Small Annexin V–Positive Platelet-Derived Microvesicles Affect Prognosis in Cirrhosis: A Longitudinal Study

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INTRODUCTION: **Microvesicles (MVs) with procoagulant properties may favor liver parenchymal extinction, then cirrhosis-related complications and mortality. In a longitudinal cohort of cirrhotic patients, we measured plasma levels of platelet-derived MVs (PMVs), endothelial-derived MVs, and red blood cell–derived MVs, expressing phosphatidylserine (annexin V–positive [AV$^+$]) or not, and evaluated their impact on Model for End-Stage Liver Disease (MELD) score and transplant-free survival.**

METHODS: **MVs were quantified using flow cytometry in plasma from 90 noninfected cirrhotic patients and 10 healthy volunteers matched for age and sex. Impact of plasma microvesicle levels on 6-month transplant-free survival was assessed using log-rank tests and logistic regression.**

RESULTS: **Microvesicle levels, mostly platelet-derived, were 2.5-fold higher in healthy volunteers compared with cirrhotic patients. Circulating small AV$^+$ PMV levels were lower in cirrhotic patients ($P = 0.014$) and inversely correlated with MELD scores ($R = -0.28$; $P = 0.0065$). During 1-year follow-up, 8 patients died and 7 underwent liver transplantation. In the remaining patients, circulating microvesicle levels did not change significantly. Six-month transplant-free survival was lower in patients with low baseline small AV$^+$ PMV levels (72.6% vs 96.2%; $P = 0.0007$). In multivariate analyses adjusted for age, ascites, esophageal varices, encephalopathy, clinical decompensation, total platelet counts, MELD score, and/or Child-Pugh C stage, patients with lower small AV$^+$ PMV levels had a significant 5- to 8-fold higher risk of 6-month death or liver transplant. Other PMV levels did not impact on survival.**

DISCUSSION: **Decreased circulating small AV$^+$ PMV levels are associated with significantly lower transplant-free survival in cirrhotic patients independently of MELD score and platelet counts.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A540, http://links.lww.com/CTG/A541, http://links.lww.com/CTG/A542, http://links.lww.com/CTG/A543, http://links.lww.com/CTG/A544

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INTRODUCTION

In cirrhotic patients, determinants of prognosis are not fully elucidated. The Model for End-Stage Liver Disease (MELD) score, although widely used to sort patients awaiting liver transplant, imperfectly predicts short-term mortality, and numerous studies have attempted to find relevant additional variables to improve prediction. At the same MELD score, patients may differ according to their liver tissue remodeling capacities. Patients with marked parenchymal extinction in the liver may have poorer prognosis compared with patients with budding cirrhotic liver, more likely to regenerate (1). Liver parenchymal extinction is consecutive to venous vascular obliteration due to thrombosis or inflammation (2). It worsens both portal hypertension and liver insufficiency, thus promoting liver-related mortality. This

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supports the beneficial role of anticoagulant therapies in experimental models of cirrhosis (3) and provides a rationale to investigate the benefit of anticoagulation in patients with advanced liver disease, as previously suggested (4).

Circulating microvesicles (MVs) are considered key vectors of intercellular communication. They are membrane-derived extracellular vesicles (between 0.1 and 1 μm in diameter) produced by budding and blebbing from the plasma membrane of nearly all cells in response to activation, apoptosis, or shear stress (5,6). MVs express cell surface markers from their parent cells which enable the characterization of their cellular origin by flow cytometry (FCM). Clinical and experimental studies have suggested that circulating MVs are implicated in liver fibrosis, portal hypertension, and coagulation disorders (7–13). A cross-sectional study reported an association between increased levels of hepatocyte-derived MVs and poor prognosis in cirrhotic patients (13). Whether MVs of other cellular origins are increased or may influence the natural history of cirrhosis remains poorly evaluated, and serial measures of circulating MVs have never been reported in cirrhotic patients. Among MVs, platelet-derived microvesicles (PMVs) represent the most abundant circulating form (14). Because of their procoagulant effects (15), PMVs may be implicated in the prognosis of cirrhosis. To date, data on PMVs offer conflicting results (8,12,13,16), perhaps because PMVs combine several components with heterogeneous properties. In particular, most published studies did not distinguish small PMVs from larger ones, which may not have the same procoagulant effect. Moreover, the role of phosphatidylserine, inconstantly expressed on the surface of PMVs, and easily detectable by annexin V labeling, deserves specific evaluation in cirrhotic patients. Phosphatidylserine indeed interacts with clotting proteins and promotes the assembly of the coagulation cascade, thus conferring strong procoagulant properties to annexin V–positive PMVs (AV^+ PMVs).

The aims of this prospective study were (i) to compare circulating MV levels in cirrhotic patients and healthy volunteers (HVs), (ii) to perform longitudinal assessment of circulating MV levels in cirrhotic patients, and (iii) to evaluate the impact of different subtypes of MVs on the prognosis of cirrhosis according to their size and their expression of phosphatidylserine.

MATERIALS AND METHODS

Study design and patients

This prospective observational study was conducted in the Hepatology Department of Besançon University Hospital between January 2014 and January 2015. We consecutively enrolled 90 cirrhotic patients until a predefined number of 30 patients was reached in each Child-Pugh class (CP-A/B/C). Liver cirrhosis was diagnosed based on clinical, biochemical, radiological, and/or histological findings. None of the patients had suspected or documented infection, portal hypertension unrelated to cirrhosis, previous organ transplant, human immunodeficiency virus infection, or experienced recent gastrointestinal bleeding (i.e., within the 2 months before inclusion). Patients with portal vein thrombosis, history of chronic heart failure, or malignancy (including hepatocellular carcinoma) were excluded, as well as those who underwent transjugular intrahepatic portosystemic shunt (TIPS) placement or received immunosuppressive agents, corticosteroids, anticoagulant, or antiplatelet agents. Ten HVs, matched to the 90 cirrhotic patients for age and sex, served as a control group. The study was performed in accordance with the Declaration of Helsinki and was approved by the local ethics committee (*Comité de Protection des Personnes* Est-II, October 21, 2013; file #13/415). Informed consent was obtained from all participants included in the study. Patients were followed up for 1 year or until death or liver transplantation. At baseline, demographics, history of liver cirrhosis, clinical, and biological data were recorded. Ultrasonography of the abdomen with portal Doppler examination was also recorded at 6 months. Cirrhosis-related complications, liver transplantation, TIPS placement, and death were recorded (see Figure S1, Supplementary Digital Content 1, http://links.lww.com/CTG/A540 for details).

MV assessment methods

Reagents and monoclonal antibodies. Fluoroisothiocyanate (FITC)-conjugated anti-Cytokeratin 18 antibody (Ab) (IgG1, Clone DC-10) was provided by Abcam (United Kingdom); Alexa-Fluor 488–conjugated anti-CD301 Ab (IgG2b Human CLEC10A/CD301, Clone 744,812) was obtained from RnD Systems (United Kingdom); phycoerythrin (PE)-conjugated anti-ASGPR1 Ab (IgG1, Clone 8D7) was provided by BD Pharmingen (France); PE-conjugated anti-CD31 Ab (IgG1, Clone IF11), PE–cyanin7-conjugated anti-CD41 Ab (IgG1 Clone P2) and PE–cyanin7-conjugated anti-$CD235a^+$ Ab (IgG1 Clone 11E4B-7-6) were obtained from Beckman Coulter Immunotech (Marseille, France). Ab solutions were centrifuged before FCM to avoid artefacts due to aggregation. The presence of phosphatidylserine at the surface of plasma MVs was assessed using FITC-conjugated annexin V (ApoScreen Annexin V-FITC; Southern Biotech, Birmingham, AL) diluted (1/5) in a solution of 1 mg/mL of binding buffer (Annexin V binding buffer 10x; Southern Biotech).

Isolation of MVs from blood samples. Blood samples were collected at inclusion (i.e., outpatient follow-up visit or hospitalization for acute decompensation) and at 6 months according to our protocol. MVs were isolated according to the International Society of Thrombosis and Haemostasis recommendations (17,18). Briefly, samples of peripheral blood were collected without tourniquet and using a 21-gauge needle. The first 5 mL were discarded to avoid the potential artefact generated by the contact phase activation. Blood was collected in 3 5-mL citrate–theophylline–adenosine–dipyridamole (3.2%) vacutainer tubes (Becton Dickinson, Le Pont de Claix, France). The tubes were maintained vertically and immediately manually transported to the laboratory (Plateforme de Biomonitoring, Etablissement Français du Sang, Bourgogne Franche-Comté, Besançon, France). Blood samples were centrifuged less than an hour after venipuncture at 2500*g* for 15 minutes, then were decantated and centrifuged at 2500*g* for 15 minutes again. All samples were frozen in liquid nitrogen, stored at −80 °C, and thawed quickly at 37 °C for 2 minutes before FCM analysis. No differences were observed according to MV size or freezing duration at −80 °C compared with fresh samples, as previously described (19).

Determination of the cellular origin of MVs. The cellular origin of plasma MVs was determined by immunostaining using selective fluorochrome-labeled Abs incubated at room temperature for 20 minutes at 4 °C in the dark with 30 μL of platelet-free plasma. The reaction was stopped by adding 150 μL of Binding Buffer in each tube (see Supplemental File S1, Supplementary Digital Content 5, http://links.lww.com/CTG/A544). To assess

the specificity of the immunostaining for MVs, we used a detergent known to solubilize the membrane phospholipids, so that MVs disappear from the analysis window (20). The mix of platelet-free plasma, antibodies, and Binding Buffer was incubated with Triton 2% (oxydant-free X-100, Interchim, Montluçon, France), at 4 °C for 20 minutes in the dark. Then, a solution of Binding Buffer 1% (150 μL) and counting beads (30 μL) was added just before cytometry analysis. We distinguished phosphatidylserine-expressing MVs using annexin V, the ligand of phosphatidylserine: MVs were thus categorized as AV^+ or not (AV-negative) MVs. Other membrane markers were used to assess the cellular origin of MVs: red blood cells ($CD235a^+$ MVs), platelets ($CD31^+/41^+$ MVs), and endothelial cells ($CD31^+/41^-$ MVs) (see Figure S2, Supplementary Digital Content 2, http://links.lww.com/CTG/A541). We did not find a reliable method to identify hepatocyte-derived MVs, since immunostaining using anti-CK18 (12), anti-CD301 (i.e., anti-ASGPR2), and anti-ASGPR1 (11) antibodies detected products which were not eliminated by Triton treatment.

Microparticle quantification by FCM. MVs were analyzed on a NAVIOS cytometer (Beckman Coulter Immunotech, Villepinte, France) (21) by an examiner unaware of the subject's status (HV, or cirrhosis, and severity of cirrhosis), and plasma levels were expressed in MVs/μL. Regions corresponding to MVs were identified in forward light scatter and side-angle light scatter intensity dot plot representation set at logarithmic gain. MV gate was standardized daily, using calibration beads (0.3, 0.5, and 0.9 μm; Megamix-Plus forward light scatter; BioCytex, Marseille, France). Small-size MVs were defined as events detected with beads <0.5 μm (19). The number of MVs was calculated on the basis of 30 μL of CytoCount beads with a known concentration of 1,022 beads/μL (DakoCytomation, Trappes, France) added to the sample just before performing FCM. Data were analyzed using Kaluza software version 1.2 (Beckman Coulter, Brea, CA). See Supplemental File S1, Supplementary Digital Content 5, http://links.lww.com/CTG/A544 for details.

Thrombogenic activity of MVs. Thrombogenic activity of MVs related to phosphatidylserine was evaluated by the functional coagulation test STA Procoag-PPL (Diagnostica Stago, Asnières sur Seine, France). A first reagent (freeze-dried citrated human plasma phospholipid-depleted) containing coagulation factors was added to 25-μL plasma including MVs and then centrifuged twice at 2500*g* for 15 minutes. After incubation, a second reagent containing activated factor X (factor Xa) was added. The coagulation time was then measured in seconds. The shorter the clotting time, the greater the procoagulant activity of MV-derived phospholipids (22).

Statistical analysis

Continuous variables were described as median and interquartile range and categorical variables as number and percentage. Comparisons of quantitative variables between the 3 CP groups were performed using the Kruskal-Wallis test. Variations of plasma MV levels between baseline and month 6 were compared using paired-samples Wilcoxon signed-rank tests. Differences between categorical variables were assessed by the χ^2 or Fisher exact test, as appropriate. Pearson correlation coefficients were used to test correlations between quantitative clinical and laboratory variables, especially the correlations between MV levels and MELD scores. Multiple testing was corrected by the Bonferroni correction when appropriate. The association between plasma MV levels and transplant-free survival was evaluated using the Kaplan-Meier model and log-rank test. Low or high plasma MV levels were defined according to their median value or the Youden index when appropriate. Bivariate analyses combining baseline MV levels and 1 relevant clinical variable impacting on 6-month transplant-free survival were conducted by logistic regression. Sensitivity analyses were performed by excluding patients with particular conditions affecting survival. A P value <0.05 was considered statistically significant. Data handling and analysis were performed with NCSS 2019 software (Kaysville, UT).

RESULTS

Study population

The main characteristics of the 90 cirrhotic patients included are reported in Table 1. Alcohol was the main cause (90%) of cirrhosis. Severe acute alcoholic hepatitis (AAH) was histologically proven in 9 patients. At baseline, the median MELD score was 14,

Table 1. Baseline characteristics of the 90 patients

Baseline characteristics	Values
Age, yr	58 [50–64]
Male sex, n (%)	69 (76.7)
MELD score	14 [7–37]
Etiology of cirrhosis, n (%)	
Alcohol	81 (90.0)
Viral hepatitis B or C	4 (4.4)
Nonalcoholic steatohepatitis	5 (5.6)
Ascites, n (%)	38 (42.1)
Encephalopathy, n (%)	7 (7.8)
Presence of SIRS, n (%)	10 (11.1)
Active alcohol consumption, n (%)	30 (33.3)
Histologically proven AAH, n (%)	7 (7.7)
Acute-on-chronic liver failure, n (%)	6 (6.6)
Overall survival at 1 yr, n (%)	82 (91)
Serum levels	
Leukocyte count (mm^{-3})	5,810 [4,580–7,310]
Platelet count (mm^{-3})	103,000 [70,000–137,000]
Prothrombin index (%)	54 [41–68]
Serum albumin (g/L)	29 [24–36]
Serum creatinine (μmol/L)	71 [59–86]
Bilirubin (μmol/L)	32 [17–70]
CRP (mg/L)	8 [4–14]

In active drinker patients with metabolic syndrome (n = 7), alcohol was considered as the main cause of cirrhosis. Among the 6 patients with ACLF, 3 had ACLF grade 1 and 3 had ACLF grade 2.
Quantitative variables are expressed as median [interquartile range].
AAH, acute alcoholic hepatitis; ACLF, acute-on-chronic liver failure; CRP, C-reactive protein; MELD, Model for End-Stage Liver Disease; SIRS, systemic inflammatory response syndrome.

45 patients had ascites and/or encephalopathy, and 3 had serum creatinine >132 μmol/L. Overall survival was 91% at 1 year. Eight deaths (7 liver-related and 1 related to cerebral stroke) occurred within the first 6 months. During follow-up, 2 patients underwent TIPS placement, 7 patients underwent liver transplantation, and 13 patients experienced at least 1 episode of cirrhosis decompensation (including 2 macrothrombosis events and 3 variceal bleeds).

Baseline microvesicle levels in cirrhotic patients and HVs

Plasma levels of all the analyzed subsets of MVs in the different groups of patients are reported in Table 2 and Figure 1. Among AV^+ MVs, we determined the proportion of total and small PMVs, endothelial-derived MVs (EMVs), and red blood cell–derived MVs (RMVs). Overall, median proportions of AV^+ PMVs, AV^+ EMVs, and AV^+ RMVs were 81%, 1%, and 9%, indicating that 9% of the total AV^+ MVs measured had unknown cellular origin. Small MVs (diameter less than 0.5 μm) accounted for 32% of total AV^+ MVs, the majority of which (27%) were PMVs. For the different cellular subtype of MVs, we detected AV-negative MVs, which constituted 77% of small EMVs, 69% of small RMVs, and only 35% of small PMVs.

Compared with HVs, cirrhotic patients had a different profile of plasma MVs characterized by lower levels of total or AV^+ PMVs and small PMVs and higher levels of small AV^+ RMVs. No significant differences were observed for EMVs or AV^+ MVs of unknown cellular origin. Total PMVs and all their subtypes (small or large PMVs, expressing or not phosphatidylserine) were positively correlated with total platelet counts (see Figure S3, Supplementary Digital Content 3, http://links.lww.com/CTG/A542). The phosphatidylserine-dependent procoagulant activity was correlated with all subtypes of AV^+ MVs. It was greater in HVs as compared with cirrhotic patients ($P < 0.0001$). When restricting the comparisons to HVs vs CP-A (i.e., compensated) cirrhotic patients, significant differences were observed only for total plasma PMVs ($P = 0.014$) and AV^+ PMVs ($P = 0.017$) and for procoagulant activity ($P = 0.0029$). There was no difference in small AV^+ PMV plasma levels between CP-A cirrhotic patients and HVs.

Sequential analysis of circulating MVs in cirrhotic patients

Circulating MVs were analyzed at 6 months in 56 patients. The flowchart of patients with measurable MVs at 6-month follow-up is shown in Supplemental Figure S1 (see Supplementary Digital Content 1, http://links.lww.com/CTG/A540). It indicates that 95% of these patients were stable or had improved their CP stage. For the remaining 5%, MELD score variations and MELD score values at 6 months did not exceed +6 and 25 points, respectively. Overall, plasma MV levels did not significantly change between baseline and 6 months in these patients. Variations in plasma levels of all MV subsets between baseline and 6 months were of small magnitude (2%–15%) without significant differences

Table 2. Baseline plasma MV levels in HVs and cirrhotic patients

	Overall, N = 100	HVs, N = 10	Cirrhotic patients, N = 90	CP-A, N = 30	CP-B, N = 30	CP-C, N = 30	P^a	P^b	P^c
AV^+ MVs	2,050 (1,327–3,658)	4,134 (2,670–5,874)	1,859 (1,258–3,359)	2,228 (1,506–2,228)	1,774 (1,176–3,968)	1,796 (1,106–2,863)	0.011	NS	NS
Small AV^+ MVs	633 (337–1,236)	1,076 (663–1,713)	568 (325–1,172)	790 (430–1,395)	660 (314–1,095)	459 (243–1,024)	0.037	NS	0.048
PMVs	2,497 (1,581–4,412)	5,086 (3,320–6,884)	2,372 (1,522–3,755)	2,538 (2035–4,240)	2,491 (1,568–4,509)	2,222 (1,153–3,283)	0.003	NS	0.031
AV^+ PMVs	1,465 (974–2,853)	3,631 (2,282–5,423)	1,410 (913–2,474)	1,637 (1,107–2,794)	1,381 (874–3,329)	1,392 (590–2,022)	0.003	NS	0.018
Small PMVs	1,214 (743–1,790)	1,789 (1,204–2,449)	1,080 (715–1,726)	1,220 (799–1,800)	1,291 (717–1,804)	903 (605–1,547)	0.015	NS	NS
Small AV^+ PMVs	406 (217–797)	798 (483–1,316)	364 (215–714)	503 (277–1,015)	378 (199–757)	264 (99–568)	0.014	0.028	0.005
EMVs	30 (22–46)	25 (18–35)	30 (22–48)	27 (19–51)	36 (26–48)	34 (19–47)	NS	NS	NS
AV^+ EMVs	10 (6–16)	9 (6–15)	10 (6–16)	9 (5–17)	11 (6–16)	10 (6–15)	NS	NS	NS
Small EMVs	20 (13–30)	13 (10–20)	21 (13–32)	16 (11–29)	22 (16–30)	23 (11–33)	NS	NS	NS
Small AV^+ EMVs	4 (3–7)	4 (3–5)	4 (3–8)	4 (3–7)	7 (4–10)	4 (1–9)	NS	NS	NS
RMVs	346 (200–682)	241 (194–332)	389 (202–701)	287 (196–445)	423 (179–720)	498 (264–887)	NS	NS	NS
AV^+ RMVs	172 (118–295)	155 (129–177)	176 (114–310)	153 (118–267)	186 (100–326)	187 (127–359)	NS	NS	NS
Small RMVs	126 (57–306)	58 (33–104)	152 (64–312)	87 (38–183)	157 (60–328)	250 (91–511)	NS	0.027	0.012
Small AV^+ RMVs	33 (16–67)	18 (10–24)	34 (16–74)	27 (14–47)	44 (20–105)	36 (14–91)	0.015	NS	0.034
Clotting time (sec)	66 (56–76)	50 (43–54)	68 (59–77)	64 (56–69)	71 (60–80)	71 (62–78)	<0.001	0.044	<0.001

Plasma levels of MVs are expressed in MVs/μL.
AV^+, annexin V–positive; CP, Child-Pugh; EMVs, endothelial-derived microvesicles; HVs, healthy volunteers; MVs, microvesicles; PMVs, platelet-derived microvesicles; RMVs, red blood cell–derived microvesicles.
[a] *P* values are for the comparison between HVs and cirrhotic patients.
[b] *P* values are for the comparison between the 3 CP stages using the Kruskal-Wallis test.
[c] *P* values are for the comparison between the 4 groups (CP-A, CP-B, CP-C patients, HVs) using the Kruskal-Wallis test.

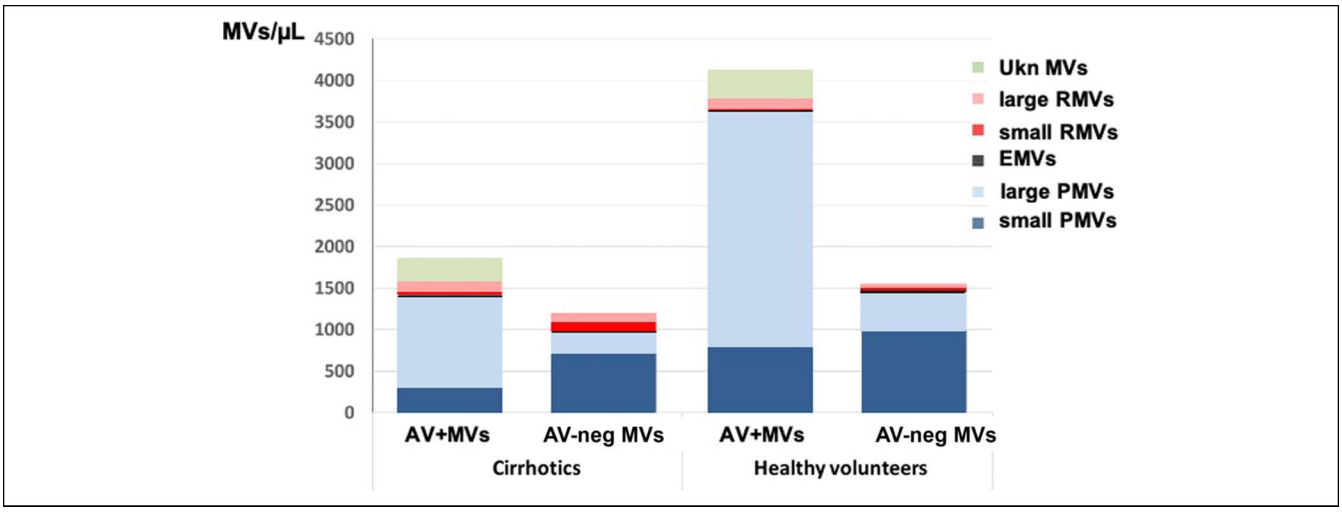

Figure 1. Baseline plasma levels of all distinguished subtypes of MVs in cirrhotic patients and HVs. The subtypes of MVs were distinguished according to their cellular origin (PMVs, EMVs, RMVs, or unknown MVs), their size (small < 0.5 μm or large MVs ≥ 0.5 μm), and their expression of phosphatidylserine (AV^+ MVs) or not (annexin V–negative). Because at least 1 marker is mandatory to detect MVs, annexin V–negative MVs of unknown origin could not be detected. In the 2 groups, PMVs are largely predominant, but plasma PMV levels were significantly higher in HVs compared with cirrhotic patients (5,086 vs 2,372/μL, $P = 0.003$), and significant differences were observed between cirrhotic patients and HVs for all subcategories of PMVs plasma levels of small AV^+ RMVs were higher in cirrhotic patients (34 vs 18/μL, $P = 0.015$). No significant differences were observed between cirrhotic patients and HVs for EMVs, which accounted for a low proportion of detectable MVs, and for AV^+ MVs with no specific cellular origin, which may include hepatocyte-derived AV^+ MVs. AV^+, annexin V–positive; EMVs, endothelial-derived microvesicles; HVs, healthy volunteers; PMVs, platelet-derived microvesicles; RMVs, red blood cell–derived microvesicles.

according to the paired Wilcoxon signed-rank test. The changes between baseline and 6-month plasma MV levels did not correlate with variations in the MELD score or CP stage, even for small AV^+ PMVs. Plasma levels of small AV^+ PMVs did not significantly change in the 5 patients who increased their MELD score by 2 points or more, in the 33 patients who decreased their MELD score by 2 points or more, in the 14 patients who decreased their MELD score by 4 points or more, and in the remaining patients with no substantial change in the MELD score.

Influence of MVs on outcomes in cirrhotic patients

Cross-sectional analysis: associations between MV levels, clinical condition, and MELD score at baseline. Baseline plasma MV levels were compared according to the presence of ascites, encephalopathy, large esophageal varices (i.e., EV grade ≥2), or clinical decompensation (i.e., ascites or encephalopathy) at inclusion. Plasma levels of small PMVs and small AV^+ PMVs did not differ. High levels of small EMVs were more frequent in patients with large EVs (85.7% vs 39.5% in patients without EVs, $P = 0.002$). High levels of small RMVs were more frequent in patients with ascites (63.2% vs 39.2%, $P = 0.025$), encephalopathy (85.7% vs 49.3%, $P = 0.045$), or clinical decompensation (64.1% vs 38.1%, $P = 0.014$). These associations were no longer significant when restricting the analyses to small AV^+ RMVs. Regarding CP stage or MELD score, only plasma levels of small AV^+ PMVs showed significant results. Plasma levels of small AV^+ PMVs decreased in parallel with the severity of CP stage (Figure 2a, Kruskal-Wallis test, $P = 0.029$) and were inversely correlated with the MELD score ($R = -0.28$, $P = 0.0065$; Figure 2b). Plasma levels of small AV^+ PMVs were thus more often low in CP-C patients (66.7% vs 41.7%, $P = 0.025$), as well as in patients with MELD score >20 (76.0% vs 40.0%, $P = 0.002$).

Longitudinal analysis. Transplant-free survival was different according to total small AV^+ MVs, but it concerned only small AV^+ PMVs. Large AV^+ MVs regardless of their cellular origin, small AV^+ MVs of other cellular origin, total PMVs, and total small PMVs (which include small AV-negative PMVs; see Figure S3, Supplementary Digital Content 3, http://links.lww.com/CTG/A542) had no prognostic impact. In patients with low levels of small AV^+ PMVs (i.e., below the median value), the 6-month transplant-free survival was 77.5% vs 95.6% in patients with high baseline levels ($P = 0.016$; see Figure S3, Supplementary Digital Content 3, http://links.lww.com/CTG/A542). The area under the receiver operating characteristic curve of small AV^+ PMVs for predicting death or liver transplant at 6 months was 0.723 (95% confidence interval [CI]: 0.563–0.831), and the best discriminant value given by the Youden index was 286/μL, while 90% sensitivity and specificity for predicting 6-month transplant-free survival were given for baseline small AV^+ PMV values greater than 506 and 71/μL, respectively. In patients with small AV^+ PMVs below 286/μL, 6-month transplant-free survival was 72.6% vs 96.2% in others ($P = 0.0007$; Figure 3). The small number of events allowed multivariate analyses with a limited number of variables. Through logistic regression analyses, the impact of small AV^+ PMVs on transplant-free survival was independent of age, ascites, encephalopathy, clinical decompensation, large EVs, CP-C stage, platelet count, or MELD score at baseline. According to the variable used for adjustment in bivariate models, the odds ratio for mortality or liver transplantation at 6 months associated with baseline small AV^+ PMV levels<286/μL ranged from 5.69 to 8.33 and their 95% CIs from 1.35 to 33.43 (Table 3). Multivariate analysis adjusted on baseline MELD score and platelet counts confirmed the significant impact of baseline small AV^+ PMV levels <286/μL on the 6-month mortality or need for transplantation (Table 4). Sensitivity

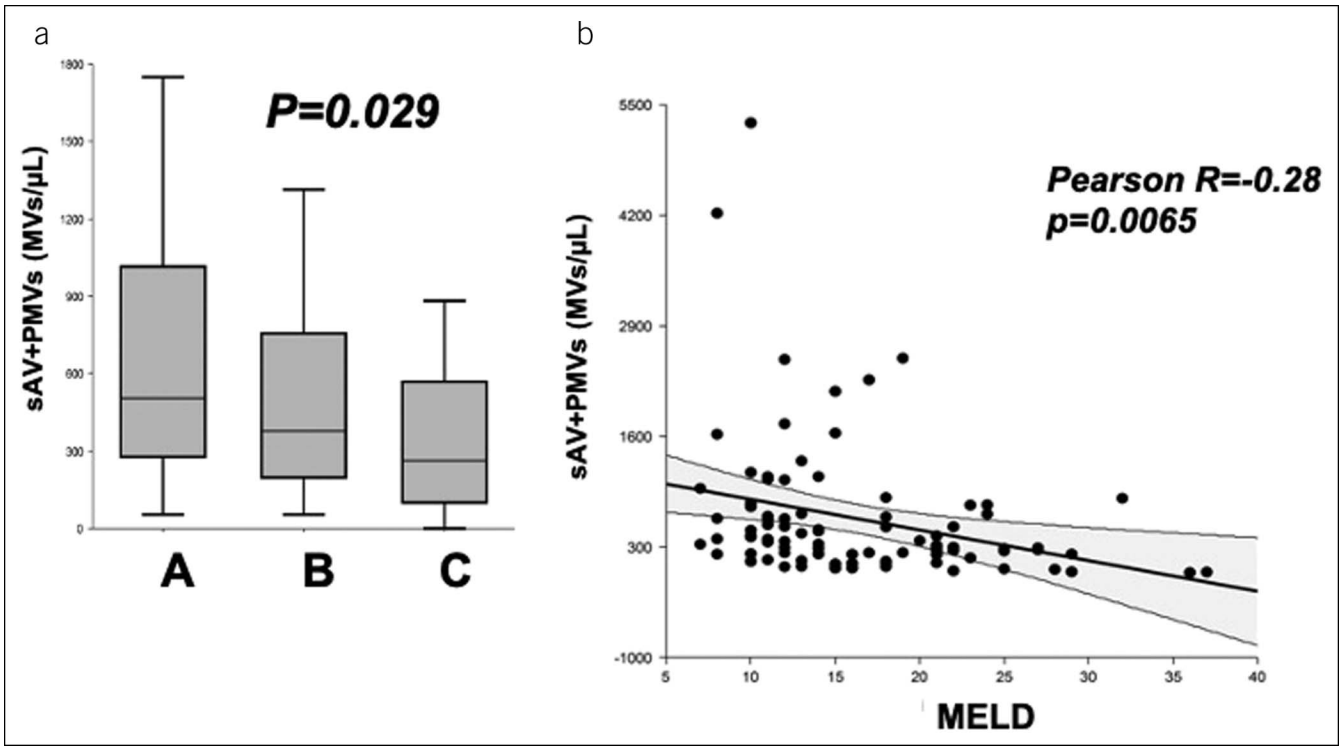

Figure 2. Plasma levels of small AV$^+$ PMVs according to CP stage (**a**) and MELD score (**b**). Plasma levels of small AV$^+$ PMVs decrease in parallel with the severity of CP stage (**a**, 503 MVs/µL in CP-A; 378 in CP-B; 264 in CP-C; Kruskal-Wallis test, $P = 0.029$) and are inversely correlated with MELD score ($R = -0.28$, $P = 0.0065$; **b**). (**a**) Boxes show IQRs and horizontal lines denote median values. The ends of the whiskers represent the lowest and the highest datapoints within 1.5 × IQR of the lower and upper quartiles. (**b**) The regression line and its confidence limits (gray zone) are given. AV$^+$, annexin V–positive; CP, Child-Pugh; IQR, interquartile ranges; MELD, Model for End-Stage Liver Disease; MVs, microvesicles; PMVs, platelet-derived microvesicles.

analyses restricted on patients without continued alcohol consumption, histologically proven AAH, renal failure, systemic inflammatory response syndrome, or acute-on-chronic liver failure showed similar significant results regarding the impact of small AV$^+$ PMVs on transplant-free survival (see Figure S4, Supplementary Digital Content 4, http://links.lww.com/CTG/A543). When none of these conditions were present at baseline, 6-month transplant-free survival was 100% in patients with high baseline small AV$^+$ PMV levels and 90% in patients with low baseline small AV$^+$ PMV levels ($P = 0.025$, see Figure S4, Supplementary Digital Content 4, http://links.lww.com/CTG/A543).

DISCUSSION

Our prospective study provides 5 relevant results: (i) the profile of circulating MVs differs between HVs and cirrhotic patients, who have similar levels of EMVs, RMVs, and MVs of unknown origin, but lower PMV levels, indicating that an increase in circulating MVs may not be necessary to induce pathogenesis; (ii) circulating MV levels remain stable in cirrhotic patients over a 6-month follow-up period, indicating that one measure might be sufficient for midterm prognostic purposes; (iii) contrary to other studies on MVs in cirrhotic patients (12,13), high MELD scores and poor prognosis were observed in parallel with a decrease in one type of MVs, suggesting that clearance rather than overproduction of certain MVs is involved in the deterioration of liver function; (iv) only a decrease in circulating small AV$^+$ PMVs was associated with high MELD scores and low transplant-free survival, whereas plasma levels of other subtypes of MVs, including other subtypes of PMVs and AV$^+$ MVs of unknown origin (which may include hepatocyte-derived MVs) had no impact, and (v) the impact of low circulating small AV$^+$ PMV levels on prognosis appears as a robust finding independent of other clinical conditions known to affect prognosis, as indicated by our bivariate and sensitivity analyses.

Our study is the first to combine double MV labeling, size discrimination, and sequential measurement of plasma MV concentrations in cirrhotic patients. This enabled us to identify and study specifically small AV$^+$ PMVs and to assess their prognostic relevance. All MVs identified in this study were confirmed as MVs by exposure to Triton, and the expression of phosphatidylserine detected by AV staining and cytometry was confirmed by the assessment of phosphatidylserine-dependent thrombogenic activity. Contrary to a previous report (13), we studied a large and well-designed cohort of cirrhotic patients, either histologically proven or clinically obvious, excluding patients with extensive (F3) fibrosis and patients with hepatocellular carcinoma, thus indicating that transplant-free survival was a reliable outcome measure of liver function in our series.

The main result of our study was the identification of the prognostic role of small AV$^+$ PMVs: Low plasma levels of small AV$^+$ PMVs were associated with the most severe stages of cirrhosis and predicted cirrhosis-related mortality. Remarkably, this finding was confirmed by our sensitivity analyzes restricted on patients without systemic inflammatory response syndrome, acute-on-chronic liver failure, AAH, ongoing alcohol consumption, or renal failure, conditions well-known to affect prognosis.

Table 3. Logistic regression analyses of mortality or liver transplantation at 6 months

	Adjustment variable			Small AV+ PMVs <286/μL		
	OR	95% CI	P	OR	95% CI	P
Age > 58 yrs	1.66	0.50–5.53	NS	8.04	2.07–31.25	0.003
Large EVs	0.29	0.03–2.60	NS	8.33	2.13–32.57	0.002
Hepatic encephalopathy	2.37	0.34–16.42	NS	8.13	2.08–31.72	0.002
Ascites	1.93	0.58–6.44	NS	7.42	1.89–28.99	0.004
Clinical decompensation	1.88	0.57–6.29	NS	7.52	1.93–29.41	0.004
Low platelet counts[a]	1.12	0.30–4.15	NS	8.00	1.91–33.43	0.0004
MELD > 20	16.32	3.76–70.70	<0.001	6.40	1.42–28.87	0.016
CP C	9.72	2.34–40.27	0.002	5.69	1.35–24.09	0.018

This table summarizes the result of bivariate analyses evaluating the impact of plasma levels of small AV+ PMVs measured at baseline on mortality or liver transplantation at 6 months. The 286 threshold was identified based on the Youden index. The second variables incorporated on the models are listed in the left column. Similar results were obtained using Cox bivariate analyses and when age, platelet counts, or MELD score was considered as continuous variables.
AV+, annexin V–positive; CP, Child-Pugh; EVs, esophageal varices, MELD, Model for End-Stage Liver Disease; OR, odds ratio; PMVs, platelet-derived microvesicles; 95% CI, 95% confidence interval.
[a]Platelet counts below its median value.

As suggested by our results, low levels of small AV+ PMVs were able to predict a 10% decrease of 6-month transplant-free survival (see Figure S4, Supplementary Digital Content 4, http://links.lww.com/CTG/A543). Because amounts of PMVs were positively correlated with platelet counts, one relevant question was whether our finding regarding small AV+ PMVs was a surrogate of low platelet counts. Indeed, thrombopoietin usually decreases in the event of hepatic insufficiency (23) and low platelet counts have been incorporated in prognostic scores in cirrhotic patients (24). In our series, however, we did not observe correlations between total platelet counts, total PMVs (which may include megakaryocyte-derived MVs), small PMVs, or large PMV levels, and MELD scores or transplant-free survival (see Figure S3, Supplementary Digital Content 3, http://links.lww.com/CTG/A542). Moreover, our multivariate analyses demonstrated an impact of baseline small AV+ PMV levels on subsequent death or liver transplantation independent on total platelet count and MELD score (Table 4). These results suggest that small AV+ PMVs are the only PMV subtype, or at least the most relevant, for predicting poor prognosis. Because low production of platelets and their derived MVs may affect all subtypes of PMVs, we hypothesize that the relationship between low circulating levels of small AV+ PMVs and poor prognosis may be the consequence of an overconsumption of small AV+ PMVs. Platelet-derived MVs are known to have procoagulant activity 50- to 100-fold stronger than activated platelets (15). Membrane exposure of phosphatidylserine, which is denser in small compared with large PMVs (25), renders this procoagulant activity more powerful. This may explain the impact of small AV+ PMVs on prognosis. Circulating small AV+ PMVs may incorporate themselves either directly into microthrombi, or into circulating cells, conferring procoagulant properties on them (26), whereas their plasma levels decrease. Microthombi may occur in liver tissue, promoting liver parenchymal extinction and worsening liver function (2), but also in the gut (4,27), rendering the intestinal barrier against bacteria less efficient and facilitating bacterial translocation, a factor that strongly contributes to cirrhosis complications (28). If confirmed, our results will help identify cirrhotic patients who may benefit from anticoagulant therapies (29). Another path for the clearance of small AV+ PMVs could involve macrophages (30) and dendritic cells (31), since MVs may be internalized by these cells in a phosphatidylserine-dependent manner, impairing their immune function (31). Hence, small AV+ PMVs could be a key factor modulating systemic inflammation, and low plasma levels of small AV+ PMVs may be a surrogate for cirrhosis-associated impaired immune function.

Another important finding comes from our sequential measurements of plasma MV levels. Interestingly, small AV+ PMV levels showed low variation between baseline and 6 months, even in the few patients with substantial changes in MELD scores. This suggests that low levels of small AV+ PMVs are either an early marker of liver impairment or even precede liver impairment, thus explaining why the prognostic impact of small AV+ PMV

Table 4. Multivariate Logistic regression analysis of mortality or liver transplantation at 6 months

	Logistic regression R^2 = 0.383; AUROC = 0.896		
Variable	OR	95% CI	P
MELD score	1.33	1.14–1.56	<0.0001
Platelet counts	1.00	1.00–1.00	NS
Small AV+ PMVs < 286/μL	7.65	1.13–51.68	0.037

This table summarizes the result of a logistic regression analysis where baseline small AV+ PMVs (categorized according to its relevant threshold), total platelet count, and MELD score (incorporated as continuous variables) were tested as predictors of 6 month transplantation or mortality. These 3 variables explained 38.3% of variance and were able to predict morality or transplantation with an AUROC at 0.896. The strong impact of the MELD score was confirmed. The significant influence of low levels of small AV+ PMVs was independent of the total platelet count.
AUROC, area under the receiver operating characteristic curve; AV+, annexin V–positive; MELD, Model for End-Stage Liver Disease; PMVs, platelet-derived microvesicles; 95% CI, 95% confidence interval.

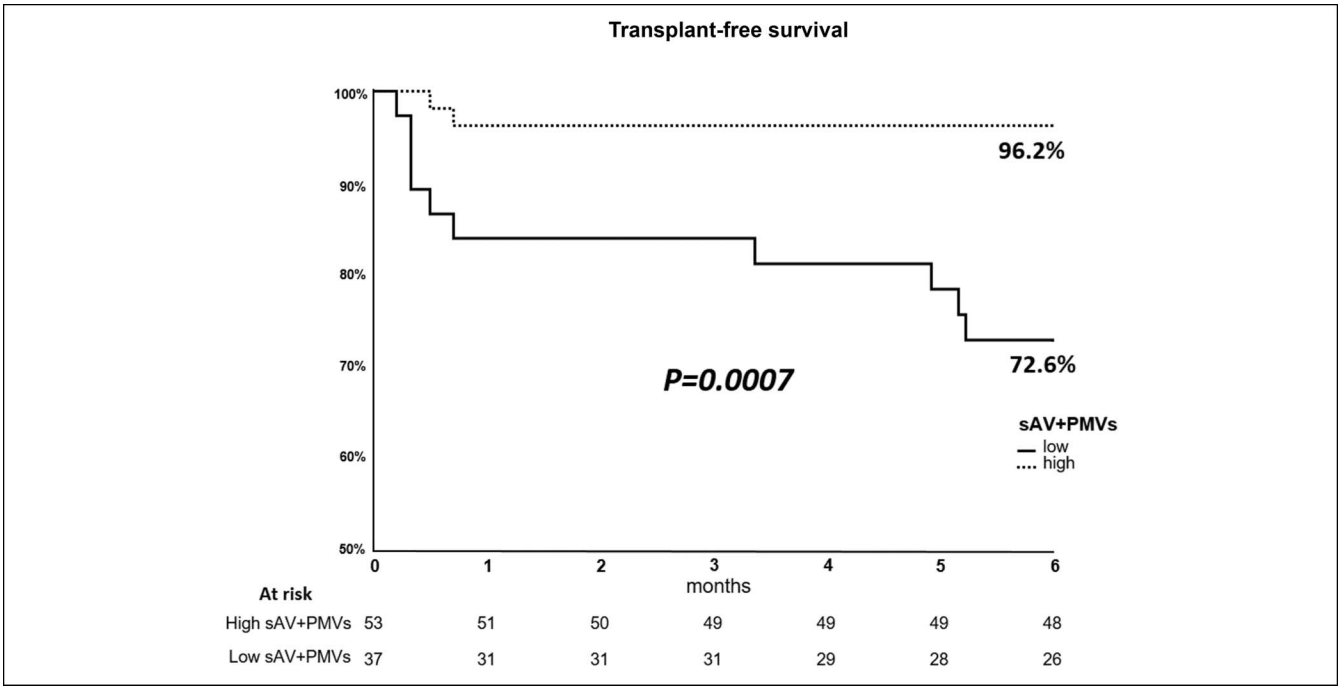

Figure 3. Six-month transplant-free survival according to baseline level of small AV^+ PMVs in cirrhotic patients. Small AV^+ PMVs are the only subtype of MVs that impact on prognosis. The area under the receiver operating characteristic curve (AUROC) of small AV^+ PMVs to predict death or liver transplant at 6 months was 0.723 (95% CI: 0.563–0.831), and the best discriminant value given by the Youden index was 286/μL. Low (solid line) and high (dotted line) levels of small AV^+ PMVs were defined according to this value. Transplant-free survival was significantly lower in patients with plasma levels of small AV^+ PMVs <286/μL. Because low plasma values of small AV^+ PMVs are associated with poor prognosis, we can hypothesize that these MVs can be captured in a phosphatidylserine-dependent manner and that this phenomenon promotes mortality. AV^+, annexin V–positive; PMVs, platelet-derived microvesicles; 95% CI, 95% confidence interval.

levels was independent of MELD score or CP stage in our multivariate analyses. This finding also suggests that a single plasma measurement of small AV^+ PMVs could be useful for predicting the need for liver transplantation within 6 months.

In this study, we also found that plasma levels of small RMVs were higher in cirrhotic patients with ascites or encephalopathy and that patients with large EVs had higher levels of small EMVs. These associations did not concern EMVs and RMVs expressing phosphatidylserine, suggesting that coagulation processes were not involved. Increased RMV release could be an adaptive strategy to modulate vascular tone by decreasing the level of bioavailable nitric oxide because of their NO scavenging capacities (32). Increased EMV release is produced by endothelium activation, which contributes to increased intrahepatic resistance (33,34). We hypothesize that RMV and EMV levels could thus be surrogate markers of clinically significant portal hypertension that deserves further investigation. In our study, follow-up was probably too short to unmask their putative prognostic value.

Our study acknowledges several limitations. First, we were unable to confirm the data previously published regarding hepatocyte-derived MVs (12,13). Our MV identification technique, more rigorous than the filtration technique used in previous studies, did not validate one simple membrane labeling by CK18 or ASGPR1 to authenticate hepatocyte MVs. Nevertheless, some AV^+ MVs derived from hepatocytes were probably detected within our MV group of unknown origin. This group had no prognostic impact in our study and was not quantitatively different in cirrhotic patients and HVs. This suggests that if hepatocyte-derived MVs actually worsen the prognosis of cirrhosis, this does not require phosphatidylserine expression. Second, the sequential measurement of MVs mainly concerned stable patients and can be considered as providing limited information. Unfortunately, the design of our study did not enable multiple quantifications of circulating MVs and serum sample collection was not guided by clinical events. It would have been interesting to investigate, e.g., how plasma levels of MVs vary in the event of ascites, hepatic encephalopathy, or acute-on-chronic liver failure. However, we found stable MVs plasma levels in 33 patients who improved and 6 patients who worsened their MELD score, suggesting that the prognostic effect associated with small AV^+ PMVs is the consequence of a slow, constant, and clinically invisible process. This underlies more dramatic events, such as bacterial infections, which are known to worsen prognosis. The hypothesis of small AV^+ PMV–induced microthrombosis perfectly corresponds to this description. However, this hypothesis was not confirmed by histological data and is only speculative. It would have been impossible for ethical reasons to biopsy these patients and impossible to identify *in situ* internalized MVs. Third, alcohol-related cirrhosis was overrepresented in our series, thus rendering our findings maybe poorly applicable in other causes of cirrhosis, in which histopathology, pathogenesis, and prognosis of portal hypertension-related events may differ. Finally, the impact of the low rate of small AV^+ PMVs on prognosis was not validated in an independent cohort. The study was originally designed as an observational study and not as a prognostic study. The cumbersome technique used to identify MVs (Triton and double labeling) was not compatible with the inclusion of several hundred patients. Our results warrant confirmation in larger series to evaluate the gain

yielded by the measurement of small AV+ PMVs compared with MELD alone for prognostic assessment.

In conclusion, cirrhotic patients have notably lower levels of circulating platelet-derived MVs. We identified a decrease in small AV+ PMVs as a powerful marker of poor prognosis, associated with a 5- to 8-fold increase in the risk of mortality or need for liver transplant within 6 months. Further studies are needed to confirm our results and the mechanisms involved.

CONFLICTS OF INTEREST

Guarantor of the article: Vincent Di Martino, MD, PhD.

Specific author contributions: conceptualization and methodology: T.T. and P.S. Investigation and data curation: D.W., E.D., N.B., G.M., S.B., and A.R. Formal analysis: D.W. and V.D.M. Writing—original draft: D.W., V.D.M., T.T., and P.S. Resources: S.B., A.R., C.L., E.G., and B.C. Writing—review and editing: D.W., V.D.M., G.M., S.B., A.R., C.L., B.C., N.B., E.D., E.G., T.T., and P.S. All authors have approved final version to be published.

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Potential competing interests: None to report.

Study Highlights

WHAT IS KNOWN

- ✓ Microvesicles (MVs) are membrane-derived extracellular vesicles which act as key vectors of intercellular communication. They are implicated in liver fibrosis progression, portal hypertension, and coagulation disorders.
- ✓ Subsets of MVs, namely hepatocyte-derived MVs, have been suggested to negatively impact on cirrhosis prognosis.

WHAT IS NEW HERE

- ✓ Through a double labeling and size discrimination of MVs, we identified a strong impact of small annexin V-positive platelet-derived microvesicles (AV+ PMVs) on the short-term prognosis of liver cirrhosis: transplant-free survival was 5- to 8-fold lower in patients with baseline low levels of small AV+ PMVs, independently of the Model for End-Stage Liver Disease score and the platelet counts.
- ✓ Sequential measures of small AV+ PMV levels showed low variation within a 6-month follow-up period, suggesting the value of this biomarker for prognostic purposes.

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