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Hepatocyte Microvesicle Levels Improve Prediction of Mortality in Patients With Cirrhosis

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Microvesicles (MVs) are extracellular vesicles released by cells following activation or apoptosis. Some MV subpopulations augment with cirrhosis severity and contribute to portal hypertension. This study aimed at determining if plasma MV levels can estimate the presence of hepatic venous pressure gradient (HVPG) ≥10 mm Hg and predict mortality in patients with advanced chronic liver disease. All patients with severe fibrosis or cirrhosis undergoing liver catheterization between 2013 and 2015 at two centers were prospectively included. We measured circulating levels of annexin V^+, platelet, leukocyte, endothelial, and hepatocyte MVs. The test cohort included 139 patients. Hepatocyte MV levels were 4.0-fold and 2.2-fold higher in patients with Child-Pugh C than in those with Child-Pugh A or B liver disease, respectively. Levels of other MV subpopulations were not influenced by liver disease severity. Hepatocyte MV levels correlated with HVPG but could not identify patients with HVPG ≥10 mm Hg. Hepatocyte MV level >65 U/L predicted 6-month mortality independently of Child-Pugh score and of Model for End-Stage Liver Disease (MELD). Patients with hepatocyte MV levels >65 U/L and MELD >15 had a higher 6-month mortality than other patients (23% versus 3%; $P = 0.001$). These findings were confirmed in a validation cohort including 103 patients. *Conclusion:* Circulating MV levels cannot identify patients with HVPG ≥10 mm Hg; by contrast, hepatocyte MV levels strongly improve prediction of 6-month mortality in patients with advanced chronic liver disease; therapies associated with decreased levels of circulating hepatocyte MV might be attractive strategies in patients with severe cirrhosis. (HEPATOLOGY 2018; 68:1508-1518).

Accurate prediction of survival in patients with cirrhosis is crucial. Indeed, liver transplantation markedly improves survival in patients unlikely to survive in the short term with medical management alone.[1] The Model for End-Stage Liver Disease (MELD) has been adopted as an objective indicator of liver disease severity.[1] However, MELD is an imperfect tool. Indeed, the present US MELD-based allocation policy is still associated with a substantial mortality on the waiting list for liver transplantation as ≈2,000 patients die annually and another 1,000 patients are removed because they are too sick for transplant.[2] Although many of these deaths are due to the shortage of donated organs, some are attributable to inaccuracy in mortality risk prediction. Microvesicles (MVs) are extracellular vesicles released into extracellular space following cell activation or apoptosis.[3] We observed that plasma levels of MVs

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CD, cluster of differentiation; HCC, hepatocellular carcinoma; HVPG, hepatic venous pressure gradient; MELD, Model for End-Stage Liver Disease; MV, Microvesicle.

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derived from hepatocytes or leuko-endothelial cells (cluster of differentiation 31–positive [$CD31^+$]/CD41) are elevated in patients with cirrhosis and increase in parallel with cirrhosis severity.[4] Moreover, plasma MVs from patients with Child-Pugh B or C cirrhosis induce vascular hypocontractility and therefore might contribute to portal hypertension and circulatory dysfunction.[4] The aim of this prospective study was to determine in two independent cohorts of patients with advanced chronic liver disease whether circulating MV levels are able to estimate the presence of clinically significant portal hypertension (i.e., hepatic venous pressure gradient [HVPG] $\geq$10 mm Hg) and to predict mortality.

Patients and Methods

PATIENTS

All consecutive patients with severe liver fibrosis or cirrhosis undergoing hepatic vein and/or right heart catheterization in two independent centers were prospectively included. The inclusion period ranged from June 2013 to March 2015 for the test cohort (Hôpital Beaujon, Clichy, France) and from January 2014 to December 2015 for the validation cohort (Hospital Clinic, Barcelona, Spain). Clinical, laboratory, and hemodynamic features were prospectively collected in both centers. Diagnosis of severe liver fibrosis (METAVIR F3) or cirrhosis was based either on histological criteria or on the combination of clinical, laboratory, morphological, and hemodynamic features.[5,6] Noninclusion criteria were a history of transjugular intrahepatic portosystemic shunt, liver transplantation, hepatocellular carcinoma (HCC) outside Milan criteria because HCC increases MV levels by itself,[7-9] active extrahepatic cancer, human immunodeficiency virus infection, primary sclerosing cholangitis and primary biliary cirrhosis because HVPG might not reflect the portosystemic gradient in patients with cholestatic liver disease,[10] Budd-Chiari syndrome, or an acute event (hepatorenal syndrome, bacterial infection, alcoholic hepatitis, variceal bleeding) within 2 weeks prior to hepatic vein catheterization. Patients on the waiting list for liver transplantation and those with HCC were seen every 3 months. Other patients were seen every 6 months. When patients did not attend the follow-up visit, they were called by phone. In the absence of an answer, we consulted the liver transplant registry and the national registry of deaths.

This study was approved by the institutional review boards of Paris North Hospitals, Paris 7 University, AP-HP (no. 11-112) and of Hospital Clinic (Barcelona, Spain). All patients included in this study gave written informed consent. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

PLATELET-FREE PLASMA PREPARATION

Platelet-free plasma was prepared at each center following a standardized protocol proposed by Lacroix and colleagues.[11] The principal investigator of this study (P.-E.R.) trained the investigators responsible for plasma preparation (O.N., G.S.-J.). Briefly, peripheral venous blood was collected within 2 hours prior to liver catheterization from the cubital vein of the patients, with a 21-gauge tourniquet needle, in 0.129 mol/L citrated tubes, after having discarded the first milliliter of blood. Tubes then remained motionless in the upright position at room temperature for a maximum of 2 hours until platelet-free plasma preparation, consisting of two successive centrifugations, each of 15 minutes at 2,500*g* at 20°C with a light brake. Aliquots

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of platelet-free plasma were then stored at −80°C until use.

To compare hepatocyte MV concentrations between hepatic and peripheral venous blood, we collected blood from the median or the right hepatic vein using a tip-curved catheter (Cook; HNB7.0-38-100-P-NS-MPA) and from the cubital vein from 10 additional patients and prepared platelet-free plasma as mentioned above.

CHARACTERIZATION OF CIRCULATING MV LEVELS

Circulating levels of annexin V^+, platelet ($CD41^+$), leuko-endothelial ($CD31^+/41^-$), pan-leukocyte ($CD11a^+$), and endothelial ($CD62e^+$ and $CD144^+$) MVs were determined on a Gallios flow cytometer (Beckman Coulter, Villepinte, France) using a technique described in detail.[4] Regions corresponding to MVs were identified in forward light scatter and side-angle light scatter intensity dot plot representation set at logarithmic gain. MV gates were defined, using calibration beads (Megamix plus FSC; Biocytex, France), as events having a diameter of 0.1-1 μm. Gates were separated into "large" (0.5-1 μm) and "small" (<0.5 μm) MVs. Events were then plotted on a fluorescence/forward light scatter dot plot to determine MV counts positively labeled by specific antibodies. Anti-CD41-phycoerythrin-cyanin 7, anti-CD31-phycoerythrin, anti-CD11a-phycoerythrin, and anti-CD144-phycoerythrin antibodies as well as their matched isotype controls were obtained from Beckman Coulter. Anti-CD62E- fluoroisothiocyanate antibodies as well as their matched isotype controls were obtained from R&D Systems Europe. Annexin V fluoroisothiocyanate was purchased from Beckman Coulter. MV concentration was assessed by comparison with a known amount of flow count calibrator beads (AccuCount Fluorescent Particles, 20 μL; Spherotech, Chicago, IL) added to each sample just before performing flow-cytometric analysis. To limit variability, all measurements of MV levels by flow cytometry were performed using a unique batch for each antibody.

We determined plasma levels of hepatocyte-derived MVs using a described technique.[4,12] Briefly, we measured circulating cytokeratin-18 levels (M65 Epi-Death ELISA; Peviva, Bromma, Sweden) before and after filtration of the plasma through two 0.2-μm filters (Ceveron MFU 500; Technoclone, Austria). The difference between soluble cytokeratin-18 levels in initial and in 0.2 μm–filtrated platelet-free plasma reflected the concentration in hepatocyte MVs (Supporting Fig. S1). We also determined plasma levels of extracellular vesicles having a size range of 0.02-0.2 μm, corresponding to small MVs and/or exosomes, further referred to as "hepatocyte exosomes." These levels corresponded to the difference between cytokeratin-18 levels in 0.2 μm–filtrated and in 0.02 μm–filtrated (Whatman 6809-1002 Anotop Syringe Filter) platelet-free plasma. Hepatocyte MV and exosome levels were expressed as units per liter, according to the manufacturer's instructions. All enzyme-linked immunosorbent assays were performed in duplicate with determination of the coefficient of variation between samples from the same patient. The results were considered adequate when the coefficient of variation was <20%. Otherwise, samples were measured again.

C-REACTIVE PROTEIN AND INTERLEUKIN-6 CONCENTRATION MEASUREMENTS

C-reactive protein (DY1707; R&D Systems Europe, France) and interleukin-6 (DY008; R&D Systems Europe) concentrations were measured in patients' plasma samples according to the manufacturer's instructions.

HEMODYNAMIC EVALUATION

In both centers, HVPG was assessed using a technique that has been described.[13,14] The principal investigator of this study (P.-E.R.) went to Barcelona to ensure consistency of HVPG measurements between the two centers. Briefly, after overnight fasting, local anesthesia was applied and an introducer was placed under ultrasound guidance using the Seldinger technique. A 7-French balloon catheter (Lemaitre Vascular, for the test cohort; Edwards Lifesciences, Irvine, CA, for the validation cohort) was inflated in the right or median hepatic vein, and wedged hepatic venous pressure was measured. Then, free hepatic venous pressure was obtained. HVPG was calculated as the difference between wedged and free hepatic venous pressures. Adequate occlusion was confirmed by injection of 5 mL of iodinated radiologic contrast medium. Permanent tracings were recorded. Clinically significant portal hypertension was defined as an HVPG ≥10 mm Hg.[13] When indicated, right heart hemodynamic measurements including pulmonary artery pressure, right atrial pressure, and pulmonary capillary wedged pressure were also performed using a

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Swan-Ganz catheter (Edwards Life Sciences). Cardiac index was measured by the thermodilution method and obtained by the average of 3-5 consecutive measurements.[15]

HISTOLOGICAL ANALYSIS

Liver tissue samples obtained from patients of the test cohort within 3 months before or after venous blood collection for MV measurement were retrospectively reviewed by an expert pathologist (V.P.) unaware of the results of MV measurements. The following features were analyzed on hematein and eosin–stained and on picrosirius-stained tissue sections using semiquantitative scoring defined *a priori*. Fibrosis was evaluated using picrosirius staining and scored according to Metavir[16]; in case of cirrhosis (F4), the Laennec scoring system was used (stage 4a, mild/definitive cirrhosis with marked septation and visible nodules, although most septa are thin; stage 4b, moderate cirrhosis with at least two broad septa and less than half of the tissue section composed of micronodules [<3 mm]; stage 4c, severe cirrhosis with at least one very broad septum or more than half of the tissue section composed of micronodules).[17] Activity was classified as absent to moderate versus severe. Presence of apoptotic hepatocytes was also evaluated.

STATISTICAL ANALYSES

Quantitative variables were expressed as median (interquartile range) and categorical variables as frequencies. Comparisons of independent quantitative and qualitative variables between groups were performed using the Mann-Whitney test and the chi-squared or Fisher exact test as appropriate, respectively. Comparisons of hepatocyte MV levels between hepatic and peripheral vein were performed using the Wilcoxon test. Spearman correlation analyses were used to evaluate the relationships between MV circulating levels and clinical and hemodynamic features. Follow-up time was defined as the period from the date of liver catheterization to 6 months after this procedure. Study outcome was evaluated using a multistate model as recommended in patients with cirrhosis[18]: data for patients who had not died were censored at the date of the last follow-up visit and coded 0; data for patients who died before liver transplantation were coded 1; liver transplantation was considered to be a competing risk event, and data were coded 2. A cumulative incidence function of death was calculated to describe the probability of death at a given time and was reported at 6 months with a 95% confidence interval. Univariate regression analyses were conducted using the Fine and Gray proportional hazards models to identify whether MV levels at baseline were associated with 6-month mortality.[19] The MV level with the best sensitivity and specificity in area under the receiver operating characteristic curve analysis (Youden's index) for death was chosen for further analyses. Each MV subpopulation achieving $P < 0.05$ was introduced into a multivariable Fine and Gray proportional hazards model with Child-Pugh score or MELD to adjust our analyses for these severity scores and determine whether these MV levels had a prognostic value independently of these scores. All statistical tests were two-sided. $P < 0.05$ was considered to be statistically significant. Statistical analyses were performed and figures were created using the SPSS statistical package 16.0 software (SPSS Inc., Chicago, IL) and GraphPad Prism 5 software, respectively. Survival analyses were performed using SAS 9.4 statistical software.

Results

PATIENTS' CHARACTERISTICS IN THE TEST COHORT

One hundred and thirty-nine patients were included in the test cohort. Their characteristics are presented in Table 1. The main cause of liver disease was excessive alcohol consumption. Indications for hepatic vein, with or without right heart, catheterization were evaluation before liver transplantation in 70 (50%) patients or before liver surgery in 7 (5%) patients or assessment of the severity and/or the cause of liver disease using a liver biopsy in 62 (45%) patients. During the 6-month follow-up, 20 (14%) patients underwent liver transplantation and 9 (6%) patients died. Causes of death were HCC-related in 2; liver-related in 5, including variceal bleeding in 1, *Klebsiella oxytoca* pneumonia in 1, acute on chronic liver failure in 1, acute alcoholic hepatitis complicated with pneumonia in 1, and complicated refractory hepatic hydrothorax in 1; and unknown in 2.

CIRCULATING MV LEVELS ACCORDING TO THE SEVERITY OF LIVER DISEASE

Hepatocyte MV levels were 4.0-fold and 2.2-fold higher in patients with Child-Pugh C than in those

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TABLE 1. Baseline Characteristics of the 242 Patients With Advanced Chronic Liver Disease

	Test Cohort (n = 139)	Validation Cohort (n = 103)	*P*
Clinical features			
Age (years)	56 (50-62)	58 (51-66)	0.047
Male gender, n (%)	107 (77)	68 (66)	0.081
Body mass index (kg/m^2)	26 (23-30)	26 (23-29)	0.901
Fibrosis, n (%)			0.006
Advanced fibrosis (F3)	10 (7)	0 (0)	
Cirrhosis (F4)	129 (93)	103 (100)	
Cardiovascular risk factors, n (%)			
Hypertension	50 (36)	23 (22)	0.024
Smoking	50 (36)	18 (18)	0.002
Diabetes	45 (32)	23 (22)	0.111
Dyslipidemia	13 (9)	8 (8)	0.818
Causes of liver disease, n (%)			
Alcohol	59 (42)	19 (18)	<0.001
Nonalcoholic steatohepatitis	37 (27)	3 (3)	<0.001
Hepatitis C	41 (29)	77 (75)	<0.001
Hepatitis B	10 (7)	4 (4)	0.405
Other	16 (12)	2 (2)	0.005
Ascites, n (%)	76 (55)	23 (22)	<0.001
HCC, n (%)	43 (31)	20 (19)	0.054
Child Pugh class A/B/C	43 (31)/52 (37)/44 (32)	66 (64)/29 (28)/8 (8)	<0.001
MELD	13 (9-17)	11 (8-14)	0.004
Large varices esophageal or history of band ligation, n (%)	26 (23)	4 (5)	<0.001
Laboratory data			
Serum sodium (mmol/L)	136 (134-138)	141 (138-143)	<0.001
Serum creatinine (μmol/L)	70 (63-86)	68 (53-81)	0.007
Serum AST (ULN)	1.81 (1.3-2.8)	1.63 (1-2.75)	0.171
Serum ALT(ULN)	1.1 (0.6-1.98)	1.3 (0.8-2.15)	0.117
Serum bilirubin (μmol/L)	33 (14-64)	24 (14-43)	0.039
Leukocytes (10^9/L)	5.1 (3.7-7.1)	4.4 (3.3-6)	0.022
Hemoglobin (g/dL)	12.0 (10.5-14.0)	13.2 (12.0-14.8)	0.012
Platelet count (G/L)	93 (68-138)	85 (59-134)	0.294
C-reactive protein (mg/L)	4.5 (1.6-8.8)	1.8 (0.4-4.1)	<0.001
Interleukin-6 (pg/mL)	9 (0-24)	0 (0-24)	0.108
Hemodynamic data			
HVPG (mm Hg)	16 (12-20)	16 (13-19)	0.279
≥10 mm Hg, n (%)	114 (85)	83 (81)	0.386
Wedge hepatic venous pressure (mm Hg)	24 (18-29)	23 (18-27)	0.231
Free hepatic venous pressure (mm Hg)	7 (5-10)	8 (6-9)	0.761
Heart rate (bpm)	72 (65-84)	63 (55-74)	<0.001
Mean arterial pressure (mm Hg)	91 (85-102)	90 (80-98)	0.046
Right atrial pressure (mm Hg)	4 (2-5)	5 (3.5-6.5)	<0.001
Mean pulmonary artery pressure (mm Hg)	13 (11-17)	15 (12-19)	0.015
Cardiac index (L/min/m^2)	3.5 (2.8-4.4)	3.4 (2.8-4.5)	0.929
Beta-blocker treatment, n (%)	69 (50)	45 (44)	0.366

Data are expressed as median (interquartile range) or number (percentage) as appropriate. Some patients had several causes of cirrhosis. Esophageal varices data were available in 111 patients in the test cohort and in 83 patients in the validation cohort. HVPG, heart rate, mean arterial pressure, right atrial pressure, mean pulmonary artery pressure, and cardiac index were available in the test cohort for 134, 139, 139, 134, 125, and 125 patients, respectively, and in the validation cohort for 103, 101, 102, 102, 47, and 46 patients, respectively.

Abbreviations: bpm, beat per minute; ULN, upper limit of normal.

with Child-Pugh A or B liver disease, respectively (Supporting Table S1 and Fig. 1A). Similar results were obtained when restricting the analysis to patients without HCC (data not shown). Hepatocyte MV levels weakly correlated with HVPG (r = 0.22, P = 0.011) and could not discriminate patients with HVPG ≥10 mm Hg from those having an HVPG below this threshold. Hepatocyte MV levels did not correlate with right heart hemodynamic values or with cardiac index but correlated with markers of systemic inflammation (leukocytes, C-reactive protein, and interleukin-6) and with MELD and its components

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and inversely correlated with serum sodium levels (Supporting Tables S2 and S3). Severe liver necroinflammatory activity and abundant liver fibrosis were associated with higher circulating levels of hepatocyte MVs (Supporting Fig. S2). There was also a trend toward higher hepatocyte MV levels in patients with apoptotic hepatocytes. In 10 additional patients (characteristics in Supporting Table S4), we compared hepatocyte MV levels in hepatic versus peripheral vein and observed 78% (27%-233%, $P = 0.037$) higher levels in hepatic than in peripheral vein from the same patients, with a strong correlation between concentrations in both sites ($r = 0.80$, $P = 0.006$).

Total soluble cytokeratin-18 levels (bound and unbound to MVs) were slightly higher in patients with Child-Pugh C than in those with Child-Pugh B liver disease (Supporting Table S1 for overall cohort; data not shown for analyses excluding patients without HCC), correlated weakly with HVPG ($r = 0.21$, $P = 0.017$) and did not significantly differ between patients with HVPG $\geq$10 mm Hg and those with HVPG $<$10 mm Hg (data not shown).

Neither circulating levels of hepatocyte exosomes nor circulating levels of annexin V^+, platelet ($CD41^+$), leuko-endothelial ($CD31^+/41^-$), pan-leukocyte ($CD11a^+$), and endothelial ($CD62e^+$, $CD144^+$) MVs measured by flow cytometry were influenced by the severity of the liver disease, except that $CD144^+$ MV levels were slightly higher in patients with Child-Pugh C liver disease in the overall cohort (Supporting Table S1) and $CD31^+/41^-$ MV levels were mildly higher in patients with Child-Pugh C liver disease without HCC (data not shown). Annexin V^+, platelet, leuko-endothelial, pan-leukocyte, and endothelial MV levels did not correlate with HVPG (data not shown) and could not identify patients with HVPG $\geq$10 mm Hg (data not shown).

FACTORS ASSOCIATED WITH 6-MONTH MORTALITY

Patients' characteristics at inclusion associated with 6-month mortality are shown in Supporting Table S5. By univariate analysis, hepatocyte MV levels were strongly associated with 6-month mortality (Table 2). A cutoff value of 65 U/L yielded the most accurate sensitivity and specificity to identify patient mortality. Patients having hepatocyte MV levels >65 U/L had a 6-month cumulative incidence of death of 18% (8%-31%) versus 1% (1%-5%) for patients having hepatocyte MV levels below this threshold (Fig. 2A).

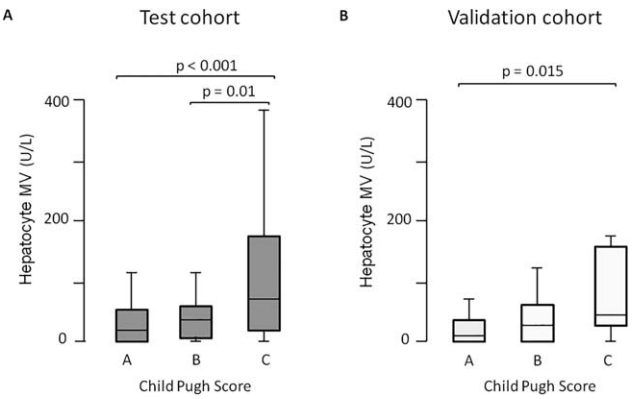

FIG. 1. Circulating hepatocyte MV levels according to the Child-Pugh score: (A) test cohort, (B) validation cohort.

Hepatocyte MV level >65 U/L still predicted 6-month mortality after adjustment for Child-Pugh score or MELD (Table 3). To further explore the added prognostic value of hepatocyte MV levels to MELD, we evaluated 6-month mortality according to the cutoff of 65 U/L and to MELD below or above 15, this threshold being recommended to list patients with end-stage liver disease for liver transplantation. Patients with hepatocyte MV levels >65 U/L and MELD >15 were clearly at higher risk for 6-month mortality than the other patients (23% versus 3%, $P = 0.001$) (Fig. 3A). Hepatocyte MV levels were also associated with 6-month liver-related mortality by univariate analysis (Supporting Table S6). Analyses adjusted on MELD and Child-Pugh score could not be performed because no liver-related death occurred in the group of patients with hepatocyte MV $\leq$65 U/L in the test cohort.

We did similar prognostic analyses using total soluble cytokeratin-18 levels and found less obvious differences (Table 2; Supporting Table S7). Hepatocyte MVs being a reflection of hepatocyte injury, we investigated the prognostic value of serum transaminase levels, but neither aspartate aminotransferase (AST) nor alanine aminotransferase (ALT) levels were associated with 6-month mortality (Supporting Table S5). Other MV subpopulations did not predict mortality except for small $CD31^+/41^-$ MV levels that were associated with mortality by univariate analysis (Table 2). When adjusting for severity scores of cirrhosis, $CD31^+/41^-$ small MV levels predicted 6-month mortality independently of Child-Pugh score but not of MELD (Supporting Table S7).

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TABLE 2. Univariate Analyses Evaluating the Association of a Circulating MV Subpopulation and Soluble Cytokeratin-18 Levels With 6-Month Transplantation-Free Survival Using Gray's Test (Transplantation Counted as Competing Risk, Death Counted as Event)

	Test Cohort			Validation Cohort		
Variable	Hazard Ratio	95% Confidence Interval	*P*	Hazard Ratio	95% Confidence Interval	*P*
Univariate analysis						
Annexin V^+ 10^3 MVs/μL	0.877	0.748-1.029	0.108	1.049	0.977-1.127	0.190
Annexin V^+ small 10^3 MVs/μL	0.831	0.656-1.053	0.125	1.068	0.985-1.159	0.112
Annexin V^+ large 10^3 MVs/μL	0.751	0.442-1.274	0.289	1.134	0.674-1.909	0.636
$CD11a^+$ 10^3 MVs/μL	0.787	0.551-1.124	0.188	0.189	0.004-9.225	0.401
$CD11a^+$ small 10^2 MVs/μL	0.704	0.326-1.521	0.798	0.064	0.000-18.559	0.342
$CD11a^+$ large 10^2 MVs/μL	0.611	0.288-1.298	0.200	0.031	0.000-985.24	0.512
$CD144^+$ 10^2 MVs/μL	0.667	0.156-2.855	0.585	**5.060**	**2.326-10.838**	**<0.001**
$CD144^+$ small 10^2 MVs/μL	0.592	0.027-12.890	0.739	**23.358**	**1.425-382.801**	**0.027**
$CD144^+$ large 10^2 MVs/μL	0.470	0.052-4.231	0.501	**9.238**	**4.566-18.69**	**<0.001**
$CD62E^+$ 10^3 MVs/μL	1.029	0.731-1.449	0.868	0.337	0.071-1.591	0.170
$CD62E^+$ small 10^3 MVs/μL	1.047	0.638-1.720	0.855	0.186	0.006-5.369	0.327
$CD62E^+$ large 10^3 MVs/μL	1.043	0.361-3.014	0.938	0.109	0.007-1.800	0.121
$CD41^+$ 10^3 MVs/μL	0.966	0.795-1.174	0.729	1.036	0.698-1.538	0.861
$CD41^+$ small 10^3 MVs/μL	0.871	0.623-1.219	0.421	0.972	0.475-1.991	0.939
$CD41^+$ large 10^3 MVs/μL	1.042	0.611-1.776	0.881	1.386	0.503-3.818	0.528
$CD31^+/41^-$ 10^3 MVs/μL	0.086	0.003-2.678	0.1622	7.434	0.952-58.018	0.056
$CD31^+/41^-$ small 10^3 MVs/μL	**0.010**	**0.000-0.732**	**0.036**	20.211	0.254-1606.147	0.178
$CD31^+/41^-$ large 10^3 MVs/μL	0.024	0.000-44.352	0.331	**61.075**	**1.500-2486.37**	**0.030**
Hepatocyte exosomes (U/L)	1.000	0.997-1.002	0.735		Not available	
Hepatocyte MVs 10^3 (U/L)	**1.998**	**1.543- 2.588**	**<0.001**	**17.440**	**5.862-51.884**	**<0.001**
Hepatocyte MVs (U/L) >65 versus ≤65*	**17.560**	**2.157-143**	**0.0074**	**6.997**	**1.614-30.335**	**0.0093**
Total soluble cytokeratin-18^+ 10^3 (U/L)	**1.544**	**1.353-1.761**	**<0.001**	**1.544**	**1.330-1.792**	**<0.001**
Total soluble cytokeratin-18^+ (U/L) >300 versus ≤300†	**11.457**	**1.405-93.412**	**0.0227**	**4.299**	**0.986-18.744**	**0.0522**
Child-Pugh score	1.451	0.918-2.292	0.1108	**2.054**	**1.566-2.694**	**<0.0001**
MELD	**1.140**	**1.019-1.274**	**0.0216**	**1.635**	**1.376-1.943**	**<0.0001**

*65 U/L: cutoff point found with Youden index (with hepatic transplantation considered as censored).
†300 U/L: cutoff point found with Youden index (with hepatic transplantation considered as censored).
Bold indicates significant associations with survival.

VALIDATION COHORT

Characteristics of the 103 patients with cirrhosis included in the validation cohort are presented in Table 1. The main cause of liver disease was hepatitis C virus infection. Indications for hepatic vein, with or without right heart, catheterization were evaluation before liver transplantation in 4 (4%) patients or before liver surgery in 19 (18%) patients or assessment of the severity and/or the cause of liver disease by a liver biopsy in 80 (78%) patients. During the 6-month follow-up, 4 (4%) patients underwent liver transplantation, and 7 (7%) died. Causes of death were acute on chronic liver failure in 2 patients, septic shock in 1, variceal bleeding in 1, hemorrhagic stroke in 1, and HCC-related in 2. The main results obtained in the test cohort were confirmed in the validation cohort, namely higher levels of hepatocyte MV levels in patients with Child-Pugh C liver disease (Fig. 1B; Supporting Table S1 for the overall cohort; data not shown for patients without HCC) and a correlation with HVPG but not with other hemodynamic values (Supporting Table S2). In the validation cohort, hepatocyte MVs levels were higher in patients with HVPG ≥10 mm Hg than in those with HVPG below this threshold (15 [0-63] versus 5 [0-15], $P = 0.015$). Hepatocyte MV levels were associated with 6-month mortality by univariate analysis (Table 2 and Fig. 2B) and after adjustment for Child-Pugh score (Table 3). Again, patients with hepatocyte MV levels >65 U/L and MELD >15 had 6-month mortality significantly higher than other groups (Fig. 3B). Hepatocyte MV levels were also associated with 6-month liver-related mortality by univariate analysis (Supporting Table S6). Analyses adjusted on MELD and Child-Pugh score were not performed due to the low number of events.

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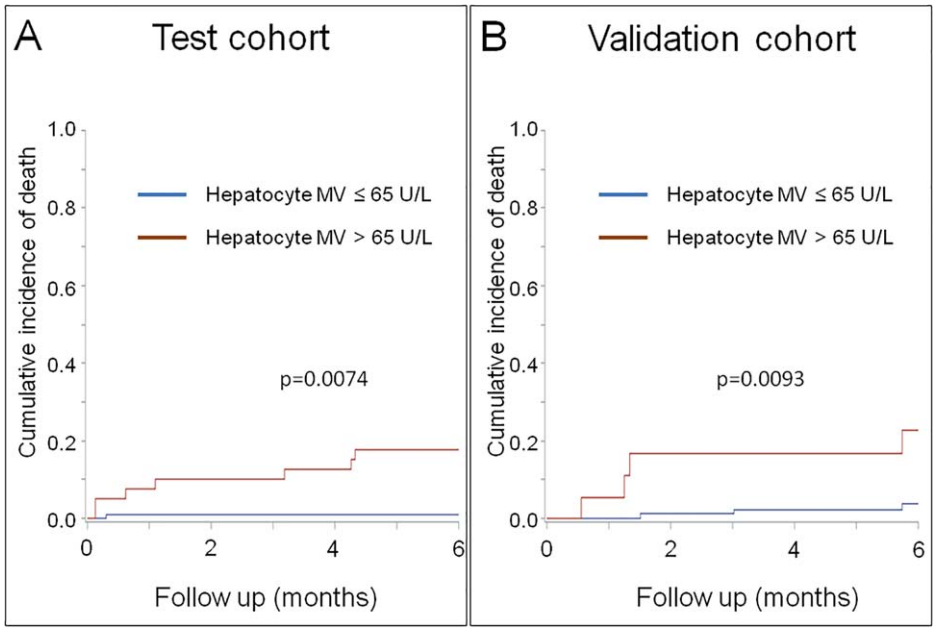

FIG. 2. Cumulative incidence of death according to circulating hepatocyte MV levels: (A) test cohort, (B) validation cohort.

As $CD144^+$ and $CD31^+/41^-$ MV levels predicted patient mortality either in the test or in the validation cohort, we performed additional analyses to gain further insight into the variables influencing circulating concentrations of these subpopulations of MVs in the 242 patients with cirrhosis. $CD31^+/41^-$ large MV levels correlated with markers of systemic inflammation (C-reactive protein and leukocytes) (Supporting Tables S8 and S9).

Discussion

MVs are emerging as a newly recognized means for conveying fundamental information from cell to cell in physiology and pathology.[20] These microstructures contain proteins, lipids, and genetic information able to modify the phenotype and function of the target cells. MVs carry specific markers of the cell of origin that make it possible to monitor their fluctuations in the circulation and thus use circulating levels as potential biomarkers. This large prospective study demonstrated that circulating hepatocyte MV levels can predict 6-month mortality in patients with cirrhosis independently of established scores, such as the Child-Pugh score or the MELD. Of note, although hepatocyte MV levels weakly correlated with HVPG, a variable of established prognostic significance, they were not able to reliably identify patients with HVPG $\geq$10 mm Hg, the threshold for so-called clinically significant portal hypertension.

TABLE 3. Analyses of the Ability of Hepatocyte MV Levels to Predict 6-Month Mortality Adjusted on Child-Pugh Score and MELD (Gray's Test, Transplantation Counted as Competing Risk, Death Counted as Event)

	Test Cohort			Validation Cohort		
Variable	Hazard Ratio	95% Confidence Interval	*P*	Hazard Ratio	95% Confidence Interval	*P*
Hepatocyte MVs (U/L) >65 versus ≤65	**12.616**	**1.922-82.810**	**0.0083**	**4.892**	**1.283-18.662**	**0.0201**
Child Pugh Score	1.244	0.852-1.818	0.2583	**2.009**	**1.472-2.740**	**<0.0001**
Hepatocyte MVs (U/L) >65 versus ≤65	**12.229**	**1.691-88.428**	**0.0131**	3.484	0.972-12.482	0.0502
MELD	1.087	0.978-1.208	0.1233	**1.604**	**1.341-1.919**	**<0.0001**

Bold indicates significant associations with survival.

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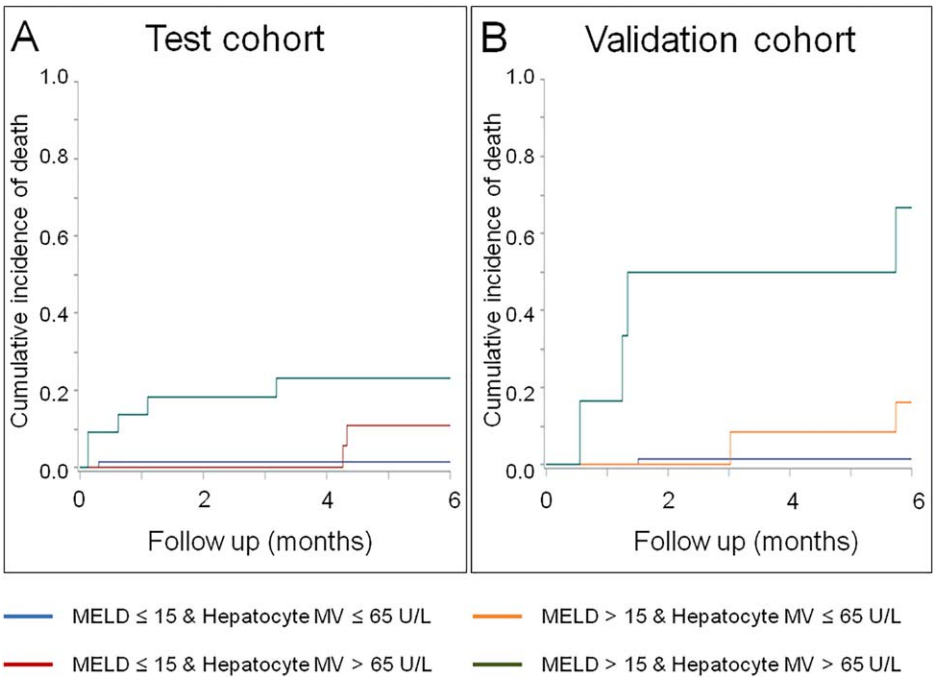

FIG. 3. Cumulative incidence of death according to MELD and circulating hepatocyte MV levels. (A) Test cohort. No death occurred in the group of patients with MELD >15 and hepatocyte MV ≤65 U/L, so this group is not represented. (B) Validation cohort. No death occurred in the group of patients with MELD ≤15 and hepatocyte MV >65 U/L, so this group is not represented.

The first major finding in this study was that circulating levels of hepatocyte MVs >65 U/L can identify, among patients with a MELD >15, those with the poorest prognosis. A limitation of our study was the limited number of events that occurred despite the large number of included patients. However, this limitation is compensated for by the confirmation of our results in an independent validation cohort, using the same cutoff, despite differences in patients' characteristics between the two cohorts, illustrating the robustness of our findings. A previous study from our group reported an increase in hepatocyte MV levels paralleling cirrhosis severity as assessed by Child-Pugh or MELD score.[3,4] However, the ability of circulating MV levels to predict outcome of patients with cirrhosis had not been directly tested. The higher ability of hepatocyte MVs to predict mortality compared to soluble cytokeratin-18 or serum AST and ALT levels suggests that these MV levels reflect an ongoing detrimental liver process and possibly an impaired ability of macrophages and of endothelial cells to clear MVs from the circulation.[3,21] The correlations of hepatocyte MV levels with markers of systemic inflammation as well as their association with severe liver necroinflammatory activity suggest that hepatocyte MVs could be released as a consequence of hepatocyte injury by inflammatory cells. An alternative or complementary hypothesis would be that hepatocyte MVs are not bystanders but rather actors that contribute by themselves to the complications of cirrhosis. The fact that hepatocyte MV levels predicted outcome only in patients with severe cirrhosis is reminiscent of the ability of circulating MVs to impair response to vasoconstrictive agents only when originating from patients with Child-Pugh B or C cirrhosis.[4] This also echoes the results of a proof-of-concept, multicenter, open-label study testing the oral pan-caspase inhibitor emricasan in patients with cirrhosis. Emricasan decreased HVPG only in patients with severe portal hypertension.[22] Apoptosis being a major stimulus of MV release, we can speculate that hepatocyte MVs mediated this effect. Hepatocyte MV levels might thus help to identify patients who will respond to emricasan. The protocol we used to prepare plasma samples with a centrifugation speed available in all medical test laboratories and a simple and highly reproducible technique to measure hepatocyte MVs makes its routine use feasible. The possibility to freeze plasma samples without altering hepatocyte MV level detection is another advantage because it facilitates shipment to a central laboratory when this is not possible locally.

The second major finding of this study is the lack of value of circulating MV levels of platelet, leukocyte, or endothelial origin to estimate HVPG or to predict

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mortality, despite very rigorous preanalytics and flow-cytometric analyses. This contrasts with previous results from our group showing that circulating levels of leuko-endothelial MVs increased with cirrhosis severity and predicted survival independently of Child-Pugh score.[4] The explanation is unlikely to be the change in flow cytometer. Indeed, to overcome the potential modifications related to the detection by newer-generation machines of smaller MVs, we analyzed separately the largest MVs corresponding to those measured with older-generation machines.[23] A more likely explanation is that in the present study we only included patients with a stable liver disease, while in our previous work exclusion criteria included only severe sepsis, HCC, or portal vein thrombosis. Patients with acute decompensation of cirrhosis were thus included. This observation provides the rationale for testing the interest of leuko-endothelial MV levels in patients with acute decompensation of cirrhosis to predict the transition to acute on chronic liver failure or death. The observation of a correlation between levels of large leuko-endothelial MVs and markers of systemic inflammation in the present study and in a previous study of our team supports this idea.[4]

In conclusion, hepatocyte MV levels improve the prediction of 6-month mortality in patients with advanced chronic liver disease when tested in the absence of an acute complication. Whether a decrease in circulating hepatocyte MV levels is an accurate surrogate endpoint for therapies that improve survival of patients with severe cirrhosis should be investigated without waiting.

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