HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Liver Progenitor Cell Markers Correlate With Liver Damage and Predict Short-term Mortality in Patients With Alcoholic Hepatitis

Pau Sancho-Bru,[1] José Altamirano,[1] Daniel Rodrigo-Torres,[1] Mar Coll,[1] Cristina Millán,[1] Juan José Lozano,[1] Rosa Miquel,[2] Vicente Arroyo,[1] Juan Caballería,[1] Pere Ginès,[1] and Ramon Bataller[1]

Alcoholic hepatitis (AH) is a severe condition developed in patients with underlying alcoholic liver disease. Ductular reaction has been associated with chronic alcohol consumption but there is no information regarding the extent of liver progenitor cell (LPC) proliferation in AH. The aim of this study was to investigate LPC markers in AH and its correlation with disease severity. Fifty-nine patients with clinical and histological diagnosis of AH were included in the study. LPC markers were assessed by real-time polymerase chain reaction (PCR) and immunohistochemistry. Standard logistic regression analysis and classification and regression trees (CART) analysis were used for statistical analysis. A microarray analysis showed an up-regulation of LPC markers in patients with AH. Real-time PCR demonstrated that *epithelial cell adhesion molecule (EpCAM), Prominin-1*, and *Keratin7* were significantly increased in patients with AH compared with normal livers ($P \leq 0.01$), chronic hepatitis C ($P \leq 0.01$), and HCV-induced cirrhosis ($P \leq 0.01$). Immunohistochemistry scores generated for Keratin7 and EpCAM demonstrated a good correlation with gene expression. *Keratin7* gene expression correlated with liver failure as assessed by model for endstage liver disease score ($r = 0.41$, $P = 0.006$) and Maddrey's discriminant function ($r = 0.43$, $P = 0.004$). Moreover, *Keratin7* (OR1.14, $P = 0.004$) and *Prominin-1* (OR1.14, $P = 0.002$), but not *EpCAM* (OR1.16, $P = 0.06$), were identified as independent predictors of 90-day mortality. CART analysis generated an algorithm based on the combination of *Keratin7* and *EpCAM* gene expression that stratified three groups of patients with high, intermediate, and low short-term mortality (89%, 33%, and 6%, respectively; area under the receiver operating curve 0.73, 95% confidence interval 0.60-0.87). Keratin7 expression provided additional discrimination potential to the age, bilirubin, international normalization ratio, creatinine (ABIC) score. *Conclusion*: LPC markers correlate positively with severity of liver disease and short-term mortality in AH patients. This study suggests that LPC proliferation may be an important feature of AH pathophysiology. (HEPATOLOGY 2012;55:1931-1941)

Alcoholic liver disease is a major cause of endstage liver disease. It ranges from fatty liver disease to steatohepatitis, cirrhosis, and eventually hepatocellular carcinoma.[1,2] Alcoholic hepatitis (AH) is an acute event in chronic alcoholic liver disease that develops in up to 20% of patients with heavy alcohol intake.[3] In its severe forms, AH leads to liver failure and a high short-term mortality rate. AH is characterized by significant hepatocellular damage, megamitochondria, hepatocyte arrest, inflammatory response, and rapid progression of fibrosis.[4] We recently developed the ABIC (Age, Bilirubin, International normalization ratio (INR), and Creatinine) score, which allows a prognostic stratification based on analytical parameters into patients with low, intermediate, and high risk of death at 90 days and 1 year.[5] The

Abbreviations: ABIC, age, bilirubin, international normalization ration, creatinine; AH, alcoholic hepatitis; CART, classification and regression tree; EpCAM, epithelial cell adhesion molecule; HCV, hepatitis C virus; KRT, keratin; LPC, liver progenitor cells; PROM1, prominin-1.

From the [1]Liver Unit, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Barcelona, Spain; [2]Pathology Unit, Hospital Clínic, Barcelona, Spain.

Received July 21, 2011; accepted January 18, 2012.

Supported by grants from the Instituto de Salud Carlos III (FIS PI080237, FIS PS09/01164 and FIS PI080126, to R.B., J.C., and P.G., respectively). P.S.-B. received a grant from Ministerio de Ciencia e Innovación, Juan de la Cierva (JCI-2009-03849) and Instituto de Salud Carlos III, Miguel Servet (CP11/00071). D.R.-T. received a grant from the Ministerio de Educación, FPU program. J.A. received a grant from Fundación Banco Bilbao Vizcaya Argentaria.

pathogenesis of AH is poorly understood and current therapies are not fully effective. Thus, there is a clear need to better understand the pathogenesis of AH in order to identify new targets for therapy and develop new therapeutic strategies for these patients.

Liver damage from any etiology induces mature hepatocytes to proliferate in order to replace the damaged tissue, allowing the recovery of liver function without any involvement of liver progenitor cells (LPCs).[6,7] However, when there is either massive liver injury or chronic liver damage that compromise the proliferative capacity of hepatocytes, progenitor cells within the Canal of Hering start to proliferate, giving rise to what is known as ductular reaction.[6-8] Little is known about the real contribution of LPC in the recovery of liver function in liver diseases. Immunohistochemistry studies performed in damaged human livers have shown that ductular reaction comprises a heterogenic population of proliferating cells, ranging from cells expressing stem cell markers with an immature phenotype, to more committed cells with an intermediate phenotype, expressing progenitor cell markers but also characteristics of both immature hepatocytes and cholangiocytes.[9-12] KRT7 is a well-known marker of ductular reaction in liver disease and it is typically expressed in LPC but also in intermediate hepatobiliary cells.[13,14] Prominin-1 (PROM1) has been described in progenitor cells from the liver and other organs and is considered a marker for hepatic cancer stem cells.[15,16] Epithelial cell adhesion molecule (EpCAM) is expressed in LPC and it has been used to isolate progenitor cells from human samples.[17,18] Moreover, EpCAM is also expressed in newly generated hepatocytes derived from progenitor cells in the regenerating liver.[19] *In vitro* studies with isolated LPC from human liver samples have demonstrated that LPC can differentiate into hepatocytes and cholangiocytes, exerting functions of mature cells.[17,20,21] However, there are no functional studies that investigated the dynamics, progression, and outcome of progenitor cell expansion during the course of liver disease, including AH.

Ductular reaction is known to be present in most chronic liver diseases but it seems especially important in acute-on-chronic events.[22,23] Moreover, factors such as fibrosis and inflammation are known to promote LPC expansion. Advanced alcoholic liver disease is characterized by an important ductular reaction due to the underlying liver injury but also to the effect of alcohol in promoting LPC proliferation.[24,25] Moreover, the extent of ductular reaction correlates with the severity of liver disease.[23,24] AH is characterized by an impairment in cell proliferation together with a massive liver damage and inflammatory response, which are key factors influencing the progression of progenitor cells. However, there is no information on the extent of ductular reaction in AH and its correlation with the severity of liver disease. In the present article we describe that in AH there is an important ductular reaction and extensive proliferation of progenitor cells. Moreover, progenitor cell markers correlate with liver disease and are good prognostic markers for short-term mortality in patients with AH.

Patients and Methods

Patients and Interventions. This study was performed with samples collected from a cohort of 69 consecutive patients admitted to the Liver Unit of the Hospital Clinic, Barcelona, from 2007 to 2010, with clinical and analytical criteria of AH. Ten patients were excluded from the study: 8 patients who did not fulfill all diagnostic criteria of AH and 2 patients with documented hepatocellular carcinoma. Finally, 59 patients with biopsy-proven AH were included. Liver biopsies were obtained by transjugular approach in all cases within 48 hours of admission and before starting treatment with corticosteroids, if indicated. Patients were managed following international guidelines and the clinical protocols approved in the Liver Unit of the hospital.[26-28] Healthy patient characteristics are depicted in Supporting Fig. 1. The study was approved by the Ethics Committee of the Hospital Clinic and all patients included in this study gave written informed consent.

Microarray Data Normalization. Markers of LPCs up-regulated in the AH microarray were identified and summarized. The microarray previously performed comprised data from 22 samples: 15 AH and 7 healthy samples.[29] Information regarding microarray analysis can be found in the Supporting Material.

Address reprint requests to: Pau Sancho-Bru, Ph.D., Institut d'Investigacions Biomèdiques August Pi i Suyner (IDIBAPS), C/ Roselló, 153, Planta 3, 08036 Barcelona, Spain. E-mail: psancho@clinic.ub.es; fax: +34 93 3129406.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.25614

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Hepatic Gene Expression Analysis. Quantitative polymerase chain reaction (PCR) was used to assess the expression of selected genes in liver human samples. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Five hundred nanograms of RNA were retrotranscribed using a high-capacity complementary DNA reverse-transcription kit (Applied Biosystems) and complementary DNA (cDNA) were then amplified using Taqman Technology (Applied Biosystems) in a final PCR volume of 10 μL using a 7900 HT instrument (Applied Biosystems). The Assay-on-Demand probes and primers for the quantification of 18s, *EpCam, PROM1*, and *Keratin (KRT)7* (Ref seq: Hs99999901_s1, Hs00158980_m1, Hs01009259_ m1 and Hs00559840_m1) were obtained from Applied Biosystems. Results were normalized to 18s rRNA expression (housekeeping gene) and gene expression values were calculated based on the $\Delta\Delta$Ct method using a pool of liver RNA as internal reference. The results are expressed as $2^{(-\Delta\Delta Ct)}$.

Histological Analysis and Development of KRT7 and PROM-1 Score. Three μm of paraffin-embedded liver sections were incubated with mouse antihuman KRT7 (1:50, DakoCytomation, Glostrup, Denmark, M7018) for 1 hour at room temperature and monoclonal mouse antihuman EpCAM (1:100, DakoCytomation, M0804) for 2 hours at room temperature. After washing in phosphate-buffered saline (PBS), sections were incubated with secondary goat antimouse antibody conjugated to horseradish peroxidase (HRP) (DAKO EnVision System-HRP, K4007) for 30 minutes at room temperature. 3,3′ Diaminobenzidine (DAB, Dako) was used as a chromogen and sections were counterstained with hematoxylin. As negative controls, all specimens were incubated with primary antibody omission under identical conditions. Dual immunofluorescent staining was performed as serial stainings with EpCAM, KRT7, and the hepatocyte marker clone OCH1e5 (M7158) (Dako) (see Supporting Material). Semiquantitative assessment of inflammation and fibrosis was performed by author R.M. on hematoxylin and eosin staining and tricromic staining, respectively. Quantification of fibrosis was performed by morphometric quantification of tricromic stained area (see Supporting Material).

To assess the degree of expression of KRT7 and EpCAM, we developed a semiquantitative score based on the nomenclature and analysis of ductular reaction described.[9,10] Staining for KRT7 was quantified according to three parameters from 0-3: ductular structures, isolated cells within the parenchyma, and intermediate hepatobiliary cells. Staining for EpCAM was quantified according to three parameters from 0-3: ductular structures, interphase cells, and hepatobiliary cells.

Table 1. Baseline Demographic, Clinical and Biochemical Characteristics of Patients with Alcoholic Hepatitis

	Study Cohort (n=59)
Age (years)	51 (46-55)
Male (%)	41 (70)
Alcohol Intake (g/day)	100 (80-120)
Corticoids (%)	24 (41)
Biochemical parameters	
Hematocrit (%)	29 (19-35)
Leukocytes $\times 10^9$/L	8.0 (6.3-11.1)
Platelets $\times 10^9$/L	65 (32-134)
Serum creatinine (mg/dL)	0.8 (0.6-1.0)
Serum albumin (g/dL)	25 (23-31)
Serum bilirubin (mg/dL)	10 (4.2-18.3)
International normalized ratio	1.6 (1.3-1.9)
AST (U/L)	132 (86-182)
ALT (U/L)	54 (34-75)
GGT (U/L)	317 (116-923)
Clinical Scores	
Maddrey's discriminant function	57 (33-77)
MELD score	29 (16-24)
ABIC score	7.65 (6.95-8.47)
ABIC class (%)	
A (<6.71)	12 (20)
B (6.71-8.99)	40 (68)
C (>9)	7 (12)
Cirrhosis (yes/no)	(42/17)
Fibrosis (F1/F2/F3-F4) n=57	(3/12/42)
Inflammation (mild/severe) n=57	(41/16)

AST, aspartate aminotranferase; ALT, alanine aminotransferase; MELD, model for end-stage liver disease; ABIC, age, bilirubin, INR, creatinine score.

Statistical Analysis. Continuous variables were described as mean (95% confidence interval [CI]) or median (interquartile range). Categorical variables were described by means and percentages. Comparisons between groups were performed using Student's *t* test or Mann-Whitney *U* test, depending on variable distribution. Differences between categorical variables were assessed by the chi-square test or Fisher's exact test, when necessary. Correlations between variables were evaluated using Spearman's *rho* or Pearson's *r*, when appropriate. The main endpoint was death from any cause at 90 days. A two-sided $P < 0.05$ was required for statistical significance. The potential role of LPC markers in the short-term prognosis of patients with AH was evaluated by univariate analysis, multivariate logistic regression analysis. A classification and regression tree (CART) analysis was performed to evaluate the interaction of LPC markers with short-term prognosis of AH patients (see Supporting Material).

Results

Patient Characteristics. The baseline clinical, demographic, and biochemical characteristics of the study

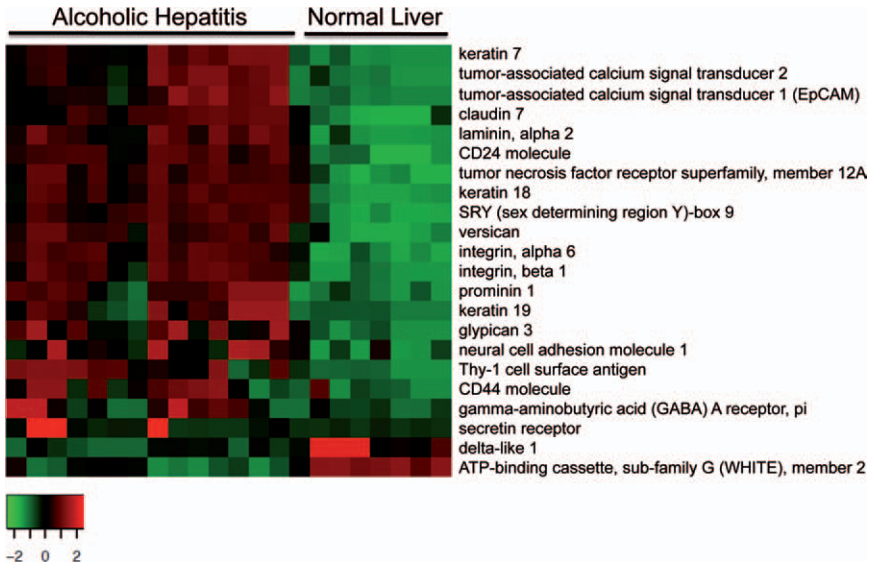


Fig. 1. Heatmap showing hierarchical clustering using expression profiles of the selected genes. Distances are measured using Pearson correlation. Results are expressed as a matrix view of gene expression data where rows represent genes and columns represent hybridized samples. The intensity of each color denotes the standardized ratio between each value and the average expression of each gene across all samples. Red pixels correspond to an increased abundance of mRNA in the indicated liver biopsy sample, whereas green pixels indicate decreased mRNA levels.

cohort are depicted in Table 1. The median alcohol consumption was 100 g/day (range, 80-120). Twenty-four patients (41%) were treated with corticosteroids. During hospitalization, 30 patients (51%) developed at least one clinical complication. These complications included bacterial infections (36%), hepatic encephalopathy (21%), in-hospital renal dysfunction (20%), and gastrointestinal bleeding (7%). The overall 90-day mortality rate was 27% (16 out of 59 patients). The main causes of death at 90 day were sepsis (7 out of 16) and multiorgan failure (9 out of 16).

Expression of LPC Markers in Patients With AH. Previous studies have shown that alcoholic liver disease is characterized by an important ductular reaction and LPC expansion.[24,25] Thus, we evaluated the hepatic expression of genes typically expressed in LPC in patients with AH and healthy subjects. Microarray analysis demonstrated an up-regulation of genes related to LPC and ductular reaction in AH samples. These genes include *KRT7, SOX9, EpCAM, KRT19, PROM1, CD44*, and others. Although some of these genes are not exclusively expressed in LPC, the marked up-regulation of a significant number of genes typically expressed in isolated LPS suggest that this cell population may be enriched in patients with AH. The results shown in Fig. 1 are a subset of genes selected from the microarray previously described.[29] Full microarray data are deposited in the NCBI Gene Expression Omnibus (GEO; access. no. GSE28619).

Detailed Analysis of Selected LPC Markers in Patients With AH and Other Liver Diseases. *KRT7, PROM1*, and *EpCAM* were selected for further analysis as representative genes described in ductular reaction. Expression of these genes was evaluated by real-time PCR in the whole series of patients with AH (n = 59), in comparison with the expression in tissue from patients with hepatitis C virus (HCV)-induced cirrhosis (HCV-CH) (n = 16), chronic hepatitis C (HCV) (n = 14), and fragments of normal tissue (n = 12). Gene expression of all three genes was significantly higher in patients with AH compared with HCV-induced liver disease, and normal livers (Fig. 2a-c). Moreover, although *KRT7, PROM1*, and *EpCAM* are expressed in different cell populations in the ductular reaction, there was a significant correlation among the expression of these markers (data not shown). We next investigated if *KRT7, PROM1*, and *EpCAM* were differentially expressed in patients with AH according to their survival rate. *KRT7* and *PROM1* expression was higher in patients who died within 90 days after admission (n = 16) compared with those who survived (n = 43) (Fig. 2d-f). There was no difference in *EpCAM* expression between patients with different survival rates (Fig. 2f). To further assess if the expression of LPC markers was associated with disease severity, we evaluated the correlation of gene expression of LPC markers with clinical prognostic scores. As shown in Fig. 2, we found a positive correlation of *KRT7*, but not *PROM-1* and *EpCAM* gene expression with the model for endstage liver disease (MELD) and Maddrey's discriminant function (Fig. 2g,h), suggesting that liver damage may promote a ductular reaction

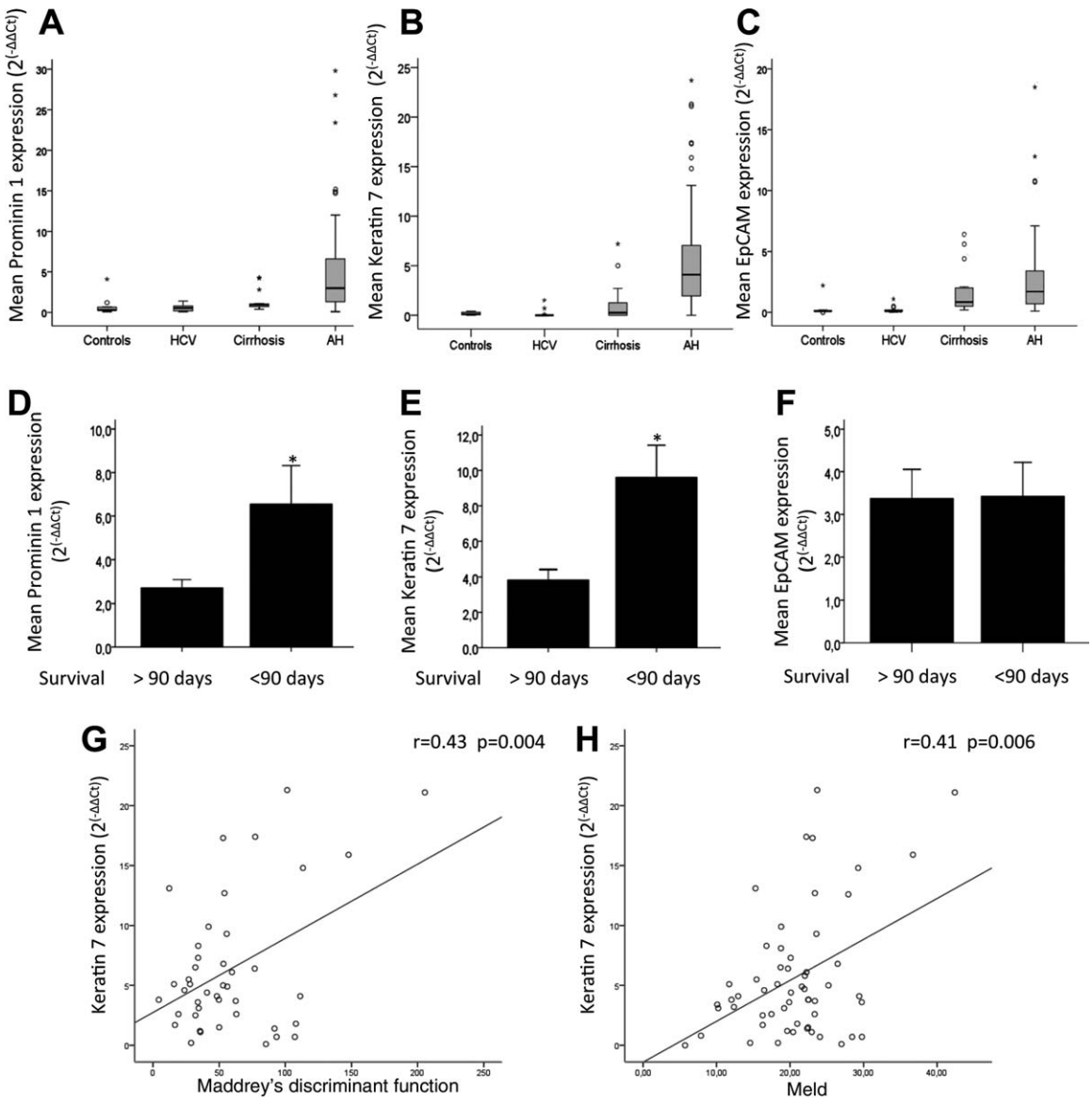


Fig. 2. Gene expression analysis of liver progenitor cell markers in liver diseases. Quantitative gene expression analysis of PROM-1 (A), KRT7 (B), and EpCAM (C) in normal livers, HCV-induced hepatitis, HCV-induced cirrhosis (cirrhosis), and AH. Expression of PROM-1 (D), KRT7 (E), and EpCAM (F) in patients with short-term survival <90 days and >90 days. Gene expression values are shown as $2^{(-\Delta\Delta Ct)}$ value. Correlation of KRT7 expression value with Maddrey's discriminant function (G) and MELD (H).

that not necessarily correlates with the expansion of more immature progenitor cell markers. These results demonstrate that AH is characterized by an important ductular reaction and that the liver damage may be an important trigger of this event.

Factors Influencing Patient Survival: Role of Progenitor Cell Markers. *KRT7, PROM1*, and *EpCAM* were found to be up-regulated in patients with poor survival rates. Thus, we evaluated if these genes are good prognostic markers of mortality in patients with AH. The univariate analysis identified serum bilirubin levels, INR, serum creatinine levels, ABIC score, and MELD at admission and *KRT7* and *PROM-1* expression in liver tissue associated with 90-day mortality. However, age, *EpCAM*, and fibrosis quantification were not associated with mortality. In the multivariate regression analysis the ABIC score, MELD, *KRT7*, and *PROM-1* were the best independent predictors of 90-day mortality (Table 2).

In order to assess if LPC markers are surrogate markers for fibrosis and inflammation, we evaluated liver fibrosis and infiltrating polymorphonuclear cells and its association with LPC markers and mortality. As shown in Supporting Fig. 2, fibrosis stage had a low but positive correlation with *KRT7* and *EpCAM* expression. Moreover, morphometric quantification of

Table 2. Factors Influencing mortality in Univariate and Multivariate Logistic Regression in Patients with Alcoholic Hepatitis

Univariate Logistic Regression	OR	CI 95%	p
At admission			
Serum bilirubin	1.07	1.01-1.14	0.02
INR	1.98	1.11-3.53	0.03
Serum creatinine	2.19	0.86-5.58	0.09
Age	1.07	0.98-1.18	0.12
ABIC score	2.85	1.84-4.43	<0.001
MELD	1.18	1.09-1.27	<0.001
Fibrosis quantification	1.03	0,95-1,12	0,43
Gene expression			
KRT7	1.18	1.08-1.28	0.001
Prominin-1	1.17	1.08-1.27	0.001
EpCam	1.09	0.93-1.28	0.26
Multivariate Logistic Regression	**OR**	**CI 95%**	**p**
ABIC score	1.75	1.00-3.06	0.04
KRT7	1.14	1.04-1.24	0.004
ABIC score	1.94	1.20-3.12	0.006
Prominin-1	1.14	1.05-1.24	0.002
ABIC score	2.25	1.36-3.71	0.001
EpCam	1.16	0.98-1.36	0.06
MELD	1.19	1.01-1.41	0.03
KRT7	1.22	1.04-1.42	0.01
MELD	1.23	1.06-1.43	0.006
Prominin-1	1.14	0.99-1.30	0.06
MELD	1.21	1.05-1.39	0.006
EpCam	1.19	0.95-1.49	0.12

INR, international normalized ratio; KRT7, keratin 7; EpCam, epithelial cell adhesion molecule; ABIC, age, bilirubin, INR, creatinine score.

fibrosis also had a positive correlation with expression of *KRT7* ($r = 0.28$ $P = 0.048$) and *PROM-1* ($r = 0.3$ $P = 0.031$) but not *EpCAM* ($r = 0.21$ $P = 0.119$). However, fibrosis was not found to be an independent predictor of mortality with an area under the receiver operating curve (AUROC) of 0.59 (95% CI: 0.41-0.75) and was unable to stratify patients based on the outcome. The degree of inflammation did not positively correlate with LPC markers (Supporting Fig. 2) nor with mortality.

In order to assess the interactions among LPC markers and their direct association with short-term survival of patients with AH, we fitted a CART model. The best decision tree (Gini's index 0.35) was constructed with the interaction of two LPC markers, *KRT7* and *EpCAM* (Fig. 3). The generated tree identified three subpopulations with different short-term prognosis (expressed as $2^{(-\Delta\Delta CT)}$): a high-risk mortality group characterized with high *KRT7* expression (KRT7 $\geq$12.9), with a 90-day mortality of 89%; a group with an intermediate mortality risk (*KRT7* between 12.9 and 3.5 and *EpCAM* $\leq$2.95), with a 90-day mortality of 33%; and a low-risk mortality group (*KRT7* <12.9 and *EpCAM* >2.95) and (*EpCAM* $\leq$2.95 and *KRT7* $\leq$3.5) with a 90-day mortality of 0% and 10%, respectively. Importantly, this CART model showed good usefulness estimated by an AUROC of 0.73 (95% CI: 0.60-0.87).

Finally, we investigated if LPC markers provide additional discrimination potential within patients classified by the ABIC score as with intermediate mortality risk (ABIC score 6.71-8.99 or ABIC B) which show a mortality rate of 30%. The AUROC for 90-day mortality using *KRT7* was 0.76 (95% CI: 0.60-0.88) (Supporting Fig. 3). We chose a cutoff value of 6.4 with a sensitivity of 60% and a specificity of 83%.

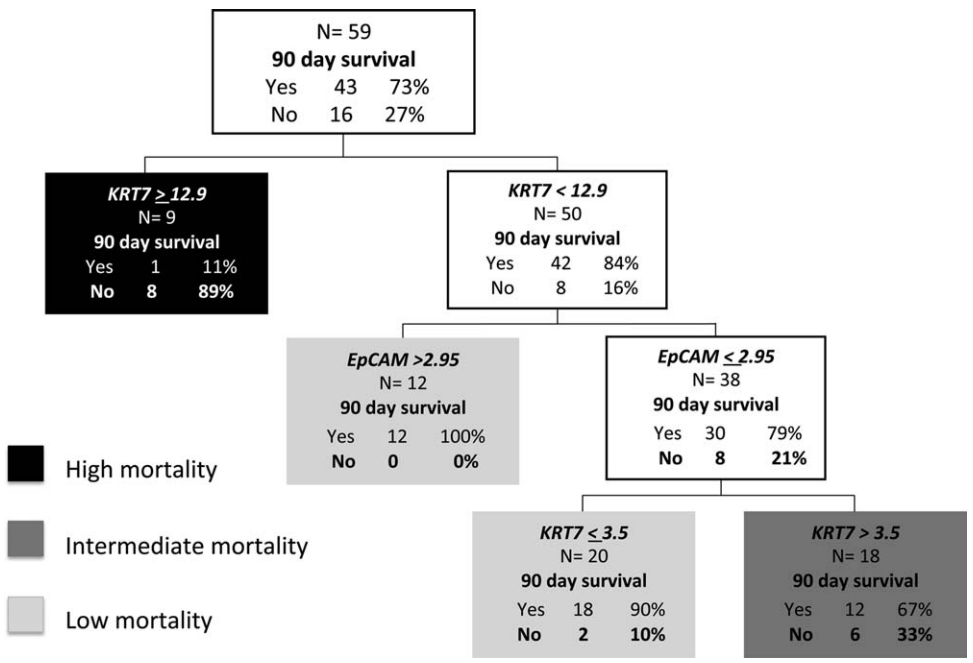
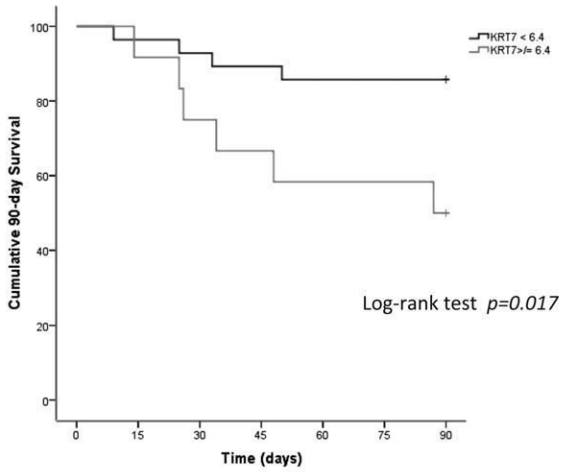


Fig. 3. Prognostic model of 90-day mortality in patients with AH generated by CART. The CART tree stratified three groups of patients with different mortality rates according to progenitor cell markers expression. Gene expression values are shown as $2^{(-\Delta\Delta Ct)}$ value. A high-risk group (15% of the patients with 89% mortality) with KRT7 $\geq$12.9 value, an intermediate risk group (30% of patients with 33% of mortality) with 3.5 $\leq$ KRT7 $\leq$12.9, and EpCAM $\leq$2.95 value, and a low-risk group (54% of patients with 6% of mortality) with KRT7 <12.9 and EpCAM >2.95 and a second group with EpCAM <2.95 and KRT7 $\leq$3.5.



At Risk n=40	day 0	day 15	day 30	day 45	day 60	day 75	day 90
Low KRT7	28	27	26	25	24	24	24
High KRT7	12	11	9	8	7	7	6

Fig. 4. Three-month survival probability of patients with moderate ABIC score according to hepatic gene expression of *Keratin7*. Kaplan-Meier curve showing the 3-month probability of survival in patients with alcoholic hepatitis and ABIC B score according to hepatic gene expression of *Keratin7 (KRT7)*.

Interestingly, using this cutoff value, we found that *KRT7* expression accurately discriminated patients with different short-term survival rates among the group of patients with an ABIC B (86% versus 50% survival in patients with KRT7 <6.4 and KRT7 ≥ 6.4, respectively; $P = 0.026$, Fig. 4).

Protein Expression of Progenitor Markers: Correlation Between Immunohistochemistry and Gene Expression. CART analysis identified *KRT7* and *EpCAM* expression as the most relevant variables to subdivide the population of patients with AH into subpopulations with different outcomes. To assess if real-time PCR gene expression correlated with protein expression and ductular reaction cells in AH samples, we performed an immunohistochemistry analysis of KRT7 and EpCAM. As described previously, we identified three main different cell populations positive for KRT7 or EpCAM according to their morphology and localization in the liver. To reflect this heterogeneity we developed a score (see Patients and Methods) to quantify the contribution of these different populations within the ductular reaction. As shown in Fig. 5, the KRT7 score positively correlated with *KRT7* gene expression ($r = 0.63$; $P = 0.003$) (Fig. 5g). The *EpCAM* score also showed a positive correlation with EpCAM gene expression ($r = 0.88$; $P < 0.001$) (Fig. 5h).

To evaluate if LPC markers reflect different progenitor cell populations or different differentiation states, we performed a dual staining for LPC markers together with a hepatocyte marker. As shown in Fig. 6 there is almost a complete overlay of EpCAM and KRT7 staining in cells of the ductular reaction, suggesting that both markers may be identifying the same cell population. Dual staining for EpCAM and hepatocyte marker has shown that cells with a membranous staining of EpCAM coexpress hepatocyte-specific mitochondrial antigen, suggesting that differentiation of EpCAM-positive cells occurs during an episode of AH. Likewise, KRT7-positive cells showing an immature hepatocyte morphology coexpressed hepatocyte markers.

Discussion

The present study describes the expression of progenitor cell markers in patients with AH and identifies them as prognostic markers predicting short-term mortality. Gene expression analysis showed an important up-regulation of progenitor cell markers in patients with AH. A further analysis of three well-known LPC markers, *KRT7, PROM1*, and *EpCAM*, in a cohort of patients with AH allowed us the possibility of stratifying patients into three groups according to their survival rate. Moreover, the combination of KRT7 expression with the ABIC score discriminated patients with intermediate ABIC values and poorly defined survival rates and stratified them according to mortality.

LPC proliferate during the course of chronic liver disease. This proliferation is particularly important in alcoholic liver disease, probably because AH causes profound hepatocellular damage and impairment of hepatocyte proliferation but also because alcohol triggers progenitor cell expansion.[24] However, little is known about the extent of progenitor cell proliferation and its role in acute-on-chronic liver failure. Our results are in accordance with previous reports describing an important ductular reaction in patients with alcoholic liver disease.[23,24] Here we show that a broad number of LPC markers are overexpressed in patients with AH, as assessed by microarray analysis. Moreover, we demonstrate that progenitor cell markers are overexpressed in patients with AH compared with HCV-induced cirrhosis, suggesting that acute-on-chronic injury may favor progenitor cell expansion. However, the group of patients with HCV-induced cirrhosis had a better liver function than the AH group, so it is plausible that LPC expansion is not a unique feature of AH. Further studies are required to determine if LPC proliferation is increased in AH compared with other etiologies of chronic liver disease.

The main innovative approach of this study is the use of CART as an alternative regression analysis

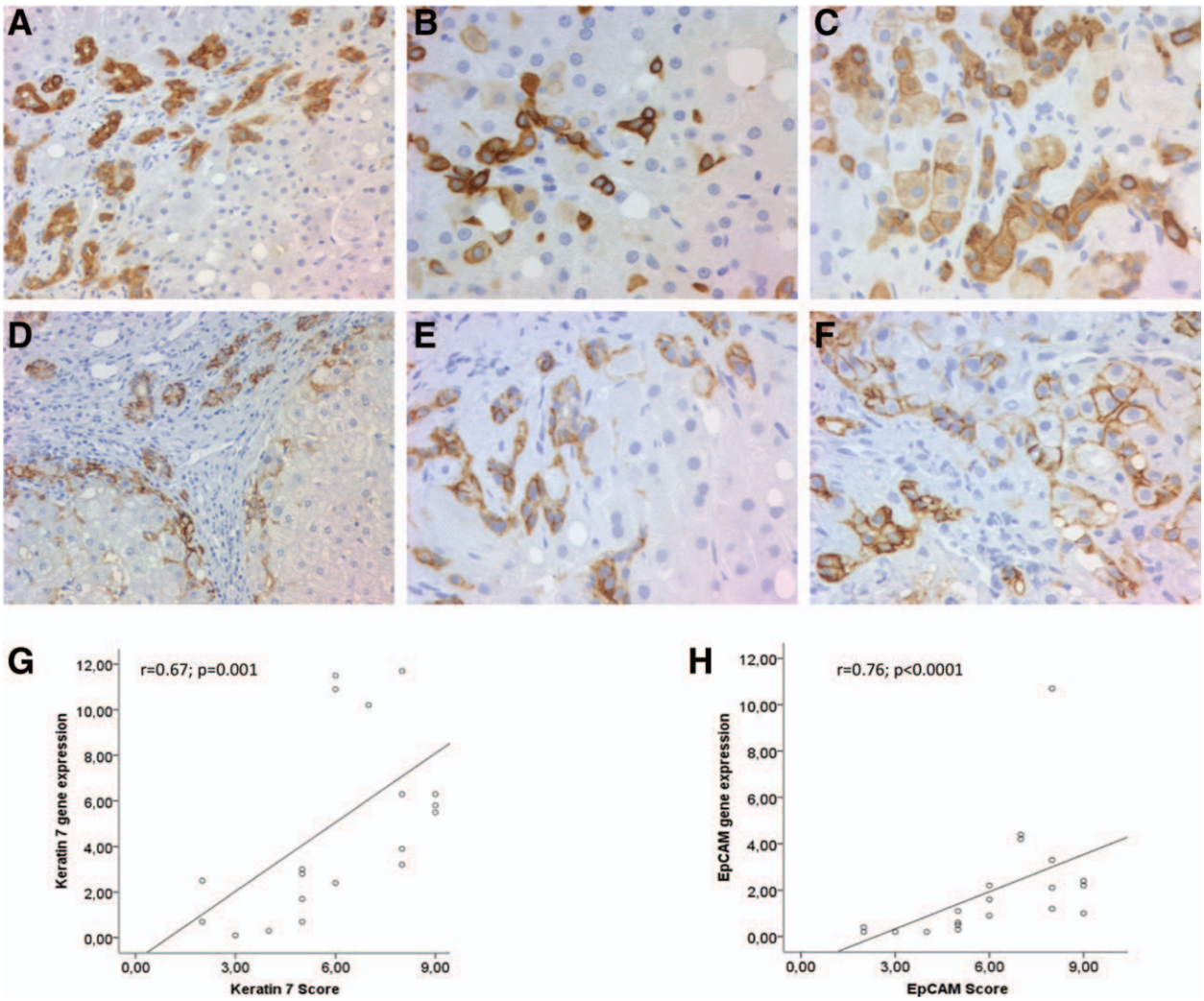


Fig. 5. Immunohistochemistry analysis of liver progenitor cell markers. Representative pictures of KRT7 staining (A-C) showing biliary structures (A) (magnification ×20), isolated cells within the parenchyma (B) (magnification ×40), intermediate hepatobiliary cells (C) (magnification ×40). Representative pictures of EpCAM staining (D-F) demonstrating the presence of ductular structures (D) (magnification ×20), cells at the interphase (E) (magnification ×40), hepatobiliary cells (F) (magnification ×40). Correlation of KRT7 expression value ($2^{(-\Delta\Delta Ct)}$) with KRT7 score (G). Correlation of EpCAM expression value ($2^{(-\Delta\Delta Ct)}$) with EpCAM score (H).

method to evaluate progenitor cells marker interactions as indicators of poor prognosis in patients with AH. The rationale behind the application of CART in this study was twofold. First, traditional logistic regression methods can be very useful in ranking the variables with statistical significance, but they contain modest information about the impact of the identified variables.[30] The elucidation of risk subgroups seems unwieldy with a traditional linear regression analysis and even if estimates for incidences are calculated for all combinations of parameters, the results still refer only to estimates. Conversely, the results of CART analysis come naturally as risk groups that are based on observed incidences and the impact of the identified parameters, providing a graphical representation in the form of a decision tree. Second, in contrast to logistic regression, CART considers not only the overall sample of patients, but also, in subsequent steps, relevant subgroups and is therefore better positioned to probe for interactions. Thus, the main benefit of CART is that it can accurately stratify prognostic subgroups based on simple combinations of variables given in semiautomatic driven software.

Several scoring systems have been developed for the prognosis assessment of patients with AH.[30-33] Those scores are based on noninvasive parameters and are reliable tools to estimate the severity and prognosis of these patients. However, it is unknown which mechanisms contribute to worsen the prognosis of patients with AH. Gene and protein expression of LPC markers are of limited applicability as prognostic score tools in clinical practice; thus, our aim was not to

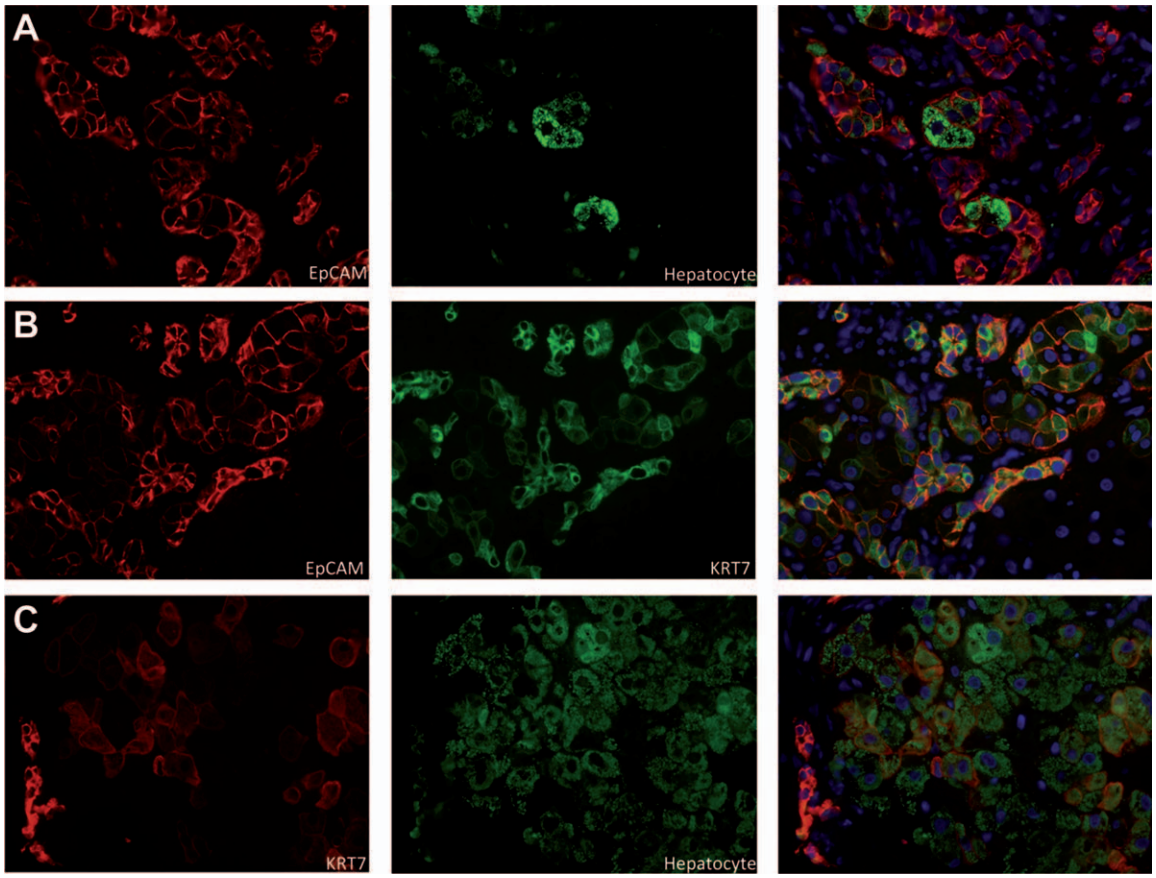


Fig. 6. Dual immunostaining of LPC markers. (A) Representative pictures of dual immunofluorescent staining of EpCAM, hepatocyte marker, and merge picture (magnification ×40). (B) Representative pictures of dual immunofluorescent staining of EpCAM, KRT7, and merge picture (magnification ×40). (C) Representative pictures of dual immunofluorescent staining of KRT7, hepatocyte marker, and merge picture (magnification ×40).

develop a clinically relevant prognostic score but to identify relevant pathophysiological mechanisms influencing the outcome in AH. We propose a simple CART model (Fig. 3) based on the combination of two LPC markers that show the importance of LPC expansion in AH and allows an accurate discrimination of three prognostic groups of patients with different mortality. First, a low-risk group with an excellent overall 90-day survival of 94%, for whom aggressive therapeutic measures may not be needed. The moderate expression of LPC markers observed in these patients probably reflects low inflammatory damage and moderate hepatocyte loss combined with an active regeneration process. Second, a high-risk group of patients with 89% mortality at 90 days. These patients with the highest KRT7 expression and poor prognosis have prominent inflammatory and necrotic damage with severe impairment of hepatocyte regeneration capacity. And third, patients with intermediate prognosis (33% mortality at 90 days) for which aggressive therapeutic approaches are required.

It is especially relevant that *KRT7* expression accurately predicts the 90-day mortality of patients with intermediate mortality as assessed by a moderate ABIC score. This finding may have potential clinical implications because the mortality of moderate ABIC is similar to that observed in the general AH patient population. By using *KRT7* expression we were able to stratify patients with an intermediate ABIC score in those having a low mortality rate (14%) and high mortality (50%). Future studies should assess KRT7 expression in a higher number of patients with moderate ABIC scores to validate these results. The possibility to identify those patients at higher risk may be useful for optimizing patient management and therapeutic decision-making. Moreover, these observations suggest that liver regeneration and LPC expansion may be an important factor influencing short-term prognosis in AH. Ductular reaction is frequently associated with fibrosis and inflammation in chronic liver disease and may be key factors promoting LPC expansion in AH. Although we show that fibrosis correlates with LPC

markers, fibrosis and inflammation are not independent predictors of bad prognosis, and for that reason are not adequate parameters to stratify AH patients according to mortality. It has been previously shown that fibrosis and inflammation trigger LPC expansion, but our results suggest that LPC markers would not be surrogate markers for fibrosis because KRT7, PROM-1, and EpCAM are independent prognostic factors of mortality in our cohort of patients.

The gene expression and immunochemistry results clearly demonstrate that there is an important expansion of KRT7-positive hepatobiliary cells in AH. It is assumed that LPC proliferate to overcome the impaired regenerative potential of a chronically damaged liver in order to restore normal liver function. Although our results clearly demonstrate an important LPC expansion, it does not lead to improved liver function, because patients with an increased number of immature hepatobiliary cells show the highest mortality rate. This observation raises the question of whether the presence of KRT7 cells correlates with more extensively damaged liver, therefore showing a higher mortality, or whether LPC proliferation has a detrimental impact on liver function. AH is a condition with a florid proinflammatory milieu, with expression of chemokines from CXC and CCL families, growth factors, and other mediators with unknown effects on LPC proliferation, differentiation, or three-dimensional organization. The possibility that in AH there is an induction of LPC proliferation but a deficient differentiation to mature hepatocytes deserves further investigation. In chronic liver disease EpCAM-positive hepatobiliary cells derive from LPC that give rise to new hepatocytes generated independently from the exhausted mature hepatocyte pool.[19] The presence of a number of EpCAM-positive hepatobiliary cells in patients with AH suggests that newly generated hepatocytes are being created. The results obtained with the dual staining of LPC markers suggest that differentiation of EpCAM- and KRT7-positive cells is not completely abrogated in AH. However, the major population of EpCAM-positive cells in samples from AH patients are small, immature cells, and only a small fraction of cells are intermediate hepatobiliary cells with an immature hepatocyte morphology. Unfortunately, the contribution of EpCAM- or KRT7-positive cells to the reconstitution of the liver parenchyma in liver regeneration and disease is not known, so from our results it is not possible to envision if the contribution of LPC may be sufficient to maintain liver function in AH patients. It is important to notice that, as observed in the CART analysis, high *EpCAM* expression in patients with intermediate *KRT7* expression identify a group of patients with a low mortality rate, suggesting a beneficial effect of EpCAM-positive cells on liver function. Whether EpCAM-positive cells contribute sufficiently to liver regeneration to maintain liver function in AH deserves further investigation.

In summary, we provide evidence that ductular reaction is a key event in AH and that LPC expansion parallels disease severity in this acute-on-chronic condition. The exploitation of a decision tree model as an alternative approach to classical regression models identified the most relevant LPC markers as prognostic markers in patients with AH. Our results suggest that LPC markers may provide additive value to clinical prognostic scores to discriminate patients with different mortality rates. This study shows a significant cellular response in AH and provides evidence for their utilization in a histological score. Moreover, the correlation of LPC markers with mortality suggest that understanding the mechanisms governing LPC proliferation and differentiation in liver disease may facilitate the design of new therapeutic approaches aimed at promoting liver regeneration in AH.

Acknowledgment: This work was performed at the Centre Esther Koplowitz. We thank Dr. Salvador Augustin for advice on statistical analysis.

References

1. Elphick DA, Dube AK, McFarlane E, Jones J, Gleeson D. Spectrum of liver histology in presumed decompensated alcoholic liver disease. Am J Gastroenterol 2007;102:780-788.
2. Teli MR, Day CP, Burt AD, Bennett MK, James OF. Determinants of progression to cirrhosis or fibrosis in pure alcoholic fatty liver. Lancet 1995;346:987-990.
3. Naveau S, Giraud V, Borotto E, Aubert A, Capron F, Chaput JC. Excess weight risk factor for alcoholic liver disease. HEPATOLOGY 1997; 25:108-111.
4. Lucey MR, Mathurin P, Morgan TR. Alcoholic hepatitis. N Engl J Med 2009;360:2758-2769.
5. Dominguez M, Rincon D, Abraldes JG, Miquel R, Colmenero J, Bellot P, et al. A new scoring system for prognostic stratification of patients with alcoholic hepatitis. Am J Gastroenterol 2008;103: 2747-2756.
6. Duncan AW, Dorrell C, Grompe M. Stem cells and liver regeneration. Gastroenterology 2009;137:466-481.
7. Zaret KS, Grompe M. Generation and regeneration of cells of the liver and pancreas. Science 2008;322:1490-1494.
8. Roskams T, Katoonizadeh A, Komuta M. Hepatic progenitor cells: an update. Clin Liver Dis 2010;14:705-718.
9. Eleazar JA, Memeo L, Jhang JS, Mansukhani MM, Chin S, Park SM, et al. Progenitor cell expansion: an important source of hepatocyte regeneration in chronic hepatitis. J Hepatol 2004;41:983-991.
10. Roskams TA, Theise ND, Balabaud C, Bhagat G, Bhathal PS, Bioulac-Sage P, et al. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. HEPATOLOGY 2004;39:1739-1745.

11. Turner R, Lozoya O, Wang Y, Cardinale V, Gaudio E, Alpini G, et al. Human hepatic stem cell and maturational liver lineage biology. HEPATOLOGY 2011;53:1035-1045.
12. Zhang L, Theise N, Chua M, Reid LM. The stem cell niche of human livers: symmetry between development and regeneration. HEPATOLOGY 2008;48:1598-1607.
13. Bateman AC, Hubscher SG. Cytokeratin expression as an aid to diagnosis in medical liver biopsies. Histopathology 2010;56:415-425.
14. Libbrecht L, Desmet V, Van Damme B, Roskams T. Deep intralobular extension of human hepatic 'progenitor cells' correlates with parenchymal inflammation in chronic viral hepatitis: can 'progenitor cells' migrate? J Pathol 2000;192:373-378.
15. Ma S, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, et al. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. Gastroenterology 2007;132:2542-2556.
16. Suzuki A, Sekiya S, Onishi M, Oshima N, Kiyonari H, Nakauchi H, et al. Flow cytometric isolation and clonal identification of self-renewing bipotent hepatic progenitor cells in adult mouse liver. HEPATOLOGY 2008;48:1964-1978.
17. Schmelzer E, Zhang L, Bruce A, Wauthier E, Ludlow J, Yao HL, et al. Human hepatic stem cells from fetal and postnatal donors. J Exp Med 2007;204:1973-1987.
18. Yamashita T, Ji J, Budhu A, Forgues M, Yang W, Wang HY, et al. EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. Gastroenterology 2009;136:1012-1024.
19. Yoon SM, Gerasimidou D, Kuwahara R, Hytiroglou P, Yoo JE, Park YN, et al. Epithelial cell adhesion molecule (EpCAM) marks hepatocytes newly derived from stem/progenitor cells in humans. HEPATOLOGY 2011;53:964-973.
20. Duret C, Gerbal-Chaloin S, Ramos J, Fabre JM, Jacquet E, Navarro F, et al. Isolation, characterization, and differentiation to hepatocyte-like cells of nonparenchymal epithelial cells from adult human liver. Stem Cells 2007;25:1779-1790.
21. Sancho-Bru P, Najimi M, Caruso M, Pawelyn K, Cantz T, Forbes SJ, et al. Stem and progenitor cells for liver repopulation: can we standardize the process from bench to bedside? Gut 2009;58:594-603.
22. Roskams TA, Libbrecht L, Desmet VJ. Progenitor cells in diseased human liver. Semin Liver Dis 2003;23:385-396.
23. Lowes KN, Brennan BA, Yeoh GC, Olynyk JK. Oval cell numbers in human chronic liver diseases are directly related to disease severity. Am J Pathol 1999;154:537-541.
24. Jung Y, Brown KD, Witek RP, Omenetti A, Yang L, Vandongen M, et al. Accumulation of hedgehog-responsive progenitors parallels alcoholic liver disease severity in mice and humans. Gastroenterology 2008;134:1532-1543.
25. Roskams T, Yang SQ, Koteish A, Durnez A, DeVos R, Huang X, et al. Oxidative stress and oval cell accumulation in mice and humans with alcoholic and nonalcoholic fatty liver disease. Am J Pathol 2003;163:1301-1311.
26. Ginès P, Lenz K, Møller S, Moore K, Moreau R, Merkel C, et al. EASL clinical practice guidelines on the management of ascites, spontaneous bacterial peritonitis, and hepatorenal syndrome in cirrhosis. J Hepatol 2010;53:397-417.
27. Garcia-Tsao G, Sanyal AJ, Grace ND, Carey W. Prevention and management of gastroesophageal varices and variceal hemorrhage in cirrhosis. HEPATOLOGY 2007;46:922-938.
28. Runyon BA. Management of adult patients with ascites due to cirrhosis: an update. HEPATOLOGY 2009;49:2087-2107.
29. Dominguez LJ, del Castillo AL, Miquel R, Colmenero J, Moreno M, Garcia-Pagán JC, et al. Identification of Fn14, a TNF receptor superfamily member, as a therapeutic target in patients with alcoholic hepatitis. HEPATOLOGY 2009;50:600A-705A.
30. Muller R, Mockel M. Logistic regression and CART in the analysis of multimarker studies. Clin Chim Acta 2008;394:1-6.
31. Dunn W, Jamil LH, Brown LS, Wiesner RH, Kim WR, Menon KV, et al. MELD accurately predicts mortality in patients with alcoholic hepatitis. HEPATOLOGY 2005;41:353-358.
32. Forrest EH, Evans CD, Stewart S, Phillips M, Oo YH, McAvoy NC, et al. Analysis of factors predictive of mortality in alcoholic hepatitis and derivation and validation of the Glasgow alcoholic hepatitis score. Gut 2005;54:1174-1179.
33. Maddrey WC, Boitnott JK, Bedine MS, Weber FL Jr, Mezey E, White RI Jr. Corticosteroid therapy of alcoholic hepatitis. Gastroenterology 1978;75:193-199.