

## Research Article

## Serum Proteoglycans as Prognostic Biomarkers of Hepatocellular Carcinoma in Patients with Alcoholic Cirrhosis

Jean-Charles Nault<sup>1,4,5,6</sup>, Erwan Guyot<sup>2,4</sup>, Christelle Laguillier<sup>2,4</sup>, Sylvie Chevret<sup>7,8</sup>, Nathalie Ganne-Carrie<sup>1,4</sup>, Gisèle N'Kontchou<sup>1,4</sup>, Michel Beaugrand<sup>1,4</sup>, Olivier Seror<sup>3</sup>, Jean-Claude Trinchet<sup>1,4</sup>, Jessica Coelho<sup>1,4</sup>, Philippe Lasalle<sup>10</sup>, Nathalie Charnaux<sup>2,4</sup>, Maryse Delehedde<sup>10</sup>, Angela Sutton<sup>2,4</sup>, and Pierre Nahon<sup>1,9</sup>

## Abstract

**Background:** Proteoglycans are involved in neoangiogenesis and transduction of oncogenic signals, two hallmarks of carcinogenesis.

**Methods:** This study sought to assess the prognostic value of serum levels of three proteoglycans (endocan, syndecan-1, and glypican-3) and VEGF in 295 patients with alcoholic cirrhosis: 170 without hepatocellular carcinoma, 58 with early hepatocellular carcinoma, and 67 with advanced hepatocellular carcinoma at inclusion. We analyzed the association between proteoglycan levels and prognosis using Kaplan–Meier and Cox methods.

**Results:** Serum levels of the three proteoglycans and VEGF were increased in patients with advanced hepatocellular carcinoma compared with those without hepatocellular carcinoma or with early hepatocellular carcinoma. In multivariate analysis, high levels of serum endocan (>5 ng/mL) were independently associated with death [HR, 2.84; 95% confidence interval (CI) 1.18–6.84;  $P = 0.02$ ], but not with hepatocellular carcinoma occurrence, in patients without hepatocellular carcinoma at baseline. High serum endocan (>5 ng/mL) and syndecan-1 (>50 ng/mL) levels were significantly associated with greater risk of tumor recurrence ( $P = 0.025$ ) in patients with early hepatocellular carcinoma treated by radiofrequency ablation. In patients with advanced hepatocellular carcinoma, high serum levels of endocan ( $P = 0.004$ ) and syndecan-1 ( $P = 0.006$ ) were significantly associated with less favorable overall survival. However, only a high level of serum syndecan-1 (>50 ng/mL) was independently associated with greater risk of death (HR, 6.21 95% CI, 1.90–20.30;  $P = 0.0025$ ).

**Conclusion:** Serum endocan and syndecan-1 are easily assessable prognostic serum biomarkers of overall survival in alcoholic cirrhosis with and without hepatocellular carcinoma.

**Impact:** These new biomarkers will be useful to manage patients with hepatocellular carcinoma developed on alcoholic cirrhosis. *Cancer Epidemiol Biomarkers Prev*; 22(8); 1343–52. ©2013 AACR.

## Introduction

In western countries, most of the hepatocellular carcinoma cases arise following cirrhosis, with an incidence

of 3% to 5% per year. The main etiologies of underlying liver disease are chronic hepatitis C, chronic hepatitis B, and alcohol consumption (1). In this setting, chronic alcohol consumption represents a public health problem and constitutes one of the main causes of cirrhosis and related hepatocellular carcinoma (2). However, hepatocellular carcinoma developing on alcoholic liver diseases remains poorly studied compared with hepatitis B- and C-associated hepatocellular carcinoma; thus, prospective follow-up cohorts of patients with alcohol-related cirrhosis are warranted. In such patients, prognosis is modeled not only by death from hepatocellular carcinoma, but also from underlying liver disease. However, difficulties in assessing the outcome in such patients warrant a search for new biomarkers easily measured in routine practice, to refine their selection and adapt therapeutic procedures (2–4). Along this line, biomarkers related to tumor progression and to the severity of the underlying liver disease need to be identified to refine prognostic predictions.

**Authors' Affiliations:** <sup>1</sup>Service d'Hépatologie, <sup>2</sup>Service de Biochimie, <sup>3</sup>Service de Radiologie, Hôpital Jean Verdier, AP-HP, Bondy; <sup>4</sup>Université Paris 13-UFR SMBH/INSERM U698, Bobigny; <sup>5</sup>Inserm, UMR-674, Génomique fonctionnelle des tumeurs solides, IUH; <sup>6</sup>Labex Immuno-oncology, Faculté de Médecine, Université Paris Descartes, Sorbonne Paris Cité; <sup>7</sup>Département de Biostatistique, Hôpital Saint-Louis, AP-HP; <sup>8</sup>Unité de Biostatistique et Epidémiologie clinique, Université Paris Diderot, INSERM, UMR-S717, Paris; <sup>9</sup>Centre de Recherche Bichat Beaujon CRB3, Université Paris 7, INSERM, U773, Paris; and <sup>10</sup>Lunginnov Campus de l'Institut Pasteur de Lille, France

**Note:** Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

**Corresponding Author:** Jean Charles Nault, Jean Verdier Hospital, APHP, University Paris 13, Avenue du 14 Juillet, Bondy 93140, France. Phone/Fax: 33-61-06-79-461; E-mail: naultjc@gmail.com

doi: 10.1158/1055-9965.EPI-13-0179

©2013 American Association for Cancer Research.

Neoangiogenesis is one of multiple pathways involved in solid tumor progression, particularly that of hepatocellular carcinoma, a highly vascularized cancer (5). Serum VEGF constitutes a classical surrogate marker of neoangiogenesis and has been associated with the prognosis of numerous solid tumors (5). In addition to neoangiogenesis, interactions with the tumor microenvironment and stimulation of the oncogenic pathway by growth factors are hallmarks of cancer (5). Proteoglycans act as pathogenic links between these biologic characteristics and seem to be satisfactory candidate biomarkers, easily measurable in sera of patients with cirrhosis and hepatocellular carcinoma (6).

Proteoglycans consist of a core protein with one or more covalently attached glycosaminoglycan chain(s) (6). Depending on the composition of the glycosaminoglycan chain(s), proteoglycans are divided into several families: heparan sulfates, chondroitin sulfates, and keratan sulfates. Proteoglycans exert various biologic functions in embryogenesis, extracellular matrix remodeling, angiogenesis, the signal transduction pathway, and the inflammatory response. Moreover, proteoglycan expression is frequently altered in cancers (6). We focused on 3 proteoglycans, endocan, syndecan-1, and glypican-3, because of their relevance to carcinogenesis.

Glypican-3, a heparan sulfate proteoglycan, is an immunohistochemical biomarker for distinguishing early hepatocellular carcinoma from preneoplastic lesions (7). The prognostic value of glypican-3 has been poorly explored compared with its diagnostic value (8). Glypican-3 is expressed in some hepatocellular carcinoma cases, characterized by poor differentiation or progenitor features, both of which are related to poor prognosis and a higher incidence of tumor recurrence (9).

Syndecan-1, another heparan sulfate proteoglycan, is involved in the migration and invasiveness of hepatocellular carcinoma cell lines and controls the cancer microenvironment by acting on growth factors and protease activities (10). Moreover, the level of shed syndecan-1 is increased in sera of patients with lung cancer and myeloma and is associated with the prognosis (11, 12). However, overexpression of syndecan-1 by malignant hepatocytes characterized by immunohistochemistry was associated with well differentiated hepatocellular carcinoma without metastasis, a subgroup of tumors with good prognosis (13). Although, increased levels of syndecan-1 in advanced hepatocellular carcinoma had been previously reported, its potential value as a diagnostic or prognostic tool has not been studied (14).

Endocan, also termed endothelial cell-specific molecule-1, is a dermatan sulfate proteoglycan secreted by endothelial cells that binds proangiogenic growth factors and regulates their activity (15, 16). It also promotes cancer cell survival, migration and invasion *in vitro*, and tumor growth *in vivo*, as described in a mouse model of human tumor xenografts (17, 18). In humans, the serum level of endocan has been identified as an independent prognostic marker in non-small cell lung cancer (19).

The aim of the present study was to assess a potential association between circulating levels of proteoglycans and VEGF with hepatocellular carcinoma occurrence and overall survival in patients with alcoholic cirrhosis, as well as with the prognosis of cirrhotic patients with hepatocellular carcinoma eligible for curative or palliative treatment.

## Materials and Methods

### Patient selection

We retrospectively assessed levels of VEGF, endocan, syndecan-1, and glypican-3 in sera of prospectively followed-up cirrhotic patients at a tertiary center (Hôpital Jean Verdier, Bondy, France). All cirrhotic patients referred to our institution for management of cirrhosis between January 2007 and December 2009 were considered. For this study, we selected patients fulfilling the following inclusion criteria: (i) biopsy-proven cirrhosis; (ii) alcoholic liver disease defined by alcohol intake of more than 50 g per day and absence of other etiologies; and (iii) a frozen serum sample collected before any treatment. Exclusion criteria included HIV infection and severe alcoholic hepatitis.

The day of inclusion was the date of the first serum collected for proteoglycan level assessment before any treatment. Clinical, biologic, endoscopic, and radiological features were recorded at inclusion.

Patients were classified into 3 groups according to the presence and status of hepatocellular carcinoma as follows:

- 1) Patients with alcoholic cirrhosis "without" hepatocellular carcinoma. Normal ultrasonography and  $\alpha$ -fetoprotein (AFP) < 100 ng/mL were necessary at baseline.
- 2) Patients with early hepatocellular carcinoma classified as Barcelona clinic liver cancer (BCLC) stage 0 (one nodule < 2 cm) or A (one nodule < 5 cm or maximum 3 nodules < 3 cm).
- 3) Patients with "advanced" hepatocellular carcinoma classified as BCLC stage B (multinodular form apart from Milan criteria), BCLC stage C (portal invasion or metastasis), or BCLC stage D (poor performance status or Child-Pugh C).

Hepatocellular carcinoma was diagnosed using either guided biopsy or Barcelona non-invasive criteria endorsed by the European Association for the Study of the Liver (EASL; ref. 3).

### Endpoints and follow-up

All cirrhotic patients without hepatocellular carcinoma at baseline were screened for hepatocellular carcinoma every 6 months using liver ultrasonography and serum AFP levels (3). The 2 main endpoints were occurrence of hepatocellular carcinoma and occurrence of liver transplantation or death.

Patients with early hepatocellular carcinoma were treated in our liver unit by radiofrequency ablation (RFA) by

**Table 1.** Characteristics of alcoholic cirrhotic patients with and without hepatocellular carcinoma

Variable	Total (n = 295)	No HCC (n = 170)	Early HCC (n = 58)	Advanced HCC (n = 67)	P
Age at inclusion <sup>a</sup>	62 (55;71)	59 (51;65)	69 (61;74)	69 (62;76.5)	<0.0001
Gender (male) <sup>b</sup>	253 (85.8)	144 (84.7)	47 (81.0)	62 (92.5)	0.13
Diabetes <sup>b</sup>	51 (17.3)	30 (17.7)	11 (19.0)	10 (14.9)	0.81
Child-Pugh A <sup>b</sup>	163 (55.3)	85 (50.0)	49 (84.5)	29 (43.3)	<0.0001
Child-Pugh B <sup>b</sup>	85 (28.8)	52 (30.6)	8 (13.8)	25 (37.3)	
Child-Pugh C <sup>b</sup>	47 (15.9)	33 (19.4)	1 (1.7)	13 (19.4)	
AFP level (ng/mL) <sup>a</sup>	4 (3;9)	4 (3;10)	4 (3.7;3)	4 (3;7)	0.66
Albumin (g/dL) <sup>b</sup>	36.7 (31.5;41.8)	37 (31.0;42.2)	39.5 (34.8;42.4)	35.1 (31.6;39.3)	0.022
Total bilirubin (mg/dL) <sup>a</sup>	1.2 (0.64;2.6)	1.4 (0.64; 2.8)	0.9 (0.61;1.6)	1.5 (0.73;3.8)	0.005
AST (>2 ULN) <sup>a</sup>	91/266 (34.2)	53/148 (35.8)	19/53 (35.8)	19/65 (29.2)	0.64
ALT (>2 ULN) <sup>a</sup>	33/266 (12.4)	18/148 (12.2)	8/53 (15.1)	7/65 (10.8)	0.77
ALP (>2 ULN) <sup>a</sup>	48/261 (18.4)	25/144 (17.4)	10/52 (19.2)	13/65 (20.0)	0.89
GGT (>2 ULN) <sup>a</sup>	188/253 (74.3)	103/143 (72.5)	34/47 (72.3)	51/64 (79.7)	0.54
Platelet count ( $\times 10^3$ /mm <sup>3</sup> ) <sup>a</sup>	125.5 (84.8;171.2)	126 (83;171)	119 (89.5;170.5)	133 (84.3;168.8)	0.92
Prothrombin time <sup>b</sup>	67 (53;80)	65 (49.3;83.0)	70 (60.8;79.3)	68 (56.5;78.0)	0.28
Creatinine ( $\mu$ mol/L) <sup>b</sup>	79 (66;95)	77 (64.3;92.0)	79 (71;93)	89 (70;107.5)	0.002
Serum endocan (ng/mL) <sup>a</sup>	4.6 (2.9;7.0)	4.5 (2.3;6.6)	4.2 (3.1;6.7)	5.8 (3.5;7.9)	0.018
Serum syndecan-1 (ng/ml) <sup>a</sup>	50.1 (26.4;103.2)	38.5 (23.9;84.0)	42.5 (25.7;58.0)	145.1 (55.5;327.5)	<0.0001
Serum glypican-3 (ng/ml) <sup>a</sup>	2.5 (1.1;5.8)	2.5 (1.2;6.7)	1.4 (0.5;2.5)	4.2 (2;9.1)	<0.0001
Serum VEGF (pg/mL) <sup>a</sup>	243 (133;448)	222.5 (110.8;368.8)	254 (143.2;400.8)	370 (173;660)	0.0016
Tumor portal thrombosis <sup>b</sup>			0 (0)	24/55 (43.6)	<0.0001
Tumor number > 1 <sup>b</sup>			9/56 (16.1)	35/60 (58.3)	<0.0001
Tumor size (mm) <sup>a</sup>			27.5 (20; 4)	60 (30;80)	<0.0001
BCLC stage 0 <sup>b</sup>			13 (22.4)	0 (0)	<0.0001
BCLC stage A <sup>b</sup>			45 (77.6)	0 (0)	<0.0001
BCLC stage B <sup>b</sup>			0 (0)	33 (49.2)	<0.0001
BCLC stage C <sup>b</sup>			0 (0)	15 (22.4)	<0.0001
BCLC stage D <sup>b</sup>			0 (0)	19 (28.4)	<0.0001

NOTE: The Fisher exact or  $\chi^2$  test was used for binary variables and the Wilcoxon or Kruskal–Wallis test for continuous variables. Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; HCC, hepatocellular carcinoma; ULN: upper limit of normal.

<sup>a</sup>Median (first and third quartiles).

<sup>b</sup>Number (%) of patients.

the same operator. All were then followed-up using abdominal CT scan and serum AFP assessment at one month and then every 3 months. Patients showing a partial response at one month defined by persistent enhancement of the lesion on CT scan could then be retreated with RFA. The 2 main endpoints were recurrence-free survival (RFS) and overall survival.

Patients with "advanced" hepatocellular carcinoma were treated and followed-up according to EASL recommendations: arterial chemoembolization for BCLC stage B, sorafenib since 2008 for BCLC stage C, and best supportive care for BCLC stage D (3, 20). Primary endpoint was overall survival. Time-to-progression was not assessed because of heterogeneity of treatment in this subgroup of patients.

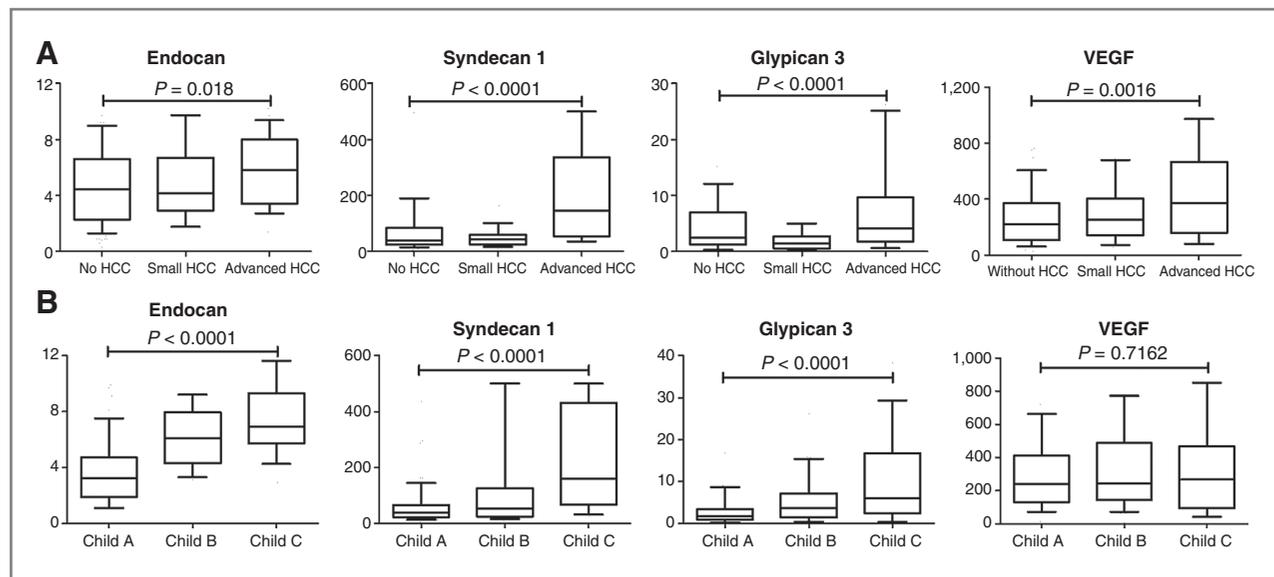
In each subgroup of patients, follow-up ended at the date of liver transplantation or death or at the last recorded visit (or information) during the 6 months before July 2010.

#### Ethics statement

All patients gave written consent for blood sampling; local ethics committee (Paris XIII) approval for the protocol was obtained.

#### Quantification of circulating proteoglycans

Blood samples were collected under fasting conditions before any treatment. After centrifugation (4,000 rpm for 15 minutes), sera were separated and stored at  $-80^{\circ}\text{C}$ . Commercial ELISA kits were used to conduct proteoglycans and VEGF quantifications in sera according to the manufacturers' instructions. Syndecan-1 sCD138 and glypican-3 ELISA kits were purchased from Gen-Probe Diacore and Cusabio Biotech Co., Ltd., respectively. Endocan serum levels were measured by ELISA kits distributed by Lunginnov and VEGF by ELISA kits from R&D Systems.



**Figure 1.** Levels of serum proteoglycans and VEGF in patients with alcohol-related cirrhosis and hepatocellular carcinoma (HCC). Levels of serum proteoglycans and VEGF according to (A) the presence and stage of hepatocellular carcinoma in the whole population and (B) Child-Pugh score in the whole population. Data were compared using the nonparametric Kruskal–Wallis test.

### Statistical analysis

Variables were compared using Fisher exact test for qualitative data and Kruskal–Wallis tests for continuous data.

Overall survival was defined by time from inclusion to death or liver transplantation; patients free of events were censored at their last visit, recorded until August 31, 2010.

For the patient group without hepatocellular carcinoma, time to hepatocellular carcinoma occurrence was defined as time between date of inclusion and date of onset of hepatocellular carcinoma. For the patient group with early hepatocellular carcinoma, RFS was defined as time between date of inclusion and date of hepatocellular carcinoma recurrence or date of death.

Distribution of time-to-failure endpoints was estimated by the Kaplan–Meier method. Levels of circulating proteoglycans (endocan, syndecan-1, and glypican-3) and VEGF were dichotomized (low level vs. high level) using mean values.

Univariate and multivariate analyses were conducted using Cox models for overall survival and RFS. Cause-specific Cox models were also fitted to estimate the influence of these covariates on time to hepatocellular carcinoma occurrence. All statistical tests were two-sided and  $P$  values  $\leq 0.05$  were considered significant. Statistical analysis was conducted using SAS 9.2 (SAS, Inc) and R 2.10.1 (R Development Core Team) software packages.

## Results

### Characteristics of patients and proteoglycan serum levels

A total of 295 patients with alcoholic cirrhosis were included in the study. Among them, 170 patients had

alcoholic cirrhosis without hepatocellular carcinoma, 58 patients had early hepatocellular carcinoma, and 67 patients had advanced hepatocellular carcinoma. Their characteristics are displayed in Table 1.

The levels of serum endocan, syndecan-1, glypican-3, and VEGF were significantly increased in patients with advanced hepatocellular carcinoma compared with patients without hepatocellular carcinoma or those with early hepatocellular carcinoma (Table 1; Fig. 1A). However, such differences were restricted to advanced hepatocellular carcinoma, whereas neither levels of serum VEGF and endocan nor levels of serum syndecan-1 significantly differed between the group of patients without hepatocellular carcinoma and that of patients with early hepatocellular carcinoma. However, the 3 subgroups of cirrhotic patients were heterogeneous in their underlying liver function, as reflected by the Child-Pugh classification (table 1). Indeed, there was a positive correlation between Child-Pugh C classification and high levels of serum endocan, syndecan-1, and glypican-3 (Fig. 1B). To eliminate this confounding factor, we focused only on Child-Pugh A patients; levels of endocan, syndecan-1, glypican-3, and VEGF levels remained significantly higher in patients with advanced hepatocellular carcinoma than in those with early hepatocellular carcinoma or without hepatocellular carcinoma (Supplementary Fig. S1A). As certain proteoglycans are implicated in microvascular dysfunction and neangiogenesis, we assessed a possible association with tumor portal thrombosis or portal hypertension. High levels of circulating proteoglycans were significantly associated with the presence of tumor portal thrombosis in patients with advanced hepatocellular carcinoma (Supplementary Fig. S1B). In contrast, in the group of patients without hepatocellular carcinoma, serum proteoglycans (except glypican-3) were

**Table 2.** Clinical and biologic variables associated with overall mortality and hepatocellular carcinoma occurrence in patients without hepatocellular carcinoma at baseline based on Cox univariable and multivariable models

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
<b>Overall survival</b>				
Gender (male)	1.02 (0.36–2.88)	0.97		
Age/10 (years)	1.11 (0.83–1.49)	0.49		
<b>Child-Pugh</b>				
A	1			
B	<b>5.36 (2.30–12.48)</b>	<b>0.0001</b>	<b>3.43 (1.26–9.37)</b>	<b>0.016</b>
C	<b>6.74 (2.73–16.63)</b>	<b>&lt;0.0001</b>	<b>4.13 (1.42–12.04)</b>	<b>0.009</b>
Creatinine >80	1.08 (0.57–2.07)	0.81		
AST (>2ULN)	0.51 (0.23–1.12)	0.092		
ALT (>2ULN)	0.43 (0.10–1.82)	0.25		
ALP (>2ULN)	0.68 (0.26–1.78)	0.43		
GGT (>2ULN)	1.47 (0.60–3.60)	0.39		
Alpha-fetoprotein/10	1.00 (0.93–1.07)	0.96		
Platelet/100	0.86 (0.51–1.46)	0.58		
Diabetes	0.76 (0.27–2.14)	0.60		
<b>Serum endocan &gt;5 ng/mL</b>	<b>5.75 (2.70–12.28)</b>	<b>&lt;0.0001</b>	<b>2.84 (1.18–6.84)</b>	<b>0.02</b>
Serum syndecan >50	1.18 (0.62–2.26)	0.62		
Serum glypican-3 >2.5	1.53 (0.80–2.93)	0.20		
Serum VEGF >240	0.72 (0.36–1.43)	0.34		
<b>Hepatocellular carcinoma occurrence</b>				
Gender (male)	1.91 (0.25–14.47)	0.53		
Age/10 (y)	<b>2.30 (1.42–3.71)</b>	<b>0.0007</b>	<b>2.47 (1.43–4.26)</b>	<b>0.0012</b>
<b>Child-Pugh</b>				
A	1			
B	2.51 (0.93–6.76)	0.068		
C	0.55 (0.07–4.39)	0.57		
Creatinine >80	1.34 (0.51–3.49)	0.55		
AST (>2 ULN)	0.77 (0.27–2.24)	0.63		
ALT (>2 ULN)	1.08 (0.24–4.82)	0.92		
PAL (>2 ULN)	1.25 (0.39–3.99)	0.70		
GGT (>2 ULN)	2.12 (0.48–9.48)	0.32		
AFP/10	1.02 (0.97–1.07)	0.39		
Platelet/100	<b>2.00 (1.07–3.72)</b>	<b>0.029</b>	<b>2.32 (1.18–4.55)</b>	<b>0.015</b>
Diabetes	1.42 (0.40–4.99)	0.59		
Serum endocan >5 ng/mL	1.73 (0.66–4.49)	0.26		
Serum syndecan-1 >50	0.88 (0.33–2.32)	0.79		
Serum glypican-3 >2.5	0.50 (0.18–1.43)	0.20		
Serum VEGF >240	1.09 (0.41–2.89)	0.86		

NOTE: In bold, variables with *P* value < 0.05 in univariate analysis.

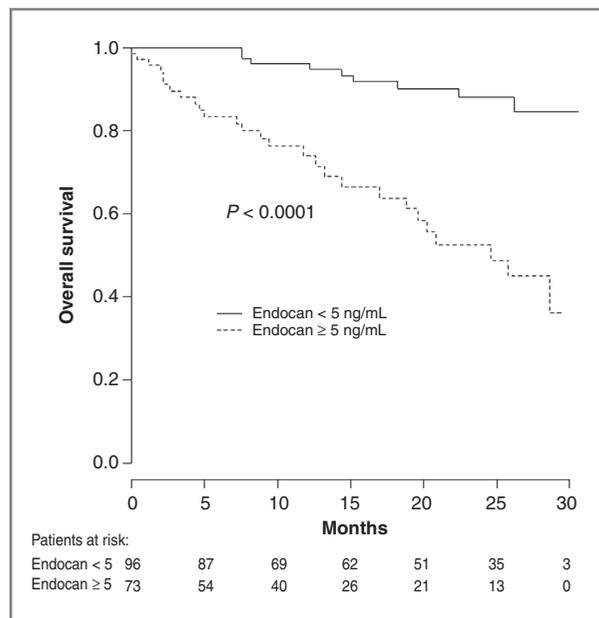
Abbreviations: ALT: alanine aminotransferase; ALP: alkaline phosphatase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; ULN, upper limit of normal.

not correlated with portal hypertension, defined by the presence of esophageal varices (Supplementary Fig. S1C).

#### **Serum endocan predicted overall survival in patients with alcoholic cirrhosis without hepatocellular carcinoma at baseline**

We next sought to assess the capacity of serum proteoglycans and VEGF to predict death or hepatocellular

carcinoma occurrence in patients without hepatocellular carcinoma at time of inclusion. Median duration of follow-up of the 170 patients without hepatocellular carcinoma was 16.8 months [interquartile range (IQR): 7.7–25.8]. Overall, 17 patients (10%) were lost to follow-up after at least one year of hepatocellular carcinoma screening, 17 patients developed hepatocellular carcinoma, and 38 either died (*n* = 30) or underwent liver



**Figure 2.** Overall survival in alcohol-related cirrhosis without hepatocellular carcinoma (HCC) at baseline according to serum levels of endocan. High levels of serum endocan were significantly associated with greater risk of death (HR, 5.75 95% CI, 2.70–12.28;  $P < 0.0001$ ) in cirrhotic patients without hepatocellular carcinoma at baseline. Patients were stratified according to the median level of serum endocan: low levels of circulating endocan (<5 ng/mL) versus high levels of circulating endocan (>5 ng/mL). The Kaplan–Meier method was used to estimate death. Numbers at risk are shown under the x-axis.

transplantation ( $n = 8$ ). In all but 5 cases, death was attributed to liver disease: advanced hepatocellular carcinoma in 8 cases and variceal bleeding and/or complications due to hepatic failure in the other 25. High levels of circulating endocan [HR, 2.84; 95% confidence interval (CI), 1.18–6.84;  $P = 0.02$ ] were chosen by the multivariate Cox model as an independent prognostic variable (Table 2 and Fig. 2), along with a high Child-Pugh score. However, none of the studied serum proteoglycans were associated with cause-specific risk of hepatocellular carcinoma occurrence in this subgroup of patients (Table 2).

### Serum proteoglycans and prognosis of patients with alcoholic cirrhosis and early hepatocellular carcinoma

We focused on the prognosis of patients with early hepatocellular carcinoma treated by RFA. Median duration of follow-up of the 58 patients with early hepatocellular carcinoma was 18.7 months (IQR: 8.9–30.0). Seven patients (12%) were lost to follow-up after at least one year of hepatocellular carcinoma screening, 28 (48%) had hepatocellular carcinoma recurrence, and 22 (38%) either died ( $n = 17$ ) or underwent liver transplantation ( $n = 5$ ). In all but 4 cases, death or liver transplantation was due to liver disease, attributable to advanced hepatocellular carcinoma in 17 cases and to variceal bleeding and/or

complications of hepatic failure in the remaining case. The median time to hepatocellular carcinoma recurrence was 16.0 months (IQR: 11.4–24.4).

Elevated levels of serum endocan (>5 ng/mL,  $P = 0.025$ ) and syndecan-1 (>50 ng/mL,  $P = 0.025$ ) were significantly associated with high risk of tumor recurrence (Fig. 3B and C). However, in multivariate analysis, both prognostic values disappeared (Supplementary Table S1).

When focusing on overall survival, we showed that only the high level of syndecan-1 (>50 ng/mL) was significantly associated with high risk of death (HR, 2.75; 95% CI, 1.15–6.57;  $P = 0.023$ ; Supplementary Table S1). In contrast with analysis of tumor recurrence, serum levels of endocan were not predictive of overall survival in the subgroup of patients with early hepatocellular carcinoma (Fig. 3A).

### Serum syndecan-1 predicted overall survival in patients with alcoholic cirrhosis and advanced hepatocellular carcinoma

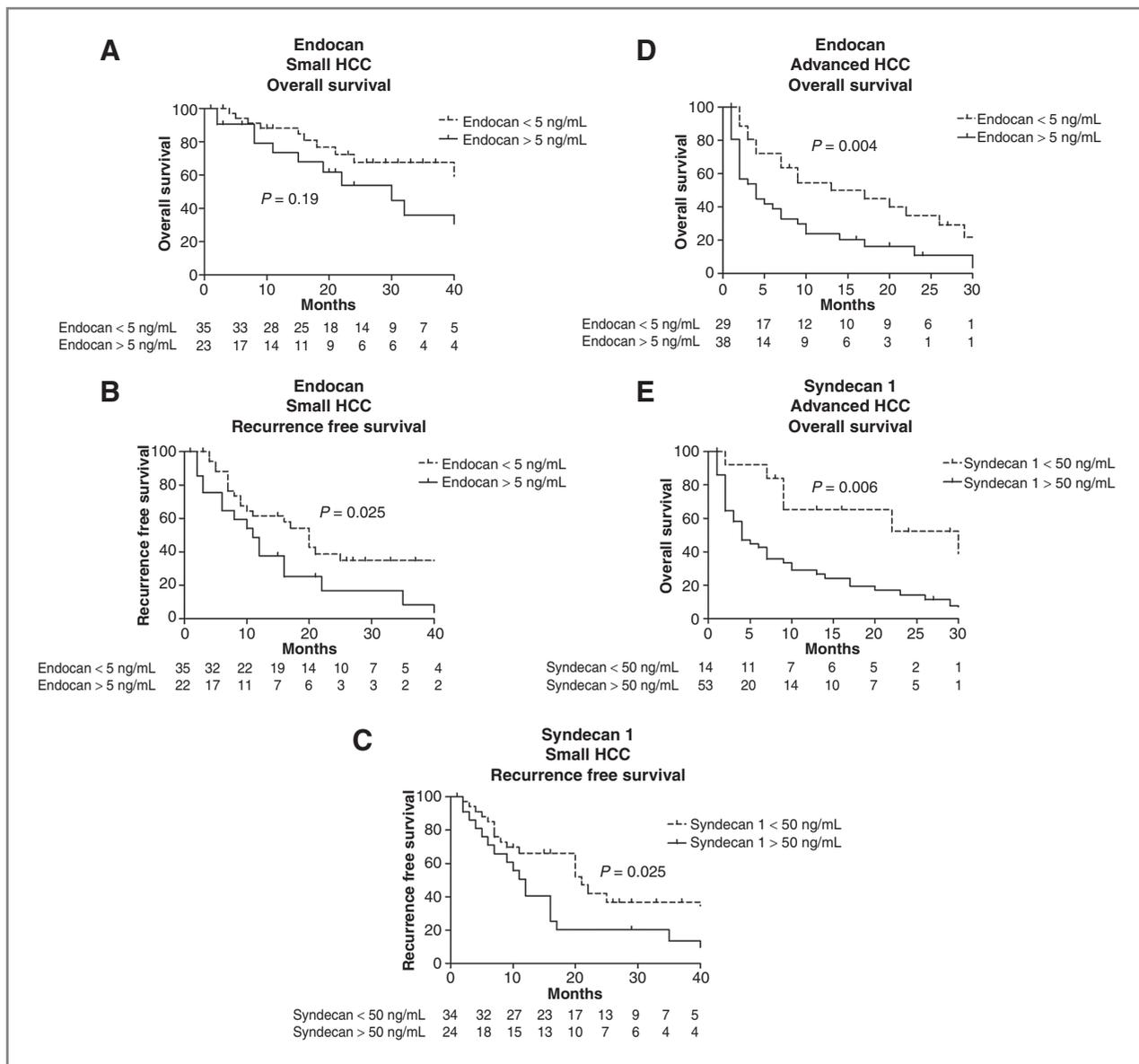
Finally, we studied the ability of serum proteoglycans and VEGF to predict death in patients with advanced hepatocellular carcinoma. Median duration of follow-up of the 67 patients with advanced hepatocellular carcinoma was 4.2 months (IQR: 1.4–13.6). Eight patients (11%) were lost to follow-up and 50 (75%) died because of hepatocellular carcinoma progression. In that group, 28 patients (41.8%) had been treated by arterial embolization, 13 (19.4%) by sorafenib, 7 (10.4%) by miscellaneous treatments, and 19 (28.4%) by best supportive care.

High levels of serum endocan and syndecan-1 were significantly associated with poor overall survival in patients with advanced hepatocellular carcinoma (Fig. 3D and E). In multivariate analysis, Child-Pugh C (HR, 6.55; 95% CI, 1.83–23.53;  $P = 0.004$ ) and a high level of syndecan-1 (HR, 6.21; 95% CI, 1.90–20.30;  $P = 0.0025$ ), together with tumor portal thrombosis (HR, 2.15; 95% CI, 0.99–4.64;  $P = 0.052$ ), were independently associated with a less favorable outcome in this subset of patients (Table 3).

### Discussion

In the present study, we analyzed the prognostic value of 3 proteoglycans (endocan, syndecan-1, and glypican-3) and VEGF in sera of 295 patients with alcohol-related cirrhosis prospectively screened for hepatocellular carcinoma and classified according to the absence or presence of early hepatocellular carcinoma and advanced hepatocellular carcinoma at the date of inclusion. While serum proteoglycans seem to be poor candidates as diagnostic biomarkers, they might be strongly related to patient prognosis. Serum levels of endocan were associated with overall survival in each subgroup of patients, and serum levels of syndecan-1 were strongly associated with overall survival in patients with advanced hepatocellular carcinoma.

In the present study, we found a significant increase in endocan, syndecan-1, glypican-3, and VEGF levels in sera



**Figure 3.** Outcome according to levels of serum proteoglycans in patients with early and advanced hepatocellular carcinoma (HCC). A, high levels of circulating endocan were not associated with higher risk of death in patients with early hepatocellular carcinoma (HR, 1.74; 95% CI, 0.75–4.04;  $P = 0.19$ ). Patients were stratified according to low levels of endocan (<5 ng/mL) versus high levels of endocan (>5 ng/mL). B, high levels of serum endocan were a risk factor for tumor recurrence in patients with early hepatocellular carcinoma (HR, 2.17 95% CI, 1.1–4.27;  $P = 0.025$ ). Patients were stratified according to the mean value of serum endocan: low levels (<5 ng/mL) versus high levels (>5 ng/mL). C, high levels of serum syndecan-1 were associated with strong risk of tumor recurrence in patients with early hepatocellular carcinoma (HR, 2.17; 95% CI, 1.1–4.28;  $P = 0.025$ ). Patients were stratified according to mean levels of syndecan-1: low value (<50 ng/mL) versus high value (>50 ng/mL) of serum syndecan-1. D, high levels of serum endocan were associated with strong risk of death (HR, 2.39 95% CI, 1.31–4.35;  $P = 0.004$ ) in patients with advanced hepatocellular carcinoma. Patients were divided according to mean levels of serum endocan: low levels (<5 ng/mL) versus high levels (>5 ng/mL). E, high levels of serum syndecan-1 were associated with poor overall survival in patients with advanced hepatocellular carcinoma (HR, 3.32; 95% CI, 1.4–7.84;  $P = 0.006$ ). Patients were stratified according to mean levels of serum syndecan-1: low levels (<50 ng/mL) versus high levels (>50 ng/mL). The Kaplan–Meier method was used to estimate overall survival and tumor recurrence for each level of proteoglycans. Numbers at risk are shown under the x-axis.

of patients with advanced hepatocellular carcinoma. This might reflect increased neoangiogenesis and microenvironment remodeling during liver cancer progression. However, because serum levels of these biomarkers were not increased in patients with early hepatocellular carcinoma, they are not useful for diagnostic purposes. Indeed,

diagnosis of early hepatocellular carcinoma is the most clinically relevant setting, as this subgroup of patients may benefit from curative treatment.

In our study, we found no association between VEGF levels and the different clinical endpoints in any of our patient subgroups. Two recent studies have reported a

**Table 3.** Clinical and biologic variables associated with overall mortality in patients with advanced hepatocellular carcinoma based on Cox univariable and multivariable models

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
<b>Overall survival</b>				
Gender (male)	1.35 (0.40–4.50)	0.63		
Age/10 (y)	0.9 (0.64–1.25)	0.52		
<b>Child-Pugh</b>				
A	<b>1</b>		<b>1</b>	
B	<b>1.61 (0.83–3.14)</b>	<b>0.16</b>	<b>3.43 (1.05–11.2)</b>	<b>0.041</b>
C	<b>4.38 (2.07–9.30)</b>	<b>0.00012</b>	<b>6.55 (1.83–23.53)</b>	<b>0.004</b>
Creatinine >80	1.19 (0.66–2.16)	0.57		
AST (>2 ULN)	1.02 (0.55–1.88)	0.96		
ALT (>2 ULN)	0.84 (0.36–1.99)	0.69		
PAL (>2 ULN)	0.52 (0.24–1.14)	0.10		
GGT (>2 ULN)	1.29 (0.60–2.78)	0.51		
AFP/10	1.0 (1.0–1.0)	0.24		
Platelet/100	0.75 (0.47–1.20)	0.23		
Diabetes	0.79 (0.37–1.70)	0.55		
<b>Tumor portal thrombosis</b>	<b>4.4 (2.33–8.33)</b>	<b>&lt;0.0001</b>	<b>2.15 (0.99–4.64)</b>	<b>0.052</b>
Number of HCC >1	0.74 (0.41–1.35)	0.33		
<b>Size of HCC/10</b>	<b>1.01 (1.01–1.19)</b>	<b>0.032</b>	<b>1.10 (0.97–1.24)</b>	<b>0.13</b>
<b>Serum endocan &gt; 5</b>	<b>2.39 (1.31–4.35)</b>	<b>0.004</b>	<b>1.16 (0.48–2.79)</b>	<b>0.74</b>
<b>Serum syndecan-1 &gt; 50</b>	<b>3.32 (1.40–7.84)</b>	<b>0.006</b>	<b>6.21 (1.90–20.30)</b>	<b>0.0025</b>
Serum glypican-3 >2.5	1.58 (0.86–2.92)	0.14		
Serum VEGF >240	1.27 (0.70–2.30)	0.43		

NOTE: In bold, variables with *P* value < 0.05 in univariate analysis.

Abbreviations: ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; GGT, gamma-glutamyl transferase level; ULN, upper limit of normal.

prognostic value of serum VEGF in advanced hepatocellular carcinoma (21, 22). However, our study differed from those in terms of etiology and severity of underlying liver disease, which could explain these differences.

In contrast, we were able to show the prognostic value of serum endocan (19). High serum endocan levels were associated with poor overall survival in patients with early and advanced hepatocellular carcinoma, but were not independent of other variables in multivariate analysis. Endocan is a proteoglycan strongly expressed by tumor endothelial cells, but poorly expressed by resting endothelial cells (16, 23). Thus, it could constitute a good surrogate marker of tumor neoangiogenesis and might be a potential therapeutic target for new antiangiogenic drugs. An antibody against endocan was used on a xenograft mouse model via tumor cells overexpressing endocan; it reduced the tumor burden in immunocompromised mice (17). In humans, expression of endocan by endothelial cells in immunohistochemistry was correlated with microvascular invasion and poor prognosis (24). However, the rate of circulating endocan has not been previously correlated with prognosis or tumor vessel expression. Here, we showed that patients with tumor portal thrombosis exhibited higher rates of serum

endocan than patients without tumor portal thrombosis. High levels of circulating endocan might therefore not only reflect macrovascular invasion, but possibly also microvascular invasion, although this hypothesis is speculative.

Interestingly, we showed that a high serum endocan level was associated with strong risk of death in patients without hepatocellular carcinoma at baseline, but was not predictive of hepatocellular carcinoma occurrence. In patients with alcohol-related cirrhosis and without hepatocellular carcinoma, the mean levels of circulating endocan (mean  $\pm$  SD, 4.8  $\pm$  3.1 ng/mL) were higher than in a control population without cirrhosis (0.77  $\pm$  0.18 ng/mL) included in previous studies (19). Along the same lines, levels of circulating endocan, syndecan-1, and glypican-3 are higher in patients with advanced liver failure than in patients with compensated cirrhosis. The underlying process explaining this overexpression remains unknown. Lipopolysaccharide and TNF- $\alpha$ , 2 classical proteins that increase during bacterial infection, could induce secretion of endocan by cultured endothelial cells (25). Endocan has been proposed as a pertinent biomarker of endothelial dysfunction in severe sepsis (25). Serum endocan may indeed reflect endothelial dysfunction induced by a

systemic inflammatory response, a pathologic process that could modify the course of cirrhosis (26). As hepatocellular carcinoma often develops in cirrhotic liver, death from the underlying disease constitutes a competitive risk of death from the tumor. In light of this, serum endocan is a potential option for refining the prognosis in addition to the classical Child-Pugh score.

In contrast to studies focusing on tumor expression (9, 27), we did not find any prognostic value for serum glypican-3, underlining the possible discrepancy between tumor expression and the serum value of glypican-3.

Finally, syndecan-1 is a proteoglycan expressed in hepatocytes and involved in proliferation, migration, and adhesion in hepatocellular and myeloma cell lines (6, 10). We found that serum syndecan-1 was independently associated with poor overall survival at advanced stages of primary liver tumors. This observation corroborates the prognostic value observed in other solid and hematologic cancers and in fundamental studies focusing on syndecan-1 in hepatocellular carcinoma cell lines (10–13).

In conclusion, our study suggests a prognostic role for serum levels of endocan and syndecan-1, respectively, in patients without and with hepatocellular carcinoma developing on alcoholic cirrhotic liver. Basic research will better characterize the role of proteoglycans in liver dysfunction and carcinogenesis. In addition to their role as functional coreceptors of chemokines and growth factors,

proteoglycans may exert other undefined roles that warrant elucidation.

#### Disclosure of Potential Conflicts of Interest

P. Lassalle is a consultant/advisory board member and M. Delehedde is employed (employment—other than primary affiliation; e.g., consulting) in Lunginnov. No potential conflicts of interest were disclosed by the other authors.

#### Authors' Contributions

**Conception and design:** J.-C. Nault, J.-C. Trinchet, N. Charnaux, M. Delehedde, A. Sutton, P. Nahon

**Development of methodology:** J.-C. Nault, S. Chevret, O. Seror, P. Lasalle, M. Delehedde, P. Nahon

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** J.-C. Nault, E. Guyot, C. Laguillier, G. N'Kontchou, M. Beaugrand, O. Seror, P. Nahon

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** J.-C. Nault, M. Beaugrand, M. Delehedde, P. Nahon

**Writing, review, and/or revision of the manuscript:** J.-C. Nault, E. Guyot, N. Ganne-Carrie, G. N'Kontchou, N. Charnaux, M. Delehedde, A. Sutton, P. Nahon

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** J.-C. Nault, E. Guyot, C. Laguillier, O. Seror, J. Coelho, P. Nahon

**Study supervision:** J.-C. Nault, N. Charnaux, A. Sutton, P. Nahon

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 14, 2013; revised May 14, 2013; accepted May 16, 2013; published OnlineFirst June 18, 2013.

#### References

- Forner A, Llovet JM, Bruix J. Hepatocellular carcinoma. *Lancet* 2012; 379:1245–55.
- Borie F, Tretarre B, Bouvier AM, Faivre J, Binder F, Launoy G, et al. Primitive liver cancers: epidemiology and geographical study in France. *Eur J Gastroenterol Hepatol* 2009;21:984–9.
- Bruix J, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, et al. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001;35:421–30.
- Llovet JM, Ducreux M, Lencioni R, Di Bisceglie AM, Galle PR, Dufour JF, et al. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2012;56:908–43.
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–74.
- Iozzo RV, Sanderson RD. Proteoglycans in cancer biology, tumour microenvironment, and angiogenesis. *J Cell Mol Med* 2011;15: 1013–31.
- Di Tommaso L, Franchi G, Park YN, Fiamengo B, Destro A, Morenghi E, et al. Diagnostic value of HSP70, glypican 3, and glutamine synthetase in hepatocellular nodules in cirrhosis. *Hepatology* 2007;45:725–34.
- Capurro M, Wanless IR, Sherman M, Deboer G, Shi W, Miyoshi E, et al. Glypican-3: a novel serum and histochemical marker for hepatocellular carcinoma. *Gastroenterology* 2003;125:89–97.
- Shirakawa H, Suzuki H, Shimomura M, Kojima M, Gotohda N, Takahashi S, et al. Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. *Cancer Sci* 2009;100: 1403–7.
- Charni F, Friand V, Haddad O, Hlawaty H, Martin L, Vassy R, et al. Syndecan-1 and syndecan-4 are involved in RANTES/CCL5-induced migration and invasion of human hepatoma cells. *Biochim Biophys Acta* 2009;1790:1314–26.
- Seidel C, Sundan A, Hjorth M, Turesson I, Dahl IM, Abildgaard N, et al. Serum syndecan-1: a new independent prognostic marker in multiple myeloma. *Blood* 2000;95:388–92.
- Joensuu H, Anttonen A, Eriksson M, Makitaro R, Alfthan H, Kinnula V, et al. Soluble syndecan-1 and serum basic fibroblast growth factor are new prognostic factors in lung cancer. *Cancer Res* 2002;62:5210–7.
- Li HG, Xie DR, Shen XM, Li HH, Zeng H, Zeng YJ. Clinicopathological significance of expression of paxillin, syndecan-1, and EMMPRIN in hepatocellular carcinoma. *World J Gastroenterol* 2005;11:1445–51.
- Metwaly HA, Al-Gayyar MM, Eletreby S, Ebrahim MA, El-Shishtawy MM. Relevance of serum levels of interleukin-6 and syndecan-1 in patients with hepatocellular carcinoma. *Sci Pharm* 2012;80:179–88.
- Sarrazin S, Lyon M, Deakin JA, Guerrini M, Lassalle P, Delehedde M, et al. Characterization and binding activity of the chondroitin/dermatan sulfate chain from Endocan, a soluble endothelial proteoglycan. *Glycobiology* 2010;20:1380–8.
- Abid MR, Yi X, Yano K, Shih SC, Aird WC. Vascular endocan is preferentially expressed in tumor endothelium. *Microvasc Res* 2006; 72:136–45.
- Scherpereel A, Gentina T, Grigoriu B, Senechal S, Janin A, Tscopoulos A, et al. Overexpression of endocan induces tumor formation. *Cancer Res* 2003;63:6084–9.
- Kang YH, Ji NY, Lee CI, Lee HG, Kim JW, Yeom YI, et al. ESM-1 silencing decreased cell survival, migration, and invasion and modulated cell cycle progression in hepatocellular carcinoma. *Amino Acids* 2011;40:1003–13.
- Grigoriu BD, Depontieu F, Scherpereel A, Gourcerol D, Devos P, Ouatat T, et al. Endocan expression and relationship with survival in human non-small cell lung cancer. *Clin Cancer Res* 2006;12:4575–82.
- Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;359:378–90.

21. Llovet JM, Pena C, Lathia C, Shan M, Meinhardt G, Bruix J. Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. *Clin Cancer Res* 2012;18:2290–300.
22. Kaseb AO, Morris JS, Hassan MM, Siddiqui AM, Lin E, Xiao L, et al. Clinical and prognostic implications of plasma insulin-like growth factor-1 and vascular endothelial growth factor in patients with hepatocellular carcinoma. *J Clin Oncol* 2011;29:3892–9.
23. Leroy X, Aubert S, Zini L, Franquet H, Kervoaze G, Villers A, et al. Vascular endocan (ESM-1) is markedly overexpressed in clear cell renal cell carcinoma. *Histopathology* 2010;56:180–7.
24. Huang GW, Tao YM, Ding X. Endocan expression correlated with poor survival in human hepatocellular carcinoma. *Dig Dis Sci* 2009;54:389–94.
25. Scherpereel A, Depontieu F, Grigoriu B, Cavestri B, Tsicopoulos A, Gentina T, et al. Endocan, a new endothelial marker in human sepsis. *Crit Care Med* 2006;34:532–7.
26. Iwakiri Y. Endothelial dysfunction in the regulation of cirrhosis and portal hypertension. *Liver Int* 2012;32:199–213.
27. Ho DW, Yang ZF, Yi K, Lam CT, Ng MN, Yu WC, et al. Gene expression profiling of liver cancer stem cells by RNA-sequencing. *PLoS ONE* 2012;7:e37159.

# Cancer Epidemiology, Biomarkers & Prevention

## Serum Proteoglycans as Prognostic Biomarkers of Hepatocellular Carcinoma in Patients with Alcoholic Cirrhosis

Jean-Charles Nault, Erwan Guyot, Christelle Laguillier, et al.

*Cancer Epidemiol Biomarkers Prev* 2013;22:1343-1352. Published OnlineFirst June 18, 2013.

**Updated version** Access the most recent version of this article at:  
doi:[10.1158/1055-9965.EPI-13-0179](https://doi.org/10.1158/1055-9965.EPI-13-0179)

**Supplementary Material** Access the most recent supplemental material at:  
<http://cebp.aacrjournals.org/content/suppl/2013/06/18/1055-9965.EPI-13-0179.DC1>

**Cited articles** This article cites 27 articles, 6 of which you can access for free at:  
<http://cebp.aacrjournals.org/content/22/8/1343.full#ref-list-1>

**Citing articles** This article has been cited by 1 HighWire-hosted articles. Access the articles at:  
<http://cebp.aacrjournals.org/content/22/8/1343.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://cebp.aacrjournals.org/content/22/8/1343>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.