GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Hepatic Stem/Progenitor Cell Activation Differs between Primary Sclerosing and Primary Biliary Cholangitis

Guido Carpino,^{*} Vincenzo Cardinale,[†] Trine Folseraas,[‡] Diletta Overi,[§] Annarosa Floreani,[¶] Antonio Franchitto,[§] Paolo Onori,[§] Nora Cazzagon,[¶] Pasquale B. Berloco,^{||} Tom H. Karlsen,^{‡**} Domenico Alvaro,[†] and Eugenio Gaudio[§]

From the Division of Health Sciences,^{*} Department of Movement, Human and Health Sciences, University of Rome "Foro Italico," Rome, Italy; the Department of Medico-Surgical Sciences and Biotechnologies,[†] Sapienza University of Rome, Latina, Italy; the Norwegian PSC Research Center,[‡] Division of Cancer, Surgery and Transplantation, Department of Transplantation Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway; the Departments of Anatomical, Histological, Forensic Medicine and Orthopedics Sciences[§] and General Surgery and Organ Transplantation,^{||} Sapienza University of Rome, Rome, Italy; the Department of Surgery, Oncology and Gastroenterology,[¶] University of Padua, Padua, Italy; and the Research Institute of Internal Medicine,^{**} Division of Surgery, Inflammatory Diseases and Transplantation, Oslo University Hospital Rikshospitalet and Institute of Clinical Medicine, University of Oslo, Oslo, Norway

Accepted for publication
November 16, 2017.

Address correspondence to
Guido Carpino, M.D., Ph.D.,
Division of Health Sciences,
Department of Movement,
Human and Health Sciences,
University of Rome "Foro Ita-
lico," Piazza Lauro De Bosis 6,
00135 Rome, Italy. E-mail:
guido.carpino@uniroma1.it.

Primary sclerosing cholangitis (PSC) and primary biliary cholangitis (PBC) are human primary cholangiopathies characterized by the damage of mature cholangiocytes and by the appearance of ductular reaction (DR) as the results of hepatic progenitor cell activation. This study evaluated the differences in progenitor cell niche activation between these two cholangiopathies. Liver tissue was obtained from healthy liver donors ($n = 5$) and from patients with PSC ($n = 20$) or PBC ($n = 20$). DR, progenitor cell phenotype, and signaling pathways were investigated by IHC analysis and immunofluorescence. Our results indicated that DR was more extended, appeared earlier, and had a higher proliferation index in PBC compared with PSC. In PBC, DR was strongly correlated with clinical prognostic scores. A higher percentage of sex determining region Y-box (SOX)9⁺ and cytokeratin 19⁺ cells but fewer features of hepatocyte fate characterized progenitor cell activation in PBC versus PSC. Lower levels of laminin and neurogenic locus notch homolog protein 1 but higher expression of wingless-related integration site (WNT) family pathway components characterize progenitor cell niche in PSC compared with PBC. In conclusion, progenitor cell activation differs between PSC and PBC and is characterized by a divergent fate commitment and different signaling pathway predominance. In PBC, DR represents a relevant histologic prognostic marker. (*Am J Pathol* 2018; 188: 627–639; <https://doi.org/10.1016/j.ajpath.2017.11.010>)

Primary sclerosing cholangitis (PSC) is a chronic cholangiopathy characterized by diffuse inflammation and fibrosis of the bile ducts.^{1,2} The progressive injury primarily involves the extrahepatic and large intrahepatic bile ducts, leading to concentric periductal fibrosis and obliterating strictures.^{1–3} The pathogenesis of PSC is still largely unknown, and the mechanisms involved in the characteristic bile duct strictures are poorly understood.^{4,5} Recently, the involvement of biliary tree stem/progenitor cells (BTSCs) was demonstrated as a determinant in the progression of duct lesions in PSC.³ BTSCs represent a stem/progenitor cell niche located within the glands of the biliary tree

(peribiliary glands).^{6–8} Massive BTSC proliferation and peribiliary gland hyperplasia were revealed in PSC but not in primary biliary cholangitis (PBC). Peribiliary gland

Supported by a research project grant from the Sapienza University of Rome, Fondo per gli Investimenti della Ricerca di Base (FIRB) grant RBAP10Z7FS_001, and Progetti di ricerca di Rilevante Interesse Nazionale (PRIN) grant 2009X84L84_001 (E.G.); FIRB grant RBAP10Z7FS_004, PRIN grant 2009X84L84_002, and a grant from Consorzio Interuniversitario Trapianti d'Organo (Rome, Italy; D.A.); a sponsored research agreement from Vesta Therapeutics (Bethesda, MD; E.G.); and a grant from the Norwegian Primary Sclerosing Cholangitis Research Center (Oslo, Norway; T.H.K.).

D.A. and E.G. contributed equally to this work.

Disclosures: None declared.

activation resulted in obliterative concentric fibrosis through the accumulation of myofibroblasts in the walls of the extrahepatic and large intrahepatic bile ducts in PSC.^{3,8}

Moreover, PSC and PBC share the appearance of ductular reaction (DR) within the liver parenchyma. DR is thought to represent the activation of the hepatic stem/progenitor cell (HpSC) niche that resides within the bile ductules.^{3,9} Interestingly, the contribution of HpSCs in human liver regeneration is variable based on disease pathogenesis.^{10,11} Different signaling pathways are involved in generating divergent responses due to the specific liver injury.^{11,12} In the HpSC niche, the neurogenic locus notch homolog protein (NOTCH) and wntless-related integration site (WNT) family pathways can be differently modulated based on disease etiology and stage.^{11,12} However, differences in HpSC response and DR phenotype between PSC and PBC have not been yet investigated.

Therefore, the aims of the present study were to compare PSC and PBC in terms of i) DR extent and HpSC activation in progressive disease stages, ii) DR phenotype and commitment, and iii) expression of signaling pathways by HpSCs.

Materials and Methods

Samples

Formalin-fixed and paraffin-embedded liver tissue from patients with PSC or PBC or from healthy livers was obtained from the Norwegian PSC Research Center, Oslo University Hospital Rikshospitalet (Oslo, Norway), from the Sapienza University of Rome (Rome, Italy), and from the University of Padua (Padua, Italy).

Specimens included i) livers with normal findings on histologic examination (controls) from liver donors ($n = 5$); ii) PSC ($n = 20$), obtained from liver biopsy samples and from explanted livers; and iii) PBC ($n = 20$), from liver biopsy samples and from explanted livers.

Informed consent was obtained from each patient, and the study protocol was conformed to the ethics guideline of the 1975 Declaration of Helsinki. The research protocol was reviewed and approved by the ethics committee at Umberto I Policlinico (Rome, Italy), and the regional committees for medical and health research ethics in southeastern Norway. No donor organs were obtained from executed prisoners or other institutionalized individuals.

Clinical Parameters

The diagnostic criteria for PSC and PBC followed the Clinical Practice Guidelines from the European Association for the Study of the Liver.¹³ Clinical and biochemical parameters were collected during biopsy or during transplantation, and, if appropriate, 1 year after ursodeoxycholic acid administration. Clinical parameters were collected to determine prognostic classes according to the Mayo Clinic's Revised Natural History Model for PSC and the Updated

Natural History Model for PBC, while the Global PBC¹⁴ and UK-PBC¹⁵ risk scores were used to stratify the risk for progression in PBC patients. Patients who presented with overlapping syndromes or concomitant liver diseases, comprising alcoholic steatohepatitis or nonalcoholic fatty liver disease, were excluded.

Light Microscopy, Histopathology, and IHC Analysis

Specimens were fixed in 10% buffered formalin for 2 to 4 hours and embedded in low-temperature–fusion paraffin (55°C to 57°C), and 3- to 4- μ m sections were stained with hematoxylin and eosin and Sirius Red/Fast Green, according to standard protocols. PSC and PBC samples were staged in accordance to the common histologic scoring systems^{16,17} and further categorized as early (not cirrhosis) or cirrhosis. The extent of fibrosis was evaluated in Sirius Red/Fast Green stains, as detailed previously.¹⁸ Briefly, Sirius Red/Fast Green–stained slides were scanned by a digital scanner (ScanScope CS System, Aperio Technologies, Inc., Oxford, UK) and processed by ImageScope software version 11.2 (Leica Biosystems, Milan, Italy). An image analysis algorithm was used to quantify the proportion of Sirius Red–stained area. The algorithm was applied on the entire section. The extent of collagen deposition was expressed as the proportion (%) of Sirius Red–stained area with respect to the total biopsy area, providing a quantitative value on a continuous scale.

For immunohistochemistry analysis, endogenous peroxidase activity was blocked by a 30-minute incubation in methanolic hydrogen peroxide (2.5%). Antigens were retrieved, as indicated by the vendor, by applying proteinase K (catalog number S3020; Dako Cytomation, Glostrup, Denmark) for 10 minutes at room temperature. Sections were then incubated overnight at 4°C with primary antibodies (Table 1). Samples were rinsed twice with phosphate-buffered saline for 5 minutes and incubated for 20 minutes at room temperature with secondary biotinylated antibody and then with streptavidin–horseradish peroxidase (LSAB + System-HRP; catalog number K0690; Dako Cytomation). Diaminobenzidine (Dako Cytomation) was used as a substrate, and sections were counterstained with hematoxylin.¹⁹ For immunofluorescence, nonspecific protein binding was blocked by 5% normal goat serum. Specimens were incubated with primary antibodies. Specimens were washed and incubated for 1 hour with labeled isotype-specific secondary antibodies (anti-mouse Alexa Fluor 546, anti-mouse Alexa Fluor 488, anti-rabbit Alexa Fluor 488, and anti-goat Alexa Fluor 546; Invitrogen, Life Technologies Ltd, Paisley, UK) and counterstained with DAPI for visualization of cell nuclei.²⁰ For all immunoreactions, negative controls (the primary antibody was replaced with preimmune serum) were also included. Sections were examined in a coded fashion by DM 4500 B Light and Fluorescence Microscopy (Leica Microsystems, Wetzlar, Germany), equipped with a Prog Res C10 Plus Videocam (Jenoptik, Jena, Germany). Immunofluorescence

Table 1 List of Antibodies Used in This Study

Name	Host	Supplier	Catalog no.	Dilution
CD326/EpCAM	Mouse	Dako Cytomation	M3525	1:100
Cytokeratin 7	Mouse	Dako Cytomation	M7018	1:100
Cytokeratin 19	Mouse	Dako Cytomation	M0888	1:100
Hep-Par1	Mouse	Dako Cytomation	M7158	1:100
Laminin	Rabbit	Dako Cytomation	Z0097	1:25
Notch1	Goat	Santa Cruz Biotechnology, Heidelberg, Germany	Sc-6014	1:50
p β -Catenin	Mouse	Cell Signaling Technology, Leiden, the Netherlands	#4176S	1:100
PCNA	Mouse	Dako Cytomation	M0879	1:100
SOX9	Rabbit	EMD Millipore, Darmstadt, Germany	AB5809	1:200
WNT1	Goat	Santa Cruz	Sc.6280	1:50
WNT3a	Mouse	Santa Cruz	Sc-136163	1:50

EpCAM, epithelial cell adhesion molecule; Hep-Par1, hepatocyte paraffin 1; Notch1, neurogenic locus notch homolog protein 1; p-, phosphorylated; PCNA, proliferating cell nuclear antigen; SOX9, sex determining region Y-box (SOX)9; WNT, wingless-related integration site family.

stains were also analyzed by confocal microscopy (model TCS-SP2; Leica Microsystems). Slides were further processed with Image Analysis System software version 7 (Delta Sistemi, Rome, Italy) and were independently evaluated by two researchers (G.C. and P.O.) in a blinded fashion.

The extent of DR was evaluated by cytokeratin (CK)-7 immunoreactivity. CK7-stained slides were scanned by a digital scanner (ScanScope CS System; Aperio Digital Pathology, Leica Biosystems) and processed by ImageScope software.^{21,22} The area occupied by CK7⁺ cells was quantified by an image analysis algorithm. The extent of DR was expressed as the percentage of the parenchymal area occupied by reactive ductules.^{21,22} Cholangiocytes lining the interlobular bile ducts were excluded from the counts. CK7-stained slides were further counterstained with Sirius Red for better visualization of the spatial relationship between DR and fibrosis.

With regard to DR phenotype, sex determining region Y-box (SOX)9 and epithelial cell adhesion molecule (EpCAM) were used as stem/progenitor cell markers; the coexpression of CK7 and CK19 was considered indicative of the maintenance of a biliary phenotype, and the expression of the hepatocyte marker hepatocyte paraffin 1 (Hep-Par1) and the loss of the biliary CK19 expression were considered as signs of hepatocyte commitment.^{11,23}

The DR proliferation index (PI) was automatically calculated as the percentage of DR cells expressing the proliferating cell nuclear antigen by specific algorithms.

For all other immunoreactions, the number of positive cells was automatically calculated by an algorithm on the entire section and, then, a semiquantitative scoring system was applied (0 = <1%; 1 = 1% to 5%; 2 = 6% to 30%; 3 = 31% to 50%; and 4 = >50%).³

Immunofluorescence stains were scanned with a digital scanner (ScanScope FL System; Aperio Technologies) and processed with ImageScope software. An algorithm was used to quantify the proportion of positive pixel area for a single fluorophore or the area with colocalization of two fluorophores.²⁴

Intermediate EpCAM⁺ Hepatocytes and Bud Definition

Buds were defined as hepatocytes, single or in clusters, immediately adjacent to cholangiocytes and located in fibrous septa (ie, in regions with parenchymal extinction). Buds were classified as levels 0 to 4. Buds of each maturation level (1 to 4) were counted on the CK19 slides.⁹ To quantitate the presence of apparent bud-derived tissue, we defined the *immaturity index* (IMI)-EpCAM as the presence of hepatocytes with EpCAM positivity.^{9,25} The presence of EpCAM⁺ hepatocytes was further confirmed by double immunofluorescence for EpCAM and Hep-Par1 and scored as: 0 = no positive cells; 1 (level 1) = single occasional; or 2 (level 2) = clusters of EpCAM⁺ hepatocytes.^{9,10} Analyses were independently performed by two different researchers (G.C. and P.O.) in a blinded fashion.

Statistical Analysis

Data are expressed as means $\pm$ SD. The *t*-test or Mann–Whitney *U*-test was used to determine the significance of differences between groups for normally or non-normally distributed data, respectively. The Pearson correlation coefficient or the Spearman nonparametric correlation was used. A *P* value of <0.05 was considered statistically significant. Analyses were performed using SPSS software version 23 (IBM, Milan, Italy).

Results

Clinical and Serologic Parameters

In PSC patients, ALP was similar between early-PSC and cirrhotic-PSC patients (Table 2). In contrast, serum bilirubin, serum albumin, and international normalized ratio were significantly altered in cirrhotic- compared with noncirrhotic-PSC patients. The cirrhotic-PSC patients

Table 2 Biochemical and Prognostic Parameters in Patients with Primary Sclerosing Cholangitis

Parameter	Early PSC (<i>n</i> = 10)	Cirrhotic PSC (<i>n</i> = 10)	<i>P</i> value
Age (y)	32.9 ± 5.5	57.13 ± 4.13	< 0.001
M/F, <i>n</i>	6/4	6/4	
Bilirubin (mg/dL)	0.95 ± 0.58	6.43 ± 5.57	< 0.05
Albumin (g/L)	44.5 ± 3.5	25.3 ± 4.1	< 0.001
AST (× ULN)	2.14 ± 0.63	3.79 ± 2.05	0.07
ALP (× ULN)	4.23 ± 2.11	1.99 ± 1.61	0.14
GGT (× ULN)	3.55 ± 1.99	2.73 ± 3.08	0.61
INR (0.8–1.2)	0.92 ± 0.04	1.9 ± 0.5	< 0.001
Mayo PSC risk	Low to intermediate	High	

Unless otherwise indicated, data are expressed as means ± SD. *P* values <0.05 are in bold.

F, female; M, male; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; INR, international normalized ratio; PSC, primary sclerosing cholangitis; ULN, upper limit of normal.

presented a high Mayo risk score, whereas noncirrhotic-PSC patients showed a low or intermediate score.

PBC patients were young adult and middle-aged women (Table 3). ALP was significantly increased in cirrhotic-PBC compared with early-PBC patients (*P* < 0.05), whereas none of the differences were significant in ALT, bilirubin, or albumin level. PBC patients, according to the Mayo Clinic's Updated Natural History Model for PBC, had an estimated probability of survival at 24 months of 99% to 100%. Liver biochemistry was evaluated 1 year after the start of ursodeoxycholic acid treatment for the calculation of the Global PBC and UK-PBC risk scores. Both the UK-PBC and Global PBC risk scores discriminated between early-PBC and cirrhotic-PBC patients (both, *P* < 0.05).

Ductular Reaction Extent and HpSC Proliferation Indexes

In livers with normal findings on histologic examination (controls), resident HpSCs can be recognized as CK7 (or

EpCAM) positive cells located in the smallest branches (canals of Hering and bile ductules) of the biliary tree at the interface with portal tracts (Figure 1A). In the noncirrhotic stages of PBC (stages I to III; early PBC) (Figure 1B), the presence of reactive ductules was revealed, and DR extent was increased (5.05 ± 0.61) compared with that in healthy livers (2.41 ± 1.67; *P* < 0.01). Cirrhotic-PBC samples (stage IV) (Figure 1, C and D) were characterized by a greater DR extent (11.90 ± 1.40) compared with those in early PBC (*P* < 0.01) and controls (*P* < 0.001). DR extent was strongly correlated with PBC stage (*r* = 0.83, *P* < 0.001).

In PSC (Figure 1, B and D), DR extent was slightly, but not significantly, increased in early-PSC biopsy samples (3.16 ± 1.15) compared with livers with normal findings on histologic examination (*P* = 0.28). Cirrhotic-PSC samples showed a greater DR extent (7.01 ± 1.96) compared with those in early-PSC stages (*P* < 0.05) and controls (*P* < 0.05). DR extent was significantly less in PSC compared with PBC samples (*P* < 0.01).

Table 3 Biochemical and Prognostic Parameters in Patients with Primary Biliary Cholangitis

Parameter	Early PBC (<i>n</i> = 10)	Cirrhotic PBC (<i>n</i> = 10)	<i>P</i> value
Age (y)	46.33 ± 11.5	46.0 ± 8.54	0.96
M/F, <i>n</i>	0/10	0/10	
Bilirubin (mg/dL)	0.63 ± 0.29	1.43 ± 1.22	0.14
Albumin (g/L)	40.3 ± 4.3	42.4 ± 4.3	0.5
AST (× ULN)	0.89 ± 0.5	1.72 ± 2.04	0.39
ALP (× ULN)	2.11 ± 1.94	5.62 ± 2.48	< 0.05
GGT (× ULN)	3.52 ± 3.54	14.83 ± 5.98	< 0.01
INR (0.8–1.2)	0.92 ± 0.04	1.9 ± 0.5	< 0.001
UK risk score, 5 years (%)	1.73 ± 1.25	9.69 ± 9.12	< 0.05
UK risk score, 15 years (%)	7.05 ± 4.3	29.47 ± 19.32	< 0.05
Global PBC score, 3 years (%)	95.93 ± 1.2	90.9 ± 3.24	< 0.01
Global PBC score, 5 years (%)	92.85 ± 2.13	84.3 ± 5.4	< 0.01
PBC score, 10 years (%)	82.47 ± 7.72	66.87 ± 8.61	< 0.01
Mayo PBC risk, 24 months (%)	99–100	99–100	

Unless otherwise indicated, data are expressed as means ± SD. *P* values <0.05 are in bold.

F, female; M, male; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; INR, international normalized ratio; PBC, primary biliary cholangitis; ULN, upper limit of normal.

Figure 1 Ductular reaction (DR) in human cholangiopathies. **A–C:** Immunohistochemistry(IHC) analysis for cytokeratin (CK)-7 in controls, primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). **A:** In control livers, CK7 immunoreaction individuates canals of Hering and bile ductules. The boxed area in the left panel corresponds with the magnified view of a canal of Hering in the right panel. **B:** In the early stages of PBC and PSC, the presence of DR is revealed. **C:** In cirrhotic samples, DR extent is greater compared with that in early stages. **D:** Differences in DR extent among groups. **E:** IHC analysis for CK7 was counterstained with Sirius Red for the visualization of the association between DR and fibrosis extent. **F:** Scatterplot showing correlation between DR and fibrosis in PBC and PSC. Data are expressed as means $\pm$ SD (**D**). * $P < 0.05$ versus control; † $P < 0.05$ versus early PSC; ‡ $P < 0.05$ versus early stages (PBC and PSC); § $P < 0.05$ versus cirrhotic PSC. Scale bars: 100 μ m (**A–C**); 200 μ m (**E**). Original magnification, $\times 40$ (**A**, right panel)

In both PBC and PSC samples, DR extent was significantly correlated with fibrosis extent (PBC: $r = 0.63$, $P < 0.01$; PSC: $r = 0.62$, $P < 0.01$) (Figure 1, E and F).

When the PI of cells within reactive ductules (DR-PI) was calculated (Figure 2), the DR-PI in cirrhotic PBC (38.4 ± 6.2) was higher in comparison with controls (5.8 ± 2.9 ; $P < 0.01$) and early-PBC samples (18.0 ± 2.1 ; $P < 0.01$). The DR-PI in early PBC was significantly higher compared with that in control specimens ($P < 0.05$). The DR-PI in cirrhotic PSC (24.4 ± 3.8) was significantly higher in comparison with those in control ($P < 0.001$) and early-PSC samples (10.0 ± 3.7 ; $P < 0.001$), and in early-PSC samples compared with normal specimens ($P < 0.05$). The DR-PI was lower in PSC compared with PBC samples ($P < 0.001$).

With regard to clinical parameters, DR in PSC samples was correlated with the Mayo PSC risk score ($r = 0.827$, $P < 0.05$) (Supplemental Figure S1A) and with bilirubin ($r = 0.841$, $P < 0.05$) and was inversely correlated with serum albumin ($r = -0.838$, $P < 0.05$). No correlation with ALP serum levels was evident in PSC patients. In PBC samples, DR was correlated with total bilirubin ($r = 0.937$,

$P = 0.001$); γ -glutamyl transferase ($r = 0.736$, $P = 0.037$); and UK-PBC risk scores up to 5 years ($r = 0.785$, $P < 0.001$), 10 years ($r = 0.820$, $P < 0.001$), and 15 years ($r = 0.847$, $P = 0.001$) (Supplemental Figure S1B) and Global PBC score ($r = 0.791$, $P = 0.019$) and Global PBC score orthotopic liver transplantation-free survival at 3 years ($r = -0.852$, $P = 0.007$), 5 years ($r = -0.850$, $P = 0.008$), and 10 years ($r = -0.833$, $P = 0.010$) (Supplemental Figure S1B). No correlation was found with the History Model for PBC from the Mayo Clinic.

Phenotype of Reactive Ductules

In control specimens with normal findings on histologic examination (Figure 3), double immunofluorescence showed that the majority of CK7⁺ cells lining the canals of Hering and the bile ductules coexpressed SOX9 (score = 3.6 ± 0.5) (Figure 3A) and CK19 (score = 4 ± 0) (Figure 3B). In cirrhotic PBC, double immunofluorescence showed that reactive ductules were mainly located within fibrous septa and that the majority of CK7⁺ cells were SOX9⁺ (score = 3.8 ± 0.4) (Figure 3A) and CK19⁺

Figure 2 Ductular reaction (DR) proliferation index (PI) in human cholangiopathies. **A:** Immunohistochemistry (IHC) analysis for proliferating cell nuclear antigen (PCNA) in control livers, primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). PCNA⁺ cells within DR are more numerous (arrows) in PBC and PSC compared with controls. PCNA expression is higher in PBC compared with PSC samples and in cirrhotic compared with early stages. **B:** Immunofluorescence for cytokeratin (CK)-7 and PCNA in late stages of PBC and PSC samples. Nuclei are displayed in blue. Immunofluorescence confirms the higher PCNA expression in CK7⁺ DR in PBC versus PSC samples (arrows). **C:** Differences in DR-PI among groups. Data are expressed as means ± SD (C). **P* < 0.05 versus control; †*P* < 0.05 versus early PSC; ‡*P* < 0.05 versus early stages (PBC and PSC); §*P* < 0.05 versus cirrhotic PSC. Scale bars: 100 μm (A); 25 μm (B).

(score = 3.7 ± 0.5) (Figure 3B). There were no or very few cells within the bile and reactive ductules that were Hep-Par1⁺ (score = 0; ie, <5%) in control and PBC samples (Figure 3C). Conversely, in cirrhotic PSC, reactive ductules were located at the interface with the fibrotic septa, and numerous cells penetrated deep into the cirrhotic nodules; variable percentages of CK7⁺ DR cells were positive for SOX9 (score = 1.2 ± 0.4) (Figure 3A) and CK19 (score = 1.65 ± 0.5) (Figure 3B), with lower values compared with controls and cirrhotic-PBC samples (both, *P* < 0.01). Interestingly, reactive ductules contained Hep-Par1⁺ cells in the cirrhotic-PSC samples (score = 1 ± 0.8) (Figure 3C) but not in the early-PSC samples (data not shown).

Hepatocyte buds (Figure 4A) were not present in controls and were more numerous in cirrhotic PSC compared with cirrhotic PBC (11.4 ± 3.7 versus 5.4 ± 3.4 ; *P* < 0.05). The immaturity index, calculated as the presence of EpCAM⁺/Hep-Par1⁺ newly derived hepatocytes (Figure 4, B and C), was higher in cirrhotic-PSC samples compared with cirrhotic-PBC samples (1.8 ± 0.5 versus 0.6 ± 0.6 ;

P < 0.01). No EpCAM⁺ hepatocytes were present in control livers (Figure 4, B and C). Few EpCAM⁺ hepatocytes were present in early stages of PSC and PBC (data not shown).

Signaling Pathways in the HpSC Niche: Expression of Laminin, NOTCH, and WNT Pathways

The presence of laminin around the reactive ductules and the expression of the NOTCH and WNT pathways by DR cells were evaluated by immunohistochemistry analysis on serial sections and confirmed by double immunofluorescence (Figures 5 and 6).

In cirrhotic PBC, the percentage of DR elements surrounded by laminin was higher (score = 2.8 ± 0.5) compared with those in the control (score = 0.5 ± 0.1) and cirrhotic-PSC samples (score = 0.6 ± 0.4) (Figure 5, A and B). NOTCH1 expression by DR was increased in cirrhotic-PBC samples (score = 3.6 ± 0.5) and in cirrhotic-PSC samples (score = 2.8 ± 0.4) compared with controls (0.5 ± 0.1) (*P* < 0.05) (Figure 5, C and D); cirrhotic-PBC

Figure 3 Phenotype of reactive ductules in human cholangiopathies. **A:** Immunofluorescence for cytokeratin (CK)-7 and transcription factor sex determining region Y-box (SOX)9 in control livers, primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). In cirrhotic PSC, ductular reaction (DR) is composed of a higher percentage of CK7⁺/SOX9[−] (blue arrows) compared with controls and cirrhotic PBC. Red arrows indicate CK7⁺/SOX9⁺ cells. The graph on the right summarizes differences in SOX9 positivity among groups. **B:** Immunofluorescence for CK7 and CK19. In cirrhotic PSC, a higher percentage of DR cells are CK19[−] compared with controls and cirrhotic PBC. Green arrows indicate CK7⁺/CK19[−] DR, and yellow arrows indicate CK7⁺/CK19⁺ DR. The graph on the right summarizes differences in CK19 positivity among groups. **C:** IHC analysis for hepatocyte paraffin 1 (Hep-Par1). Reactive ductules contain Hep-Par1⁺ cells (arrows) in cirrhotic PSC, but not in controls or cirrhotic PBC. Arrowheads indicate negative DR cells. The graph on the right summarizes differences in Hep-Par1 positivity among groups. Data are expressed as means ± SD. **P* < 0.05 versus other groups. Scale bars = 50 μm.

samples showed significantly higher values with respect to those in cirrhotic PSC (*P* < 0.05). The study of the WNT pathway revealed that the expression of WNT1 (Figure 6, A and B), WNT3a (Figure 6, C and D), and phosphorylated β-catenin (Figure 6, E and F) were higher in cirrhotic-PSC samples (2.8 ± 0.9 , 2.0 ± 1.0 , and 3.3 ± 1.0 , respectively; all, *P* < 0.05) and in cirrhotic-PBC samples [0.6 ± 0.5 (*P* < 0.01); 0.2 ± 0.5 (*P* < 0.01); and 1.5 ± 1.0 (*P* < 0.05)] compared with controls (0, 0, and 0.5 ± 0.1); cirrhotic-PSC samples showed significantly higher values compared with cirrhotic PBC (all, *P* < 0.05). Results are visually summarized by the heat map in Figure 6G.

Discussion

The main results of the present study indicate that i) DR in PBC is more expansive, appears earlier, and has a higher PI compared with PSC; ii) DR extent correlates with fibrosis

and clinical prognostic risk scores both in PSC and PBC patients; iii) the phenotype of reactive ductules differs between PSC and PBC, with a predominant biliary phenotype in PBC and prominent signs of hepatocyte commitment in PSC; and iv) the observed differences in fate commitment were associated with signaling pathways in HpSC niche, as characterized by lower levels of laminin and NOTCH1 and higher WNT/β-catenin pathway expression in PSC compared with PBC.

DR is composed of anastomosing strands of tortuous ductules, and its presence is thought to be the result of HpSC activation and proliferation.^{26,27} HpSCs are facultative stem cells that reside in the human liver and can differentiate toward hepatocytic or cholangiocytic lineages.^{28–30} Under normal conditions, they are mostly quiescent cells and do not take part in the renewal of liver parenchyma because mature hepatocytes and cholangiocytes have high proliferative capabilities.^{28–30} In chronic biliary diseases, a variety of insults induce the apoptosis of

Figure 4 Features of hepatocyte commitment in hepatic stem/progenitor cell (HpSC) compartment in human cholangiopathies. **A:** Immunohistochemistry (IHC) analysis for cytokeratin (CK)-19 in control (CTR) livers, primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). The evaluation of hepatocyte buds indicated a higher prevalence in cirrhotic PSC (arrows) compared with controls and cirrhotic PBC. The graph on the right summarizes differences in hepatocyte bud number among groups. **B:** IHC analysis for epithelial cell adhesion molecule (EpCAM). EpCAM⁺ hepatocytes are rarely present in normal and cirrhotic-PBC samples, whereas clusters of EpCAM⁺ hepatocytes are frequently observed in cirrhotic-PSC samples (arrows). The graph on the right summarizes differences in EpCAM⁺ hepatocytes (Heps) among groups. **C:** Immunofluorescence for EpCAM and hepatocyte paraffin 1 (Hep-Par1). Nuclei are displayed in blue. Immunofluorescence confirms the presence of clusters of EpCAM⁺/Hep-Par1⁺ hepatocytes in PSC (yellow asterisks) but not in PBC (white asterisks). Dashed lines individuate the interface between the portal tract (PT) and the liver parenchyma. Data are expressed as means $\pm$ SD (A and B). * $P < 0.05$ versus other groups. Scale bars: 50 μ m (A and B); 100 μ m (C).

cholangiocytes and determine the impairment of cholangiocyte replicative capabilities^{4,19,31}; thus, the secondary proliferative pathway of HpSCs is activated.⁸ Accordingly, a prominent DR has been described in chronic biliary diseases.^{11,32} However, our results indicate that the presence, extent, and phenotype of DR significantly differs between PBC and PSC. In PBC, a prominent DR appeared at an early stage, and its extent was correlated with the disease stage; contrarily, in PSC, DR did not correlate with the histologic stage and was less prominent in comparison with PBC. Accordingly, the HpSC PI was higher in samples obtained from PBC compared with PSC patients matched for the same histologic stage.

Interestingly, DR extent was correlated with fibrosis extent in both PBC and PSC samples; DR is considered a main driver of fibrogenesis,³³ and a strict correlation with fibrosis severity has been described in several chronic

hepatic diseases, thus individuating patients with a worse prognosis and an increased risk for mortality.^{34,35} The activation of HpSCs is supported by a peculiar niche composed of hepatic stellate cells, macrophages, and defined extracellular matrix compounds.^{12,33,36,37} Proliferating HpSC can trigger profibrogenetic pathways acting on hepatic stellate cells and macrophage activation states.³⁶ In this context, our results suggest that DR extent is associated with fibrosis extent in both PBC and PSC; thus, the degree of HpSC activation, more than its phenotype, may influence the progression to cirrhosis and to a more aggressive course of the disease.

In PBC, histologic staging according to Ludwig predicts clinical evolution.^{38,39} Interestingly, our data indicate that DR extent strongly correlates with prognostic clinical scores predicting PBC outcome and ursodeoxycholic acid response (ie, Global PBC and UK-PBC risk scores); thus, the

Figure 5 Laminin and neurogenic locus notch homolog protein 1 (NOTCH1) expression in human cholangiopathies. **A:** Immunohistochemistry (IHC) analysis for laminin in controls, primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). In cirrhotic PBC but not in controls and cirrhotic PSC, ductular reaction (DR) is surrounded by a laminin-rich matrix (arrows). Vessels represent positive controls (arrowheads). **B:** Immunofluorescence for cytokeratin (CK)-7 and laminin shows laminin (arrowheads) around DR (arrows) in PBC. Nuclei are displayed in blue. **C:** IHC analysis for NOTCH1. NOTCH1⁺ DR cells (arrows) are more numerous in cirrhotic PBC compared with controls and cirrhotic PSC. **D:** Immunofluorescence for NOTCH1 and sex determining region Y-box (SOX)9 confirms high NOTCH1 (green arrows) expression in SOX9⁺ (red arrows) cells in cirrhotic PBC. Nuclei are displayed in blue. Scale bars: 100 μm (A and C); 50 μm (B and D).

evaluation of DR extent in liver biopsy may represent an additional important histologic prognostic marker.

In PSC, the Ludwig and other histologic staging systems are considered clinically relevant, given their association with transplant-free survival and time to liver transplantation.⁴⁰ However, although considered a progressive cholangiopathy, spontaneous stage regression evaluated by applying the Ludwig scoring system on paired liver biopsies has been observed in a proportion (nearly 15%) of PSC patients.³⁹ This aspect accounts for the debated utility of histologic evaluation as a reliable end point in clinical trials in PSC.⁴¹ Although DR extent was correlated with the Mayo risk score, our observations exclude a possible clinical utility in patients with early histologic lesions and in patients with low Mayo risk scores.

Besides DR extent, HpSC phenotype differed between PSC and PBC; a stem/biliary (CK19⁺/SOX9⁺/Hep-Par1⁻) phenotype was prominent in cirrhotic PBC, with few signs of hepatocyte commitment. Conversely, the loss of CK19/SOX9 expression within DR, the Hep-Par1 positivity in reactive ductules, the presence of numerous hepatocyte buds, and EpCAM⁺ hepatocytes indicated that signs of hepatocyte commitment were frequently observable in cirrhotic PSC. Interestingly, the proliferation of reactive ductules preceded the appearance of Hep-Par1⁺ DR cells

and the accumulation of EpCAM⁺ hepatocytes. Taken together, our results indicate a different pattern underlying HpSC activation in PBC and PSC. In PBC, the HpSC compartment is activated early, highly proliferative, and shows signs of an undifferentiated phenotype or biliary fate commitment.^{34,42} In PSC, HpSCs are activated later, have a low proliferation rate, and show signs of hepatocyte commitment.⁹

The different patterns of HpSC activation putatively reflect differences in the primary target of damage.¹ PBC is characterized by an autoimmune attack targeting the cholangiocytes lining interlobular bile ducts and leading to their progressive loss.⁴³ Initially, this loss is compensated by the proliferation of residual mature cholangiocytes⁴⁴; then, cholangiocyte proliferative capabilities are impaired and cholangiocyte apoptosis predominates.^{19,43} Thus, HpSCs proliferate to compensate for this progressive loss.¹¹ Conversely, PSC mainly affects septal and area intrahepatic bile ducts and the extrahepatic biliary tree.^{1,4} As recently demonstrated, the primary damage to these portions of the biliary tree triggers the proliferation of BTSCs located within peribiliary glands.³ PSC lesions progress to concentric periductal fibrosis with subsequent duct stenosis (strictures), which may determine the development of secondary intrahepatic cholestasis. In this context, the late activation of the

Figure 6 Wingless-related integration site family (WNT)/ β -catenin expression in human cholangiopathies. **A:** Immunohistochemistry (IHC) analysis for WNT1 in primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC). WNT1⁺ (arrows) cells in ductular reaction (DR) are more numerous in cirrhotic PSC compared with cirrhotic PBC. **B:** Immunofluorescence for WNT1 and cytokeratin (CK)-7 confirms WNT1 (red arrows) expression by CK7⁺ DR (yellow arrows) in cirrhotic PSC. Nuclei (NU) are displayed in blue. **C and D:** Immunofluorescence for WNT3a and sex determining region Y-box (SOX)9 shows fewer WNT3a⁺/SOX9⁺ DR cells (arrows) in cirrhotic PBC compared with cirrhotic PSC. Nuclei are displayed in blue. **E:** IHC analysis for phosphorylated (p) β -catenin shows higher expression (arrows) by DR in cirrhotic PSC compared with cirrhotic PBC. **F:** Immunofluorescence for p β -catenin and CK7 confirms the cytoplasmic/nuclear p β -catenin expression by CK7⁺ DR (arrows) in cirrhotic PSC. Nuclei are displayed in blue. **G:** The heat map shows differences in IHC analysis expression of laminin, NOTCH1, WNT1, WNT3a, and p β -catenin by DR. Color range refers to the used semiquantitative scoring system, as follows: 0 = <1%; 1 = 1% to 5%; 2 = 6% to 30%; 3 = 31% to 50%; and 4 = >50%. Data are expressed as means $\pm$ SD in each cell (**G**). **P* < 0.05 versus control; [†]*P* < 0.05 versus PSC; ^{††}*P* < 0.05 versus PBC. Scale bars: 100 μ m (**A**, **C**–**E**); 50 μ m (**B** and **F**).

HpSC compartment seems to indicate a secondary response of the hepatic parenchyma to cholestasis more than a primary reaction to the biliary damage; this corresponds with the signs of HpSC commitment toward hepatocytic lineage, tentatively supporting hepatocyte regeneration. Accordingly, with regard to biochemical serum markers of liver damage, DR was correlated with markers of cholestasis both in PSC and PBC, but it was correlated with a reduction in albumin levels only in PSC patients.

The expression of the components of the NOTCH- and WNT-signaling pathways within the HpSC compartment has been studied. The activation of NOTCH and WNT pathways sustains HpSC proliferation.^{12,36} In particular,

NOTCH signaling is implicated in the commitment toward cholangiocyte fate, whereas WNT can trigger differentiation toward the hepatocyte lineage.^{12,36} Furthermore, a crucial element for the HpSC response is represented by extracellular matrix composition, in which the degradation of the collagen matrix, coupled with production of a laminin-rich niche, leads to HpSC expansion and inhibits hepatocyte differentiation.^{34,37} Our results indicate that DR cells in PSC were surrounded by a niche with low laminin content and with higher expression of WNT pathway elements compared with PBC samples, corresponding to the presence of hepatocyte commitment signs in PSC.³⁷

The different activation of HpSC and BTSC niches in PBC and PSC may also have implications in the risk for primary liver cancers. PSC, but not PBC, is an established risk factor for cholangiocarcinoma.^{1,45} The association between PSC and cholangiocarcinoma may be due to the activation of the BTSC niche, which takes place in PSC but not in PBC.³ In PSC, large intrahepatic bile ducts and the extrahepatic bile duct lesions are characterized by BTSC proliferation, metaplasia, and dysplasia.²⁸ On the other hand, PBC patients have an increased risk for hepatocellular carcinoma occurrence, and this risk has been associated with advanced histologic stage and nonresponse to ursodeoxycholic acid treatment.^{46,47} This association seems to be in accordance with the prominent DR observed in cirrhotic-PBC patients, the correlation with prognostic clinical scores, and the assumed role of HpSCs in hepatocellular carcinogenesis.^{48,49} Moreover, the activation of signaling pathways within the niche is in agreement with the well-established role of the NOTCH and WNT deregulation in liver tumor development.^{50–52}

In conclusion, our results indicate a different pattern of HpSC activation in the context of human primary cholangiopathies, which reflects the involvement of different portions of the biliary tree as the primary target of damage. These aspects may have implications in the pathogenesis of different cholangiopathies. Interestingly, the evaluation of DR in biopsy samples may individuate patients with progressive fibrosis and could have prognostic value, especially in PBC. Future studies may reveal the role of the HpSC-activation patterns in furnishing valuable information in the clinical management of cholestatic diseases.

Acknowledgments

We thank Melissa Kerr for proofreading the manuscript.

G.C. conceived, designed, and performed experiments, analyzed data, and wrote the manuscript; V.C. and D.O. analyzed data, performed experiments, and wrote the manuscript; T.H.K., T.F., A.Fr., N.C., P.O., P.B.B., and A.Fl. acquired data and wrote the manuscript; and D.A. and E.G. conceived and designed experiments, interpreted data, provided financial support, and edited the manuscript. G.C. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.11.010>.

References

- Lazaridis KN, LaRusso NF: Primary sclerosing cholangitis. *N Engl J Med* 2016, 375:1161–1170
- Hirschfield GM, Karlsen TH, Lindor KD, Adams DH: Primary sclerosing cholangitis. *Lancet* 2013, 382:1587–1599
- Carpino G, Cardinale V, Renzi A, Hov JR, Berloco PB, Rossi M, Karlsen TH, Alvaro D, Gaudio E: Activation of biliary tree stem cells within peribiliary glands in primary sclerosing cholangitis. *J Hepatol* 2015, 63:1220–1228
- Tabibian JH, O'Hara SP, Splinter PL, Trussone CE, LaRusso NF: Cholangiocyte senescence by way of N-ras activation is a characteristic of primary sclerosing cholangitis. *Hepatology* 2014, 59:2263–2275
- Fickert P, Pollheimer MJ, Beuers U, Lackner C, Hirschfield G, Housset C, Keitel V, Schramm C, Marschall HU, Karlsen TH, Melum E, Kaser A, Eksteen B, Strazzabosco M, Manns M, Trauner M: Characterization of animal models for primary sclerosing cholangitis (PSC). *J Hepatol* 2014, 60:1290–1303
- Cardinale V, Wang Y, Gaudio E, Carpino G, Mendel G, Alpini G, Reid LM, Alvaro D: The biliary tree: a reservoir of multipotent stem cells. *Nat Rev Gastroenterol Hepatol* 2012, 9:231–240
- Carpino G, Cardinale V, Onori P, Franchitto A, Berloco PB, Rossi M, Wang Y, Semeraro R, Anceschi M, Brunelli R, Alvaro D, Reid LM, Gaudio E: Biliary tree stem/progenitor cells in glands of extrahepatic and intrahepatic bile ducts: an anatomical in situ study yielding evidence of maturational lineages. *J Anat* 2012, 220:186–199
- Lanzoni G, Cardinale V, Carpino G: The hepatic, biliary, and pancreatic network of stem/progenitor cell niches in humans: a new reference frame for disease and regeneration. *Hepatology* 2016, 64:277–286
- Stueck AE, Wanless IR: Hepatocyte buds derived from progenitor cells repopulate regions of parenchymal extinction in human cirrhosis. *Hepatology* 2015, 61:1696–1707
- Katoonizadeh A, Nevens F, Verslype C, Pirenne J, Roskams T: Liver regeneration in acute severe liver impairment: a clinicopathological correlation study. *Liver Int* 2006, 26:1225–1233
- Spee B, Carpino G, Schotanus BA, Katoonizadeh A, Vander Borgh S, Gaudio E, Roskams T: Characterisation of the liver progenitor cell niche in liver diseases: potential involvement of Wnt and Notch signalling. *Gut* 2010, 59:247–257
- Boulter L, Govaere O, Bird TG, Radulescu S, Ramachandran P, Pellicoro A, Ridgway RA, Seo SS, Spee B, Van Rooijen N, Sansom OJ, Iredale JP, Lowell S, Roskams T, Forbes SJ: Macrophage-derived Wnt opposes Notch signaling to specify hepatic progenitor cell fate in chronic liver disease. *Nat Med* 2012, 18:572–579
- European Association for the Study of the Liver: EASL clinical practice guidelines: management of cholestatic liver diseases. *J Hepatol* 2009, 51:237–267
- Lammers WJ, Hirschfield GM, Corpechot C, Nevens F, Lindor KD, Janssen HL, Floreani A, Ponsioen CY, Mayo MJ, Invernizzi P, Battezzati PM, Pares A, Burroughs AK, Mason AL, Kowdley KV, Kumagi T, Harms MH, Trivedi PJ, Poupon R, Cheung A, Lleo A, Caballeria L, Hansen BE, van Buuren HR: Global PBC Study Group: Development and validation of a scoring system to predict outcomes of patients with primary biliary cirrhosis receiving ursodeoxycholic acid therapy. *Gastroenterology* 2015, 149:1804–1812. e1804
- Carbone M, Sharp SJ, Flack S, Paximadas D, Spiess K, Adgey C, Griffiths L, Lim R, Trembling P, Williamson K, Wareham NJ, Aldersley M, Bathgate A, Burroughs AK, Heneghan MA, Neuberger JM, Thorburn D, Hirschfield GM, Cordell HJ, Alexander GJ, Jones DE, Sandford RN, Mells GF: UK-PBC Consortium: The UK-PBC risk scores: derivation and validation of a scoring system for long-term prediction of end-stage liver disease in primary biliary cholangitis. *Hepatology* 2016, 63:930–950
- Ludwig J, Dickson ER, McDonald GS: Staging of chronic non-suppurative destructive cholangitis (syndrome of primary biliary cirrhosis). *Virchows Arch A Pathol Anat Histol* 1978, 379:103–112
- de Vries EM, de Krijger M, Farkkila M, Arola J, Schirmacher P, Gotthardt D, Goeppert B, Trivedi PJ, Hirschfield GM, Ytting H,

- Vainer B, Buuren HR, Biermann K, Harms MH, Chazouilleres O, Wendum D, Kemgang AD, Chapman RW, Wang LM, Williamson KD, Gouw AS, Paradis V, Sempoux C, Beuers U, Hubscher SG, Verheij J, Ponsioen CY: Validation of the prognostic value of histologic scoring systems in primary sclerosing cholangitis: an international cohort study. *Hepatology* 2017, 65:907–919
18. Della Corte C, Carpino G, De Vito R, De Stefanis C, Alisi A, Cianfarani S, Overi D, Mosca A, Stronati L, Cucchiara S, Raponi M, Gaudio E, Byrne CD, Nobili V: Docosahexanoic acid plus vitamin D treatment improves features of NAFLD in children with serum vitamin D deficiency: results from a single centre trial. *PLoS One* 2016, 11:e0168216
19. Onori P, Alvaro D, Floreani AR, Mancino MG, Franchitto A, Guido M, Carpino G, De Santis A, Angelico M, Attili AF, Gaudio E: Activation of the IGF1 system characterizes cholangiocyte survival during progression of primary biliary cirrhosis. *J Histochem Cytochem* 2007, 55:327–334
20. Carpino G, Renzi A, Cardinale V, Franchitto A, Onori P, Overi D, Rossi M, Berloco PB, Alvaro D, Reid LM, Gaudio E: Progenitor cell niches in the human pancreatic duct system and associated pancreatic duct glands: an anatomical and immunophenotyping study. *J Anat* 2016, 228:474–486
21. Carpino G, Nobili V, Renzi A, De Stefanis C, Stronati L, Franchitto A, Alisi A, Onori P, De Vito R, Alpini G, Gaudio E: Macrophage activation in pediatric nonalcoholic fatty liver disease (NAFLD) correlates with Hepatic progenitor cell response via Wnt3a pathway. *PLoS One* 2016, 11:e0157246
22. Nobili V, Carpino G, Alisi A, Franchitto A, Alpini G, De Vito R, Onori P, Alvaro D, Gaudio E: Hepatic progenitor cells activation, fibrosis and adipokines production in pediatric nonalcoholic fatty liver disease. *Hepatology* 2012, 56:2142–2153
23. Turner R, Lozoya O, Wang Y, Cardinale V, Gaudio E, Alpini G, Mendel G, Wauthier E, Barbier C, Alvaro D, Reid LM: Human hepatic stem cell and maturational liver lineage biology. *Hepatology* 2011, 53:1035–1045
24. Carpino G, Cardinale V, Gentile R, Onori P, Semeraro R, Franchitto A, Wang Y, Bosco D, Iossa A, Napoletano C, Cantafora A, D'Argenio G, Nuti M, Caporaso N, Berloco P, Venere R, Oikawa T, Reid L, Alvaro D, Gaudio E: Evidence for multipotent endodermal stem/progenitor cell populations in human gallbladder. *J Hepatol* 2014, 60:1194–1202
25. Yoon SM, Gerasimidou D, Kuwahara R, Hytioglou P, Yoo JE, Park YN, Theise ND: Epithelial cell adhesion molecule (EpCAM) marks hepatocytes newly derived from stem/progenitor cells in humans. *Hepatology* 2011, 53:964–973
26. Gadd VL, Skoien R, Powell EE, Fagan KJ, Winterford C, Horsfall L, Irvine K, Clouston AD: The portal inflammatory infiltrate and ductular reaction in human nonalcoholic fatty liver disease. *Hepatology* 2014, 59:1393–1405
27. Forbes SJ, Newsome PN: Liver regeneration—mechanisms and models to clinical application. *Nat Rev Gastroenterol Hepatol* 2016, 13:473–485
28. Miyajima A, Tanaka M, Itoh T: Stem/progenitor cells in liver development, homeostasis, regeneration, and reprogramming. *Cell Stem Cell* 2014, 14:561–574
29. Huch M, Gehart H, van Boxtel R, Hamer K, Blokzijl F, Verstegen MM, Ellis E, van Wenum M, Fuchs SA, de Ligt J, van de Wetering M, Sasaki N, Boers SJ, Kemperman H, de Jonge J, Ijzermans JN, Nieuwenhuis EE, Hoekstra R, Strom S, Vries RR, van der Laan LJ, Cuppen E, Clevers H: Long-term culture of genome-stable bipotent stem cells from adult human liver. *Cell* 2015, 160:299–312
30. Espanol-Suner R, Carpentier R, Van Hul N, Legry V, Achouri Y, Cordi S, Jacquemin P, Lemaigre F, Leclercq IA: Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. *Gastroenterology* 2012, 143:1564–1575.e7
31. Alvaro D, Invernizzi P, Onori P, Franchitto A, De Santis A, Crosignani A, Sferra R, Ginanni-Corradini S, Mancino MG, Maggioni M, Attili AF, Podda M, Gaudio E: Estrogen receptors in cholangiocytes and the progression of primary biliary cirrhosis. *J Hepatol* 2004, 41:905–912
32. Libbrecht L, Roskams T: Hepatic progenitor cells in human liver diseases. *Semin Cell Dev Biol* 2002, 13:389–396
33. Williams MJ, Clouston AD, Forbes SJ: Links between hepatic fibrosis, ductular reaction, and progenitor cell expansion. *Gastroenterology* 2014, 146:349–356
34. Dubuquoy L, Louvet A, Lassailly G, Truant S, Boleslawski E, Artru F, Maggioro F, Gantier E, Buob D, Leteurtre E, Cannesson A, Dharancy S, Moreno C, Pruvot FR, Bataller R, Mathurin P: Progenitor cell expansion and impaired hepatocyte regeneration in explanted livers from alcoholic hepatitis. *Gut* 2015, 64:1949–1960
35. Guldiken N, Kobazi Ensari G, Lahiri P, Couchy G, Preisinger C, Liedtke C, Zimmermann HW, Ziol M, Boor P, Zucman-Rossi J, Trautwein C, Strnad P: Keratin 23 is a stress-inducible marker of mouse and human ductular reaction in liver disease. *J Hepatol* 2016, 65:552–559
36. Boulter L, Lu WY, Forbes SJ: Differentiation of progenitors in the liver: a matter of local choice. *J Clin Invest* 2013, 123:1867–1873
37. Lorenzini S, Bird TG, Boulter L, Bellamy C, Samuel K, Aucott R, Clayton E, Andreone P, Bernardi M, Golding M, Alison MR, Iredale JP, Forbes SJ: Characterisation of a stereotypical cellular and extracellular adult liver progenitor cell niche in rodents and diseased human liver. *Gut* 2010, 59:645–654
38. Burak KW, Angulo P, Lindor KD: Is there a role for liver biopsy in primary sclerosing cholangitis? *Am J Gastroenterol* 2003, 98:1155–1158
39. Angulo P, Larson DR, Therneau TM, LaRusso NF, Batts KP, Lindor KD: Time course of histological progression in primary sclerosing cholangitis. *Am J Gastroenterol* 1999, 94:3310–3313
40. de Vries EM, Verheij J, Hubscher SG, Leflang MM, Boonstra K, Beuers U, Ponsioen CY: Applicability and prognostic value of histologic scoring systems in primary sclerosing cholangitis. *J Hepatol* 2015, 63:1212–1219
41. Ponsioen CY, Chapman RW, Chazouilleres O, Hirschfield GM, Karlsen TH, Lohse AW, Pinzani M, Schrupf E, Trauner M, Gores GJ: Surrogate endpoints for clinical trials in primary sclerosing cholangitis: review and results from an International PSC Study Group consensus process. *Hepatology* 2016, 63:1357–1367
42. Lanthier N, Rubbia-Brandt L, Lin-Marq N, Clement S, Frossard JL, Goossens N, Hadengue A, Spahr L: Hepatic cell proliferation plays a pivotal role in the prognosis of alcoholic hepatitis. *J Hepatol* 2015, 63:609–621
43. Invernizzi P: Primary biliary cirrhosis. Foreword. *Semin Liver Dis* 2014, 34:253–254
44. Alvaro D, Mancino MG, Glaser S, Gaudio E, Marziani M, Francis H, Alpini G: Proliferating cholangiocytes: a neuroendocrine compartment in the diseased liver. *Gastroenterology* 2007, 132:415–431
45. Hrad V, Abebe Y, Ali SH, Velgersdyk J, Al Hallak M, Imam M: Risk and surveillance of cancers in primary biliary tract disease. *Gastroenterol Res Pract* 2016, 2016:3432640
46. Cavazza A, Caballeria L, Floreani A, Farinati F, Bruguera M, Caroli D, Pares A: Incidence, risk factors, and survival of hepatocellular carcinoma in primary biliary cirrhosis: comparative analysis from two centers. *Hepatology* 2009, 50:1162–1168
47. Trivedi PJ, Lammers WJ, van Buuren HR, Pares A, Floreani A, Janssen HL, Invernizzi P, Battezzati PM, Ponsioen CY, Corpechot C, Poupon R, Mayo MJ, Burroughs AK, Nevens F, Mason AL, Kowdley KV, Lleo A, Caballeria L, Lindor KD, Hansen BE, Hirschfield GM; Global PBC Study Group: Stratification of

- hepatocellular carcinoma risk in primary biliary cirrhosis: a multi-centre international study. *Gut* 2016, 65:321–329
48. Govaere O, Komuta M, Berkers J, Spee B, Janssen C, de Luca F, Katoonizadeh A, Wouters J, van Kempen LC, Durnez A, Verslype C, De Kock J, Rogiers V, van Grunsven LA, Topal B, Pirenne J, Vankelecom H, Nevens F, van den Oord J, Pinzani M, Roskams T: Keratin 19: a key role player in the invasion of human hepatocellular carcinomas. *Gut* 2014, 63:674–685
 49. Tummala KS, Brandt M, Teijeiro A, Grana O, Schwabe RF, Perna C, Djouder N: Hepatocellular carcinomas originate predominantly from hepatocytes and benign lesions from hepatic progenitor cells. *Cell Rep* 2017, 19:584–600
 50. Boulter L, Guest RV, Kendall TJ, Wilson DH, Wojtacha D, Robson AJ, Ridgway RA, Samuel K, Van Rooijen N, Barry ST, Wigmore SJ, Sansom OJ, Forbes SJ: WNT signaling drives cholangiocarcinoma growth and can be pharmacologically inhibited. *J Clin Invest* 2015, 125:1269–1285
 51. Guest RV, Boulter L, Dwyer BJ, Kendall TJ, Man TY, Minnis-Lyons SE, Lu WY, Robson AJ, Gonzalez SF, Raven A, Wojtacha D, Morton JP, Komuta M, Roskams T, Wigmore SJ, Sansom OJ, Forbes SJ: Notch3 drives development and progression of cholangiocarcinoma. *Proc Natl Acad Sci U S A* 2016, 113:12250–12255
 52. Schulze K, Imbeaud S, Letouze E, Alexandrov LB, Calderaro J, Rebouissou S, Couchy G, Meiller C, Shinde J, Soysouvanh F, Calatayud AL, Pinyol R, Pelletier L, Balabaud C, Laurent A, Blanc JF, Mazzaferro V, Calvo F, Villanueva A, Nault JC, Bioulac-Sage P, Stratton MR, Llovet JM, Zucman-Rossi J: Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. *Nat Genet* 2015, 47:505–511