007$ ) and with non-POPH group 1 PAH (44.05 ng/mL [30.25–53.60];  $P = 0.03$ ). Patients with POPH and non-POPH group 1 PAH did not have significantly different CV MIF levels ( $P = 0.5$ ; Fig. 5).

### MIF as a screening test for POPH

We evaluated whether CV MIF levels could improve the screening for POPH among patients with liver disease. In controls ( $n = 26$ ) and patients with incident POPH ( $n = 13$ ) with adequate estimates of TR gradient by echocardiogram, CV MIF and TR gradient had similar testing characteristics (area under the curve [AUC]: 0.77 vs. 0.75). Combined, MIF and TR gradient had an AUC of 0.91 ( $P = 0.18$ , compared with TR gradient alone; Fig. 6). Additionally, the presence of either MIF >60 ng/mL or TR gradient >50 mmHg by echocardiogram had a 92% sensitivity and 92% specificity for the diagnosis of POPH with a positive predictive value of 86% and negative predictive value of 96%. With these cutoffs, a screening algorithm of MIF in conjunction with echocardiogram could have avoided 24 right heart catheterizations in the control group.

### Treatment response and outcomes

Six patients with POPH underwent repeat catheterizations with sample collection before and after initiation of PAH therapy. The relationship between the absolute change in CV MIF and the absolute change in PVR after initiation of PAH therapy is depicted in Figure 7 ( $r = 0.71$ ,  $P = 0.14$ ). Pulmonary hemodynamic characteristics and CV MIF levels before and after initiation or uptitration of PAH therapy are detailed in Table 2.

Three patients with POPH died during the study period, and one patient was lost to follow-up. The 3 deaths occurred at 20.1 months, 8.4 months, and 0.7 months after study enrollment and diagnostic right heart catheterization. Causes of death were liver failure, sepsis, and unknown. All 3 patients who died had PAW MIF levels >100 ng/mL. High CV MIF levels were not associated with mortality, but high

PAW MIF levels were associated with an increased risk of death in patients with POPH (Fig. 8). Five patients have undergone liver transplant (8.9, 16.1, 18.6, 4.9, and 6.7 months after study enrollment), and all are alive at 12.5, 6.2, 1.2, 12.1, and 0.4 months after transplant.

### Lung MIF expression

To evaluate MIF expression in the lungs, we performed immunohistochemical tests using archived lung biopsies. MIF was expressed predominantly in the endothelium and alveolar macrophages of both controls and patients with PH (Fig. 9). Because of a lack of available specimens, we could not evaluate MIF expression in subjects with POPH.

### DISCUSSION

This study demonstrates that MIF is a promising novel biomarker of POPH disease presence and severity in patients with liver disease. We found that MIF was higher in both the systemic and pulmonary circulations of patients with POPH compared with controls and correlated with hemodynamic indices of disease severity. More importantly, the combination of circulating MIF >60 ng/mL or TR gradient >50 mmHg had an excellent negative predictive value of 96% to identify patients with liver disease who may not need a right heart catheterization to rule out POPH, and high pulmonary MIF levels were associated with an increased risk of death. Additionally, our results suggest that MIF production in the pulmonary circulation, rather than impaired hepatic clearance, may play a role in disease pathogenesis.

Earlier investigations of biomarkers in POPH have consisted of predominantly small studies that relied on echocardiogram rather than invasive hemodynamic testing for differentiation of case patients versus controls or did not correlate biomarker levels with disease severity or treatment response.<sup>18–20</sup> In contrast to these studies, we enrolled patients with well-characterized liver disease and pul-

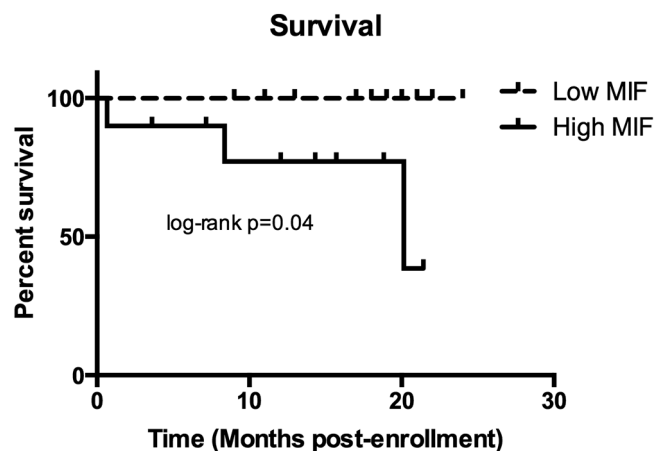


Figure 8. Kaplan-Meier survival curves for patients with POPH with high and low pulmonary macrophage migration inhibitory factor (MIF) levels above and below the median for the cohort (49.59 ng/mL) are depicted. Patients with high pulmonary MIF levels had poorer survival (log-rank  $P = 0.04$ ).

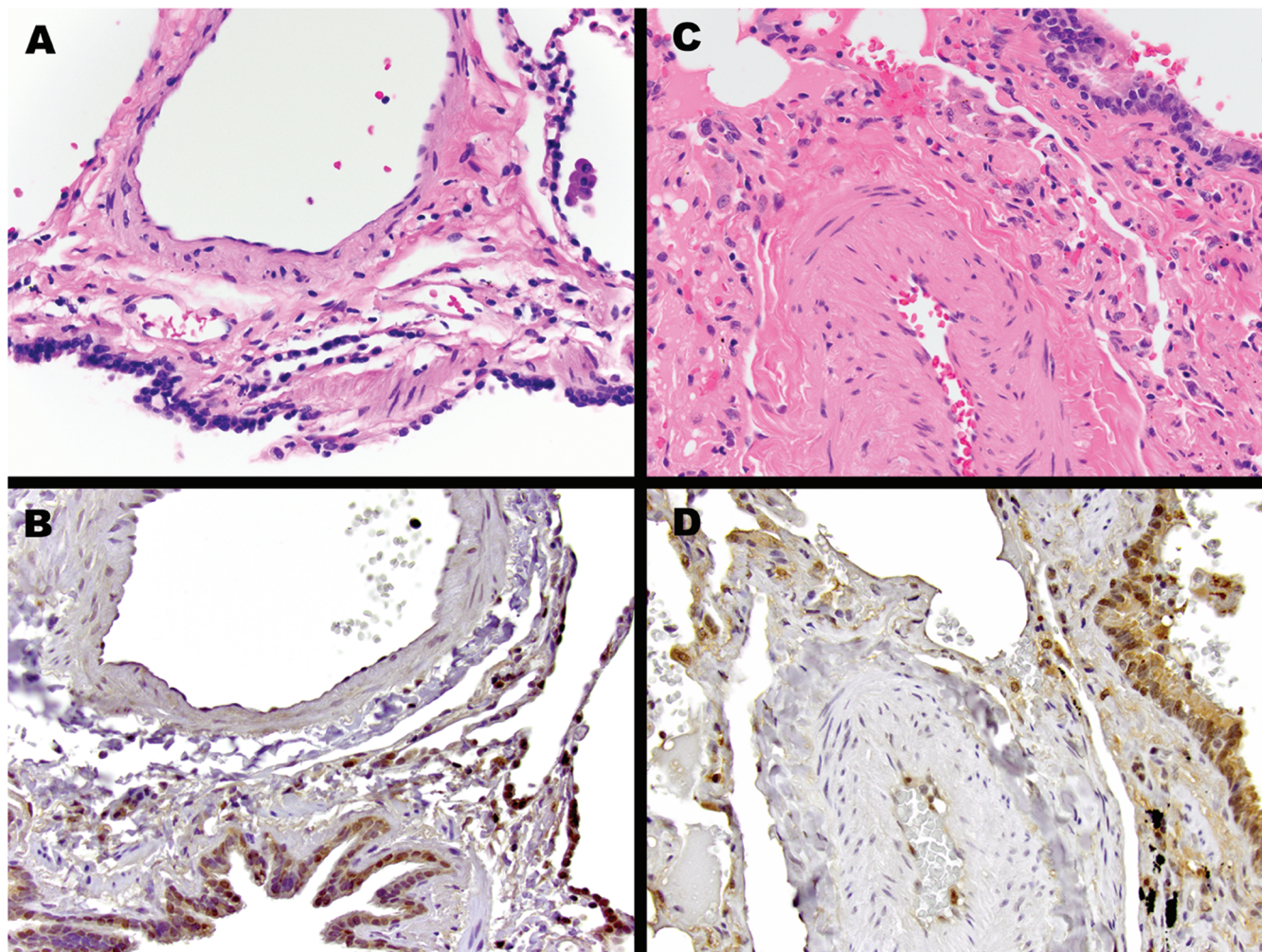


Figure 9. Macrophage migration inhibitory factor (MIF) immunohistochemistry. Representative pulmonary parenchyma from control patient without pulmonary arterial hypertension (PAH) or lung disease (A and B) and patient with PAH (C and D). Compared with the control lung, the pulmonary artery from the patient with PAH demonstrates thickening and hypertrophy of the muscular medial layer. MIF immunostaining for both patients with PAH and control patients show expression in airway-lining ciliated bronchial epithelial cells, pulmonary macrophages, pneumocytes, and endothelial cells. Of note, the vascular smooth-muscle cells are negative. Hematoxylin and eosin stain (A, C) and MIF immunostain (B, D) images are presented at 400× original magnification.

monary hemodynamics and obtained samples at the same time as right heart catheterization, allowing biomarker levels to be temporally correlated with hemodynamics and disease severity. Interestingly, we found that MIF levels were strongly correlated with PVR and inversely correlated with CO but were not significantly correlated with mPAP. Of note, a diagnosis of POPH and elevated PVR, and not mPAP, are predictors of outcome in PAH,<sup>21</sup> and MIF may play a pathogenic role in pulmonary vascular derangements described in POPH. We also found that circulating MIF levels in conjunction with TR gradient had a 96% NPV for the diagnosis of POPH. Given the current recommendation to screen all patients being considered for liver transplantation for POPH,<sup>8</sup> this finding has important clinical implications. A screening algorithm combining MIF and echocardiogram with an excellent NPV could poten-

tially decrease the number of right heart catheterizations in this group of patients, who often have an increased bleeding risk. Last, although the number of deaths was small, high pulmonary MIF levels were associated with an increased risk of death.

Despite significant advances in our knowledge regarding clinical and genetic risk factors for POPH,<sup>11,22</sup> its pathogenesis remains poorly understood. Studies have identified decreased prostacyclin synthase expression,<sup>23</sup> higher serum estradiol levels, and genetic variations in aromatase in patients with POPH,<sup>22</sup> but the mechanistic link between these findings and the pathogenesis of POPH remains unknown. MIF, a critical upstream regulator of inflammation, inhibits prostacyclin synthase expression in animal models of hypoxia-induced pulmonary hypertension and is associated with pulmonary artery smooth-muscle cell proliferation.<sup>24,25</sup> Studies involving other



diseases have also shown that MIF can activate aromatase and increase estradiol levels.<sup>26</sup> Additionally, MIF has been implicated in the pathogenesis of several autoimmune diseases, including autoimmune liver disease,<sup>27-30</sup> which is a known risk factor for POPH.<sup>11</sup> MIF levels in peripheral blood samples are elevated in patients with idiopathic PAH, where MIF contributes to inflammation and endothelial dysfunction,<sup>15</sup> but this is the first study to identify higher circulating MIF levels in the pulmonary circulation of patients with PAH.

We also found that patients with POPH had an increased transpulmonary gradient of MIF compared with controls and similar RA MIF levels, suggestive of increased pulmonary production rather than decreased hepatic clearance. The source and potential stimuli for MIF production in POPH, however, remains to be determined. We found that MIF expression was prominent in alveolar macrophages, vascular endothelium, and lymphocytes in controls and patients with PAH, but we did not appreciate significant immunostaining in vascular smooth muscle cells, suggesting that smooth muscle is unlikely to be a source for higher circulating MIF levels in PAH.

Our study had several limitations. First, our sample size was small. Compared with similar studies of biomarkers in POPH, however, it is one of the largest studies of patients with POPH to date.<sup>18-20</sup> In addition, although we found that CV MIF levels were similar to PAW MIF levels within individuals, potentially because of a prolonged half-life within the circulation, we did not specifically measure MIF levels in peripheral blood samples. We focused on assessing the importance of CV levels of MIF (compared with levels from other compartments) to describe a biomarker that may have a practical applicability in the future as a repetitive measure in clinical practice. We expect that CV MIF levels would be similar to MIF levels in peripheral blood samples, but this would need confirmation in order for MIF to be a practical screening test. MIF can also be upregulated in a variety of other conditions, including hypoxia and inflammatory and autoimmune diseases.<sup>31,32</sup> Although we did not find significant differences in potential confounders, such as peripheral oxygen saturation, prevalence of hepatocellular carcinoma, or autoimmune liver disease between patients with POPH and controls (Table 1), it is possible that the presence of other conditions in which MIF levels are elevated could confound its utility as a screening test. Last, although we found that elevated pulmonary MIF levels were significantly associated with increased mortality, it should be noted that this was an exploratory post hoc analysis and not the primary outcome of our study. Because we did not routinely measure other prognostic PAH markers, such as 6-minute walk distance and brain natriuretic peptide, we were also unable to assess whether MIF levels correlated with these traditional markers of disease severity and prognosis.

Future studies to determine the correlation of MIF with hemodynamic response to PAH therapy and liver transplant are warranted, as is validation of MIF in conjunction with echocardiogram as a screening test for POPH in a larger, prospective cohort of patients with liver disease. Additional studies are also necessary to determine whether other differentially expressed biomarkers, such as hepatocyte growth factor and leptin, play a role in POPH disease pathogenesis or add synergistic value to the screening of patients with liver disease for POPH.

In conclusion, MIF is a novel biomarker of POPH disease presence and severity and may play an important role in disease pathogenesis. In addition, MIF is a promising screening tool for the presence of POPH in the high-risk population of patients with liver disease and portal hypertension and may also be an important prognostic indicator.

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