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AIM AND SCOPE

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WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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REVIEW

Glycogenic hepatopathy: A narrative review

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Abstract

Glycogenic hepatopathy (GH) is a rare complication of the poorly controlled diabetes mellitus characterized by the transient liver dysfunction with elevated liver enzymes and associated hepatomegaly caused by the reversible accumulation of excess glycogen in the hepatocytes. It is predominantly seen in patients with longstanding type 1 diabetes mellitus and rarely reported in association with type 2 diabetes mellitus. Although it was first observed in the pediatric population, since then, it has been reported in adolescents and adults with or without ketoacidosis. The association of GH with hyperglycemia in diabetes has not been well established. One of the essential elements in the pathophysiology of development of GH is the wide fluctuation in both glucose and insulin levels. GH and non-alcoholic fatty liver disease (NAFLD) are clinically indistinguishable, and latter is more prevalent in diabetic patients and can progress to advanced liver disease and cirrhosis. Gradient dual-echo MRI can distinguish GH from NAFLD; however, GH can reliably be diagnosed only by liver biopsy. Adequate glycemic control can result in complete remission of clinical, laboratory and histological abnormalities. There has been a recent report of varying degree of liver fibrosis identified in patients with GH. Future studies are required to understand the biochemical defects underlying GH, noninvasive, rapid diagnostic tests for GH, and to assess the consequence of the fibrosis identified as severe fibrosis may progress to cirrhosis. Awareness of this entity in the medical community including specialists is low. Here we briefly reviewed the English literature on pathogenesis involved, recent progress in the evaluation, differential diagnosis, and management.

Key words: Glycogenic hepatopathy; Diabetes mellitus; Hepatomegaly; Mauriac syndrome; Elevated liver enzymes; Liver biopsy; Gradient dual-echo MRI

Core tip: Glycogenic hepatopathy (GH) is considered as a benign reversible condition. Elevation in transaminases is a common finding in patients with diabetes mellitus, and non-alcoholic fatty liver disease (NAFLD) and GH are the two primary underlying pathologies in most cases. It is essential to distinguish NAFLD from GH as this can progress to advanced liver disease. However, recent studies have identified the varying degree of fibrosis in glycogen hepatopathy as well, further emphasizing the need for future studies. We briefly reviewed the literature on mechanisms involved in the development of GH, evaluation of these patients, recent progress made on diagnostic tests and management.

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INTRODUCTION

GH is a rare clinical condition that develops due to excessive accumulation of glycogen in the hepatocytes predominantly seen in pediatric patients and young adults with poorly controlled type 1 diabetes mellitus (T1DM), and rarely observed in a patient with type 2 diabetes mellitus (T2DM)[1-48] (Tables 1-3). GH is characterized by transient liver dysfunction with elevated liver enzymes and associated hepatomegaly caused by a reversible accumulation of excess glycogen in hepatocytes. The collection of glycogen seen on a liver biopsy is critical for the diagnosis. It is an underrecognized entity, and awareness of this clinical condition by clinicians, including gastroenterologists, is low.

GH was initially described in 1930 by Pierre Mauriac in a pediatric patient with poorly controlled T1DM (brittle DM), who presented with hepatomegaly, cushingoid features, and poor growth and development, a condition known as Mauriac Syndrome[30,45]. Since then, more cases have been reported without the full spectrum of Mauriac Syndrome, both in adults and children with T1DM and rarely in T2DM. Various terms have been used to describe this entity, including hepatic glycogenosis, glycogen hepatopathy, glycogen storage hepatomegaly and hepatic glycogen storage[2,4-7,9,14,47]. In 2006, Torbenson and colleagues proposed the term "Glycogenic Hepatopathy" due to hepatocyte glycogen overload but without showing extrahepatic features of Mauriac Syndrome. This term has been used universally since then to describe this unique pathologic feature[44].

The pathophysiology of GH is incompletely understood, and clinical characteristics have not been fully characterized yet. It is believed to be the consequence of recurrent fluctuations in glucose level with hyperglycemia, hypoglycemia, and hyperinsulinization. In addition to glycogen accumulation, steatosis with varying degree of fibrosis may be evident in liver biopsy[38,45]. Intensive insulin regimens with glycemic control provide full resolution of clinical symptoms, laboratory abnormalities, and histological abnormalities.

GH remains underrecognized in adults, as most clinicians mistake it for NAFLD, a more common hepatic abnormality associated with DM. The clinical or radiological distinction between GH and NAFLD is difficult. It is essential that primary care physicians and other specialists be aware of this entity, as differentiating this condition from NAFLD is vital, given its associated comorbidities such as cardiovascular disease, progressive liver fibrosis, and cirrhosis. The focus of this review is to provide an update on recent development in understanding the pathophysiology and the progress in the noninvasive evaluation of differentiating nonalcoholic fatty liver disease from glycogenic hepatopathy.

LITERATURE SEARCH

A literature search was conducted using PubMed using search terms "glycogen" (MeSH Terms) OR "glycogen" (All Fields) AND ["liver diseases" (MeSH Terms) OR "liver" (All Fields AND) "diseases" (All Fields)] OR "liver diseases" (All Fields) OR "hepatopathy" (All Fields)". A total of fifty-one studies involving adolescents or adults were included for this review.

INCIDENCE

Much of the knowledge on GH that has been accumulated over the decades since GH was first reported in 1930, are from case reports, case series, a retrospective cohort study, or more recently, a case-control study[45,46]. It has been estimated that about 8% of American adults have elevated serum aminotransferases[49]. Following a negative initial serologic evaluation, 90% of patients with chronically elevated abnormal aminotransferases are due to NAFLD[50]. The prevalence of liver disease among people with diabetes is estimated to be 17% to 100%[51], with nonalcoholic fatty liver disease and hepatic glycogenosis being the predominant pathologies.

True incidence and prevalence of GH are unknown. Sixty-two percent of the reported cases are female patients, indicating a slight female predominance. Thirty-eight percent of reported cases are male patients, with most cases occurring in adolescence. Based on published reports in English literature, approximately 98% of GH cases were reported in T1DM, while the remaining 2% is caused by T2DM. The incidence of this syndrome has decreased significantly with the introduction of long-acting insulin and better control of

Table 1 Summary of the major case reports in English (pub med indexed) on Glycogenic hepatopathy in type 1 diabetes mellitus

Ref.	Study design	Age/sex	HbA1C	Keto acidosis	Hepatomegaly	AST/ALT/ALP/T bili	Normalization of LFTs
Ruschhaupt *et al*[1], 1970	Case report	13/M	NA	Yes	Yes	9/14/115/128/NA	5 d
Berman *et al*[2], 1973	Case report	21/F	NA	Yes	Yes	UK	Expired (5 d)
Olsson *et al*[3], 1989	Case report	15/F	NA	Yes	Yes	535/417/570/NA	10 d
		20/F	NA	No	Yes	1117/1235/941/NA	18 mo
		24/F	NA	Yes	No	323/276/429/NA	NA
Munns *et al*[4], 2000	Case reports	13/M	14.1	No	Yes	132/135/170/NA	4 wk
		17/F	13.3	No	Yes	102/147/175/NA	2 wk
		16/F	12.2	No	Yes	567/316/196/NA	2 wk
Fridell *et al*[5], 2007	Case report	18/F	NA	Yes	Yes	Elevated/N	After pancreatic transplant
		21/M	NA	No	Yes	Elevated/N	After pancreatic transplant
Cuthbertson *et al*[6], 2007	Case report	19/F	12.2	Yes	Yes	NA/800/132/N	NA
Sayuk *et al*[7], 2007	Case report	19/F	8.1	Yes	Yes	98/49/147/0.5	NA
		37/M	16.0	Yes	Yes	226/399/326/0.9	NA
Bassett *et al*[8], 2008	Case report	10/F	10.1	No	No	143/147/137/N	NA
Abaci *et al*[9], 2008	Case report	16/M	11.1	No	Yes	66/58/431/0.8	3 mo
Hudacko *et al*[10], 2008	Case report	20/F	13.3	No	Yes	249/383/NA/NA	5 mo
Sweetser *et al*[11], 2010	Case report	27/M	15.0	No	Yes	6720/2549/529/1.7	3 mo
Van Den Brand[12], 2009	Case report	29/F	15.3	No	No	2500/1000/316/NA	NA
Saxena *et al*[13], 2010	Case report	33/F	13.7	No	No	428/404/205/N	12 mo
Bua *et al*[14], 2010	Case report	17/F	12.0	No	Yes	138/164/NA/NA	12 mo
Aljabri *et al*[15], 2011	Case report	13/F	13.0	No	Yes	NA/500/137/N	3 mo
Dantuluri *et al*[16], 2012	Case report	14/F	11.0	No	Yes	Elevated	Few days
Lin *et al*[17], 2012	Case report	10/ F	12.8	Yes	Yes	121/194/185/NA	6 mo
Messeri *et al*[18], 2012	Case report	31/F	10.3	No	Yes	225/258/375/0.54	2 mo
Murata *et al*[19], 2012	Case report	21/M	6.2	Yes	No	119/122/NA/NA	25 d
Cha *et al*[20], 2012	Case report	22/F	13.8	No	Yes	1028/365/346/0.5	8 wk
		26/F	12.9	Yes	Yes	914/307/189/0.5	4 wk
		20/F	13.6	No	No	1310/346/132/0.2	16 wk
Saadi T[21], 2012	Case report	18/M	11.0	No	Yes	144/92/123/1.4	3 mo
Saikusa *et al*[22], 2012	Case report	13/M	12.0	No	Yes	100/191/NA/NA	NA
Imtiaz *et al*[23], 2013	Case report	19/F	14.6	Yes	Yes	NA/199/139/NA	5 mo
Butts *et al*[24], 2014	Case report	13/F	8.8	Yes	Yes	NA/113/NA/NA	NA
Jeong *et al*[25], 2014	Case report	13/F	10.7	Yes	Yes	105/84/NA/NA	3 mo
Martin *et al*[26], 2014	Case report	13/M	10.4	Yes	Yes	3969/1196/NA/NA	16 d
Parmar *et al*[27], 2015	Case report	21/F	NA	Yes	Yes	4202/973/N/N	1 wk
Xu *et al*[28], 2015	Case report	22/M	14.6	No	Yes	331/223/223/0.3	NA
Garcia-Suarez *et al*[29], 2015	Case report	28/F	10.5	No	Yes	1600/534/N/N	NA
Atmaca *et al*[30], 2015	Case report	19/M	NA	Yes	Yes	603/570/921/NA	3 wk
Irani *et al*[31], 2015	Case report	19/M	12.0	Yes	Yes	505/345/145/N	NA
Brouwers *et al*[32], 2015	Case report	19/F	9.5	No	Yes	Elevated	NA
		19/F	13.3	No	Yes	Elevated	NA
		17/M	12.7	No	Yes	Elevated	NA
		20/F	14.7	No	Yes	Elevated	NA
Charndrasekaran *et al*[33], 2015	Case report	5/F	16.1	Yes	Yes	53/20/NA/0.7	NA
Silva *et al*[34], 2016	Case report	21/F	9.0	Yes	Yes	110/120/190/0.74	Few days
		20/F	10.1	Yes	Yes	270/423/179/1	Few days
		29/F	10.9	No	Yes	910/461/172/0.35	3 mo
		22/M	15.7	Yes	Yes	226/109/79/0.64	2 wk
Deemer *et al*[35], 2016	Case report	18/F	11.3	Yes	Yes	NA/36/120/N	NA
Saracho *et al*[36], 2016	Case report	14/M	12.8	Yes	Yes	Elevated	NA
Chandel *et al*[37], 2017	Case report	12/F	10.5	Yes	Yes	690/356/158/0.2	3 mo
Ikarashi *et al*[38], 2017	Case report	21/M	11.7	No	Yes	2723/956/NA/NA	1 mo
		24/F	11.0	Yes	Yes	658/216/NA/NA	Few days
		19/F	13.6	Yes	Yes	737/266/NA/NA	Few days
		29/F	16.5	Yes	Yes	469/243/NA/NA	6 mo
Maharaj *et al*[39], 2017	Case report	23/F	12.3	Yes	Yes	127/199/160/2.7	Few days
Al Sarkhy *et al*[40], 2017	Case report	6/M	11.6	Yes	Yes	367/205/221/N	Few days
Shah *et al*[41], 2017	Case report	31/F	NA	Yes	Yes	627/185/280/0.7	NA
Omer[42], 2017	Case report	14/M	11.0	Yes	Yes	Normal	NA

HbA1C: Hemoglobin A1c; AST: Aspartate transferase; ALT: Alanine transferase; ALP: Alkaline phosphatase; T bili: Total bilirubin; M: Male; F: Female; NA: Not available.

Table 2 Summary of the major studies in English (pub med indexed) on Glycogenic hepatopathy in type 1 diabetes mellitus

Ref.	Study design	Age/sex	HbA1C	Keto acidosis	Hepatomegaly	Liver abnormality	Normalization of LFTs
Chatila *et al*[43], 1996	Retrospective Study	Mild Fibrosis (14%)	11patients (8-A and 3-P)				
		41/F	NA	NA	No	20/45/1.4 x ULN/0.35	Few days to weeks
		21/F	NA	NA	No	282/404/0.4 x ULN/0.28	Few days to weeks
		58/F	NA	NA	Yes	35/56/3.2 x ULN/0.40	Few days to weeks
		70/F	NA	NA	Yes	76/NA/NA/1.28	Few days to weeks
		36/F	NA	NA	No	40/86/2.1 x ULN/0.28	Few days to weeks
		46/F	NA	NA	Yes	22/20/1.7 x ULN/N	Few days to weeks
		23/F	NA	NA	Yes	265/410/9.5 x ULN/0.40	Few days to weeks
		19/F	NA	NA	Yes	344/532/2.6 x ULN/0.8	Few days to weeks
		13/M	NA	NA	Yes	940/910/2 x ULN/0.30	Few days to weeks
		13/M	NA	NA	Yes	85/NA/0.3 x ULN/0.48	Few days to weeks
		15/M	NA	NA	Yes	NA/NA/1.9 x ULN/0.08	Few days to weeks
Torbenson *et al*[44], 2006	Retrospective Study	14 patients					
		19/F	NA	Yes	Yes	97/83/80/NA	NA
		1/2M	13.5	Yes	Yes	49/47/182/NA	NA
		22/F	NA	No	Yes	48/77/62/NA	NA
		8/M	NA	No	Yes	Elevated/NA	NA
		15/F	NA	No	NA	Normal	NA
		22/M	16	Yes	Yes	1100/360/251/NA	NA
		25/M	10.8	NA	NA	1629/1128/298/NA	NA
		16/M	NA	Yes	NA	Elevated/NA	NA
		20/M	9.9	No	yes	N/120/147/NA	NA
		18/F	10.8	No	NA	N/57/N/NA	NA
		28/M	NA	No	NA	1099/1544/384/NA	NA
		34/M	10.1	No	Yes	N/NA/N/NA	NA
		16/M	NA	No	Yes	1413/1354/476/NA	NA
		23/F	NA	No	Yes	255/224/307/NA	NA
Fitzpatrick *et al*[45], 2014	Retrospective	31 patients	11(Mean)	Yes-all	76/76/NA/NA(M)	NA	14 (73%) Fibrosis
		16M/15F	12Mild and 2Bridging Fibrosis				
		Median age 15					
Mukewar *et al*[46], 2017	Case control study	20 patients	11.4(Mean)	88.90%	301/308/170/0.5(M)	8 mo	10% Mild fibrosis but no bridging fibrosis
			55%				
		16F/4M					
		24-A, 12-P					

T1DM: Type 1 diabetes mellitus; HbA1C: Hemoglobin A1c; AST: Aspartate transferase; ALT: Alanine transferase; ALP: Alkaline phosphatase; T bili: Total bilirubin; M: Male; F: Female; NA: Not available; A: Adults; P: Pediatrics.

blood sugar, but unfortunately, it still exists[52].

PATHOGENESIS

The hallmark of the GH is severe fluctuation in levels of glucose and administration of supraphysiologic levels of insulin to control the hyperglycemia. While first described in association with recurrent hyper- and hypoglycemia with ketoacidosis in T1DM, it has since been reported without ketosis or acidosis in patients with T2DM and with variable insulin requirements[3,4,8-16,42]. The excess glycogen accumulation has also been reported in diabetic patients without previous episodes of hypoglycemia[44,53]. Rapid onset of hyperglycemia causing GH was reported in a case by Murata and colleagues in which the initial Hemoglobin A1C was only 6.2%; however, the patient presented with severe hyperglycemia with ketoacidosis and blood glucose of 1495 mg/dL[19].

Physiologically, the liver takes up glucose after feeding and either utilizes it for fuel or stores it as glycogen. Stored mainly in the liver and skeletal muscles, glycogen is a polymer of glucose that acts as a reservoir for glucose. The hepatic glycogen level is maintained by the balance between glycogenosis and glycogenolysis. High glucose levels cause an influx of glucose into the hepatocytes *via* facilitated diffusion through the glucose transporter 2 (GLUT2), independent of insulin[43]. Once the glucose is present within the hepatocytes, the enzyme glucokinase irreversibly phosphorylates glucose to glucose-6-phosphate, trapping it within the hepatocytes (Figure 1). Subsequent treatment of hyperglycemia with the high dose of insulin enhances further conversion of trapped glucose to polymerize into glycogen[43]. Glucose-6-phosphate is converted to glycogen by the enzyme glycogen synthase, which exists in an active dephosphorylated form and in an inactive

Table 3 Summary of the major case reports in English (PubMed indexed) on Glycogenic hepatopathy in type 2 diabetes mellitus

Ref.	Study design	Age/sex	Type of DM/insulin	A1C	Keto acidosis	Hepatomegaly	AST/ALT/ALP/T bili	normalization fibrosis of LFTs
Olson *et al*[3], 1989	Case report	39/M	Type 2/insulin	NA	Yes	No	429/764/1882/NA	21 d
Tsujimoto *et al*[47], 2006	Case report	41/M	Type 2/insulin	10	No	Yes	1064/1024/202/2.3	17 d
Umpaichitra[48], 2016	Case report	15/M	T2DM/metformin	6.5	No	Yes	245/330/N/N	18 mo

T2DM: Type 2 diabetes mellitus; HbA1C: Hemoglobin A1c; AST; Aspartate transferase; ALT: Alanine transferase; ALP: Alkaline phosphatase; T bili: Total bilirubin; M: Male; F: Female; NA: Not available.

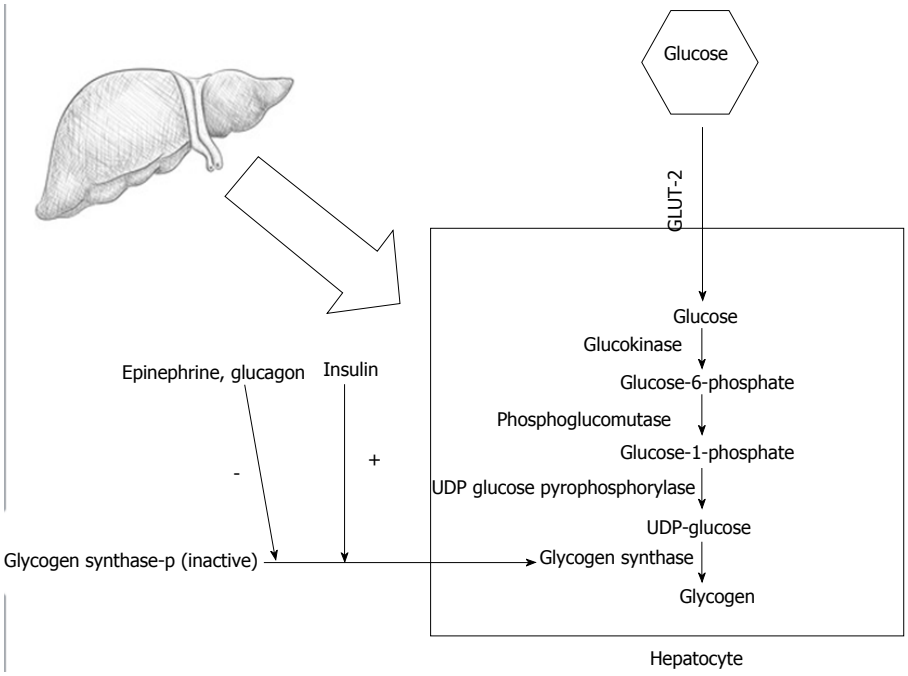

Figure 1 Steps of glycogenesis; effects of hyperglycemia and insulin treatment in glycogenic hepatopathy and formation of glycogen.

phosphorylated form. The active dephosphorylated structure of glycogen synthase is produced by the action of a phosphatase enzyme. The phosphatase enzyme is stimulated by elevated glucose and insulin levels. Glycogen production persists for some time after insulin levels have declined while inhibiting the glycogenolysis[15]. Hepatomegaly can sometimes develop within days to weeks and can improve rapidly once the hyperglycemia is controlled[54].

The reason for excessive accumulation of glycogen in hepatocytes with GH in contrast with the reduced glycogen stores observed in diabetes mellitus (DM) patients is unknown. Even more intriguing is why only a small subset of patients with DM develops GH. GH was first reported after short-acting insulins became available for the treatment of DM. Initially when short-acting insulins were used for the treatment of DM, large doses of insulin were required to control hyperglycemia, causing consequent hypoglycemia. This vicious cycle of excessive doses of short-acting insulin and subsequent administration of glucose to counteract the resulting hypoglycemia could have led to the continued accumulation of glycogen in the liver, a proposed mechanism by various authors[3,19,54]. These findings are infrequently observed today due to widespread use of long-acting insulin and possibly the decreased frequency of hypoglycemic events in this patient cohort.

Another hypothesis is that there is a defect in the genes that code for the proteins which regulate glycogen synthase or glucose 6-phosphatase activity. Although minor abnormalities have been identified in enzymes that control glycogen metabolism, these changes were not considered enough to explain hepatic glycogen storage in GH in the setting of DM[4]. A study by MacDonald and colleagues of an adolescent boy with Mauriac syndrome identified a mutation in PHKG2, is the catalytic subunit of glycogen phosphorylase kinase[55]. Expression of the mutant PHKG2 in a human liver cell line inhibited the enzyme activity of the phosphokinase complex and increased glycogen levels[55]. The mother of the boy with Mauriac syndrome also had the mutant PHKG2 but did not have diabetes or hepatomegaly. These findings do not explain all the cases of GH in adults, as most cases resolved with optimizing the hyperglycemia. Tomihira and colleagues investigated the gene structure of phosphorylase enzyme to define

Table 4 Summary of the published articles in English (PubMed indexed) on Glycogenic hepatopathy without DM

Ref.	Pathology	Study design	Age/sex	Hepatomegaly	AST/ALT/ALP/ T bili	Normalization	Fibrosis of LFTs
Resnick *et al*[56], 2011	Dumping syndrome	Case report	2/M	No	NA/199/NA/NA	16 mo	No fibrosis
Kransdorf *et al*[57], 2015	Anorexia nervosa	Case report	26/F	NA	75/101/108/	NA	No fibrosis
Iancu *et al*[58], 1986	Steroids use	Retrospective	6 mo-14 yr	Yes	Normal	3-5 d	No fibrosis

AST: Aspartate transferase; ALP: Alanine transferase; ALT: Alkaline phosphatase; T bili: Total bilirubin; M: Male; F: Female; NA: Not available.

possible mutations; however, they could not determine a probable gene defect that might cause hepatic glycogenosis[53]. Although no direct evidence is available so far, Berman MC hypothesized that the cause of GH is excess glycogen deposits in an intracellular location inaccessible to normal metabolic mechanisms, a phenomenon like that occurring in type Ⅱ glycogenesis in which glycogen deposits within the lysosomes, rendering them unavailable for phosphorylation activity[2]. In patients with a classic presentation of Mauriac syndrome, the wide fluctuations between hyper- and hypoglycemia-a pattern suggestive of under- and over-insulinization with secondary hyperadrenalism-possibly caused the cushingoid features. The pathogenesis of growth retardation in those initial cases reported in children was thought to be multifactorial[52].

The association of ketoacidosis in T1DM and GH not well understood. There could be some mechanisms account for excess deposition of glycogen or failure to mobilize during periods of hypoglycemia. Hormones like adrenaline, cortisol, or growth hormones released due to hypoglycemia, could synergistically act and release large quantities of non-esterified fatty acids from adipose tissue. This high concentration of free fatty acids inhibits glucose oxidation in muscles, and they may have similar effects on liver promoting excess storage of glycogen[2]. Other theory is that extremely low inorganic serum phosphate level in diabetic ketoacidosis may limit recovery from the acute diabetic state by acting as rate limiting factor for hexokinase and phosphokinase reactions. Depletion of intracellular inorganic phosphate for which there is an absolute requirement may be responsible for limitation of phosphorylase activity. Poorly controlled T1DM patients would be expected to have similar recurrent bouts of hypophosphatemia with the consequent entry of large amounts of glucose and inorganic phosphate into the cells[2].

GH in T2DM

The exact mechanism of development of GH in T2DM with insulin resistance is poorly understood and has yet to be clarified. Upon review of previously reported cases of GH, most cases were found in patients who were on an insulin regimen for T1DM, except two instances reported in T2DM[3,47,48] (Table 3). Pathophysiology of GH in T2DM is unclear and could have a different mechanism than that seen in T1DM. Umpaichitra V reported a case of GH in an adolescent male with T2DM whose liver enzymes returned to normal after treatment with metformin[48]. In T2DM, metformin is typically the first-line of treatment. The fact that the patient was in the early stages of diabetes and had not decompensated enough to cause total insulin resistance might explain the preserved effect of insulin on glycogenesis[48]. The mechanism which metformin may have alleviated the glycogenesis, in this case, is also not precise. It was possible that once he was started on metformin, his hepatic gluconeogenesis decreased and glucose uptake in peripheral tissues increased, possibly leading to a paucity of glucose as a substrate for glycogenesis[48].

OTHER DISORDERS ASSOCIATED WITH GH

Excessive hepatic accumulation of glycogen causing GH occurs not only in patients with DM but also in other conditions, including dumping syndrome after gastrectomy, anorexia nervosa, high-dose glucocorticoid use, azathioprine use, and insulin overdose[56-58] (Table 4).

Dumping syndrome after gastric bypass

One of the inherent functions of insulin is to stimulate glycogenesis. Hyperglycemia and hyperinsulinemia are thought to be the etiology for hepatic glycogen deposition in dumping syndrome. Dumping syndrome is a complication of gastric surgery, such as gastric bypass surgery. In patients who had gastric surgery, chyme rapidly "dumped" from the stomach into the small bowel without complete digestion. Resnick *et al*[56] reported a case of a toddler fed *via* gastrostomy tube, who developed GH secondary to Dumping syndrome after Nissen fundoplication. Like GH, it involves fluctuation between hyperglycemia from the rapid nutrition glucose load and hyperinsulinemia. The pathophysiology of Dumping syndrome can resemble that of GH seen in people with diabetes, eventually causing the hepatic accumulation of glycogen.

Anorexia nervos

Anorexia nervos (AN) is characterized by the obsessive fear of gaining weight, distorted body image, and a significantly low body weight. Lisa Kransdorf and colleagues reported a case of anorexia nervosa with glycogen deposition with elevated liver enzymes[57]. It has been reported that 1 in 10 patients with AN will have abnormal liver enzymes. A series of cases of AN with liver failure was reported by Rautou *et al*[59]; about

50% of them presented with hypoglycemia. Again, this lack of glycogen deposits supports the hypothesis that hepatic glycogen accumulation is potentially a protective mechanism. Hepatic glycogenosis is thought to be an adaptive response that protects against potentially fatal hypoglycemia in malnutrition. It is essential to be aware of this association when treating patients with AN.

Short-term high dose steroid therapy

Steroids promote elevation in glucose, gluconeogenesis, and glycogen deposition. Current available evidence regarding GH with steroid use was gathered from pediatric patients. Hepatic glycogen deposition has been described in several experimental models. In a study by Iancu *et al*[58] 141 patients had received steroid therapy: 13% had hepatomegaly, and three patients were noted to have glycogenic hepatopathy. It is unclear whether they had any elevation in liver enzymes. Hepatomegaly resolved soon after discontinuing the steroid therapy in all patients with hepatomegaly. Several mechanisms were involved in the pathogenesis of GH in steroid use. Following hyperglycemia induced by steroids, increased glycogen deposition occurs by the activation of phosphorylase by glucose and the subsequent activation of glycogen synthase. Glycogen synthase is activated by insulin, which increases following steroid-induced hyperglycemia. The knowledge that the hepatomegaly in patients taking steroids could be GH-related will aid in the management of these patients.

Insulin overdose

Excessive insulin levels associated with exogenous insulin administration augments the glycogen deposition. This is evident from a case reported by Tsujimoto *et al*[47] in which a patient with T2DM self-administered a massive dose of long-acting insulin in a suicide attempt. He became hypoglycemic and was later administered a large dose of intravenous glucose to counteract the persistent hypoglycemia. Prior to the suicide attempt, his baseline liver enzymes were initially normal. After the administration of insulin and intravenous glucose the patient developed acute glycogen storage hepatomegaly. His liver enzymes increased significantly to more than 30 times the upper limit of normal.

CLINICOPATHOLOGICAL FEATURES

Clinical manifestations

Clinical presentation of GH varies from asymptomatic patients with elevated liver enzymes to various symptoms associated with hyperglycemia. Patients can present with symptoms of diabetic ketoacidosis (DKA), such as polyuria, polydipsia, marked dehydration, as well as abdominal pain, nausea, or vomiting or may occasionally present with signs of acute hepatitis, jaundice, and pruritus. Pediatric patients may present with extreme hepatomegaly along with growth failure and delayed puberty. The rapid enlargement of the liver causes stretching of the liver capsule, resulting in visceral pain. Although a few reported cases had normal liver sizes on imaging studies, hepatomegaly was observed in more than 90% of the reported cases, with varying degrees of elevation in transaminases and rarely an elevation in alkaline phosphatase (ALP) level. Excessive glycogen with swollen hepatocytes eventually could cause sinusoidal compression and subsequent ascites[34]; therefore, ascites can also be a part of the clinical presentation, albeit rarely[34,44]. Physical examination of patients with GH generally exhibits tender hepatomegaly without splenomegaly.

Biochemical features

Most patients with GH present with hepatocellular abnormality with a predominant elevation in aspartate transaminase (AST) and alanine transaminase (ALT) levels, although a mixed or predominantly cholestatic pattern can rarely occur[3,11,20,30,44]. For patients not experiencing a DKA episode, laboratory studies may show mild elevations in ALT, AST, and ALP. Marked elevations of transaminases and sometimes in the range of more than 100 folds' increase from normal were reported in several reports, mimicking acute hepatitis[26]. However, liver synthetic functions were usually preserved. No histological evidence was identified that suggested the increased enzymes were due to liver necrosis. Elevations in liver enzymes were thought to be due to enzyme leakage from hepatocyte membrane injury, not cell death. However, the exact mechanism is unknown. AST elevations were found to be significantly higher than the ALT elevations. Although ischemic hepatitis is the result of hypotension and hypoperfusion, subjects with inadequate perfusion without the hypotension can also produce a significant elevation in transaminases without liver necrosis[26]. Likewise, patients with DKA will have severe dehydration, which could contribute to these massive elevations in transaminases on top of the GH causing enzyme elevation. In a case-control study by Mukewar *et al*[46] analyzing 36 patients with T1DM, more than half of patients with GH had recurrent episodes of DKA, and these patients had higher levels of HbA1c than patients with TIDM without GH. Patients with GH could have elevated levels of plasma lactate, with or without the presence of a DKA episode, although the mechanism is still poorly understood[35,45]. In a retrospective review by Fitzpatrick of 31 patients with Mauriac syndrome, almost half the patients had elevated lactic acid level despite no signs of DKA[45]. One of the proposed theories is that a reduction in gluconeogenesis in the liver may raise lactate levels in the body. Therefore, lactic acidosis could be secondary to reduced gluconeogenesis and a lack of conversion of pyruvate to glucose[35].

The elevation in amylase and lipase levels less than three times the upper limit of normal without the evidence of acute pancreatitis on imaging has been

seen in some patients with GH[34]. Elevation in these enzymes even in the presence of abdominal pain should not be considered diagnostic of acute pancreatitis, since high levels of amylase and lipase can be seen in patients with DKA without evidence of pancreatitis on CT scan[34]. Hence in patients with GH, elevations of amylase and lipase less than three times the upper limit of normal may be nonspecific yet present. Additionally, antinuclear antibody was positive in some cases, showing a mostly homogeneous speckled pattern. These may be nonspecific as well, as there were no reports of any associated systemic disease[18,26,38,41,48].

DIAGNOSIS

Laboratory evaluation

No single serologic test can diagnose GH. Most patients with GH will have elevated transaminases. It is challenging to diagnose GH solely based on clinical features alone, and liver biopsy is crucial for definitive diagnosis. Typically, the increase in liver enzymes is transient and normalizes in a short period ranging from few days to few weeks[38]. Several laboratory tests may be done initially to rule out other causes of hepatomegaly and chronic hepatitis. Because there is an association between T1DM and autoimmune hepatitis, testing for autoimmune antibodies, such as ANA, anti-smooth muscle antibody and antimitochondrial antibody is essential. The signs and symptoms of Wilson disease often begin during the teenage years and should be ruled out. Glycogen storage diseases (GSDs) are caused by congenital deficiencies in various enzymes and clinically present in the neonatal period or infancy. These patients may already start to present with hypoglycemia and hepatomegaly during infancy[60]. As expressed by Umpaichitra, glucagon stimulation test in children can be tried to rule out GSD[48]. As an up-regulator of glycogenolysis and a down-regulator of glycogenesis, glucagon would facilitate the breakdown of glycogen to allow the release of glucose into the bloodstream. Patients who can demonstrate an increase in serum glucose levels after stimulation by glucagon efficiently rule out a glycogen storage disease[48]. However, the best modality to accurately diagnose a GSD is next-generation genetic sequencing.

Imaging studies

Different imaging studies can be used to support or refute the diagnosis of GH. One of the principal differential diagnoses is NAFLD. In patients with GH, abdominal ultrasound would show hepatomegaly with uniform echogenicity, indicative of glycogen storage, like the fatty change seen in NAFLD. Ultrasound of abdomen is not a useful modality to differentiate NAFLD from GH, as the mildly bright liver can be observed in both. A bright liver compared to the spleen in CT scan imaging can be the clue to diagnosing GH. The liver density on CT scan of the abdomen in patients with GH is increased (hyper dense), compared to a patient with NAFLD in whom it is decreased (hypodense); this subtle difference can give a clue to GH, as reported by Sweetser[11].

Neither CT abdomen nor liver US is a useful test for the definitive diagnosis of GH; however, gradient dual-echo MRI sequence was reported to be able to distinguish fat deposition from an edematous condition such as acute tissue injury; both of which appears as low-density areas on CT. MRI imaging in GH shows low intensities on T2 weighted images. Gradient dual-echo MRI is a powerful tool to distinguish GH from NAFLD. T1 weighted gradient-dual-echo MRI images with in-phase and opposed-phase conditions could efficiently differentiate hepatic glycogen from the fat seen in NAFLD[11,22]. If there is no significant difference in the signal intensities between the two phases then the results are not consistent with intrahepatic fat storage (NAFLD) and are more consistent with GH[19,22] (Figure 2). A few recent reports have shown the presence of varying degrees of fibrosis in patients with GH[45,46]. Presence or absence of liver fibrosis in GH patients can be measured using noninvasive tests such as Fibroscan. Magnetic resonance elastography may have a role in evaluating the degree of fibrosis with GH and needs further studies. Several authors have also demonstrated elevated hepatic glycogen concentration in subjects with glycogen storage disease using 13C MRS (Magnetic Resonance Spectroscopy), which has the advantage of assessing the entire liver while avoiding the risks of liver biopsy[61]. However, these tests may not yet be readily available in most centers.

Liver biopsy and Histological features

A liver biopsy typically shows swollen hepatocytes with an accumulation of glycogen in the cytoplasm. Associated steatosis may be mild to absent in most cases. Although some authors recommended the therapeutic trial of intensive insulin therapy for four weeks preceding any invasive investigations, liver biopsy is the gold standard for diagnosing GH. A hematoxylin and eosin (HE) stain of the biopsy specimen in a patient with GH would show pale and enlarged hepatocytes with prominent plasma membranes, increased cytoplasmic volume, and numerous glycogenated nuclei, which are empty nuclei with ring-like chromatin elements (Figures 3 and 4). Sinusoidal compression by the swollen hepatocytes can produce a paved appearance to the liver parenchyma. The architectural structure of the liver parenchyma likewise remains intact. Furthermore, an addition of diastase to the Periodic-Acid Schiff (PAS) stained specimen would cause enzymatic breakdown of glycogen in the hepatocytes, causing these hepatocytes to turn into "ghost cells"[41] (Figure 4). Histological examination of hepatocytes from patients with GH normally does not exhibit significant portal inflammation, steatosis, or significant fibrosis. In fact, only a minority of cases reported had fibrosis, which was minimal except two cases reported by Fitzpatrick and colleague showed bridging fibrosis[44-46]. In a review

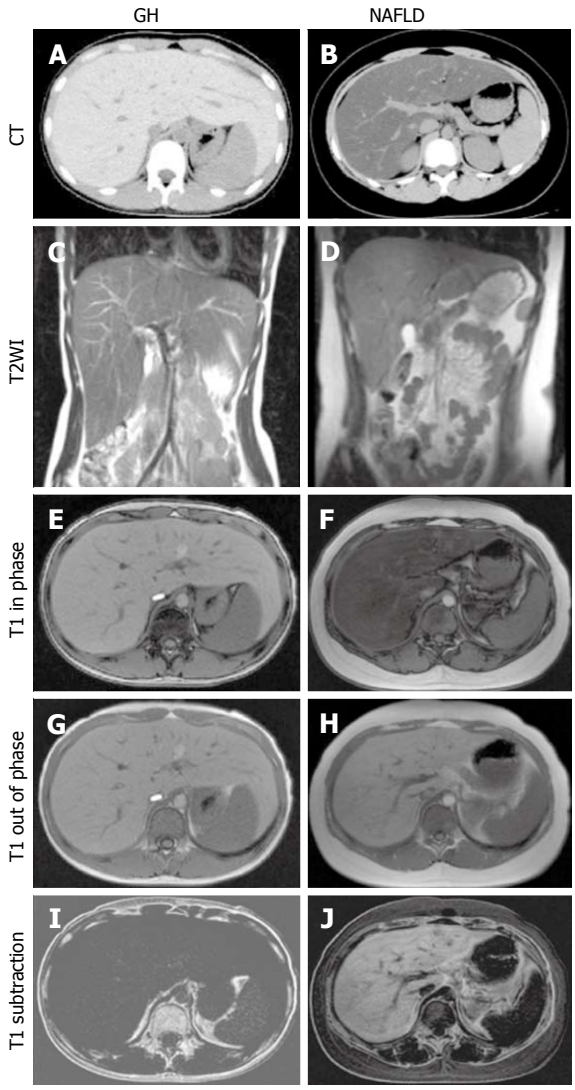

Figure 2 Computed tomography (CT) and magnetic resonance imaging (MRI) of (A, C, E, G, I) glycogenic hepatopathy (GH) in a 13-year-old girl and (B, D, F, H, J) non-alcoholic fatty liver disease (NAFLD) in a 13-year-old boy. On CT, GH was (A) high density, but NAFLD was typically (B) low density; On T2-weighted imaging (T2WI), both enlarged livers were (C) 19.1 cm and (D) 16.8 cm along the right midclavicular line; On gradient dual-echo MRI, the GH liver was iso-intense between the (E) in-phase and (G) out-of-phase images, namely; I: Low intensity on subtraction. The NAFLD liver, however, had low intensity on the (F) in-phase image, and high intensity on the (H) out-of-phase image, namely; J: High intensity on subtraction. With permission from Saikusa *et al*[22], John Wiley and Sons publications.

of cases of GH by Torbenson *et al*[44], mild steatosis was noted in 14% of cases, mild steatohepatitis in 7% of cases, and mild fibrosis in 14% of cases with GH. Histopathology of a case reported by Shah *et al*[41]. also showed GH with focal portal tract fibrosis. Future studies warranted to further assess for development of fibrosis in GH as it can progress to cirrhosis.

DIFFERENTIAL DIAGNOSIS

For patients with DM who present with hepatomegaly and elevated transaminase levels, there are several differential diagnoses apart from glycogenic hepatopathy, including NAFLD. Among T2DM patients, hepatic enlargement with elevated transaminases is usually the result of NAFLD, while GH is the most likely pathology in patients with T1DM[25]. Other potential causes of liver damage in people with diabetes include celiac disease and autoimmune hepatitis. Hepatitis A, Hepatitis B, Hepatitis C, hemochromatosis, and Wilson's disease should all be considered in the list of differential diagnoses, depending on extent and pattern of liver abnormality on presentation (Table 5).

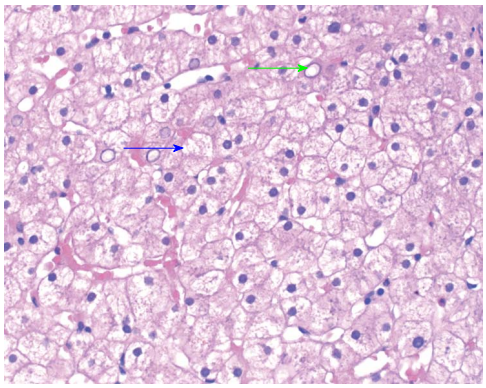

Figure 3 Percutaneous liver biopsy section of a patient with glycogenic hepatopathy. HE stain showing enlarged hepatocytes with cytoplasmic pallor with reddish pink globules consistent with glycogen accumulation (blue arrow), and prominent glycogenated nuclei (green arrow).

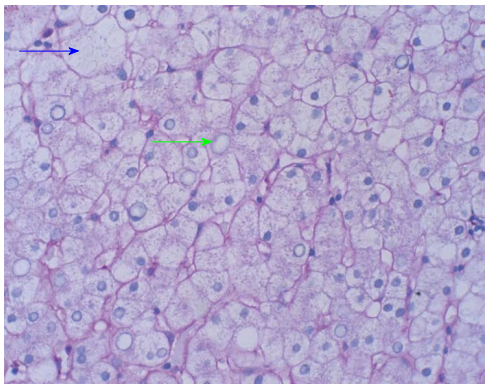

Figure 4 D-Periodic-Acid Schiff stain remove glycogen leaving empty looking cytoplasm (blue arrow) and nuclei (green arrow).

NAFLD

The most common etiology for elevated live enzymes in general population including patients with DM is NAFLD. NAFLD is more typically found in obese adults with T2DM secondary to insulin resistance, while GH is more common in patients with lower body mass index and pediatric patients. Upon assessment of laboratory studies from a patient with NAFLD, steatohepatitis would show mild elevations in AST, ALT, and ALP, just like in most cases of GH. CT scan imaging of a patient with NAFLD would show hepatomegaly with decreased liver density, while the gradient-dual-echo MRI would show low intensity in phase and high

Table 5 Summery of the main differential diagnosis for glycogenic hepatopathy

	Main etiology	Clinical presentation	Imaging characteristics	Key, liver biopsy pathological features	Diagnosis	Management	Cirrhosis
GH	Acquired excessive glycogen deposition in the liver mostly seen in patients with T1DM	Hyperglycemia with hyperglycemic symptoms; could be asymptomatic. Liver enzyme elevation is mild to extreme range in some case	US and CT shows increased echogenicity. Dual-Echo MRI; iso-intense between the in-phase and out-of-phase images, and low intensity on subtraction	Glycogen deposition in the cytoplasm with swollen hepatocytes, with or without mild steatosis and fibrosis. Diastase digestion of glycogen cause hepatocytes to turn into "ghost cells"	Radiologic and liver biopsy	Optimal control of DM	May have mild fibrosis, severe fibrosis is very rare and seen in only a few reported cases
GSD	Inborn errors of glucose and glycogen metabolism results in abnormal deposition of glycogen	Presentation varies depend on types of GSDs They will have manifestations of a liver, kidney, and skeletal muscle involvement with hypoglycemia, hepatomegaly, muscle cramps, and weakness, *etc*	Like GH	Findings vary in different type of GSDs; Nonspecific histologic findings to PAS positive glycogen deposition which could be diastase sensitive or resistant	Biochemical tests and molecular testing	Symptomatic treatment to dietary changes to maintain the blood glucose level and pharmacologic therapy in different types of GSDs. May need liver transplantation in selected cases	Some GSD can progress to cirrhosis
NAFLD	Hepatic steatosis	Most patients asymptomatic and some may have minor symptoms, Liver enzymes elevation usually < 5 times upper limit of normal	US and CT shows increased echogenicity. Dual-Echo MRI; low intensity on the in-phase image, and high intensity on the out-of-phase image and high intensity on subtraction.	Steatosis with or without lobular inflammation and hepatocyte ballooning. May have varying degrees of fibrosis	Radiologic and liver biopsy	Lifestyle modification and pharmacologic therapies. May need liver transplant in advanced cirrhosis	Can progress to cirrhosis
Hepatosclerosis	Is a hepatic manifestation of microangiopathic disease seen in long -standing DM	Often clinically silent, Serum aminotransferases are normal or minimally elevated. ALP, Total bilirubin may be elevated	No specific imaging characteristics	Extensive dense perisinusoidal fibrosis	Liver biopsy	Unknown	Unknown
AIH	Chronic Hepatitis of unknown etiology	Spectrum of clinical manifestations ranges from asymptomatic patients to those with considerable symptoms, and rarely presents with acute liver failure	No characteristic imaging features, may show cirrhotic liver in advance case	Interface hepatitis and portal lymphoplasmacytic infiltrate with varying degree of fibrosis	Characteristic biochemical tests and liver biopsy	Glucocorticoid monotherapy or in combination with immunomodulators. Rarely may require liver transplantation	Can progress to cirrhosis
Hemochromatosis	Autosomal recessive disorder. Mutations cause increased iron absorption and excessive deposition in the liver, heart, pancreas, and pituitary	Asymptomatic or chronic liver disease with elevated transaminases, skin pigmentation, DM, arthropathy, impotence and cardiac enlargement, *etc*	MRI is most sensitive and can estimate iron concentration in the liver. Dual-Echo MRI; demonstrates decreased signal intensity in the affected tissues on the in-phase images compared with the out of- phase images (opposite of steatosis)	A liver biopsy will reveal iron overload. Presence of cirrhosis can be determined	Biochemical tests including genetic testing, radiologic, and liver biopsy	Phlebotomy or Chelation therapy if unable to tolerate phlebotomy	Can progress to cirrhosis

Wilson disease	Autosomal recessive disorder with impaired cellular copper transport and impaired biliary copper excretion results in accumulation of copper most notably the liver, brain, and cornea.	Predominantly hepatic, neurologic, and psychiatric manifestations. Elevated transaminases mild to moderate and ALP may be markedly subnormal	US, CT, may show signs of cirrhosis and normal caudate lobe which is contrary to other types cirrhosis	Vary largely from fatty changes to cirrhosis and occasionally fulminant hepatic necrosis. Can be stained for copper	Biochemical tests and slit lamp examination with or without genetic testing and liver biopsy	Treatment with a chelating agent. Some cases may require liver transplantation	Can progress to cirrhosis
Acute viral hepatitis A, B, C, D and E. Rarely, HSV, VZ, EBV and CMV	Hepatitis A and E are transmitted by feco- oral route; Rest of the viruses spread either by sexual contact, contact with body fluids or blood or from birth from an infected mother	Many of the symptoms are nonspecific; May have marked elevation in transaminases often > 15 times the normal	US or CT findings are nonspecific; Could be used to rule out other causes	Liver biopsy shows hepatocyte necrosis with a portal, periportal and lobular lymphocytic infiltration; Plasma cells present during resolving phase	Diagnosis by biochemical tests	Treatment conservative or antiviral therapy	Acute infection may progress to chronic, and that may progress to cirrhosis

PAS: Periodic-acid Schiff; T1DM: Type 1 diabetes mellitus; US: Ultrasound; CT: Computed tomography; GH: Glycogenic hepatopathy; GSD: Glycogen storage disease; NAFLD: Non-alcoholic fatty liver disease; AIH: Auto immune hepatitis; DM: Diabetes mellitus; ALP: Alkaline phosphatase; HSV: Herpes simplex virus; VZ: Varicella zoster virus; CMV; Cytomegalovirus.

intensity out of phase[22]. Histological examination of a liver biopsy specimen from a patient with NAFLD could likewise show macro vesicular steatosis, mild lobular and portal inflammation, and varying degrees of fibrosis. Evidence of hepatocellular injury and fibrosis would indicate increased severity and progression to actual steatohepatitis. NAFLD is likewise more likely to progress to fibrosis, cirrhosis and or hepatocellular carcinoma. Because of the potential for progressive liver disease and liver failure in NAFLD, it is crucial to distinguish NAFLD from GH, generally considered a more benign and reversible condition.

Hepatosclerosis

Hepatosclerosis is an underrecognized form of hepatic diabetic microangiopathy that needs to be differentiated from GH. It occurs in patients with T1DM more often than in T2DM and is associated with severe microvascular disease in other organs. Hepatosclerosis is characterized by an indolent course and is seen predominantly in female diabetic patients, although actual prevalence of this remains unknown. Generally, ALP is elevated with or without an elevation in total bilirubin, and serum aminotransferase levels may be normal or minimally elevated[62]. Liver biopsy may show extensive, dense, perisinusoidal fibrosis, and immunostaining reveals basement membrane components in a perisinusoidally distribution[62]. It is unclear whether hepatosclerosis plays any role in pathogenesis of NAFLD or cirrhosis in patients with DM. Long-term follow-up and future studies are required to examine the natural history and to explore treatment options for this form of microvascular complication involving the liver.

GSD

GSD is a group of inherited disorders in which excess glycogen accumulates in different tissues including liver, skeletal muscles, or both due to the deficiency of enzymes that regulate glycogenolysis or gluconeogenesis. Glycogen accumulation is not only caused by mutations in enzymes directly involved with glycogen catabolism or glucose metabolism, but it can also be caused by mutations in proteins that have an indirect impact on glycogen metabolism. It is vital to differentiate GH and GSDs with genetic testing, as management would vary vastly between the two pathologies. The hepatocytes in both conditions are markedly swollen and filled with glycogen, while the subtle difference can be the presence of higher cytoplasmic clumping of glycogen in GSD[44]. A clinical parameter such as the response to diabetic control in patients with poorly controlled DM is an important component to distinguish GH from GSD[44]. Management for GSDs revolves around management of complex carbohydrate intake and surveillance for complications, whereas management for GH centers on proper glycemic control with insulin and prevention of hyperglycemic episodes.

Drug induced liver injury and GH

Should a diabetic patient be on additional medications and the course of management be complicated by continued elevations in transaminases despite fluid resuscitation and treatment with insulin, it would be wise to drug induced liver injury (DILI) along with associated GH. Maharaj *et al*[39] published the case of a patient who initially presented with DKA, hepatomegaly, and elevated transaminases but was later diagnosed

with GH accompanied by DILI. He had a past medical history of T1DM and bipolar disorder that was being treated with the antipsychotic drugs paliperidone and asenapine. The author recommended DILI as a possibility whenever GH is suspected with concomitant use of hepatotoxic medications.

MANAGEMENT, PROGNOSIS AND FOLLOW UP

Once the diagnosis is made, improved glycemic control is the mainstay of management. Although there has not yet been any research in support of any pharmacological treatment strictly targeting or preventing glycogen deposits in the liver, it is regarded that resolution of the hyperglycemia would cause a decrease in transaminases level back to normal. GH can resolve with both clinical and biochemical resolution as quickly as it develops within days to weeks with good glycemic control[40]. Due to the autoimmune destruction of beta-islet cells in the pancreas, type 1 diabetics are unable to secrete endogenous insulin inherently. As such, exogenous insulin therapy is necessary to maintain their serum glucose levels. The mainstay of treatment for GH is strict control of glucose levels, close supervision of Hemoglobin A1C and prevention of recurrence of episodes of DKA. Prognosis with improved glycemic control is excellent. Reversal of GH has also been reported following pancreatic transplantation in people with diabetes, further consolidating the fact that it is entirely reversible by treating the diabetes[18].

Pathology results of majority of the cases reported in the literature did not show any significant fibrosis of the liver; however, further long-term studies are required to assess for the consequence of the mild fibrosis identified in some reports, and few cases of bridging fibrosis as severe fibrosis may further progress to cirrhosis. Despite showing a benign clinical course with strict glycemic control, GH could recur and relapse with uncontrolled glycemic levels[38]. Therefore, patients with a history of GH may still need to be followed up for any relapse of symptoms if persistent control of hyperglycemia is not maintained.

CONCLUSION

In conclusion, GH should be one of the differential diagnoses upon examination of a patient with uncontrolled DM presenting with elevated transaminases and hepatomegaly. GH should not be overlooked as a differential diagnosis solely due to its underrepresentation in diabetic patients. Compared to other liver diseases associated with DM, GH is a favorable diagnosis due to its benign nature and good prognosis.

Future progress is required in understanding the biochemical defects underlying GH and development of fibrosis. Further research is needed for an ideal noninvasive, rapid diagnostic test to avoid the extensive workup and associated costs in evaluating suspected cases of GH. For now, a more aggressive pursuit of liver biopsy in the evaluation of elevated transaminases could identify additional cases of GH, allowing for continued elucidation of prevalence and natural history of this entity. Clinicians should also continue to pool patient data from case studies of patients with GH, to better understand the underlying risk factors and characteristics of this disease.

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REVIEW

Hepatitis C virus: Morphogenesis, infection and therapy

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Abstract

Hepatitis C virus (HCV) is a major cause of liver diseases including liver cirrhosis and hepatocellular carcinoma. Approximately 3% of the world population is infected with HCV. Thus, HCV infection is considered a public healthy challenge. It is worth mentioning, that the HCV prevalence is dependent on the countries with infection rates around 20% in high endemic countries. The review summarizes recent data on HCV molecular biology, the physiopathology of infection (immune-mediated liver damage, liver fibrosis and lipid metabolism), virus diagnostic and treatment. In addition, currently available *in vitro*, *ex vivo* and animal models to study the virus life cycle, virus pathogenesis and therapy are described. Understanding of both host and viral factors may in the future lead to creation of new approaches in generation of an efficient therapeutic vaccine.

Key words: Hepatitis C virus; Transmission; Molecular biology; Pathogenesis; *In vitro* and *ex vivo* models of hepatitis C virus infection; Treatment

Core tip: Brief overviews on epidemiology of hepatitis C virus (HCV), virus morphology and the virus life cycle are presented. A special attention was focused on *in vitro* and *in vivo* models that are currently used to study the HCV infection. In fact, extensive use of existing models and creating a new ones is a way to reveal important events in the virus-cell interaction. In particular, the models might shed light on the mechanisms behind virus induced pathogenesis and chronicity, and by that contribute to the development of new drugs and prophylactic vaccine. Recently, multiple therapies with a pan-genotypic activity appeared on the market. The new agents (third generation) and new inhibitors (entry inhibitors, release inhibitors) being studied, should allow to cure most of the patients in the mid-term, if they will have equal access to the therapy.

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INTRODUCTION

The hepatitis C virus (HCV) is a major blood borne human pathogen. There are approximately 120-130 million or 3% of the total world population that are HCV infected (Figure 1). According to World Health Organization (WHO), annually there are about 3-4 million new cases of infection[1,2]. HCV is considered a major public health issue, since the virus is the etiological factor of chronic hepatitis that frequently progress to a cirrhosis and hepatocellular carcinoma (HCC). In developed countries, the most important route of HCV transmission is intravenous drug abuse, whereas in resource-poor countries invasive procedures or injection-based therapies with contaminated instruments are the predominant source of new infections[3].

Without treatment, most of the acute infections progress to chronic ones, followed by liver disease, such as cirrhosis and HCC. Alcohol abuse and the metabolic syndrome are the main cofactors influencing the progression to advanced liver disease and HCC[4]. Each year, about a third of liver transplantations are performed on patients with complications associated with the HCV infection, with decompensated cirrhosis or HCC[5]. In the next decade, an increase in the burden of hepatitis C is expected because of aging of the currently infected population[6,7]. During that period, the number of HCV-related cirrhosis cases is estimated to increase by 31% and HCC by approximately 50%[6] with an additive effect due to the occurrence of the metabolic syndrome[8,9]. Thus, HCV infection represents a major public health issue that should be addressed with strong policy interventions to effectively identify and treat HCV infected patients.

There are seven genotypes (gt 1-7) and numerous subtypes of HCV. The rate of infection and subtype prevalence are country depend. The difference between the infection rate in low and high endemic countries is about 20% (Figure 1)[10,11]. For example, North America, Western Europe and North and Australia's have the lowest HCV prevalence. Conversely, in Asian and African countries the virus prevalence is high. The highest virus prevalence was registered in Egypt, where 22% of population is infected[12]. It has been postulated that the epidemic has been caused by extensive iatrogenic transmission during the era of parenteral-antischistosomal-therapy mass-treatment campaigns before 1985[13].

MOLECULAR BIOLOGY OF HCV

HCV morphology and parameters

HCV is a small, enveloped, positive single-stranded RNA virus that belongs to the *Flaviviridae* family, genus *Hepacivirus*. Analysis of viruses from plasma and from cell culture supernatant indicated that enveloped particles are icosahedral and 56-65 nm[14,15] in diameter, while and the viral core is about 45 nm[14,15]. Viral spikes on the membrane of the virion are about 6 nm[14] and they are formed by heterodimers of E1 and E2 glycoproteins. In fact, the population of the extracellular HCV particles is heterogeneous. Particles are pleomorphic and size, buoyant density, and infectivity might differ significantly[14,15]. A large majority of particles is non-infectious. Interestingly, the buoyant densities of infectious particles isolated from serum and from cell culture medium are different. A significant amount of the particles are associated with cellular lipoproteins making that a hallmark mark of HCV[16,17]. The pattern of virus-associated lipoproteins might differ and several of those are associated with HCV most frequently: low density lipoproteins (LDL), very low-density lipoproteins (VLDL) and apolipoproteins (Apo) A1, B, C and E (Figure 2)[18]. The viral particles associated with lipoproteins are called "lipoviral particles (LVP)". More details on LVP are given in "Virus assembly and release" section. Detailed update on HCV-associated lipoproteins is given in the review by Grassi *et al*[19].

Viral genome

The genome of HCV is approximately 9600 nucleotides long (Figure 3A). It contains two highly conserved untranslated regions (UTR) 5'-UTR and 3'-UTR that are flanking a single open reading frame (ORF). Dependent on the genotype, the ORF might contain from 9030 to 9099 nucleotides and it is coding for a single polyprotein precursor of 3010 to 3033 amino acids (aa), respectively (Figure 3B and C)[20,21]. Translation occurs in the endoplasmic reticulum and it is initiated by IRES at the 5' UTR[22].

5'-untranslated region

The 5'-UTR is highly conserved including special site to control the HCV genome replication and the viral polyprotein translation. The region is 341 nucleotides long and it contains four distinct domains (Ⅰ-Ⅳ). The first 125 nucleotides of 5'UTR spanning the domains Ⅰ and Ⅱ have been shown to be essential for the viral RNA replication. The domains Ⅲ-Ⅳ composes an internal ribosomal entry site (IRES) involved in ribosome binding which can initiate viral polyprotein translation in a cap-independent manner[20]. The initiation of HCV protein synthesis requires the ordered assembly of ribosomal pre-initiation complexes, beginning with the association of the small (40S) ribosomal subunit with a messenger RNA (mRNA). The cap-independent translation begins with the 40S ribosome binding and the scanning to the initiation codon which is followed by association with the 60S ribosomal subunit to form an active 80S ribosome[21,22]. The HCV IRES is folded into highly structured domains which are called Ⅱ to Ⅳ. The mutational analysis of the IRES domains suggested that a structural integrity was necessary for an efficient protein synthesis both *in vitro* and *in vivo*[23-25].

Table 1 Overview of the size of hepatitis C virus proteins

Protein	No. of aa	aa position n ref. seq.	MW of protein, kDa
Core immature	191	1-191	23
Core mature	174	1-174	21
F protein of ARF protein	126-161		Approximately 16-17
E1	192	192-383	35
E2	363	384-746	70
p7	63	747-809	7
NS2	217	810-1026	21
NS3	631	1027-1657	70
NS4A	54	1658-1711	4
NS4B	261	1712-1972	27
NS5A	448	1973-2420	56
NS5B	591	2421-3011	66

No. of aa: Number of amino acid; MW: Molecular weight; kDa: KiloDalton; ref.seq.: Reference sequence (HCV strain H77 ; accession number NC_0041).

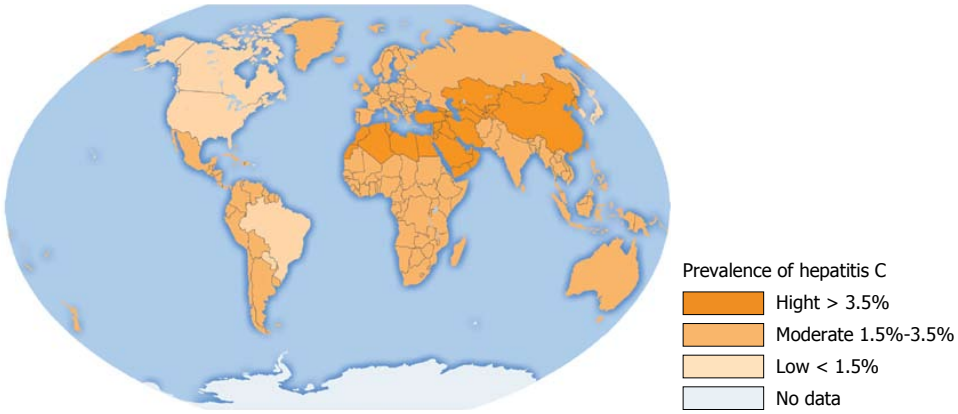

Figure 1 Hepatitis C virus infection in the World. Analysis of seroprevalence[2].

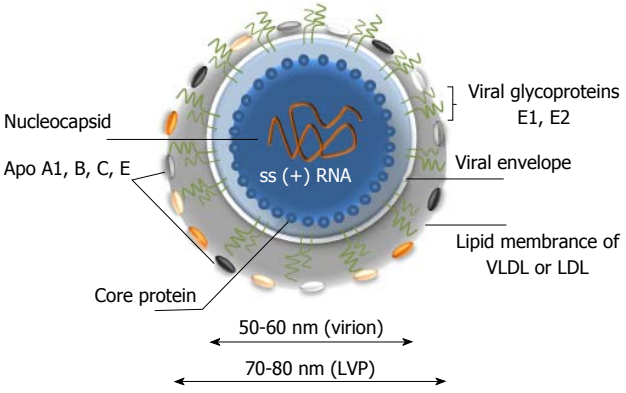

Figure 2 A model of hepatitis C virus lipoviral particle. Lipid membrane formed by low density lipoproteins (LDL) and very low-density lipoproteins (VLDL) on the surface of the virion (given in grey). Viral core is given in blue and viral RNA is shown in orange. Heterodimers of glycoproteins E1 and E2 are partially embedded in the lipid bilayer and are forming 6 nm long spikes (projections) on the surface of the virion[14-19]. As a result of association with LDL and VLDL, the morphology of the virion is not icosahedral. Depending on the viral source, the shape and size of the particles might vary.

3'-untranslated region

The 400 nucleotides long 3'-UTR region is thought to playing a crucial role in the HCV replication. The region is highly conserved and it is divided into three functionally parts: A variable sequence of 40 nucleotides, a variable internal poly (U/UC) rich tract of 30 to 80 nucleotides (depending on the HCV strains), which is followed by a highly conserved 98-nucleotide X-tail containing three stable stem loop (SL) structures called: 3'SL1, 3'SL2, 3'SL3[25].

Viral proteins

The translation occurs in the endoplasmic reticulum and it is initiated by IRES at the 5'UTR[25]. A single polyprotein precursor is processed by cellular and viral proteases into ten proteins (Figure 3B). Three structural proteins (core, E1, E2) are located at the amino-terminal part of the polyprotein and are essential components of the virions. Seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) are located in the remaining part of the polyprotein and these proteins are involved in particle morphogenesis, RNA replication and in regulation of cell functions (Table 1). It is worth mentioning, that the structural and non-structural proteins of HCV are multifunctional. A brief characterization of the protein is given below.

Core protein (p22)

The core protein of HCV is translated as an immature protein of 22 kDa, that it is composed of 191 amino acids (aa). It is excised from the polyprotein in the

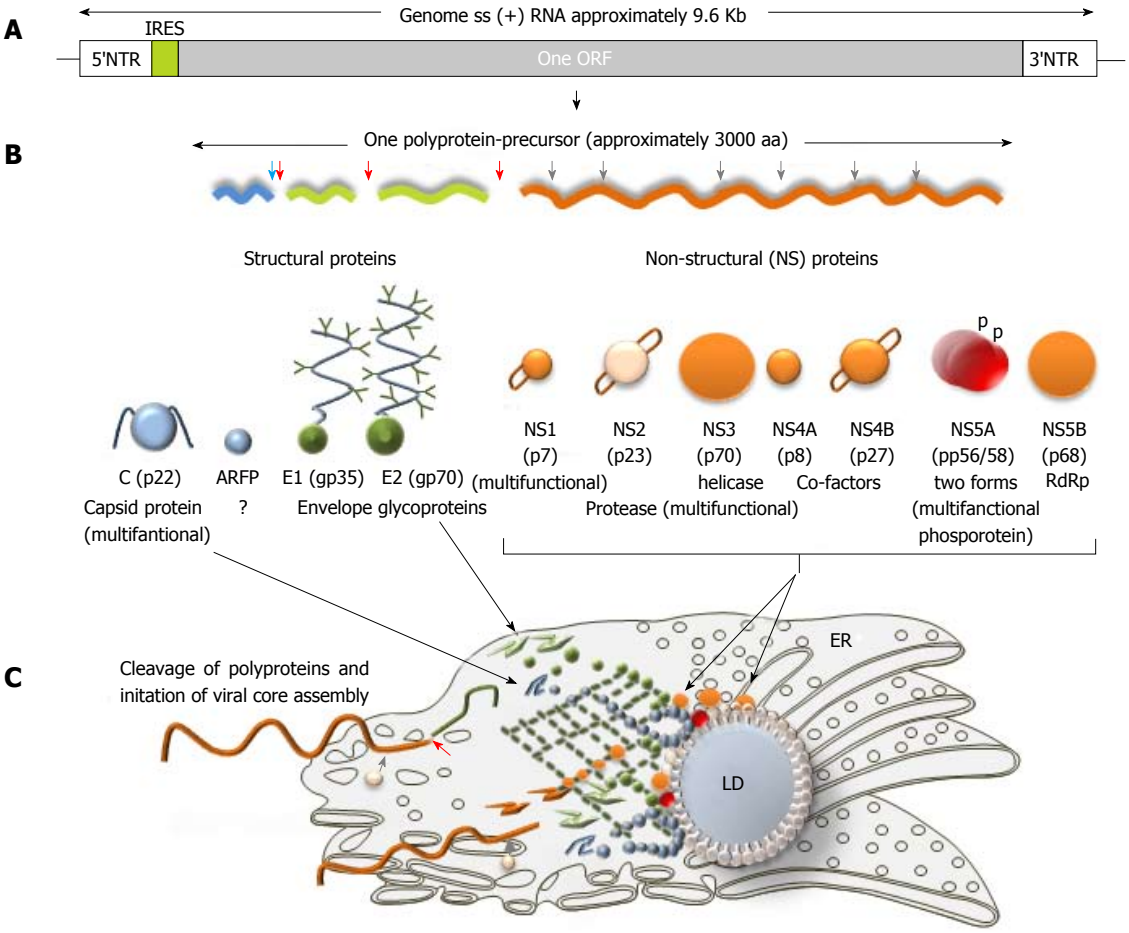

Figure 3 Hepatitis C virus genome, polyprotein precursor and the initial steps of core assembly in endoplasmic reticulum. A: Being structurally identical, the genomes of seven hepatitis C virus (HCV) genotypes demonstrated approximately 30% of sequence diversity[140]. Two non-translated regions (5'- and 3'-NTR) are flanking a single open reading frame (ORF) shown in grey; B: Polyprotein precursor composed of about 3000 amino acids translated from a single ORF. Three structural proteins - a core protein (shown in blue) and two envelope proteins (shown in green) and seven non-structural (NS) proteins NS1, NS3, NS4A, NS4B, NS5A, and NS5B (shown in orange) that are involved in cleavage, assembly, transcription and some other functions are shown. Alternative reading frame protein (ARFP) that overlaps with the core protein sequence is given in blue. Serine protease NS2 is given in pale yellow. Transmembrane fragments in non-structural proteins are shown as staples; p- phosphoprotein; C: A model of initial steps of the virion core assembly in the endoplasmic reticulum (membranous web is given in dark green) on lipid droplet (LD). Polyprotein precursor is cleaved by cellular C-terminal signal peptidase (red arrow) and cellular signal peptidase (blue arrow) to release capsid protein. NS3-NS4A serine protease cleaves the remaining proteins, while NS2-NS3 (grey arrow) protease cleaves itself. Pre-assembled cores are transported to the LD, where the final steps of assembly take place[183].

endoplasmic reticulum (ER) by a cellular signal peptidase (SP)[26]. An additional cleavage of the immature protein results in the mature 21 kDa core protein. The core protein contains three domains. The first domain spans the N-terminal region of 117 aa (aa 1-117). It contains mostly basic residues and there are two short hydrophobic regions. This domain is involved in binding to the viral RNA. The second domain between the aa 118 and 174 is more hydrophobic and less basic. This domain is engaged in the creation of links with the lipid droplet (LD). LDs are intracellular structures that are used for the lipid storage (Figure 3C)[18] . The third domain is localized between the aa 175 and aa 191 and it contains the signal sequence for the ER membrane translocation of E1 ectodomain[27]. *In vivo*, the mature core proteins are believed to form homo-multimers that are accumulated mainly at the ER membrane and can be self-assembly into the HCV-like particles[28]. During the viral capsid assembly, the protein also interacts with the HCV RNA. Besides that, the core protein poses regulatory functions during the RNA translation. Analyses of HCV core protein expression indicate that additionally it may be involved in several processes in cells such as apoptosis, lipid metabolism [HCV-related (mainly gt 3) fatty liver] and the development of HCC[29]. Interestingly, the core protein can induce the redistribution of LDs by the regeneration and the regression of these organelles in specific intracellular domains[30]. This phenomenon is also linked to the assembly of HCV particles. LDs are associated with the HCV core protein at the second domain and the interaction seems to trigger steatosis[30]. It has been shown, that if the core protein is mutated, the association with LDs might be disrupted. A decrease of this association has a negative effect on the HCVcc production. The HCV core protein was also shown to be involved in the Ca^{2+} regulation[31]. Thus, it is not only a

basic structural element of HCV, but it is an active player in a significant amount of additional processes, including signaling pathways regulation[17,30,31].

Envelope proteins E1 (gp35) and E2 (gp70)

Glycoproteins E1 (gp35 kDa, 192 aa long) and E2 (gp70 kDa, 363 aa long) are type 1 transmembrane proteins that are forming the envelope of the virus particle (Figures 2 and 3B). E1 an E2 are cleaved from the precursor in ER by the cellular SP. The glycoproteins contained large hydrophilic ectodomains and 30 aa long transmembrane domains (TMD). The TMD is responsible for an anchoring of the envelope proteins in the membrane of the ER and their ER retention[32]. The ectodomains of both glycoproteins are heavily glycosylated. The E1 has 4-5 and the E2 - 11 putative N-glycosylation sites, respectively. Interestingly, the glycosylation sites are rather conserved in each genotype[33], but the number of glycosylation sites varies between the genotypes. It's worth mentioning, that some of the glycan's are engaged in folding and formation of the E1-E2 heterodimer complexes on the surface of the virion. These complexes are essential for the interaction with cellular receptors and helps to promote the virus-to-cell fusion[34].

The HCV E2 contains two hypervariable regions HVR 1 and HVR2 which are the most mutable parts of the HCV genome. The first 27 amino acids of E2 are attributed to the HVR1 sequence. It is suggested that a prominent heterogeneity of the region could help the virus to evade from the immune system pressure and to develop a chronic infection[35]. It is worth mentioning, that an infectious clone lacking the HVR1 was still capable to infect chimpanzee, but with a largely reduced efficiency, thus supporting the HVR1 engagement in the cell infection[36]. The HVR2 includes seven aa (91-97) and this region was shown to contribute to the HCV E2 receptor binding[37]. The high variability of the HVRs reflects the exposure of these domains to HCV-specific antibodies. In fact, the E2-HVR1 is the most frequent target for neutralizing antibodies[38,39]. However, the combination of a viral mutation with the selective pressure of the humoral immune response leads to the viral escape *via* epitope alterations[40]. Moreover, the association of virions with lipoproteins and the presence of a glycan shield (possessed by the E1-E2 heterodimers), reduce the antibody access to the "sensitive" neutralizing epitopes[24,41,42]. This protection is representing a serious obstacle on the way to obtain broadly neutralizing antibodies.

Non-structural protein NS1 (p7)

The NS1 (also called "p7") is a small 63 aa long protein. It is located between E2 and NS2 proteins and it is linked to both structural and non-structural proteins. Its cleavage is mediated by the cellular SP. Interestingly, the E2-p7-NS2 precursor can also be detected[43,44]. It is suggested, that such a precursor may regulate the kinetic of the HCV infection; however, up to now the precise role of this protein in the HCV life cycle remains obscure. NS1 has two transmembrane domains (TMDS) embedded in the ER membrane and the C-terminal TMD of NS1 can act as a signal sequence to promote the translocation of NS2 to the ER lumen[45]. The protein can also form the ion channel which has an important role in the HCV infection. This channel can be blocked by the antiviral drug[45]. The recent studies demonstrate that NS1 acts to prevent the acidification which is required for the production of HCV particles[46]. In addition, the protein appears to be essential during assembly and release of infectious particles as shown on different genotypes[47]. Based on that, NS1 may be considered as a potential target for new antivirals[48,49].

Non-structural protein NS2 (p23)

The NS2 is a hydrophobic transmembrane protein of 23 kDa composed of 217 aa. It is a cysteine protease which is required for the HCV infectivity[50]. The N-terminal residues of NS2 can form 3 or 4 transmembrane helices which are inserted in the ER membrane. The crystal structure has been determined. The C-terminal residues of NS2 with the 181 aa long N-terminal domain of NS3 play an important role in the function of the NS2/3 cysteine protease[51]. The NS2/3 protease is spanning from the amino acid 810 to amino acid 1206. It has been shown that the NS3 zinc-binding domain could stimulate the activity of the NS2 protease[52]. The well-known function of NS2 is the auto-cleavage at the NS2/3 site[53]. Oem *et al*[53] suggested that the expression of HCV NS2 results in the up-regulation of the fatty acid synthase transcription. In fact, this may implicate the role of NS2 in the prompting HCV induced steatosis[54]. There data are in favor of the protein engagement in the viral assembly and release[54].

Non-structural protein NS3 (p70)

The NS3 is a 70 kDa protein composed of 631 aa. It is cleaved at its N-terminus by the viral NS2/NS3 autoprotease. The C-terminal part of NS3 (442 aa) has an ATPase/helicase activity, and it catalyses the binding and unwinding of the viral RNA genome during the viral replication[55,56]. The N-terminal part (189 aa) of the NS3 protein has both serine protease and NTPase activities[56]. The NS3 along with the non-structural protein NS4 is involved in cleavage of the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions[57,58]. A direct interaction between NS3 and NS5B is mediated through the protease domain of NS3. It is suggested, that these proteins may act together during the HCV replication[59]. The HCV protease NS3/4A can also cleave some cellular targets involved in the innate immunity, such as MAVS (an antiviral signaling protein), which can indirectly activate the nuclear factor-kappa B (NF-κB) and the IFN regulatory factor 3 to induce type Ⅰ interferon[60,61]. The protein NS3/NS4A can interfere with the adaptive immunity. In addition, the NS3/NS4A protease is

essential for the viral infectivity, thus, it is a promising target for antivirals[61-64]. In 2011, two potent NS3/NS4A inhibitors, boceprevir[61] and telaprevir[62] were approved by FDA and EMA and were used in combination with IFN α and ribavirin. However, several resistance-associated mutations within the NS3/NS4A coding region have been observed.

Non-structural protein NS4A (p8)

The NS4A is an 8 kDa proteins that is composed of 54 aa. As indicated above, it is a co-factor required for the NS3 protease activity. The N-amino-terminal domain of NS4A is hydrophobic and the deletion analysis shows that NS4A was required for the ER targeting of NS3[65-68]. In addition the NS4A interaction with NS5A is essential for the phosphorylation of NS5A[68]. Besides its important role in HCV replication, NS4A can also contribute to the viral pathogenesis by influencing some cellular function[66]. Interestingly, NS4A was detected not only on the ER, but also on the mitochondria either alone or together with NS3 in the form of NS3/4A polyprotein. It has been shown, that the NS4 expression altered the distribution of mitochondria and caused damage which leads to the host cell apoptosis[66].

Non-structural protein NS4B (p27)

The NS4B is a 27 kDa protein of 217 aa. It has four membrane spanning domains which are important in recruitment of the other non-structural viral proteins[68]. By electron microscopy, it has been shown, that NS4B induced a tight structure termed "membranous web". All viral proteins were found to be associated with this membranous web that formed a viral replication complex ("factory") in HCV infected cells[67]. The polymerization activity and lipid modification of NS4B are important for the induction of the specialized membrane structure involved in the viral RNA replication[68,69]. NS4B is likely not engaged in the virus replication, but may contribute to the assembly and the release of the virus particles[69].

Non-structural protein NS5A (p56/p58)

The NS5A is a 56 kDa phosphoprotein of 485 aa (Figure 3). In HCV infected cells, the protein is present in two differently phosphorylated forms: phosphorylated (56 kDa) and hyper phosphorylated (58 kDa). NS5A is multifunctional and it contributes to the HCV replication, virus pathogenesis, modulation of cell signaling pathways, virus propagation and the interferon response[70,71]. The N-terminal amphipathic helix of the NS5A protein is conserved in different genotypes and it is necessary for the membrane localization[72-76]. Mutations in NS5A enhanced the capacity of the sub genomic HCV RNA replication in cell culture systems[77]. It was shown that the N-terminal part of NS5A contained a new zinc-coordination motif which affected the HCV replication[70]. NS5A has a potential role in mediating the IFN response. It contains a so-called "interferon-α sensitivity-determining region" (ISDR, aa 237-276)[78]. ISDR can be used to predict the resistance and the sensitivity of HCV to the IFN treatment. NS5A was also showed to be associated with a variety of cellular signaling pathways including apoptosis[70]. NS5A is already used as a target for the direct acting antivirals.

NS5B protein (p66-68)

The NS5B is a 591 aa long protein and it is located at the C-terminus of the precursor. NS5B is a RNA-dependent RNA polymerase containing the GDD motif in its active site[79]. The NS5B initiates synthesis of the HCV negative-strand RNA. The crystal structure of NS5B showed a typical 'right hand' polymerase shape with finger, palm and thumb sub domain. Since NS5B lacks the "proof-reading'' function[79], numerous mutants might be generated during transcription. Because of its key role in the virus replication, the NS5B protein is considered as a potential target for the antiviral drug[80].

F protein, ARFP

In addition to ten proteins described above, the frameshift (F) or alternate reading frame protein (ARFP), or "core+1" protein has been reported[81-83]. The ARFP is the result of a -2/+1 ribosomal frameshift between codons 8 and 14 of the adenosine-rich region encoding the core protein. ARFP ends have different stop codons depending on the genotype. Thus, its length may vary from 126 to 161 amino acids. In the case of genotype 1a, the protein contains 161 amino acids. However, the situation with ARFP is more complicated, since alternative forms of this protein such as ARFP/DF (double-frame shift) in genotype 1b, and ARFP/S (short form) were recently described[83]. ARFP is a short-living protein located in the cytoplasm[84] in associated with the endoplasmic reticulum[85]. Detection of anti-ARFP antibodies in sera of HCV-positive subjects indicates that the protein is expressed during infection[86], but is likely not involved in the virus replication. Some findings suggested engagement of ARFP in the modulation of dendritic cells function and stimulation of the T cell responses[87]. The implication of ARFP in the viral life cycle remain to be elucidated.

HCV TRANSMISSION ROUTES

The HCV in blood and blood products is the main source of infection. However, the transmission routes of HCV might be different and ountry dependent. The iatrogenic transmissions are: the blood transfusion of unscreened products[88], the transfusion of clotting factors or other blood products[89,90], the organ transplantation, the reuse of medical instruments used in invasive settings (*e.g.*, needles, infusion sets, syringes, catheters) in Egypt before 1985, hemodialysis, endoscopy, intravenous drug use[91-95]. The sexual way of transmission is controversial. Nevertheless, the risk may increase when favoring conditions such as sexually transmitted infections, the frequencies and the type of sexual activity are taking

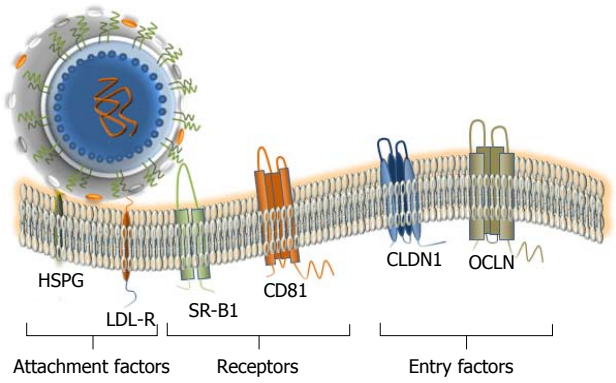

Figure 4 Attachment factors, receptor and entry factors utilized by hepatitis C virus. At least six membrane proteins are essential for the virus attachment and entry. Heparan sulfate proteoglycan (HSPG) - a type of glycosaminoglycan (GAG) and low density lipoproteins (LDL)-receptor that is promoting the LDL endocytosis are considered as binding factors for hepatitis C virus (HCV). Scavenger Receptor class B member 1 (SR-B1) and CD81 that are ubiquitously expressed on the cell surface, are considered as true receptors. The receptor–viral cargo complex is then moving to the cell-cell contacts (not shown), where the interaction with the tight-junction proteins Claudin 1 (CLDN1) and Occludin (OCLN) takes place. Both proteins are required at the late stage of the entry. Additional interaction partners that are likely engaged in the HCV entry are described in the text. The docking of the LVP to the cellular membrane is shown.

place[94-101].

The rate of HCV mother-to-child transmission is about 4.3%, but it is much higher, 22.1% among the human immunodeficiency virus (HIV) co-infected mothers[102,103]. The mother-to-child transmission generally occurs at delivery, but also *in utero*, if associated with high risk factors, such as high maternal HCV RNA levels and/or HIV co-infection. Interestingly, vaginal *vs* cesarean section delivery, amniocentesis and breast-feeding did seem to increase the transmission risk[102-108]. Because of a passive transfer of anti-HCV antibodies at, or after 18 mo of ages, perinatal transmission diagnosis as serum HCV RNA and/or anti-HCV antibodies should be performed twice in infants (6 mo and 1 year of age)[109] during this period, as recommended by the European pediatric HCV network[110]. It should be emphasized, that the INFL3 CC (IL28B) genotype is likely associated with a spontaneous clearance of the HCV genotype 1(gt1) infection[104].

According to the World Health Organization, thousands of new cases of the HCV infection (as a result of an occupational exposure *via* skin injury), are registered annually[111]. Most of these cases occurred during surgery in emergency departments, and routine medical procedures[112]. Seroconversion rates in infected individuals are ranging from 0% to 10.3% (+/- 0.75%)[113,114]. For instance, after accidental needle stick, seroconversion rate was reported as 1.8%[115]. During a 5-year period (2008-2012), a total of 16 HCV outbreaks resulting in 160 outbreak-associated cases and more than 90000 at-risk persons notified for screening were reported by the Centers for Disease Control (CDC) and Prevention[115]. Among dentists[116] and surgeons[117], an eye protection should be reinforced because of possible HCV transmission by splashes of blood and other body fluids[117]. Cosmetic procedures and/or acupuncture, as well as circumcision are extensively associated with HCV transmission, but the risk seems to be small[118,119]. Lacking of sterile techniques and/or nonprofessionally performed tattoos or piercings, especially before the mid-1980s, raised the HCV transmission rate significantly[120-126].

HCV GENOTYPES/SUBTYPES DEPENDENT TRANSMISSION

The HCV genome demonstrated a prominent genetic diversity. Seven genotypes (gt 1-7) and 67 subtypes (a, b, c, *etc*.) have been described. The genotypes are characterized by a distinct geographic distribution and clinical manifestations[127,128]. For example, the genotype 1 is prevalent in Americas, Japan and Europe[129]. Indeed, the HCV genotype prevalence also differs according to the transmission route and the age of infected individuals. For instance, the HCV gt 3a and 1a are highly represented among intravenous drug users and the HCV gt 1b is frequent among patients who received blood transfusions[130-134]. In Japan, the HCV gt 1b is the most prevalent[1] while, the infected population is generally older than in the United States[2], and an iatrogenic transmission is a predominant risk factor for the HCV acquisition.

HCV LIFE CYCLE

Binding and entry

The HCV entry initiates the viral replication cycle. Virus that is a complex and multi-step process that is not completely understood. However, the main principal steps and the principal cell surface interaction partners are known. At least, four cellular entry factors and two specific receptors are required for a successful virus entry (Figure 4). Since many interactions should take place, the binding to the cell surface is a relatively slow process which should be well coordinated[135-141]. The initiation of HCV entry seems to be similar to that of many other viruses, that utilize for attachment the glycosaminoglycans (GAGs)[141]. Earlier, it was proposed that E1 and E2 are involved in these interactions[141]. However, the HCV particle interacts with lipoproteins and in this regard, it is more likely that the apolipoprotein E is responsible for the interaction with GAGs[141]. Furthermore, due to the nature of the viral lipoprotein particle, it was proposed that the LDL receptor is required in the initial stage of attachment. However, this interaction leads the particle to a degradation pathway[141]. After the completion of docking, the viral particle interacts with specific cellular receptors. It was shown that the envelope glycoprotein E2 interacts with two receptors a scavenger receptor B1 (SR-B1), and with CD81 (tetraspanin family protein -TSPAN28)[142-144]. It is likely, that interaction of HCV with SR-B1 comes first[142]. In this regard, it remains to be determined whether SR-B1

and CD81 form a complex that facilitates the entry. It is noteworthy that the interaction with CD81 alone can activate signaling pathways that may be important for the viral infectious cycle[141] and the interaction between CD81 and the E2 glycoprotein appears to be essential for initiating the adsorption[141]. Next, the receptor complex with attached virion is moving to the tight junction, where the interaction with the proteins claudin-1 (Cldn1) and occludin (OCLN) is likely taking place. However, there is no experimental evidence indicating a direct interaction between the viral particle and the tight junction proteins. Thus, these proteins cannot be considered as classical receptors. Finally, other cellular factors such as epidermal growth factor receptor[145] and the Niemann-Pick C1-like 1 cholesterol uptake receptor[146] are likely involved in the HCV entry. It has been shown that CD81 and Cldn1 proteins may interact with the surface of hepatocytes[147], suggesting that CD81-Cldn1 form a complex that is implicated in the HCV entry. Although the tight junction proteins are involved in the HCV entry, the role of cell polarization in viral entry remains controversial. Interestingly, a live imaging of the fluorescent HCV particle in Huh7.5 cells showed that the particle was first attached on filopodia and then migrated towards the main body of the cell using presumably an actin-dependent transport mechanism[148]. It is important to note that the hepatocyte is a polarized cell with multiple apical and basolateral poles presenting a unique organization that is observed only in the liver. Another characteristic of HCV is its ability to spread by cell-to-cell contacts[149]. The essential role of the E1, E2 transmembrane domains in the viral entry[150] and the involvement of different regions of the ectodomain of E2 in the viral assembly were demonstrated[151]. It has been shown that glycans on the envelope proteins may be involved in different stages of the viral infectious cycle[42,152,153]. Furthermore, the N-linked glycan shield of the viral glycoproteins is essential to protect against the neutralization of the virus by antibodies[154].

An additional interaction partner of the CD81 receptor, called "EWI-2wint" was identified[155]. It was demonstrated that EWI-2wint is a natural inhibitor of the HCV entry, which by an interaction with the CD81 receptor can prevent CD81-E2 interaction. Later on, the regions involved in the interaction between CD81 and EWI-2wint were identified[156] and studies in living cells at a single molecule level proved that EWI-2wint reduces the mobility of CD81 at the plasma membrane[157,158]. Finally, it was reported that the enrichment of the plasma membrane in ceramides has an inhibitory effect on the viral entry[159].

Post-entry events

It was shown by a traffic monitoring that after binding to the cells surface, the virus particles enter into the cells using clathrin dependent endocytic pathway[160,161]. The viral particles are transported to the early endosomes expressing RAB5A where the merger took place. This process requires an acidification of the compartment[162]. The virus capsid is then released and destroyed, while the viral RNA is released to the cytoplasm. The RNA is used for both processes the replication and the polyprotein translation (Figure 5). The RNA translation occurs in the endoplasmic reticulum (ER) and it is initiated by a binding of the 5'UTR IRES to the ribosome. The primary translation product is approximately 3000 amino acid long polyprotein precursor which contains structural and non-structural proteins of the HCV. Then, the polyprotein is cleaved by the host and viral proteases into structural and non-structural proteins.

Several studies evidenced that the HCV infection results in ER stress and autophagy responses and that HCV can regulate the autophagy pathway. In fact, the autophagy machinery is required to initiate the HCV replication and suppression of autophagy inhibits this process[163-171]. Interestingly, it has been demonstrated that HCV induces autophagosomes *via* a Class Ⅲ PI3K-independent pathway and uses autophagosomal membranes as sites for its own RNA replication[171]. For the HCV RNA replication, polarized positive HCV RNA genome synthesizes a negative strand by the NS5B RNA-dependent RNA polymerase. The newly synthesized negative RNA strand may further act as a template to synthesize the positive strand of the viral RNA[172,173].

Viral assembly and release

A assembly of the virion is another multi-step process and certain steps of it remains obscure. It is known that the particle assembly occurs within the ER in a close proximity or directly on the surface to LD[170] (Figure 5). After the proteolytic cleavage of the polyprotein precursor, NS5A initiates the early phase of the viral particle formation by the interaction with the core protein and its C-terminal serine cluster determines the NS5A-core protein interaction[174].

The viral RNA released from the viral core is recruited to the replication complex within the membranous web. The HCV RNA-dependent RNA polymerase (NS5B) has no proofreading mechanism to correct errors during the strand synthesis. This propensity for error during replication results in an accumulation of the HCV quasispecies, those are closely related, but genetically somehow distinct. The newly synthesized positive-sense viral RNA is transported to the site of the core assembly and is encapsulated. However, the mechanism that engaged in RNA delivery to the capsid during assembly is not known. The nucleocapsids with RNA are presumably enveloped by budding into the lumen of the ER. So, after the nucleocapsid formation, it is associated with the envelope protein forming an immature particle and secreted from cell through the cytoplasmic membrane[173,174]. However, it remains unclear how the coupling with lipoproteins and apolipoproteins is regulated, since the pattern of lipoproteins on the virus particle might differ significantly. Next, the virions are

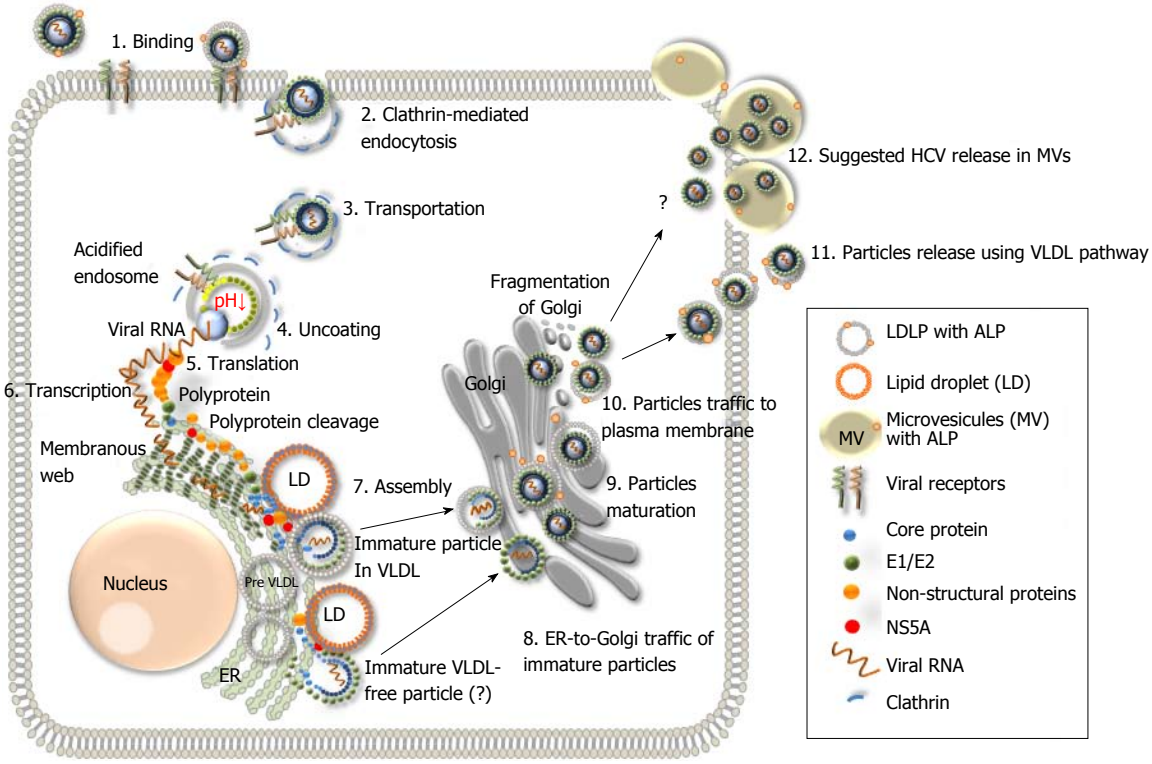

Figure 5 Schematic representation of the hepatitis C virus life cycle. (1) The lipoviral particles (LVP) binds to entry factors and receptors on the surface of hepatocyte; (2) The virus enters into the cell by a clathrin-mediated endocytosis; (3) Transportation of the virus in endosome; (3) Acidification of endosome, uncoating of the virion and dissociation of the viral core; (4) Release of the viral RNA; (5 and 6) Translation and replication of the viral RNA in the ER in the convoluted membrane structure called the membranous web (shown in dark green); (7) Cleavage of the protein precursor by cellular and viral proteases, assembly of the core on the surface of the lipid droplet (LD) and recruitment of the newly synthesized viral RNA to the viral core during the formation. The mechanism of the viral RNA recruitment to the site of the assembly is not known. It is also unclear whether the core maturation is finalized at this stage; (8) The viral nucleocapsid egresses into the lumen side of the ER, likely interacted with very low-density lipoproteins (VLDL) and translocated to Golgi. It is not clear if some VLDL-free particles can also be produced (?). Details of traffic machinery are not well defined; (9) Final maturation of viruses in Golgi and virus induced partial fragmentation of Golgi; (10) The hepatitis C virus (HCV) complex with VLDL is directed to the plasma membrane using the VLDL secretory pathway; (11) Release of particles from the cell (adapted from Ref.[16,19,135]); and (12) The HCV release in MV cannot be excluded as another pathway for the virus release during active replication.

transported through the Golgi compartment to finalize the maturation. Recently, it has been shown, that HCV can induce the fragmentation of Golgi[175]. Thus, it might be another important indication of the virus-induced pathogenesis. However, if the fragmentation of Golgi is significant, it may cause not only destructions of Golgi, but also provoke the cell death. Definitely, it might be not advantageous for the virus. It cannot be excluded, that this phenomenon might take place predominantly during the active replication of the virus. In this regard, it might be useful to investigate the HCV-induced Golgi destructions, as a possible trigger of liver necrosis.

Virus release and extracellular particles

The detail mechanism of the HCV release requires more comprehensive investigation. It is well established that secretion of the HCV particle depends on both the VLDL and the apolipoprotein (apoB) presence[176-187]. So, when the maturation is finished, the virions are transported to the plasma membrane using the VLDL pathway[145]. Particles isolated from cell-cultures and from patient sera are pleomorphic, immature and most of them are non-infectious[170]. In this regard, in the virus preparations from human plasma examined by electronic microscopy (EM), it was difficult to reveal "perfect" mature particle[170]. The association with fragments of cellular membranes (or other cellular debris) and/or an irregular lipoprotein content might be the reasons of the problem. Anyhow, it is evident, that a classical "assembly-maturation-release" processing line of HCV is facing problems. A diverse buoyant density of infectious particles is reflecting the problems. The analysis of LVP from plasma by sucrose gradient centrifugation demonstrated, that the buoyant density of the virus-contained fractions is diverse (1.10 g/mL to 1.16 g/mL)[170]. Positioning of virus-containing fraction on the gradient depends on the initial material that was used for the virus isolation. The infectious particles isolated by isopicnic centrifugation from cell-culture were mostly present in the fraction with a density 1.14 g/mL and examined by cryo EM, when were about 60 nm in diameter[170]. Significantly more virus-associated cellular impurities including lipoproteins, are associated with viruses isolated from patient sera and that influence the virus density and the particles are significantly less dense[177,178]. It is considered that the observed density

fluctuation is a result of irregular content of lipoproteins tightly associated with virion. In fact, this diversity might have an additional reason. It cannot be excluded that *in vivo* the virus particles might in addition be transported by extracellular vesicles. In fact, the size of putative transporters might be large enough to carry not a single, but numerous viral particles. For example, membrane derived microvesicles (MVs) can be 500-1000 nm in diameter, so as large as lipid droplets[184,185]. MVs are present in the fractions of different densities, indicating a diversity of diameter and/or cargo[186].

Analysis of cell supernatant by gradient density centrifugation demonstrated that the MVs subpopulations are different, depending on the cell line. It is known, that MVs are intensively released by primary hepatocytes and immortalized cells of hepatic origin[186]. The release from hepatocytes might be increased by an undergoing lipotoxicity[187]. Thus, MVs are actively used by the hepatocytes as delivery system and the amounts of MVs might be increased in response to lipotoxicity and likely other complications. In this regard, we speculate that during acute phase of infection (or high rate of replication) the virus can trigger the MV release and that might be an additional pathway for the virus. How MVs containing virus particles can influence HCV distribution along the density gradient? In fact, MV containing different amount of viruses may contribute to observed diversity of buoyant densities of HCV, especially when plasma (or serum) is investigated.

It is known, that the diffusion of spherical particle through a viscous liquid is dependent on the particle diameter. It is described by Stokes-Einstein equation:

$$D = \frac{kT}{6\pi\eta r}$$

D = diffusion constant, k = Boltzmann constant, T = temperature (K), η = solvent viscosity, r = radius of spherical particle.

Another parameter that influences the diffusion is the mass of spherical particle and the equation counting these parameters looks as follows:

$$nD = \frac{6\pi\eta\alpha}{M}$$

where: D = diffusion; α = radius of the spherical particle; M = mass of the particle; η = viscosity of the medium.

So, the diffusion of a spherical particle is decreased with the increase of diameter and mass. Thus, if MVs besides the regular cargo (mRNA, miRNA, modulators, peptides, proteins) have in addition a different particle load, it might explain why MVs of the same sizes might be recovered in fractions with different densities, and vice versa. Thus, when supernatants (or plasma) are examined by density gradient centrifugation, MVs of different size and different virus loaded, can increase the amount of virus-containing fractions. The expected distribution of lipoproteins, LVP from human plasma in a sucrose gradient and the suggested positioning of MVs with different virus load are given below (Figure 6, adapted from[170] with modifications).

What might be the advantage, for the virus to use the MVs secretory pathway? Since the MV are released by budding from the plasma membrane, it allows the virus to acquire additional protection from the "rough environment". Second, if MV is loaded with numerous particles, it might be the way to increase the probability of a successful infection of distant cells by creating a high "local MOI" after MV distruction. That effect might be even more prominent, if the MV loaded with viruses would be endocytosed by the cell. We suggest, that the MV pathway might be used during active virus replication of the virus. The possible implication of MVs for the virus delivery might be interesting to investigate using *in vitro* models.

MODELS TO STUDY HCV

The progress in HCV research is completely dependent on model systems[188]. The most frequently used models (Figure 7) for investigation of HCV are described below.

HCV replicon systems

The HCV genome was identified in 1989 by cloning it from infected chimpanzee, while in humans the amounts were too low for detection[189]. The first complete full-length HCV cDNA clone was constructed from the HCV strain H77 (genotype 1a). The HCV RNA transcribed from this clone was found to be infectious after intrahepatic injection in a chimpanzee. The HCV viremia was detected at week 1 and increased from 1×10^2 genomes/mL to 1×10^6 genomes/mL at week 8[190,191]. Then, several full-length HCV RNAs were synthesized and were shown to be infectious in chimpanzees[190-193]. However, these HCV clones were found to replicate inefficiently *in vitro*. This limitation was resolved by the group of Prof. Ralf Bartenschlager, when subgenomic HCV replicon, cloned from the HCV genome was constructed[73]. After transfection of this subgenomic clone into the Huh7 cells, it was found that in drug-resistant cells a high-level of the HCV RNA replication occurred. The replication of the HCV subgenomic replicon was confirmed in several cell lines, but the hepatocarcinoma cell line Huh7 was the most permissive. The interferon treatment of the replicon inoculated Huh7 cell clones, made them more permissive to support both subgenomic and full-length HCV replication, (these so-called cured cell lines are Huh7.5 or Huh 7.5.1 cells)[15,194]. Afterwards, several studies demonstrated that the virus and host factors were important for the HCV replication in cells. Some mutations in the wide-type (wt) consensus sequence efficiently enhanced the HCV replicon replication, some mutations in the non-structural protein efficiently contributed to the replication and the adaptation to the host cells[195-198]. The mechanism of improved replication caused by adaptive mutations is still unknown. Although

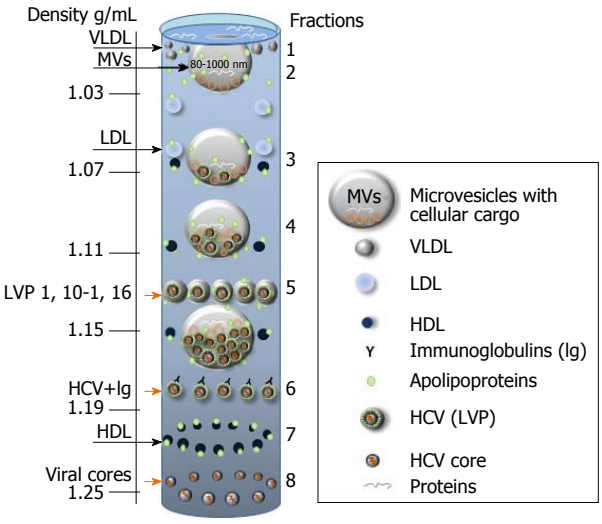

Figure 6 Putative distribution of lipoproteins (LDL, VLDL, HDL), lipoviral particles and viral cores after density gradient centrifugation of plasma from infected individual. The picture is based on the known buoyant densities of analyzed elements[15,170,184-187]. The density values (g/mL) are given on the left. The initial material that has been used for the virus isolation, contributes greatly to the associated lipid content of the virions and, as a consequence, it influences the buoyant density[177,183-187]. Because of an irregular protein to cholesterol amounts, high density lipoproteins (HDL) of 5-15 nm in diameter can be detected in the different fractions (1.06-1.21 g/mL). The family of HDL is given as dark blue spheres. The buoyant density of microvesicles (MVs) may vary from 1.02 g/mL to 1.16 g/mL, depending on MVs diameter and cargo (miRNA, tRNA, fragments of mRNA and proteins). If hepatitis C virus (HCV) is released in MVs with similar diffusion parameters (size and mass), the buoyant density of vesicle should be viral cargo-dependent. Fractions 1: Single cellular or viral proteins "floating" on the surface, very low-density lipoproteins (VLDL, diameter 50-75 nm, density 0.95-1.006 g/mL); Fraction 2: Low density lipoproteins (LDL, diameter 18-25 nm, density 1.019-1.06 g/mL) and virus-free MVs with a low cellular cargo content; Fraction 3: MVs that carry few viral particles, possible overlap with LDL and HDL; Fraction 4: MVs with a higher viral load, possible overlaps with HDL and with densely loaded virus-free MVs; Fraction 5: Lipoviral particles (LVP, diameter 60-80 nm, density 1.10-1.16 g/mL) and MVs with a significant viral and/or mixed load. Overlaps with HDL, virus-loaded MVs and "dense" virus-free MVs are possible; Fraction 6: Lipid-free virions with attached immunoglobulins, possible overlap with HDL; Fractions 7: HDL with a high protein load; Fraction 8: Non-enveloped viral cores that might overlap with dense HDL. Small grey spheres - VLDL. Light blue spheres - LDL. Orange arrows indicated fractions of the gradient that are most likely to contain viruses and viral cores.

these replicons with adaptive mutations could replicate with a high efficiency, they were not able to produce infectious particles *in vitro*.

This problem was solved with development of the genotype 2a infectious clone JFH-1. A selectable HCV replicon was constructed containing the full-length HCV cDNA and showed to produced infectious particles *in vitro* and *in vivo*[199]. Based on this infectious clone, several different genotypes chimeric clones (genotype 1a, 1b, 2a and 3b) where the non-structural genes have been replaced by those of JFH-1 were constructed and were shown to be infectious in Huh-7 cells. The most efficient construct is the genotype 2a/2a clone which consists of J6CF and JFH-1 derived sequence[200]. The HCV replicon is remarkably valuable for studying the HCV replication and for testing of new antiviral drugs. The HCV subgenomic replicons containing reporter genes (luciferase, secreted alkaline phosphatase and chloramphenicol transferase) facilitated the study of the HCV infection. This high-throughput screening assay allowed the visualization and tracking of the HCV replication complex in living host cells without affecting the HCV replication[47,201].

HCV pseudotype virus particles

The HCV pseudotyped particles were constructed with chimeric genes expressing HCV (genotype 1a) envelope E1 and E2 proteins (HCVpp) and the transmembrane and cytoplasmic tail of vesicular stomatitis virus G protein. The pseudotyped particles allowed a detailed study of the role of HCV receptors in the early steps of HCV infection (adsorption, and viral entry) in Huh7 cells and primary human hepatocytes[202-204]. The system is useful for testing of new antiviral drugs[203].

The HCV subgenomic replicon and the HCV pseudotyped particles (HCVpp) have markedly improved studies on the HCV infection. However, the high replication rate in cells was not correlated with amount of released infectious virions. However, the main disadvantage of these *in vitro* systems is that the viruses released from these cells are not infectious. The major reason of that might be that the adaptive mutations enhancing HCV replication rates are deleterious for HCV particles assembly and release. The problem was solved when a genotype 2a subgenomic replicon was established in the Huh-7 cell lines. It contained a full-length genotype 2a clone JFH-1 derived from serum of a Japanese patient with fulminant hepatitis. The replication rate was about 20 times greater than Con1 (gt1b) when transfected into Huh-7 cells. In addition, this replicon could replicate efficiently without amino acid mutations[47]. In 2005, Wakita *et al*[200] continued to transfect this JFH-1 clone into Huh-7 cell lines and found high levels of intracellular HCV RNA replication and protein expression. By means of passages in infected cells, they demonstrated that JFH-1 transfected cells had continuous HCV replication. In this study, it was also shown, that the HCV viral particles (HCVcc) secreted in the culture supernatant had a density of approximately 1.15-1.17 g/mL. The released particles have a spherical morphology and a diameter of about 55 nm. The JFH-1 infectivity could be neutralized by anti-CD81 antibody, suggesting the important role of CD81 in HCV entry. In addition it was found that the secreted particles could also infect chimpanzee[191]. Later, several groups observed that JFH-1 could efficiently infect and replicate without adaptive gene mutation in different cell types and HCV infectious particles could be produced in the culture supernatant[25,199,205-209]. Zhong *et al*[192] established a robust highly infectious *in vitro* system with JFH-1 and Huh-7.5.1 cells. The advantage of this model, compared to that established by the group of Prof. Wakita, was the possibility of HCV to undergo serial

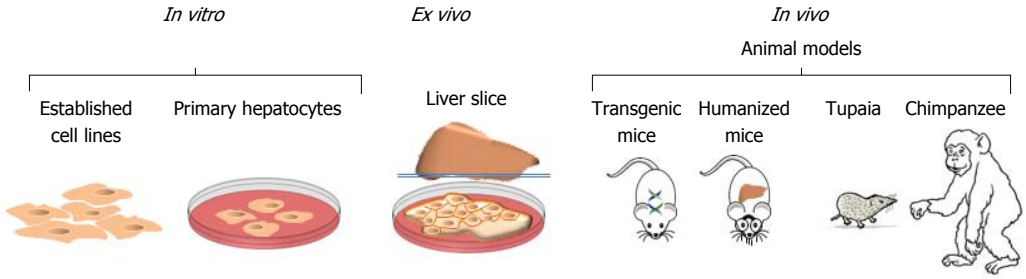

Figure 7 ***In vitro*, *ex vivo* and *in vivo* models to study hepatitis C virus.**

passages without losing of infectivity. Although JFH-1 *in vitro* system provides a powerful tool to study antiviral drugs and vaccines, it has some limitations: HCV JFH-1 derived from an exceptional case of HCV-related fulminant hepatitis belongs to genotype 2a which is not the dominant genotype worldwide. That is why since 2005, different chimeric JFH-1 clones were constructed to transfect Huh-7.5 cells, based on intergenotypic genotype replicons such as J6/JFH-1 (genotype 2a/2a), S52/JFH1 (genotype 3a/2a) and sa13/JFH-1 (genotype 5a/2a)[209,210], but they are less infectious than HCV JFH-1. The reason why JFH-1 is more permissive to Huh-7.5 cell lines than other genotypes is still not fully understood. In summary, the HCVcc model allowed to understand the entire life cycle of HCV and that serves as a background for development of numerous antivirals[211].

Animal models

Chimpanzee could be a good model to study the HCV infection[194,212]. However, these animals are rare, difficult to handle, very costly and limited in their use by ethical issues[212]. Few data obtained using chimpanzee model indicated a specific immune response at the acute phase of infection, resulting in inconsistent specific neutralization and viral eradication.

Tupaia (Treeshrew, *Anathana ellioti*) is potentially a good model to study the HCV infection, but the instability of the infection and its low level limit the use of these animals.

The immunotolerized rat model supports the HCV replication. Histological and biochemical evidence of infection were present, but viremia was relatively low as compared with viral load in humans[188].

Mouse models compared with other animal models, have some advantages, such as producing animals in a short time (short gestation period), lower breeding cost and their small size making them easy to manipulate[188,213-217]. Heterotopic liver graft mouse model seemed to be suitable to evaluate the putative effect of anti-HCV drugs, but the short and low viremia and loss of the liver graft were limiting factors. Hepatic repopulation mouse model as the chimeric urokinase-type plasminogen activator/severe combined immunodeficient disorder mouse model has many similarities to human systems, and so is the most successful small animal model for HCV infection. The main limitation of the model is the absence of a normal immune system, which may be overcome by combining the model with a human hemato-lymphoid system, the value and reproducibility of which has yet to be established[218].

A mouse combined human immune system/human liver chimeric Rag2-g(c) model was developed in order to study immune system and liver pathogens. This 'Hu-HEP' model was used to study hepatocytes derived from human induced pluripotent stem cells. The main limitations of these models are the low number of animals that can be generated and the high cost[219-221].

Recent study demonstrated that hepatocyte-like cells differentiated from human embryonic stem cells and patient-derived induced pluripotent stem cells could be engrafted in the liver parenchyma of immune-deficient transgenic mice carrying the urokinase-type plasminogen activator gene driven by the major urinary protein promoter. This efficient engraftment and *in vivo* HCV infection of human stem cell-derived hepatocytes provide a model to study chronic HCV infection in patient-derived hepatocytes, action of antiviral therapies, and the biology of HCV infection[222-225].

In vitro models

Established human hepatocarcinoma cell line Huh-7 and its derivatives support the HCV replication. However, as every transformed cell line, they resemble the primary cells only partially. Thus, results of experiments performed on these cells might not be always appropriated[222].

The primary human hepatocytes and human fetal hepatocytes are more clinically and physiologically relevant. Thus, they are used to test the susceptibility of HCV to drugs and drug-metabolizing enzymes. Another *in vitro* model, the micropatterned co-cultures of primary human hepatocytes surrounded by a supportive stroma that expressed all known HCV entry factors. The cells could be infected by HCV pseudotyped particles and HCVcc, albeit with low viral titers. Using this method in combination with the highly sensitive luminescence-based and fluorescence reporter systems, the efficiency of anti-HCV therapeutics has been evaluated[222].

Recent studies show that both embryonic[223] and induced pluripotent[224,225] stem cells can be differentiated into hepatocytes, that are phenotypically similar to

human fetal liver. Study of genetic defects that impact the HCV infection could be performed in a human iPS-derived hepatocyte-like cell-based model[225]. Induced human liver-like cells supported the entire life cycle of HCV genotype 2a reporter virus. Produced infectious particles were able to infect HuH-7.5 cells and secretion of TNF-α, and IL-28B/IL-29 was detected in cell culture supernatants. Thus, the evaluation of antiviral drugs using these cells was possible[222].

Ex vivo model

Analysis of precision-cutting adult human liver slices from infected or non-infected individuals represents another promising model. In fact, this model allowes to maintain the tridimensional structure of liver and analyse gene and protein expression.It is worth mentioning, that using this model, it was demonstrated for the first time, the ability of primary isolates (as well as JFH-1, H77/C3, Con1/C3) to undergo *de novo* viral replication with the production of high titer infectious virus. Thus, this approach allowed to validate the efficiency of the new antiviral drugs[226,227].

PATHOGENESIS

Immune-mediated liver damage

The HCV causes damage to the liver cells, but the exact mechanism of this phenomenon is unknown. It is believed, that the damage is largely mediated by the host immune response. In immunocompetent and immunocompromised patients with little, or no intra-hepatic damage, including inflammation, high levels of the HCV replication have been reported[133,228,229]. In about 30% of HCV liver transplanted patients, despite the high levels of HCV replication, a recurrent hepatitis is developed one year after transplantation [230]. However, high levels of an intrahepatic HCV replication are usually tolerated by the host immune system. A lympho-mononuclear infiltrate represented mainly by $CD8^+$ T cells expected to play a major role in the viral containment, though other subsets, such as $CD4^+$ T and natural killer (NK) cells, and regulatory T cells (Treg) are considered[231]. The intrahepatic $CD4^+$ and $CD8^+$ T cells can recognize HCV structural and nonstructural antigens[232,233]. However, why in most patients the immune response cannot resolve the infection remains obscure. In fact, cytotoxic $CD8^+$ T cell-mediated killing could be blunt by a predominant Treg response[234].

The liver fibrosis is caused by inflammatory cells of the intrahepatic infiltrate secreting cytokines and chemokines to activate hepatic stellate cells (HSC) to secrete collagen[235]. HSCs may exist as several different phenotypes with distinct molecular and cellular functions and features, each of which contributes significantly to the liver homeostasis and the disease. The quiescent stellate cells are critical to the normal metabolic functioning of the liver. The liver injury provokes the transdifferentiation of quiescent stellate cells to their activated phenotype, leading to a metabolic reprogramming. That increases the autophagy (to fuel the metabolic demands), the amplification of parenchymal injury and the development of 'classic' phenotypic features of activated HSCs/myofibroblasts. Through these changes, the activated stellate cells drive the fibrotic response to injury and the development of cirrhosis. As liver injury subsides, the activated stellate cells can be eliminated by one of three pathways: Apoptosis, senescence or reversion to an inactivated phenotype. The senescent stellate cells are more likely to be cleared by the NK cell-mediated cell death while the inactivated stellate cells remain 'primed' to respond to further liver injury. Reduction in the number of activated stellate cells contributes to the regression of fibrosis or cirrhosis and the liver repair in most, but not all patients. The relative inputs of these three pathways on the fibrosis regression are not clearly defined[233]. The HCV-specific $CD8^+$ T cells expressing PD-1, a marker for exhaustion, were found at the time of the acute phase[236]. The acquisition of a memory phenotype and the recovery of an efficient $CD8^+$ T cell function declined with HCV infections PD-1 expression, whereas, when HCV persisted and the HCV-specific $CD8^+$ cells remained dysfunctional, high levels of PD-1 were maintained, the PD-1 ligation likely provides an overall inhibitory signal to Tregs cells shown in a study of PD-1 expression on Tregs in humans during the chronic HCV infection. In response to HCV antigens, the PD-1 blockade enhanced an interleukin-2 (IL-2)–dependent proliferation of intrahepatic Tregs and enhanced the overall ability of Tregs to inhibit T-effector cells. An increase in Treg proliferation with PD-1 blockade was linked to this effect[237]. A typical model of the wound-healing response to a persistent liver injury is the hepatic fibrosis occurring in the chronic hepatitis C[238]. Cytokines and chemokines capable of activating hepatic stellate cells to secrete collagen are secreted by the inflammatory cells of the intrahepatic infiltrate[239,240]. Thus, the fibrogenesis seems to be linked to the HCV expression through indirect mechanisms, mediated by a virally driven inflammation, but the direct role of viral factors in the disease progression should be investigated in more details. The cell injury, such as an oxidative stress and a steatosis, may be induced specifically by several viral proteins alone which could directly activate the hepatic stellate cells[241,242]. These observations may explain why some patients with chronic hepatitis C with normal liver enzymes and a minimal/mild inflammation may present a significant liver fibrosis as shown by the histology of liver biopsies. Overall, up to 70% of patients with a HCV cirrhosis will demonstrate a reversibility on follow-up biopsies[243-248]. Moreover, a reduced portal pressure and a decreased all-cause mortality are improved when the reversal occurs[245]. It is to point out that approximately 10% of HCV patients present a persistent or even progressive fibrosis following SVR, which might reflect other concurrent underlying liver diseases, especially a

nonalcoholic fatty liver disease (NAFLD)[248]. The stellate cells can respond to cytokines and growth factors after priming stimuli. Then the proliferation, contractility, fibrogenesis, matrix degradation and proinflammatory signaling are enhanced. Now, it is clear that there are disease-specific pathways of fibrosis, without all activated cytokine pathways[249,250]. This is especially relevant to NAFLD, where there are many convergent pathogenic routes[248-250]. Importantly, different families of inflammatory cell types and their subsets may either promote or inhibit fibrosis[249-252].

Alterations of lipid metabolism

Lipids are required for the HCV replication and particles assembly. As mentioned above, HCV can modify the host serum lipid profile and this(ese) modification(s) can provoke the steatosis[253]. The steatosis is more frequent and more severe in patients with HCV gt 3 and it is correlated with a high HCV RNA levels. On one hand, in HCV-infected patients, the steatosis can be considered as a marker of the liver disease progression[254] and, on the other hand, as an indication of the reduced response to therapy[254]. However, if it is not metabolic or alcoholic steatosis, an efficient antiviral therapy is capable to reduce it[255,256].

Extrahepatic replication of HCV

The apparent presence of the HCV genomes in extra-hepatic sites of patients infected with HCV has been shown. Using sensitive PCR assays, the HCV was revealed in leukocytes and there are evidences that these cells may represent a reservoir of the virus after treatment[257,258]. Interestingly, the pool of the HCV quasi-species differs between the plasma and peripheral blood monocytes, suggesting an independent spread of HCV within different cell types[259,260]. The infection of B-cells from non-Hodgkin's lymphoma by HCV has also been demonstrated. A cell line established from transformed lymphocytes supported the HCV replication and also enable production of infectious viral particles capable to infect peripheral blood B cells[261]. The significance of these extrahepatic HCV reservoirs is not well understood, although one could speculate that the leukocyte compartments might represent an additional route by which HCV can directly manipulate the immune system and also another means by which the virus avoids the eradication.

Extrahepatic manifestations associated with the HCV infection

At least, one clinically significant extrahepatic manifestation occurs in 38% to 76% of HCV infected patients with chronic HCV[262,263]. The most frequent associated pathology is a mixed cryoglobulinemia. It is detected in 19% to 50% of HCV infected patients, while only 15% of them are symptomatic. The cryoglobulins are immunoglobulins which precipitate at a temperature below 37 ℃. They are produced by HCV activated B cells. The cryoglobulins deposed in small and medium vessels are the cause of systemic vasculitis which can manifest in level joint, skin, renal or peripheral nerves[262]. Other observed extrahepatic manifestations are the following: lymphoma, thyroid disorders, diabetes, xerostomia and xerophthalmia[263-265]. The HCV infection cure leads to a gradual decrease of the cryoglobulin level in serum, followed by the remission of cryoglobulin-related symptoms and pathologic lesions[265]. Interestingly, as a result of treatment the incidence of type 2 diabetes is also reduced by approximately two thirds[264,265].

CLINICAL MANIFESTATION OF HCV INFECTION

Acute hepatitis

The acute hepatitis C is asymptomatic in 90% of infected people[266]. In some cases, asthenia, fever and muscle, and joint pain can appear. While, signs of jaundice are not frequent. Acute hepatitis C is characterized by a transient increase in the rate of serum transaminases. The first detectable virus marker is viral RNA that appears one to two weeks after exposure. Then seven-eight weeks later, the anti-HCV IgG response can be detected[266-268]. In 20% of cases, hepatitis C is resolved spontaneously through the innate and adaptive immunity[269]. The viral RNA becomes undetectable within three to four months after infection. Various factors could promote the viral clearance. Similarly, hepatitis acute symptoms would reflect a significant immune response of the host. The gene polymorphism of interleukin (IL) 28B also influences the host immune response[268,269]. The fulminant hepatitis C is exceptional[268].

Chronic infection

In about 80% of cases[268], the immune system is not capable to eradicate the HCV during the acute phase of infection. When the viral replication persists for more than six months after acute infection, the hepatitis is considered chronic. At the stage of chronic hepatitis, most patients are asymptomatic and may have no non-specific symptoms such as fatigue, arthralgia or myalgia. The transaminase levels may be moderately increased or even normal[270]. The long-term evolution of chronic infection is variable. The factors that accelerate the disease progression are the following: acquisition of more than 40 years, male gender, co-infection by HIV, higher body mass index, fatty liver and alcohol consumption[269]. After 10 to 30 years, about 20 to 30% of patients develop a cirrhosis. The cirrhosis may be associated with a liver failure, as a decompensation following a portal hypertension (ascites, gastrointestinal bleeding, *etc*.). In cirrhotic patients, the risk of death from complications is 4% per year, and their risk of developing an hepatocellular carcinoma (HCC) is 1 to 5% per year. Thirty-three percent of patients with HCC die within one year after diagnosis[269,270]. Overall, among patients with cirrhosis, the 5-year survival rate is 50%.

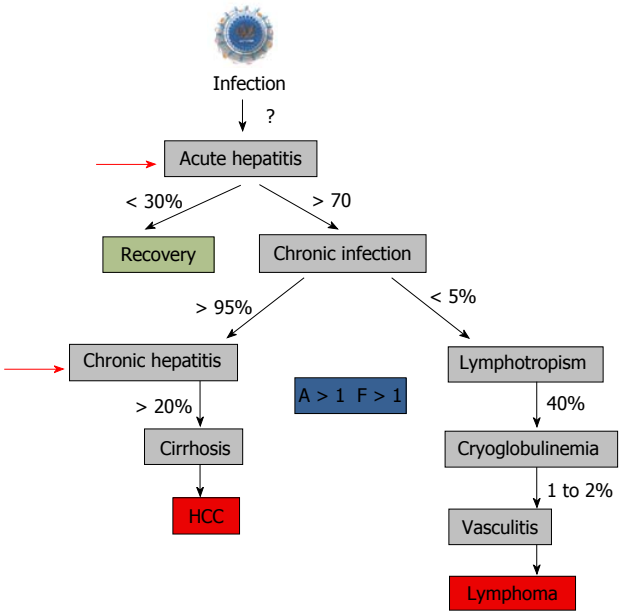

Figure 8 Natural history of hepatitis C virus infection and start to treat. The recommendation to treat chronic hepatitis is usually a "significant" fibrosis as defined by a Child–Pugh score (A to C) and a fibrosis grade (F) greater than 1 by the Metavir scoring system, with usually a significant necrotic-inflammation as defined by an activity stage greater than 1 by the Metavir scoring system[288,289]. The Child–Pugh score employs five clinical measures of liver disease: Total bilirubin, Serum albumin, Prothrombin time, Ascites, Hepatic encephalopathy. The letter F refers to the scars of the liver caused by the aggression. It is classified from F0 to F4: F1, F2 are minimal to moderate fibrosis, F3 corresponds to a pre-cirrhotic stage and F4 corresponds to cirrhosis. Red arrows indicated the time to start treatment[290].

In fact, the decompensated cirrhosis is the leading cause of liver transplantation[268,269] (Figure 8).

DIAGNOSTIC

For HCV diagnosis both serologic and nucleic acid-based tests were developed[270,271]. Serologic tests are sufficient when chronic hepatitis C is expected, with a sensitivity of more than 99% if using the 3rd generation assays. Positive serologic results require additional HCV RNA or (with slightly reduced sensitivity) HCV core antigen measurements in order to differentiate between chronic hepatitis C and resolved HCV infection from the past. When an acute hepatitis C is considered, a serologic screening alone is insufficient, because mature anti-HCV antibodies are developed late after transmission of the virus.

Morphological methods like immunohistochemistry, *in situ* hybridization or PCR from liver specimens play no relevant role in the diagnosis of hepatitis C because of their low sensitivity, poor specificity and low efficacy compared to serologic and nucleic acid-based approaches.

HCV core antigen assay

Recently, a new quantitative HCV core antigen assay (Architect HCV Ag, Abbott Diagnostics) was approved by the EMA. This assay comprises 5 different antibodies targeted the HCV core. The test is highly specific (99.8%), equally effective for different HCV genotypes, and shows a relatively high sensitivity for the determination of chronic hepatitis C (corresponding to 600-1000 IU/mL HCV RNA). However, HCV core antigen correlated well, but not fully linearly, with HCV RNA serum levels, and false-negative results might be obtained in patients with an impaired immunity[271-273]. Another study has shown that the HCV core antigen quantification could be an alternative to the HCV RNA quantification for on-treatment antiviral response monitoring[274]. Here, a HCV core antigen below the limit of quantification at treatment 1 wk was strongly predictive of RVR, whereas patients with a less than 1 log10 decline in HCV core antigen at treatment 12 wk had a high probability of achieving nonresponse. The new HCV core antigen assay could be a cheaper, though somewhat less sensitive, alternative for nucleic acid testing.

Nucleic acid testing for HCV

Since the HCV RNA is detectable within a few days of infection; the nucleic acid-based tests are efficient in an early diagnostic of acute hepatitis C and should be considered as mandatory. The HCV RNA measurement is furthermore important in determination of the HCV genotype, selection of treatment strategy, therapy duration and evaluation of the treatment success[274]. For a number of antiviral combination therapies, the HCV RNA follow-up studies are essential to define the outcome of the treatment and further therapeutic strategies, if necessary. Traditionally, the tests should be repeated 24 wk after treatment completion to assess whether a sustained virologic response (SVR) has been achieved. However, as the probability of a virologic relapse is similar after 12 and 24 wk, the new time point for assessment of final virological treatment outcome is 12 wk after the end-of-treatment[275,276]. Both qualitative and quantitative PCR-based detection assays are available. Qualitative PCR tests are sensitive and are used for initial diagnostic of hepatitis C, for screening of blood and organ donations and for confirming SVR after treatment completion (Table 2). Quantitative reverse transcriptase (RT) real-time PCR-based assays can detect and quantify the HCV RNA over a very wide range, from approximately 10 IU/mL to 10 million IU/mL. The measurements are essential in the treatment monitoring when the virus load is gradually reducing.

HCV genotyping

HCV genotyping is mandatory for every patient who considers antiviral therapy[277]. For DAA-based therapies, the determination of HCV genotypes and even subtypes is important because of significantly distinct barriers to resistance on the HCV subtype level. However, the importance for the HCV genotyping may decline with the availability of highly and broadly effective all oral combination therapies in the future. Both direct sequence

Table 2 Commercially available hepatitis C virus RNA detection assays

Assay	Distributor	Technology	Detection limits (IU/mL)	Genotypes	Approval status
Qualitative HCV RNA detection assays					
Amplicor™ HCV 2.0	Roche molecular Systems	PCR	50	All	FDA, CE
Versant™ HCV	Siemens Medical Solutions Diagnostics	TMA	5-10	All	FDA, CE
Quantitative HCV RNA detection					
Amplicor™ HCV Monitor 2.0	Roche molecular Systems	PCR	500 to approximately 5.10^5	All	CE
HCV SuperQuant™	National Genetics institute	PCR	30 to 10^7	All	U.S only
Versant® HCV RNA 3.0	Siemens Medical Solutions Diagnostics	bDNA	615 to 8.10^6	All	FDA, CE
Cobas® AmpliPrep/High pure system/Cobas® TaqMan®	Roche molecular Systems	Real-time PCR	15 to 10^7	All	FDA, CE
Abbot RealTime™ HCV	Abbot Diagnostics	Real-time PCR	10 to 10^7	All	FDA, CE
Artus HCV QS-RGQ assay	Qiagen	Real-time PCR	34 to 10^8	All	CE
Versant® HCV 1.0 kPCR assay	Siemens	Real-time PCR	10 to approximately 10^7	-	CE

HCV: hepatitis C virus; TMA: Transcription-mediated amplification of RNA; bDNA: Branched DNA hybridization assay.

analysis and reverse hybridization technology allow the HCV genotyping. Initial assays were designed to analyze exclusively the 5'UTR, which was burdened with a high rate of misclassification especially on the subtype level. Current assays were improved by additionally analyzing the coding regions, in particular the genes encoding core protein and the NS5B, both of which provide non-overlapping sequence differences between the genotypes and subtypes[278,279].

TREATMENT

All people with confirmed chronic HCV infection should be offered a high-quality care as soon as possible. Simultaneously, the screening and management of alcohol use is essential to prevent the progression to cirrhosis[278,280]. The modelling suggests that the treatment early in the course of HCV disease for all or specific populations could prevent the disease progression and onward transmission[281-283]. In fact, not all people with chronic HCV infection will progress to fibrosis[284]. So, medical authorities of a country to should decide when to start anti-HCV treatment. It should be emphasized that the transmission has been documented among people recently cured who lacked access to prevention services[285]. More research is needed to determine cost-effective eligibility criteria for both key and other populations that maximize reductions in the HCV-related morbidity, mortality, and transmission in different epidemiological contexts.

The WHO guidelines recommend the prioritizing treatment among people with an advanced fibrosis or cirrhosis in order to prevent a liver cancer[286]. The HCV co-infection in people living with HIV increases mortality while HIV has been shown to accelerate the progression of HCV disease[287,288]. Therefore, people with HIV/HCV co-infection may also warrant treatment prioritization. The stage of HCV disease could be estimated through liver biopsy (METAVIR system) identifying individuals needing an immediate treatment[288]. However, the liver biopsy test is expensive and can lead to complications such as infections, excessive bleeding, pain, or accidental injury to other organs. The use of liver function tests and platelet counts to determine the degree of liver fibrosis, such as the aminotransferase-to-platelet ratio index and the Fibrosis-4 score could be a non-invasive alternative and a more useful approach for gauging treatment eligibility across different tiers of health systems[289,290]. Monitoring systems should be put in place for people who do not initiate treatment immediately.

In the course of the last two decades, treatment of the HCV infection has significantly improved. For nearly 15 years, the combination of pegylated interferon alfa and ribavirin (PR) allowed a moderate sustain virologic response (SVR). Cure of the infection was achieved in 45% of genotype 1 and 65% of genotype 3 and around 85% of genotype 2 infected patients[291]. Development of the direct-acting antiviral drugs (DAAs) targeting viral proteins (NS3/4A protease, NS5B polymerase with nucleotide and non-nucleotide inhibitors, NS5A viral replication complex) was achieved due to the better understanding of the HCV life cycle. It revolutionized the treatment of chronic hepatitis C[292,293]. The classes of oral anti-HCV inhibitors and their characteristics are shown (Table 3). The combination of PR with the first-generation protease inhibitors demonstrated a high antiviral effectiveness (75% of SVR, but restricted to genotypes 1) with substantial adverse effects for the first-generation protease inhibitors. In 2011, telaprevir and boceprevir obtained a market approval. In 2012, the recommendations were approved for their use in HCV mono- and in 2013 for HCV/HIV co-infected infected patients. Then, SVR rates increased from 75% to 90% while reducing treatment duration, adverse effects, and the number of pills, thanks to the combination of PR with second-generation protease inhibitors[63,294,295]. From 2015 the standard of care is a combination of DAAs. Sofosbuvir, daclatasvir and the sofosbuvir/ledipasvir combination are part of the preferred regimens in the WHO guidelines, and can achieve cure rates above 95%[63]. These medicines are much more effective, safer and better-tolerated than the older therapies. In the first studies, excellent results were obtained on a group of so-called "easy-to-treat patients" and in

Table 3 Selected directly acting antiviral agents and host targeting agents in the pipeline[63]

Drugs name	Company	Target/active site	Phase
NS3/4A protease inhibitors			
Vaniprevir (MK-7009)	Merck	Active site/macrocyclic	Ⅲ
Voxilaprevir GS-9857	Gilead	Active site	Ⅲ
Glecaprevir (ABT-493)	Abbvie	Active site	Ⅲ
IDX21437	Idenix	Active site	Ⅱ
Sovaprevir (ACH-1625)	Achillion	Active site/macrocyclic?	Ⅱ
Nucleoside analog NS5B polymerase inhibitors (NI)			
MK-3682 (formerly IDX20963)	Merck	Active site	Ⅱ
ACH-3422	Achillion/Janssen	Active site	Ⅱ
Non- Nucleoside analog NS5B polymerase inhibitors (NNI)			
Beclabuvir (BMS-791325)	Bristol-Myers Squib	NNI site 1/Thumb 1	Ⅲ
Setrobuvir (ANA598)	Anadys/Roche	NNI site 4?/palm 1	Ⅱ
NS5A inhibitors			
BMS-824393	Bristol-Myers Squibb	NS5A protein	Ⅱ
PPI-461	Presidio	NS5A protein	Ⅱ
PPI-668	Presidio	NS5A protein	Ⅱ
Pibrentasvir (ABT-530)	Abbvie	NS5A protein	Ⅲ
ACH-2928	Achillion	NS5A protein	Ⅰ
Ruzasvir (MK-8408)	Merck	NS5A protein	Ⅱ
Host targeting agents			
SCY-635	Scynexis	Cyclophilin inhibitor	Ⅱ
Miravirsen	Santaris	miRNA122 antisense NA	Ⅱ
RG-101-	Regulus	miRNA122 antisense NA	Ⅱ
TT-0034	Tacere Therapeutics	RNA interference with HCV	Ⅱ

Table 4 Selected directly acting antiviral agents and host targeting agents whose development has been stopped or temporarily halted[63]

Drugs name	Company	Target/Active site
NS3 /4A protease inhibitors		
Ciluprevir BILN 2061	Boehringer Ingelheim	Active site/macrocyclic
Narlaprevir (SCH900518)	Schering-Plough	Active site/linear
PHX1766	Pheromix	Active site
Danoprevir (R7227)	Roche/InterMune	Active site/macrocyclic
Faldaprevir (BI201335)	Boehringer Ingelheim	Active site/linear
Telaprevir (VX-950)	Vertex	Withdrawn
Boceprevir (SCH503034)	Merck	Withdrawn
Nucleoside analogue NS5B polymerase inhibitors (NI)		
Valopicitabine (NM283)	Idenix/Novartis	Active site
R1626	Roche	Active site
Mericitabine (R7128)	Roche/Pharmasset	Active site
GS-938	Gilead	Active site
IDX184	Idenix	Active site
Non-nucleoside NS5B polymerase inhibitors (NNI)		
BILB 1941	Boehringer Ingelheim	NNI site 1/thumb
MK-3281	Merck	NNI site 1/thumb 1
VX-759	Vertex	NNI site 2/thumb 2
VX-222	Vertex	NNI site 2/thumb 2
VX-916	Vertex	NNI site 2/thumb 2
ABT-072	AbbVie	NNI site 3/palm 1
HCV-796	ViroPharma/Wyeth	NNI site 4/palm 2
Filibuvir (PF-00868554)	Pfizer	NNI site 2/thumb 2
IDX375	Idenix	NNI site 4/palm 2
Tegobuvir (GS-9190)	Gilead	NNI site 4/palm 2
GS-9669	Gilead	NNI site 3/palm 1
Deleobuvir	Böhringer	NNI site 3/palm 1
Host targeting agents		
NIM811	Novartis	Cyclophilin inhibitor
Alisporivir (Debio-025)	Novartis	Cyclophilin inhibitor

small groups of patients have been confirmed in the phase Ⅲ studies and in "difficult-to-treat patients" [such as in check of previous regimens (including protease inhibitors), cirrhotic patients, liver or kidney transplanted patients, HIV infected patients or multi-drug treated patients, at an increased risk of the drug interaction][63]. Nevertheless, viral variants resistant to DAAs can emerge during the antiviral therapy[295-298]

(Table 4). The estimation of a putative virus resistance profile prior to an antiviral therapy can help to select the optimal treatment regimen for individual patients[295,296].

CONCLUSION

A prominent genetic diversity of HCV in combination with non-trivial replication cycle poses serious problems in virus research and therapy of virus-associated diseases. However, during the last several years a significant progress in understanding of the HCV molecular biology and pathogenesis has been achieved. In many aspects that allows to making a great step forward towards antivirals design, that result in DAA therapy. However, even with the new drugs the HCV-associated problems, of both fundamental and applied origin, are not solved. In fact, the resolution of these problems is going hand in hand.

First, in the absence of direct cytopathic effect, the mechanism of lipid deregulation, that results in liver pathologies, is not completely understood. Second, the system of the HCV control during latency and putative triggers of virus active replication were poorly investigated. Third, design of prophylactic vaccine is still in its infancy. Finally, the access to drugs and therapies for the people leaving in the third world is very restricted. Even if SVR rates > 90% are reached, nearly 10 million people will be living without treatment options[63]. Indeed, an easy access to diagnosis and treatment is still missing to drug users, people in difficult socio-economic situations, migrants, prisoners, because of themselves or health policy.

Therefore, fundamental studies on virus-cell interactions and studies directing towards development of the prophylactic vaccine should be intensified.

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REVIEW

Cell fusion in the liver, revisited

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Abstract

There is wide agreement that cell fusion is a physiological process in cells in mammalian bone, muscle and placenta. In other organs, such as the cerebellum, cell fusion is controversial. The liver contains a considerable number of polyploid cells: They are commonly believed to originate by genome endoreplication, although the contribution of cell fusion to polyploidization has not been excluded. Here, we address the topic of cell fusion in the liver from a historical point of view. We discuss experimental evidence clearly supporting the hypothesis that cell fusion occurs in the liver, specifically when bone marrow cells were injected into mice and shown to rescue genetic hepatic degenerative defects. Those experiments-carried out in the latter half of the last century-were initially interpreted to show "transdifferentiation", but are now believed to demonstrate fusion between donor macrophages and host hepatocytes, raising the possibility that physiologically polyploid cells, such as hepatocytes, could originate, at least partially, through homotypic cell fusion. In support of the homotypic cell fusion hypothesis, we present new data generated using a chimera-based model, a much simpler model than those previously used. Cell fusion as a road to polyploidization in the liver has not been extensively investigated, and its contribution to a variety of conditions, such as viral

infections, carcinogenesis and aging, remains unclear.

Key words: Cell fusion; Hepatocytes; TdTomato; Lineage tracing; Chimeras; Extracellular vesicles

Core tip: About 70% of hepatocytes are polyploid, arising either from genome duplication without division (endoreplication) or from cell fusion. Experiments with chimeric mice containing two cell populations each bearing a different genetic marker had shown that some liver cells express markers of both genomes, suggesting that cell fusion occurred. Here, we review the data in the literature and describe new experiments using a chimeric model that confirms that cell fusion contributes to liver polyploidy. We argue that the role of cell fusion in pathological conditions, such as viral hepatitis and neoplastic transformation, is worth further study.

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INTRODUCTION

Mammalian cells are usually diploid, with the exception of mature gametes, which are haploid. Interestingly, a few tissues contain polyploid cells, such as muscle cells, osteoclasts, hepatocytes, megakaryocytes and trophoblasts[1]. A common feature in all these tissues is the presence of a diploid progenitor cell that at some point during the differentiation/maturation process becomes polyploid. Polyploidization can be explained by two main mechanisms: endoreplication and cell fusion. Endoreplication occurs when the genome is duplicated without cell division, whereas cell fusion occurs between two different cells, either of the same or of a different identity. In the latter case, the genomes from two different cell types-which can come even from different species-fuse within the same membrane and, therefore, coexist within the same cell[2]. For both mechanisms, the outcome is a polyploid cell.

POLYPLOIDY IN NORMAL CELLS

It is generally recognized that polyploidization in normal organisms is adaptive since it helps specialized cells acquire the ability to perform new, specific functions[3]. For example, osteoclasts are large multinucleated cells that perform the difficult task of resorbing bone matrix. This specialized function cannot be accomplished, for example, by mononucleated TRAP$^+$ osteoclasts as this leads to osteopetrosis, a disease in which bone is not degraded[4]. Among the other types of polyploid cells, there is agreement that muscle cells and trophoblasts are products of cell fusion. In contrast, megakaryocytes are polyploid cells generated by genome duplication followed by aborted cytokinesis[5].

Polyploid liver cells are usually considered to be formed by endoreplication of the genome[6,7]. This conclusion was originally based on the seminal work of Mintz and colleagues, who pioneered the use of chimeric mice to study gene expression[8]. In the chimeric mouse, cells with two different genomes coexist in a single organism. If a cell fuses with another having a different genome, the resultant cell will contain markers of both. In their studies, Mintz and colleagues concluded that fusion occurred in muscle but not in the liver[8-10]. However, they had to rely mainly on the analysis of isoforms expressed by tissue-specific enzymes because single-cell markers were not yet available. Contrary to osteoclasts, trophoblasts and muscle cells, whose nuclei maintain their individuality, polyploid liver cells can be bi- or mono-nuclear.

Specific cell cycle genes, such as Cdk1, are involved in polyploidy formation and maintenance in liver[11]. Interestingly, after partial hepatectomy, quiescent hepatocytes can start proliferating again: deletion of cyclin-dependent kinase 1 (Cdk1), 2 (Cdk2), cyclin E1 or E2 individual genes does not limit liver regeneration, but concomitant ablation of Cdk2 and Cyclin E1 reduces liver regeneration, suggesting partial overlapping function of some cell cycle genes[11,12].

TRANSDIFFERENTIATION AND FUSION: A DEBATE NOT YET SETTLED

The study of cell fusion in the liver began while investigating the existence of lineage transdifferentiation. The possibility of cell transdifferentiation was raised at the end of the last century in a paper published in Science[13] in which the authors, after having transplanted neural stem cells transgenic for the beta-galactosidase (βgal) gene into wild-type mice, detected βgal$^+$ cells in peripheral blood. Their interpretation was that neural cells had transdifferentiated into cells of the hematological lineage. This plasticity was surprising to many but, because the report was published just after the birth of Dolly the sheep, looked plausible[14]. We must emphasize here that that work had nothing to do with the reprogramming approach reported several years later by Yamanaka, who, in contrast, obtained reprogramming by forced expression of intracellular transcription factors[15]. The plasticity of neuronal stem cells was claimed by Bjornson *et al*[13] to occur by simple exposure to endogenous factors present *in vivo*.

Essentially, studies on plasticity were performed by transplanting cells from a mouse transgenic for an easily detectable marker gene (*e.g.*, βgal) into a non-

transgenic animal. The appearance of βgal-marked cells in an organ different from the tissue of origin was interpreted to be the result of transdifferentiation. The Science paper in which cells of the nervous system were suggested to acquire a hematological fate, was rapidly followed by other examples of transdifferentiation involving cells of several other lineages, including blood, brain, muscle, kidney and heart[16-25].

Due to the high potential for translation to the clinic, bone marrow cells (BMCs) escalated to center stage. BMCs are easily obtained, extensively investigated, routinely transplanted and well-characterized in humans. If simple transplantation protocols allowed the rescue of degenerative defects in organs such as brain, kidney or liver, we would have a sort of panacea in hand. Unfortunately, although many clinics-mostly in the United States-still advertise these kinds of treatments[26], transdifferentiation as originally proposed in the Science paper has not been confirmed by subsequent, more controlled studies[27-32]. Indeed, although a limited transdifferentiation capacity of some cells cannot be completely ruled out, more-recent studies have shown that transdifferentiation is often an experimental artifact. As stated above, transdifferentiation was claimed to occur if, after a given lineage (for example, hematopoietic cells) expressing a reporter gene was transplanted into a wild type mouse, cells of other lineages (for example, brain) were found to coexpress the reporter gene with accepted markers of their lineage. While βgal was initially used as a marker, most subsequent papers exploited fluorescent reporter genes that could be easily traced *in vivo*. It was assumed that all fluorescent cells found in a normal, non-transgenic, mouse had to be the progeny of transgenic donor cells: Hence, if they were found in other organs, they must have derived from original cells that had acquired a new fate by transdifferentiation.

In addition to trivial technical artifacts, cell fusion was raised to explain some of these results: in the experimental design discussed above, fusion between any cells of the host with transplanted donor cells could have provided the former with the reporter gene. It is difficult to discriminate between the two possibilities-transdifferentiation and cell fusion-with simple marker analysis.

As mentioned above, transdifferentiation of BMCs would be an attractive approach for regenerative medicine. Heart, brain and liver are heavily affected by degenerative genetic diseases that have a huge impact on human health; they would all greatly benefit from cell fusion-based therapies using exogenous cells, if that mechanism indeed occurs *in vivo*. Certainly, exogenous cells could provide defective endogenous ones with the missing genetic component while maintaining the differentiation status of the mature cell.

With regard to the liver, several reports in which BMCs were transplanted into recipient mice in the hope of inducing hepatocyte transdifferentiation showed that cells bearing donor-derived cellular markers could be found in host livers[18-21]. These "transdifferentiated" cells increased in number when the host livers were either injured (partial hepatectomy) or affected by a chronic degenerative genetic defect. However, the results were challenged by scientists who were unable to reproduce the transdifferentiation of hematological cells into non-hematological ones[33-36]. Cell fusion was shown to occur *in vivo*, so several reports investigated fusion events in a variety of other models. In the liver, the most spectacular experiments were performed by Grompe's group on the classical model of fumarylacetoacetate hydrolase (Fah) deficiency[37]. Mice recessive for a Fah mutation are models for tyrosinemia type Ⅰ, a severe genetic disease leading to liver failure in humans. Grompe and coworkers showed that bone marrow transplants in these mice led to the generation of liver cells bearing the donor marker, and demonstrated that this event was not due to transdifferentiation of hematological into hepatic lineage cells. Instead, these marker-carrier liver cells originated from cell fusion between donor bone marrow and resident hepatocytes, leading to polyploid cells that were not easily distinguishable from true hepatocytes in that the latter could also be polyploid. Due to the growth advantage shown by normal hepatocytes over diseased ones, the approach was so efficient that several mice were essentially cured. Results were confirmed by further studies[38-40], which also pointed to macrophages as the hematological cell responsible for fusion[41,42]. These results are in agreement with macrophages being physiologically prone to cell fusion[43]. In a review of 77 published studies on the generation of hepatocytes by hematopoietic cells transplanted in liver, the authors concluded that cell fusion was the mechanism involved[44]. Cell fusion is enhanced by the presence of liver injury or chronic disease, such as in the Fah model, since in a well-controlled study in which BMCs were injected into normal recipients, only 7 out of 470000 liver cells examined bore donor markers as a result of cell fusion[34]. In addition to BMCs, other types of cells, such as mesenchymal or amniotic stem cells and cells differentiated from pluripotent stem cells, can fuse with cells in injured livers, even when injected into a different species[45]. Human umbilical cord blood cells have also been reported to fuse with hepatocytes of immunocompromised mice[46], although no evidence of cell fusion was reported in other studies[47-49]. Moreover, cell fusion and transdifferentiation have been claimed to coexist[50].

The cell fusion-based explanation was found to hold also in other similar experimental settings[34,37,39,51,52] (reviewed in[27,53,54]). However, the possibility that at least in some cases, especially when an injury is applied to the recipient organ, bone marrow donor cells could be directed toward a different fate has not been completely ruled out, since several reports of well controlled differentiation have been published[55-64].

CELL FUSION IN THE NORMAL LIVER

The discovery that cell fusion can cure a degenerative disease of the liver prompted Grompe's group to investigate whether cell fusion occurs also in the disease-free state. The experimental plan to address this was as follows: they transplanted 1 × 10^5 wild-type ($Fah^{+/+}$) hepatocytes into each of four $Fah^{-/-}$/βgal+ recipients. After more than 80% of the liver was repopulated, 1 × 10^5 hepatocytes were serially transplanted into each of two $Fah^{-/-}$ recipients and the liver was again repopulated to a donor contribution of more than 80%. Then they analyzed 3 × 10^7 Fah^+ hepatocytes, but were unable to find a single $Fah^+/\beta gal^+$ cell. They concluded that the frequency of cell fusion, if any, was very low[41].

It must be taken into considerations that the protocol involved damaged livers and injections of adult cells. However, although complex, the approach looks suitable to address the question of cell fusion in the disease-free liver. The only caveat is that, if the originally transplanted wild-type cells were mature hepatocytes (the age of the mice used was not specified), then it is possible that they represent polyploid cells that were already fully differentiated and functional and, therefore, less prone to fuse. This is because at this stage they have already achieved the benefits of being large cells with multiple genomes.

Apart from the original studies by Mintz and colleagues already cited, other studies investigating whether cell fusion occurs in the normal liver are lacking. Cell fusion has occasionally been reported to occur in hepatocytes or in hepatic tumor lines cultured *in vitro*[65-67]. Yet, this does not prove that the mechanism is physiologically relevant *in vivo*. For these reasons, Faggioli and coworkers devised and implemented a relatively simple but straightforward protocol based on chimeric mice, as originally proposed by Mintz's group[8-10]. Embryo-derived mouse chimeras are mice born from embryonal cells carrying different genomes[68]. They can be created either by morula aggregation or by injection of embryonic stem cells (ESCs) into blastocysts, and they can be exploited for the study of cell fusion. If each of the two aggregated morulae contains a different reporter gene, then cells positive for both reporters will definitively be fused cells.

Faggioli *et al*[71] reasoned that by aggregating morulae from two different strains of transgenic mice expressing either green fluorescent protein (GFP)[69], or the βgal protein (Rosa 26 mouse[70]), the outcome would be animals that display two genetically distinct liver cell populations, each bearing a single marker (either GFP or βgal); any cell displaying both markers must be the result of cell fusion. With the appropriate controls, they identified three populations: $GFP^+/\beta gal^-$; $GFP^-/\beta gal^+$; and $GFP^+/\beta gal^+$. The percentage of double-positive cells in the chimeric samples was estimated to be about 25%.

The authors confirmed their results with two other independent strategies. Briefly, they performed PCR amplification on single hepatocytes with primers specific for each reporter gene, finding cells displaying both markers only in chimeric mice, in a percentage close to 10% of cells bearing at least one marker. In addition, the authors used fluorescent *in situ* hybridization (FISH) to investigate the sex chromosome content of hepatocytes in XY◀═▶XX chimeric mice. They reasoned that, if fusion occurred between a female and a male cell, some binucleated cells containing Y chromosome(s) only in one of the two nuclei would be detected. Similarly, if mononucleated polyploid hepatocytes were analyzed, they should contain only X chromosomes in various numbers in the case they derived from the XX component of the chimeric mouse, or as many X as Y chromosomes if derived from an XY cell. In contrast, if the mononucleated polyploid hepatocytes were products of a cell fusion event between a female and a male cell, an unbalanced complement of X and Y chromosomes would be found. In the end, sex chromosome patterns were detected that were clearly indicative of cell fusion in binucleated as well as mononucleated hepatocytes in about 5%-10% of cells[71].

These results are at odds with those presented by Willenbring *et al*[41]. This discrepancy could be explained by the different approaches used, since that of Faggioli *et al*[71] mimics normal liver development, while the one used by Willenbring *et al*[41] involves the injection of exogenous hepatocytes into damaged liver and complex transplantation experiments. As mentioned before, this could ultimately lead to underestimation of fusion events in the latter study.

We are not aware of recent studies investigating cell fusion in normal liver, although replication of the chimera studies would not be too time consuming. Apparently, fusion is neither considered to occur frequently nor to be of physiological relevance. For this reason, while performing a study on the role of cell fusion in cancer[72], we addressed cell fusion with an even simpler approach based on the production of chimeric Cre: tdTomato mice. Morulae derived from mice transgenic for Cre recombinase under the control of a constitutive promoter were fused to morulae from mice transgenic for an inactive floxable tdTomato gene that is activated only if Cre recombinase is expressed in the same cell (Figure 1A). Cells from the two morulae will develop independently and no cell will be tdTomato-positive unless fusion with a Cre-containing cell has occurred (see the schematic representation in Figure 1B).

This approach-which has been widely used for lineage and transplantation studies-has the advantages of having an undetectable background if cell fusion does not occur, no interference between the two fluorescent reporter genes, and simple assessment in liver sections with well-validated tdTomato-specific antibodies. In addition, leakiness of the promoter, which sometimes occurs in Cre-based conditional mice, does not affect this model.

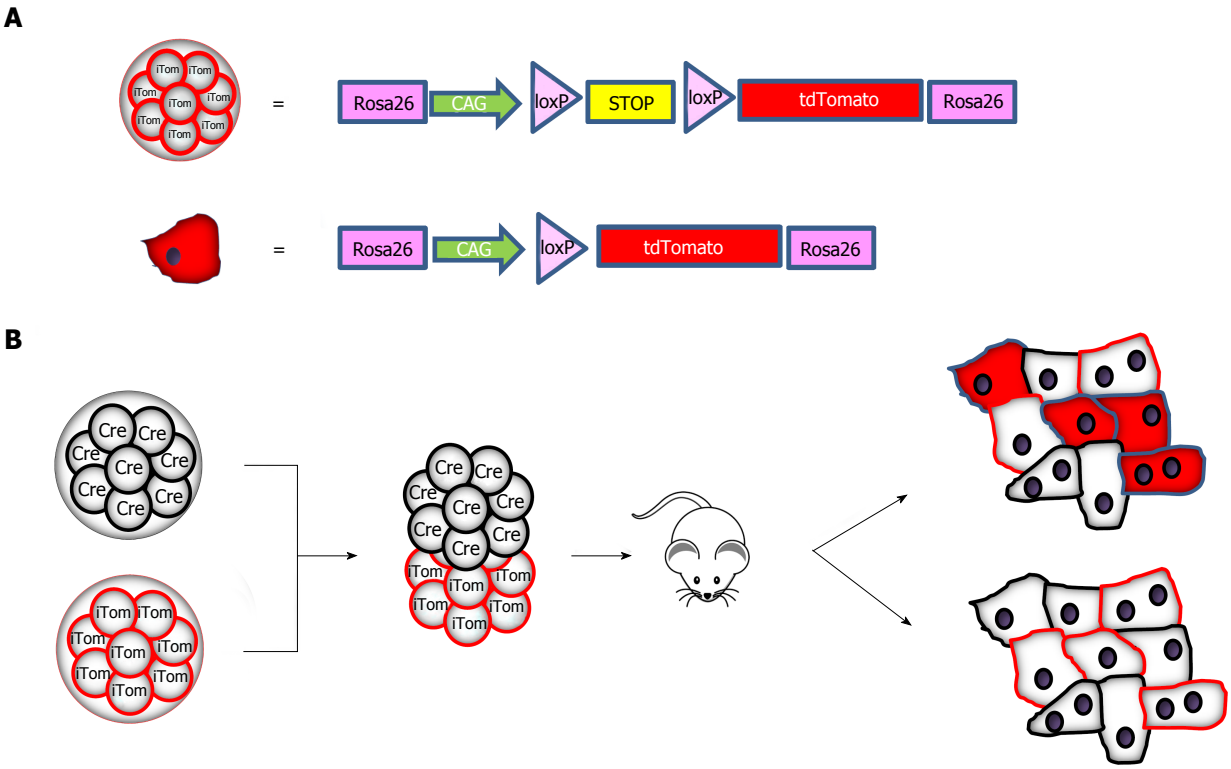

Figure 1 Schematic representation of the Cre: TdTomato approach here described. A: Depiction of the transgene carried by the tdTomato mouse (JAX 007905). The transgene is inserted in the Rosa26 locus and contains a Tomato gene (tdTomato) under the control of a constitutive promoter. Between the promoter and the gene there is a stop cassette flanked by two loxP sequences in the same orientation that prevents tdTomato transcription in this configuration. If the stop cassette is removed by exposure to the Cre recombinase, the tdTomato gene becomes expressed and its expression is maintained through the cell life; B: The generation of mouse chimeras by aggregation of one Cre+ morula (Cre, black-circled cells) derived from CMV-Cre mice (JAX 006054) with a tdTomato one (iTom, red-circled cells) is shown. The aggregated morulae are transferred to females and the progeny analyzed at three months of age. tdTomato expression should occur only if a Cre+ cell fuses with a tdTomato one (full red hepatocyte), whereas if no fusion occurs only tdTomato-negative cells are seen.

Analysis performed to date on two chimeric mice has clearly identified the presence of tdTomato-positive cells in the liver (Figure 2). Positivity was not detected in wild-type mice or in inactive tdTomato mice. As expected, the progeny of tdTomato-Cre mice crosses were positive in all tissues.

Fused cells are distributed all over the liver parenchyma, but are often found in clusters. This is in keeping with cell fusion occurring in cells maintaining their proliferative capacity, giving rise to a progeny that expands but remains in close proximity to their original location. This is in agreement with other studies showing hepatocytes originating form clonally derived clusters in postnatal liver[73,74].

However, the devil is in the detail and we are always at risk of artifacts[75]. In the chimeric experimental design, coexpression in the same cell of two reporter genes originally expressed independently by two distinct cells is commonly accepted as proof of a fusion event. This assumption was used in our original work on cell fusion[71]. However, the detection of fluorescence is prone to artifacts caused by endogenous background fluorescence, a phenomenon especially marked in liver; in the case of βgal, endogenous enzymatic activity can also lead to misinterpretation. In addition, it has become increasingly appreciated over the last ten years that transfer of materials-including RNA and proteins-between cells *via* extracellular vesicles is a frequent phenomenon[76-78]. Therefore, it cannot be excluded that in the Cre-tdTomato approach aforementioned, RNA encoding Cre recombinase or tdTomato could have been transferred from the Cre^+ cell to the tdTomato one, and thus activating the reporter locus leading to expression of the reporter protein. Even the transfer of a few RNA or protein molecules over a very short period of time can activate the tdTomato gene, which then would become permanently expressed. However, the Cre-Lox and GFP systems have been widely used, in general giving consistent results for expression and expected specificity. Unfortunately, with the technologies available to date there is no way of discriminating fusion events from vesicle-mediated transfer *in vivo* while maintaining physiological conditions. In this regard, it is worth mentioning that several recent papers analyzing the fate of GFP^+ cells transplanted into mouse retina have reported the detection of GFP^+ cells that did not originate from the donor[79-81]. This suggests that GFP activity was leaked into the intracellular space and absorbed by endogenous cells or was transferred to them by extracellular vesicles–fusion can be excluded since retinal cells were normal in size and not polyploid. This is troubling if true, and some lineage

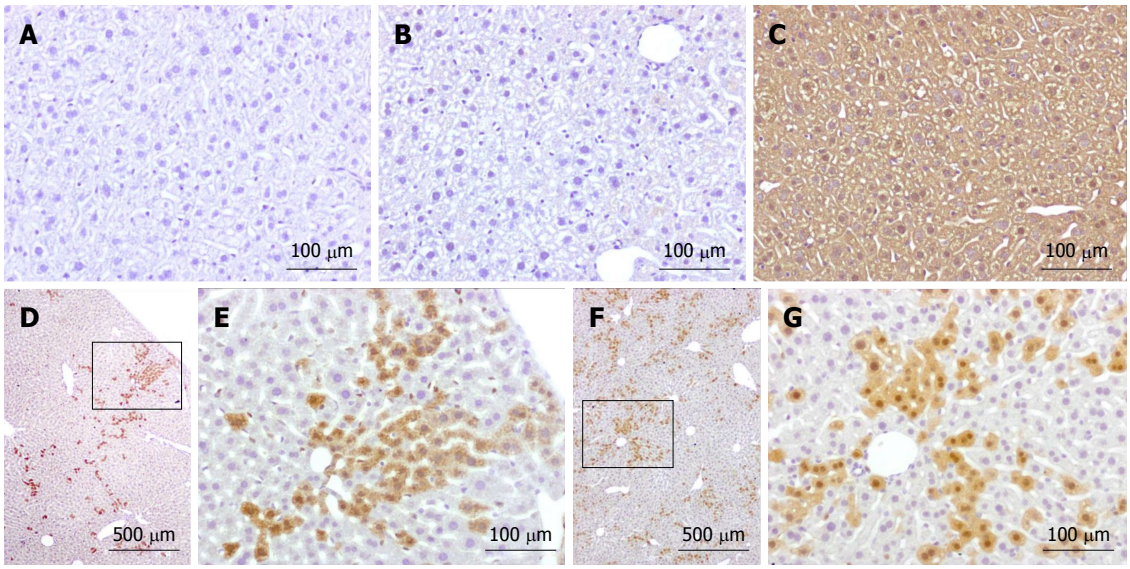

Figure 2 Immunohistochemical analysis of tdTomato expression in livers of chimeras and controls. For tdTomato detection, 3 μm slices were stained with anti-RFP antibody (ab124754: Abcam, Cambridge, United Kingdom) diluted 1:100 in PBS containing 0.05% Tween 20. Detection was performed using Mach 1 HRP-polymer (Biocare Medical, Concord, CA, United States) incubation followed by the revelation with Betazoid DAB (Biocare Medical). A: C57BL/6J wild-type mouse; B: Inactive tdTomato mouse; C: Cre+/tdTomato+ double transgenic mouse; D-G: Cre:tdTomato chimera 1 and 2, respectively. Wild type and Inactive Tomato mice are completely negative; Cre+/tdTomato+ are completely positive due to activation of the tdTomato gene in all cells. In the chimeras, many cells are negative, but a fraction shows clear expression of the tdTomato gene, which has been activated by the co-expression of Cre in fused cells. E and G: higher magnification views of the boxed area in D and F respectively.

or transplantation studies based on the detection of reporter genes should be carefully re-examined.

Techniques based on *in situ* hybridization with probes specific for sex chromosomes can be used to demonstrate cell fusion[71], since the presence of an XY nucleus as well as an XX one in a binucleated cell should definitively be due to cell fusion. This technique-which does not allow the analysis of live cells-has been used in studies on the ploidy of hepatocytes, with the caveat that the analysis might be complicated by the aneuploidy shown by some normal human and murine liver cells[82-85]. In any case, it will be difficult to investigate cell fusion in man: in theory, transplantation of male hepatocytes in female hosts performed for regenerative liver diseases could detect cell fusion, but this is a very rare occurrence and would require biopsies or post-mortem examination.

CONCLUSION

Cell fusion in the liver is still controversial. Thus, replication of previous studies with appropriate mouse chimeras is welcomed. Endoreplication and cell fusion are not mutually exclusive, as suggested by Gentric and Desdouets[86]. We strongly believe that fusion in the liver should be studied in order to confirm and explain this phenomenon. If established, this will open several new lines of investigation. For example, is cell fusion or endoreplication preferred in different contexts, or are they interchangeable? What is the fusion potential of hepatocytes with a DNA content higher than 4n? Are there hepatocytes with unbalanced or uneven-n chromosome numbers, and are there fusion products between one diploid and one tetraploid cell? Does cell fusion occur in species other than rodents, and particularly in man? Can fused cells participate in the ploidy reduction occurring after partial hepatectomy? Are HBV or HCV infections, which are themselves fusogenic viruses, able to change hepatocyte ploidy and binuclearity[87], or do other metabolic stresses[88] affect endoreplication or fusion? Does cell fusion play a role in HCV-mediated liver carcinogenesis[89]?

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REVIEW

Management of bacterial infection in the liver transplant candidate

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Abstract

Bacterial infection (BI) is a common cause of impairment of liver function in patients with cirrhosis, especially in the liver transplant candidates. These patients share an immunocompromised state and increased susceptibility to develop community and hospital-acquired infections. The changing epidemiology of BI, with an increase of multidrug resistant strains, especially in healthcare-associated settings, represents a critical issue both in the waiting list and in the post-operative management. This review focused on the role played by BI in patients awaiting liver transplantation, evaluating the risk of drop-out from the waiting list, the possibility to undergo liver transplantation after recovery from infection or during a controlled infection.

Key words: Cirrhosis; Portal hypertension; Bacterial infection; Liver transplantation

Core tip: Bacterial infection (BI) is a common cause of impairment of liver function in patients with cirrhosis, especially in the liver transplant candidates. BI may play a detrimental role in patients awaiting liver transplantation, increasing the risk of drop-out from the waiting list.

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INTRODUCTION

The liver is actively involved in inflammatory response against bacteria, and plays a central role in the regulation of immune defense, bacterial clearance, acute-phase protein, cytokine production and metabolic adaptation to inflammation[1]. Conversely, sepsis-induced hypoxic hepatitis and cholestasis make hepatic dysfunction an independent predictor of mortality during bacterial infection (BI)[2,3].

Cirrhosis is *per se* an immunocompromised state which predisposes to the development of BI, and sepsis-related death[4]. It's characterized by an immunodeficient state due to an impaired response to pathogens at different levels of the immune system, involving innate and adaptive cell dysfunction[5]; this condition coexists with a persistent stimulation of immune system, with enhanced serum levels of pro-inflammatory cytokines[6,7]. The severity of this inflammatory state correlates with severity of liver dysfunction[8,9]. Moreover, other superimposed conditions - such as impaired gut microbiota and intestinal barrier dysfunction - further increase the risk of BI[10]. In a study by Rasaratnam *et al*[11], when patients were treated with selective intestinal decontamination, their hepatic venous pressure gradient decreased by a mean of 2.43 mmHg, further strengthening the hypothesis that bacteria contribute to the hyperdynamic circulation and portal hypertension in cirrhosis.

Sepsis-related organ damage in cirrhosis is characterized by both an excessive inflammatory response and a decrease in the hepatic capacity of tolerance[12]. This further increases circulatory dysfunction, with splanchnic vasodilation and organ hypo-perfusion[13,14], leading to worsening of portal hypertension (*via* activation of neurohumoral pathways) and fluid retention[15]. Development of BI is a common trigger of extra-hepatic organ failures, in particular acute kidney injury[16], hepatic encephalopathy[17], coagulopathy[18], adrenal insufficiency[19] and respiratory failure[20].

CHANGING EPIDEMIOLOGY OF BACTERIAL INFECTION IN DECOMPENSATED CIRRHOSIS

In the past decades, there have been several improvements in the management of cirrhosis and its complications, such as hepatocellular carcinoma and portal hypertension. However, a significant proportion of patients with decompensated cirrhosis still need liver transplantation (LT), which represents the only effective therapeutic option.

In this setting, development of BI could significantly impair the natural history of the liver transplant candidate[21,22]. Preventive and therapeutic strategies for most of the complications of cirrhosis are well-defined; nevertheless, even if risk factors for the onset of BI in decompensated patients awaiting LT are well-known, they remain poor preventable.

The protean epidemiology of BI in cirrhosis depends on several factors, such as site of infection, setting of BI development, and local epidemiology.

Spontaneous bacterial peritonitis (SBP) and urinary tract infections are the most frequent BI in cirrhosis, followed by pneumonia, skin and soft tissue infections, bloodstream infections (BSI)[12,23]. SBP is mainly due to bacterial translocation - especially gram-negative strains[24], however epidemiology is rapidly changing. A multicenter study from Portugal[25], evaluating patients with severe liver dysfunction (median Child-Pugh class C-10; MELD score 19) recently showed an increase in gram-positive bacteria (GPB, 42%) at diagnosis of SBP; notably, one out of three SBP episodes occurred during hospitalization. This has determined the adoption of new antibiotic strategies: Piano *et al*[26] demonstrated that the combination of broad-spectrum antibiotics, meropenem plus daptomycin, was significantly more effective than conventional therapy (ceftazidime) in the treatment of nosocomial SBP (86.7% *vs* 25%; $P < 0.001$).

BSI represent another common cause of BI in cirrhosis[12], mainly due to gram-positive strains[27], because of the high number of invasive procedures and quinolone prophylaxis. However, there's an increasing prevalence of gram-negative bacteria (GNB) as the cause of BSI; Bartoletti *et al*[28] showed in a multicenter observational study on 312 cirrhotic patients in Italy, an equal distribution between GNB and GPB (53% *vs* 47%) at diagnosis of BSI.

In the last decades, clinical practice in Hepatology has dramatically changed as a consequence of the implementation of the liver transplant programs. Cirrhotic patients are nowadays frequently admitted to the ICU and undergo many diagnostic and therapeutic invasive procedures. This is associated with a higher risk of secondary infections caused by nonclassical pathogens. Fernandez *et al*[29] included 572 BI, 39% of which were nosocomial, reporting an increase in the rate of GPB infections associated with the increasing use of invasive procedures during hospitalization and in the ICU. More recently, Merli *et al*[30], collected 173 episodes of BI requiring hospitalization or occurred during hospitalization in 424 patients with cirrhosis; BI episodes were further divided into three classes (community acquired, hospital acquired, health-care acquired). GNB were more frequent in community acquired and health-care acquired infections, while GPB in hospital-acquired ones. *Enterobacteriaceae* (44.3%) (particularly *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*) and *Enterococci* (*Enterococcus faecium* and *Enterococcus faecalis*) (19.7%), were the pathogens most frequently responsible for infection.

Table 1 Risk factors of bacterial infection in cirrhosis

Risk factors for bacterial infection in cirrhosis
Impairment of liver function
Child-Pugh score[36-38]
MELD score ⩾ 15[40]
Low serum albumin[39]
Alcohol related disease[45,51]
Total ascitic fluid protein concentration < 15 g/L[84]
ICU admission[39,85]
Variceal bleeding[41,86]
Blood transfusion requirements
Mean arterial pressure
Severity of bleeding
Malnutrition[40]
Invasive procedures[29]
ERCP in PSC patients or with incomplete drainage[87]
Hospitalization[29,40,43,44]

MELD: Model for end stage liver disease; ICU: Intensive care unit; ERCP: Endoscopic retrograde cholangiopancreatography; PSC: Primary sclerosing cholangitis.

The spreading of multidrug resistant (MDR) bacteria-related infection is alarming worldwide[31,32]. In the past, patients with cirrhosis remained largely unaffected by this phenomenon because they were rarely admitted to the ICU. Nowadays, frequent hospitalizations, development of LT programs and antibiotic prophylaxis made cirrhosis at high risk of MDR infections[33]. A multicenter study in Italy[34] reported a 27% (83/395) prevalence of MDR infections, mainly due to GNB (extended-spectrum β lactamase *E. Coli* and Carbapenems resistant *K. Pneumoniae*). Another study from Greece[35] reported 19% MDR infection rate amongst patients with SBP, being MDR bacteria associated with healthcare-acquired infections and with patients at higher MELD score (28 *vs* 19, $P = 0.012$). Also in the above-mentioned study by Merli *et al*[30], MDR infections occurred more frequently during hospitalization (56% in hospital acquired/ healthcare-associated infections *vs* 22% in community acquired infections, $P = 0.008$).

RISK FACTORS FOR BACTERIAL INFECTION IN END STAGE LIVER DISEASE

Patients with end stage liver disease, listed for LT, have the highest risk for BI development. This might be due to frequent hospitalizations, severity of liver dysfunction and multiorgan failure (Table 1). Correlation between the development of BI and severity of liver disease has been widely demonstrated[36]. In a Japanese group of patients with cirrhosis and hepatocellular carcinoma[37], incidence of BI rose from 2.3% in Child-Pugh class A patients to 25.6% in Child-Pugh class C patients. In a further study[38], even if patients with cirrhosis were younger and had less cardiopulmonary comorbidities than patients without cirrhosis, they had higher rates of septic shock at hospital admission, and equal mortality. Moreover, bacteremia and mortality increased with the severity of liver disease (5.6%, 20.5%, 33.3% and 0%, 8.5%, 38.9% in Child-Pugh classes A, B, and C, respectively; $P = 0.038$).

Furthermore, low serum albumin, ICU admission, and GI bleeding, are other independent predictors of BI development, according to Deschenes *et al*[39]. Merli *et al*[40] analyzing 54 BI episodes occurred in 50 patients, showed that, at multivariate analysis, a MELD score ⩾ 15 (OR: 2.8, 95%CI: 1.3-6.1), history of previous infection within 12 mo (OR: 4.7, 95%CI: 2.2-10.6), and a diagnosis of malnutrition (OR: 4; 95%CI: 1.5-10) were independent predictors for infections and sepsis.

Variceal bleeding is another predictor of BI onset; Tandon *et al*[41], reviewing the available literature on patients admitted for GI bleeding without receiving antibiotic prophylaxis, showed that 242 out of 552 (44%) developed an episode of BI. Severity of GI bleeding, according to blood transfusion requirements (HR: 1.22; 95%CI: 1.01-1.47), mean arterial pressure (HR: 0.96; 95%CI: 0.93-0.99) were independent predictors for BI onset[42]. In another Spanish study[29], 126 patients underwent at least one invasive procedure, comprising variceal sclerotherapy or banding, surgical intervention, trans-jugular intrahepatic portosystemic shunt, having a higher probability of developing BI due to GPB.

Sinclair *et al*[43] showed that 43% of LT candidates required at least one hospitalization within 1 year; moreover, a significant proportion of hospitalized patients (> 45%) required repetitive hospitalisations. Patients with cirrhosis have 4 to 5-fold higher probability to develop a BI episode during hospitalization than non cirrhotic population[42,44].

The role of etiology of underlying liver disease as a risk factor for BI development is debated[45]. Alcohol abuse is associated with increased intestinal permeability, dysbiosis and increased bacterial translocation[46]. Furthermore, Legionella and Mycobacterium tuberculosis infections are significantly more prevalent in patients with alcohol abuse[47,48]. In the setting of cirrhosis, several studies reported a higher rate of BI in patients with alcoholic etiology when compared with non-alcoholic[49,50]. Sargenti *et al*[51] evaluating characteristics of 398 BI in 633 cirrhotics (363 alcoholic, 270 nonalcoholic), reported a similar occurrence of BI between groups, but pointed out that alcohol related disease was significantly associated with bacterial pneumonia and GPB.

IMPACT OF BACTERIAL INFECTIONS IN LIVER FUNCTION

Worsening of liver function is frequently observed in patients with infection, especially in those with sepsis, being itself a trigger for multiorgan failure, and development of Acute on Chronic Liver Failure (ACLF)[52-54].

In the above-mentioned study by Merli *et al*[40], Child–Pugh and MELD scores worsened in 62% of patients after infection; moreover, onset of ascites, hepatic encephalopathy, hyponatremia, hepatorenal

syndrome, were more frequent in patients with infection as compared with those who were not infected.

Prognosis of BI significantly correlates with the severity of liver disease and with the severity of extra-hepatic organ involvement[54,55]. A systematic review[21] considering 11987 patients with an episode of BI from 178 different studies, reported 1-, 3-, and 12-mo mortality of 30.3%, 44%, and 63%, respectively, and almost half of patients surviving at 1 mo died within a year. Renal failure, stage of cirrhosis (according to Child-Pugh score), age and severe sepsis were the factors independently associated with death. Several studies confirmed the critical role of renal failure in patients with cirrhosis and BI[56,57]. Mortality increased with the occurrence and severity of acute kidney injury and with the outcome of renal failure (15% 90-d mortality after complete recovery, 40% after partial renal recovery, and 80% in patients without renal recovery or progression). According to the study by Cazzaniga *et al*[58], systemic inflammation and fulfillment of SIRS criteria, are other factors significantly associated with mortality, since in-hospital mortality of these decompensated patients with MELD score > 18 rose from 12% to 43%. Dionigi *et al*[22] retrospectively evaluated prognosis of patients who were hospitalized in a tertiary center in the United Kingdom; they demonstrated that in-hospital mortality rate was higher in those patients who had infection at admission and/or developed infection during hospitalization (HR: 5.02; 95%CI: 2.75–9.16; P < 0.001).

IMPACT OF BACTERIAL INFECTION IN THE LIVER TRANSPLANT CANDIDATE

The onset of BI usually determines a further worsening of liver function and multiorgan failure, with high probability of death or drop-out from the WL[59,60]. Reddy *et al*[61] prospectively evaluated the outcome of 136 patients after an episode of BI developed while awaiting LT: 42% were delisted or died, 35% underwent transplantation, and only 24% achieved transplant-free survival within 6 mo. As expected, those who remained in the waiting list had a lower MELD score compared to those who either received a transplant or died/delisted; furthermore, those patients who underwent LT after BI recovery had a significant higher survival than those without LT (95% *vs* 5%; P < 0.001). At univariate analysis, the number of organ failures was the main factor that predicted death or delisting, whereas MELD score did not differentiate between those who were ultimately transplanted *vs* those who were delisted. Mounzer *et al*[62] showed that, 38% of patients who had experienced an episode of SBP before waiting list admission, were subsequently removed from the list or died.

Regarding patients who fully recovered from an episode of BI, the study by Sun *et al*[63] showed that recipients with pre-transplant BI (n = 32) within 12 mo before LT had a higher MELD score (median 25 *vs* 22, P < 0.05) at transplant, higher time of post-LT intubation (3 d *vs* 2 d, P = 0.05), and longer post-transplant hospitalization (29 d *vs* 20 d, P = 0.05). However, post-transplant mortality was not different between groups (9.4% *vs* 2.9%) and was not associated with pre-LT infection. Lin *et al*[64] retrospectively analyzed the outcome of 34 living donor LT candidates who had experienced an episode of BI within 4 wk prior surgery, which was effectively treated (*e.g.*, disappearance of symptoms and signs suggestive of sepsis, normalization or improvement of laboratory and/or imaging findings after antibiotic therapy). The post-operative outcome was compared with 20 patients with pre-LT ACLF without infection. The only difference between groups was the longer total hospital stay (89.0 d *vs* 65.5 d, P = 0.024), whereas post-LT ICU stay, one-year survival, and post-LT infection rates were similar between groups. Few data are available on the possibility to offer a standardized MELD exception after recovery from infection[65-67]. A possible scenario is the onset of recurrent episodes of cholangitis in PSC candidates; in a study by Goldberg *et al*[68], 300 patients who received MELD exception points for an increased risk of waitlist mortality, had a lower proportion of death/drop out (20.0% *vs* 1.3% P < 0.001); however, this non-standardized exception has not been further confirmed.

Several studies recently investigated the outcome of patients who underwent LT under "controlled" infection. In an Italian study[69], 84 patients were considered eligible for LT after disappearance of symptoms and signs suggestive of severe sepsis/septic shock. The overall post-LT 90-d mortality, septic shock, and sepsis as cause of death were not significantly different between infected and not-infected LT recipients; however, patients with previous infection had in the post-operative course higher rates of infections (40% *vs* 36%, P = 0.003) and post-transplant MDR strains (26% *vs* 13%, P = 0.005). Artru *et al*[70] recently demonstrated that ACLF grade 3 patients were transplanted in France after they had recovered from an episode of BI according to a subjective criterion of "controlled sepsis" for at least 24 h within transplant; the authors demonstrated an excellent 1-year post-LT survival (83.8%), not different than that observed in patients with no ACLF or with lower stages of ALCF.

MDR bacteria colonization represents another important issue in the setting of WL, because of the risk of spreading of BI in the post-operative course and/or after the introduction of immunosuppression. Giannella *et al*[71] prospectively evaluate the role of carbapenems resistant K. *Pneumoniae* (CR-KP) colonization (*e.g.*, presence of MDR bacteria in the rectal swab in absence of symptoms and signs of active infection) in 237 patients awaiting LT, of whom 11 (4.6%) were positive at the time of LT. Hospital admission, higher MELD at LT, prior antibiotic exposure, post-operative complications, and ICU length of stay were the factors associated with the CR-KP active infection after LT. In addition, the same group, performing a multicenter prospective

study on CR-KP carriers[72], not only in the setting of LT, demonstrated that the number of additional colonization sites was an independent risk factor for invasive infection.

In conclusion, BI significantly modify the natural history of patients with cirrhosis listed for LT. Severe BI in a sick and frail patient can produce a multiorgan failure comprising further deterioration of liver function. Even if this can increase priority in the WL, this gain in priority should be used only after adequate control of infection. To date, standardized definition of "controlled infection" is lacking. As for other patients with severe ACLF in the WL[73,74], prioritization rules in the respect of distributive justice, definition of the ideal timing for LT and definition of delisting criteria have to be refined in the next future.

MEDICAL PROPHYLAXIS OF BACTERIAL INFECTION

Antibiotic prophylaxis in patients with decompensated cirrhosis is standard of care in patients with recent gastrointestinal bleeding[75], and in those with high risk of SBP(*e.g.*, Child-Pugh > 9, serum bilirubin > 3 mg/dL and impaired renal function), or in secondary prophylaxis for SBP[24].

Antibiotic prophylaxis after upper GI bleeding reduces the incidence of in-hospital infections, re-bleeding rate within 7 d (7% *vs* 34%), and 28-d mortality (13% *vs* 35%, $P = 0.04$).

However, some concerns about long-term prophylaxis has been recently raised, since it's been associated with high prevalence of MDR BI, before and after LT. Tandon *et al*[76] evaluating 110 episodes of BI (30% hospital acquired), reported 47% of antibiotic resistance and a significant association between previous exposure to systemic antibiotics and antibiotic-resistance. Infections due to MDR bacteria are associated with an increased risk of septic shock, acute kidney injury, and death, in the post-transplant setting[44]. Furthermore, antibiotic use has been identified as the strongest predictor of invasive post-transplant fungal infection, associated with a 60% mortality[77].

Even if several studies suggested the need to stratify patients who need antibiotic prophylaxis, both after variceal bleeding and after an episode of SBP, no robust data are available to date[12,23,78,79].

Patients with cirrhosis admitted to ICU could be at higher risk of BI. Recently, a metanalysis on prognosis of cirrhotics admitted to ICU showed that acute kidney injury and sepsis as indications to ICU admission were the only factors significantly associated with mortality[80]. Another retrospective study[81] on 42 patients who underwent LT from the ICU, showed that pre-LT intubation was a factor significantly associated with post-LT pneumonia ($P = 0.02$).

On the contrary, patients who recover liver function while in the WL (*e.g.*, after viral eradication/suppression), history of BI would not be a sufficient factor for administering long-term antibiotic prophylaxis. In addition, the spreading of MDR bacteria will reduce the potential role of antibiotic mono-prophylaxis with quinolones or cephalosporines.

Given the crucial role played by dysbiosis in BI in patients with cirrhosis, several studies assessed the role of intestinal decontamination. Grat *et al*[82] evaluated the fecal microflora in 40 LT candidates, showing that abundance of several species (*e.g.*, *Bifidobacterium* and *Enterococcus*) significantly correlated with the severity of liver disease. In systematic review and metanalysis, Safdar *et al*[83] compared parenteral (*e.g.*, cephalosporins/quinolones), topically applied or non-absorbable antibiotic strategies (polymyxin, gentamicin, and nystatin) for intestinal decontamination. The Authors found an association between selective decontamination and reduction of GNB infections ($P = 0.001$), however studies were underpowered and heterogeneous.

CONCLUSION

BI represent a turning point in the natural history of cirrhosis, being the first cause of development of ACLF, and significantly affecting the outcome of patients listed for LT. These patients are at the highest risk of infection, because of frequent hospitalizations and contacts with healthcare facilities, immune dysregulation, end-stage liver disease. SBP, pneumonia and bloodstream infection represent the commonest sites of BI. In such cases, early institution of empirical antibiotic therapy is mandatory, to reduce infection-related mortality. However, empirical antibiotic therapy should take into account the changing epidemiology of infections, related both to an increase of gram positive strains and to MDR bacteria.

In the setting of LT, patients should be considered suitable for transplant after resolution of infection. However, according to recent studies, selected patients with "controlled infection" should be considered for transplant, since this condition does not impair the post-transplant outcome[69,70]. Antibiotic prophylaxis is the standard of care in cirrhotic patients with gastrointestinal bleeding or with previous episodes of SBP. However, it should be considered also in other settings with a high prevalence of BI, as in patients listed for LT, admitted to ICU and requiring intubation, because of a higher risk of post-LT pneumonia.

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REVIEW

Digital liver biopsy: Bio-imaging of fatty liver for translational and clinical research

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Abstract

The rapidly growing field of functional, molecular and structural bio-imaging is providing an extraordinary new opportunity to overcome the limits of invasive liver biopsy and introduce a "digital biopsy" for *in vivo* study of liver pathophysiology. To foster the application of bio-imaging in clinical and translational research, there is a need to standardize the methods of both acquisition and the storage of the bio-images of the liver. It can be hoped that the combination of digital, liquid and histologic liver biopsies will provide an innovative synergistic tri-dimensional approach to identifying new aetiologies, diagnostic and prognostic biomarkers and therapeutic targets for the optimization of personalized therapy of liver diseases and liver cancer. A group of experts of different disciplines (Special Interest Group for Personalized Hepatology of the Italian Association for the Study of the Liver, Institute for Biostructures and Bio-imaging of the National Research Council and Bio-banking and Biomolecular Resources Research Infrastructure) discussed criteria, methods and guidelines for facilitating the requisite application of data collection. This manuscript provides a multi-Author review of the issue with special focus on fatty liver.

Key words: Biobank; Bio-imaging; Fatty liver; Genomics; Liver biopsy; Liver cancer; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Magnetic resonance; Radiomics; Ultrasound

Core tip: The manuscript provides an extended expert review on the issue of bio-imaging of the liver with special focus on fatty liver and the need for a new integrated approach to bio-banking.

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INTRODUCTION

Autopsy, from the Greek autopsía, namely seeing with our own eyes or direct examination after death, gave birth to modern pathology during the Italian Renaissance when autopsies were performed by such artists as Leonardo da Vinci to paint and sculpt the human body and by such medical doctors as Benivieni to understand the causes of death. Two and half centuries later, the father of anatomical pathology Morgagni executed hundreds of autopsies and released the monumental five-volume On the Seats and Causes of Disease[1]. The first liver aspiration biopsy on a living patient was performed in 1883 by Paul Ehrlich, but the breakthrough that allowed the spread of liver biopsy as a diagnostic tool in clinical practice was the simple and effective technique invented by Menghini in 1958[2]. The combination of liver histology from conventional biopsy and circulating biomarkers (liquid biopsy) helps in generating an understanding of the interplay between genes and epigenetic factors in physio-pathogenic processes, providing a bimodal approach for the study and understanding of liver physio-pathology. The liver biopsy specimen however, provides a random sampling of just 1/150000th of the liver, and as such is subject to considerable sampling errors in cases with an inhomogeneous distribution of the pathologic lesion[3]. Moreover, it is obtained by an invasive procedure, unsuitable for the repeated sampling necessary to follow the *in vivo* dynamics of many physio-pathologic mechanisms. In addition, in the process of fixation and staining, the melting of intracellular fat results in artifactual ghost droplets. In short, histology provides a dead, isolated frame from a living film that runs throughout the liver, and this has deeply limited the advancement of knowledge regarding the physiopathology of fatty liver.

Modern biomedical imaging techniques offer an attractive non-invasive option for the *in vivo* study of liver physiopathology and have the capacity to provide detailed anatomical and biochemical information on the whole organ, thereby overcoming the limit of sampling error. The combination of image-based digital liver biopsy with liver histology and liquid biopsy could yield a new multimodal approach to the study of the fatty liver in clinical pathology. To foster this approach in clinical and translational research there is a need to standardize the methods of both acquisition and storage of bio-images of the liver.

ACQUISITION OF HEPATIC BIO-IMAGES

Biomedical tomographic images are rich in information that is increasingly subject to quantitative radiological assessment. A major development in computer-aided

research and diagnosis in recent years is based on computing multiple image features from volumes of interest (VOIs) or regions of interest (ROIs) of an organ and/or pathological tissue, and linking them to clinical or physio-pathological features, namely radiomics[4-6]. Automatic and semiautomatic image algorithms are mandatory for computing large numbers of image features from standardized VOIs and or ROIs for 2D/3D[7-12]. After computation, the image features can be used in correlation studies with physio-pathological and clinical characteristics and to build predictive models[13,14]. Because many of the computed features are correlated, appropriate machine learning methods are useful for establishing sustainable pattern recognition analysis[15-17].

In parallel to these developments, a great deal of new knowledge about the physiopathology of fatty liver disease has been accumulated in recent years, revealing the complexity of the mechanisms involved in non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) and the severity of ultrasonographic findings in nonalcoholic fatty liver disease reflects the metabolic syndrome and visceral fat accumulation[18-20]. The most recent guidelines and expert opinions for the management of NAFLD patients call for a new, systems medicine approach to the study of the interplays between the major physiology systems that control the vital relations of the liver with the environment, brain and nervous system, endocrine system, digestive system (gut and microbiota) and immune system[19]. New concepts for patient stratification are needed to identify different clinically significant profiles within the generic context of metabolic-syndrome[18]. This is especially important given that NAFLD affects almost one third of the general population, is an emerging cause of liver related mortality, and no reliable predictors of disease progression and response to therapy are yet available that can be applied on a large scale.

In keeping with this premise, we propose the digital liver biopsy as a new paradigm for full exploiting the information contained in multi-modality or multi-contrast conventional and molecular liver imaging, with the aim of establishing a comprehensive platform of liver radiomics, capable of capturing the heterogeneity of liver pathology associated with intra-hepatic fat accumulation. Central to our vision is that digital liver biopsies be archived together with stored specimens from both liquid and histologic biopsies in a national bio-imaging repository network including reference hepatology units and an accredited biobank hub[21]. This will foster the advancement of liver radiomics analysis, incorporating multiple texture and intensity-based features to identify those consistent with morphological transformations and highly correlated with clinic-pathologic characteristics. These imaging biomarkers will increase the potential of translational and clinical research and properly address several unmet needs for precision medicine[22-25]. The approach also encourages studies combining new imaging techniques with liver and blood metabolomics, as well as the analysis of the interplay between specific genes and the epigenetic factors conditioning their expression; opening a very interesting new way to target the dynamics of the pathogenic processes involved in NAFLD/NASH. While we hope that these new studies will identify different aetiologies, new diagnostic and prognostic biomarkers, and therapy targets; the underlying aim is to establish a basis for better patient stratification in respect to both prevention and outcome prediction that can support personalized treatment of NAFLD. The starting points for such work are the imaging technologies that are already well-established in clinical routine or well-advanced as imaging biomarkers. From current translational research and clinical practice there are several relevant PET/TC developments, as well as offshoots of ultrasound (US) and magnetic resonance (MR) imaging techniques[24-27].

LIVER ELASTOGRAPHY FOR ASSESSMENT OF FIBROSIS

Liver stiffness has been measured using magnetic resonance elastography (MRE), ultrasound-based transient elastography (TE, Fibroscan), and acoustic radiation force impulse imaging techniques in order to assess fibrosis for defining different degrees of the disease and predicting clinical outcomes[28-32]. Despite their many advances, these techniques have several limitations that must be taken into account[33-36]. TE for instance, is hampered by the presence of significant fat and/or fluid between the chest wall and the liver. These result in unreliable TE measurements in about a quarter of obese patients: A figure that likely can be reduced using a larger probe, at least in non-severely obese individuals[35,36]. Lee *et al*[37] compared the diagnostic performance of transient elastography (TE) with acoustic radiation force impulse imaging (ARFI) for staging fibrosis in nonalcoholic fatty liver disease (NAFLD). Liver stiffness correlated with fibrosis stage ($P < 0.05$) and the area under the ROC curve of TE (kPa) was slightly better than ARFI (m/s), namely 0.757 *vs* 0.657. MRE is more accurate than TE, especially for detection of initial stages of liver fibrosis, but it is influenced by iron overload and requires additional hardware[38]. Other limitations of MR elastography are technical, such as ensuring coupling of the driver to the abdomen, and ensuring that wave interference and attenuation do not compromise the stiffness values in some parts of the liver. Despite these obstacles and the limited clinical use seen at present due to expense and restricted availability, it is expected that MRE will come to represent the "gold standard" for tissue stiffness measurement[39,40].

The most important limitation of liver stiffness measures is that they are surrogates of liver fibrosis rather than a metric of fibrosis itself. Moreover, fibrosis is just one of the three major pathophysiological vectors, that determine liver stiffness; the others being congestion

and inflammation[33,34]. The impact of congestion can easily be ruled out in patients without right heart failure through examination under standard fasting conditions, but there remains a need for additional clinical and pathological information and/or biomarkers to precisely characterize the different and relative contributions of inflammatory and/or fibrotic processes to liver stiffness in the single patient[34].

MAGNETIC RESONANCE RELAXOMETRY FOR ASSESSMENT OF FIBROSIS

Contrast in MR images is the product of exponential relaxation processes, the time constants of which (T1, T2, and T2*) are determined by the physico-chemical environment of the tissues. While T2 values are typically dominated by tissue water content, the use of T2 measurements for characterizing liver inflammation is complicated by iron sequestration and storage in hepatocytes. In fact, the use of T2 shortening (seen with spin-echo sequences) is well established for estimating high levels of iron overload, while T2* shortening (evident with gradient echo sequences) being more iron-sensitive is used in characterizing low levels of iron content. Similarly, several studies[24,41,42] have reported that liver T1 varies with degree of fibrosis. A number of fast imaging approaches have been developed that permit whole-liver T1 and T2 mapping within a small number of breath-holds, raising the potential for their clinical use. As yet however, these techniques are not widely available and need to be validated for use as surrogate endpoints in clinical trials[43].

Building on these components, a multi-parametric MR strategy has recently been established[26] that includes T1 mapping for the *in vivo* characterization of fibrosis/inflammation, T2* mapping for liver iron quantification, and proton magnetic resonance spectroscopy (1H-MRS) for liver fat quantification (see below) without the intravenous injection of contrast agents. A good agreement with histology was shown in a cohort of patients with chronic liver disease of different etiologies[26]. Excitingly, other emerging MR biomarkers, including magnetization and saturation transfer, intra-voxel incoherent motion imaging, and perfusion mapping, most of which are well established in neurological imaging, offer potential to further characterize fibrosis, but remain to be optimized and adapted to liver imaging.

MAGNETIC RESONANCE SPECTROSCOPY FOR ASSESSMENT OF STEATOSIS

Sensitivity to the subtle differences in magnetic fields affecting atoms of a given species due to their different positions within a molecule or in different molecules has rendered magnetic resonance spectroscopy (MRS) one of the most powerful tools in biochemistry. In the face of the complex chemical environment of the liver, and in the modest static magnetic fields available for *in vivo* use, however, the scope for MRS is greatly limited. Nonetheless, in expert hands, it is considered a gold standard technique for *ex vivo* evaluation of tissue fat content and commonly used as an *in vivo* reference standard[45-47]. In terms of analysis, MRS is straightforward. One simply integrates the area under the water and the individual fat component peaks of the hydrogen spectrum, and then calculates the ratio of fat signal to the total hydrogen signal.

Although the acquistion of the hydrogen spectrum is relatively fast, there are a number of difficulties with this technique. Firstly, it measures only in a small region of the liver, and so lacks information on heterogeneity within the liver. Second, the positioning of the volume is subject to uncertainty due to patient respiration – in particular differences in breath-hold position between planning and acquistion can produce large errors in the fat fraction estimation[48]. Third, the presence of iron accumulation can affect the reliability of the measurement. One approach for overcoming the limit of a single sampling volume is to perform MRS in multiple voxels simultaneously so that an image can be formed (chemical shift or spectroscopic imaging)[47]. Relative to conventional MRS however, spectroscopic imaging is time consuming, and requires specialized pulse sequences that are not uniformly available across centres. More importantly, the resolution of the images remains rather poor, and contamination errors due to blurring reduce the accuracy of fat fraction measurements. In fact, at least one report has suggested that neither MR spectroscopy nor chemical shift imaging provide adequate accuracy for clinical decision-making[46].

PROTON-DENSITY FAT FRACTION MEASUREMENT

When observed in weak magnetic fields ($\leqslant$ 1T), the MR spectrum of fat is largely reduced in its primary peak associated with hydrogen bound to carbon along the backbone of the fat molecule (triglycerides). This observation has given rise to a widely used method of creating water and fat images in MRI that was initially proposed by Dixon[48]. Because of their different resonant frequencies, the water and the primary fat peak cycle in and out of phase after excitation. The difference between an image formed when the peaks are in-phase and one formed when they are out-of-phase is proportional to the quantity of fat present and one can readily calculate a fat fraction. A significant limitation of this "two-point" Dixon (or in-phase: out-phase) technique is ambiguity as to which (*i.e.*, water or fat) is the dominant component. Uncorrected, the assumption that water dominates leads to significant errors when the fat fraction exceeds 50%[45]. In the liver, a further complication of the two-point Dixon technique is associated with the presence of iron, which can be increased several hundredfold in patients with iron overload[44]. At elevated iron contents, the MR signal decays significantly between the in and

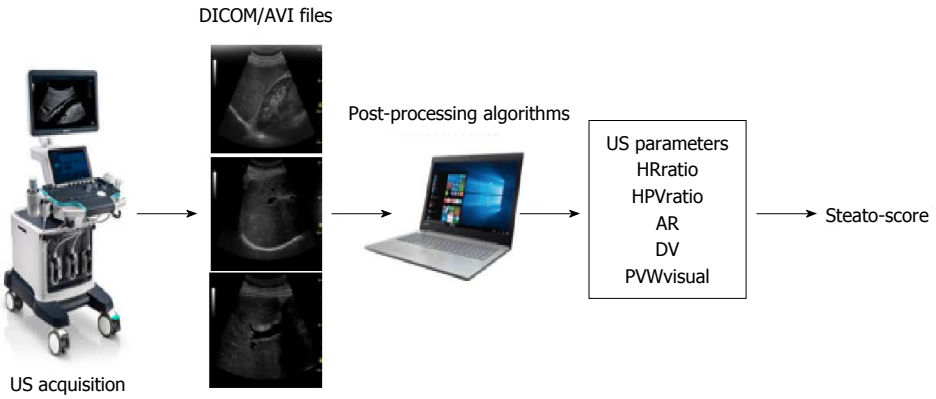

Figure 1 Quantitative multi-parametric assessment of intrahepatic fat by ultrasound. Workflow of the image acquisition, processing and data elaboration leading to a score provided by an algorithm (Steato-score)[65]. US: Ultrasound; AR: Attenuation rate; DV: Diaphragm visualization.

out of phase echoes, creating an artificial elevation of the fat fraction (or reduction if the out of phase echo is acquired first).

At higher magnetic field strengths, and in the presence of good magnetic field shimming, the hydrogen spectrum becomes more complex as the different physical-chemical environments occupied by hydrogen within fats contribute additional frequencies (chemical shifts) relative to water. As not all of the peaks are in-phase (nor out-of-phase) at any given time, the additional peaks produce variability in the fat fraction estimates when using the two-point Dixon technique[48]. To deal with the errors due to the contributions from the minor fat peaks in the hydrogen spectrum, Yu *et al*[49] proposed the use of multi-component model fitting to multi-echo MRI data to produce proton density fat fraction (PDFF) measurements. After a rapid evolution to further account (up to certain limits) for ambiguities in water or fat dominance, T2* effects associated with iron content, and the possibility to perform quantification with or without phase information[50-54], PDFF has emerged as the preferred MR imaging strategy for fat quantification[55].

Although PDFF measurements[56] have been reported to better correlate with biochemical analysis (Folch method[57]) than with the conventional histopathology grading of fat content, there is a relatively good concordance between MRI-PDFF and liver histology[58,59]. Relative to the conventional Dixon technique, PDFF measurements offer increased accuracy and repeatability, and can be used to assess the full range of fat values with less risk of inversion between water and fat values. The fact that the entire liver can be examined with PDFF scans provides a considerable advantage over MR spectroscopy for measurement stability as a high number of voxels contribute to estimates of fat fraction in the liver as a whole or in individual lobes thereof. Achieving this improved performance however, requires care in the choice of imaging parameters acquisition, and software specifically adapted to the fitting process.

There are now numerous PDFF software solutions that offer varying degrees of support, cost and availability for clinical and research use. Most free-ware solutions for example, do not offer support either in use of the software, or for the optimization of imaging parameters and data preparation. The maturity, cost and support for commercially available software is varied, and their reliability and accuracy are still being established in practice.

INTRAHEPATIC FAT MEASURED BY ULTRASOUND

US imaging has many advantages over MRI/MRS since it is simple, quick, less expensive and well tolerated. Moreover, the equipment is relatively widely available and, if needed, can be brought to the patient and conventional US is already widely used for diagnosis and management of liver pathologies. However, while US provides reliable qualitative diagnosis of liver steatosis when liver fat is above roughly 20%[60-62], its quantitative assessment is considered unreliable due to operator dependence. As described above, US can be used to measure shear wave propagation as an indicator of liver stiffness in fibrosis[58,59] and the Controlled Attenuation Parameter (CAP) score provided by Fibroscan (Echosens, Paris, France) was the first widely available quantitative US measure of intrahepatic fat in clinical practice[63,64]. It is based upon a single parameter, the US beam attenuation rate along a beam transmission line.

There is now a trend to adopting multi-parametric strategies to better assess steatosis quantitatively. One such approach is based on the acquisition of common scan projections (both subcostal longitudinal and oblique views) available with commercial US systems[65]. Two recordings (clips of 5 s) allow the quantification of 5 different parameters associated with intrahepatic fat accumulation (Figure 1). These include the hepatic-renal ratio (HR_{ratio}) and the hepatic-portal vein ratio (HPV_{ratio})[60,61] that describe the echogenicity of the liver parenchyma in comparison with renal cortex and inner portal vein, respectively. In addition, the attenuation rate (AR) takes into account the reduction of US beam penetration[61], while grades are assigned to diaphragm

visualization (DV, assessing the degree of diaphragm line visualization) and portal vein wall visualization (PVW_{visual}, assessing blurring of the portal vein walls). An algorithm (Steato-score) combining these five parameters showed good diagnostic performance discriminating presence (⩾ 5%) or absence (< 5%) of steatosis relative to ^{1}H-MRS with an area under the receiving operator characteristic (ROC) curve of 0.98 and roughly 90% specificity and sensitivity[65].

In a further refinement, the five parameters (HR_{ratio}, HPV_{ratio}, AR, DV, PVW_{visual}) will be combined with texture analysis of the liver parenchyma in the images to discriminate multiple steatosis classes using machine learning approaches. Taking ^{1}H-MRS values as ground-truth, the diagnostic performance of advanced algorithms will extend the quantification of higher intrahepatic fat levels by ultrasound[65-67].

PET TO STUDY LIVER METABOLISM

Positron emission tomography (PET) is able to image *in vivo* biochemical processes quantitatively; molecules labeled with positron-emitting radioisotopes are used in trace quantities (*i.e.*, without pharmacological effect) to visualize and measure rates of biochemical processes *in vivo*. Among the various PET-tracers, the fluorinated glucose analogue [^{18}F]-2-fluoro-2-deoxy-D-glucose (FDG), is the most widely used for metabolic, neurologic, and oncologic research as well as in clinical practice. Inflammatory and cancer cells are both metabolically active and show an increased uptake of FDG which is subsequently phosphorylated to FDG-6-phosphate and trapped intracellularly because most of FDG-avid cells, including inflammatory cell, do not express glucose-6-phosphatase. Hepatocytes on the other hand, do express this enzyme, and release FDG back into the blood.

Several studies have examined the potential of this difference in FDG kinetics in the study of fatty liver[68-70], comparing hepatic FDG uptake in NAFLD patients to controls using the semi-quantitative measurement of standardized uptake value (SUV). The results however, are mixed with some reporting a negative correlation between liver FDG uptake and NAFLD[69], others a positive[70] and others no association[69]. These conflicting results may be the consequence of the many factors, both physiological and technical that can affect SUV values[71,72]. Interestingly, FDG PET studies have nonetheless shown a link between vascular inflammation and NAFLD[73] as well as that liver SUV in patients with NAFLD may be a prognostic factor for cardio-cerebrovascular events[74,75].

From a metabolic point of view the options on PET tracers are manyfold providing a role to play in the study of NAFLD pathology. A first example is the study of hepatic metabolism in the pathogenesis of the metabolic syndrome and its clinic-pathologic correlates (*e.g.*, diabetes, hypertension and obesity). A negative correlation between higher hepatic glucose uptake HGU and liver fat content was reported in overweight subjects with type 2 diabetes[76]. These results support the notion that NAFLD is epiphenomenon of insulin resistance. The liver is an insulin sensitive organ[77] and thus, hepatic glucose uptake is reduced in subjects with insulin resistance.

Other aspects of liver metabolism such as fatty acid uptake, oxidation, and perfusion are also amenable to study with PET techniques. In a recent study in morbidly obese subjects, Immonen and coworkers, using a palmitate analog, [^{18}F]-fluoro-6-thiaheptadecanoic acid (^{18}F-FTHA), found morbidly obese subjects to have elevated hepatic fatty acid uptake (HFU) before bariatric surgery that decreased after surgery, though it did not normalize relative to lean subjects. Intriguingly, HFU and liver fat content in these patients were not related[78]. Iozzo *et al*[79] using ^{11}C-palmitate-PET scanning showed that obese subjects have higher rate of fatty acid oxidation than lean subjects. According to these authors, the assessment of fatty acid oxidation is of major importance in characterizing subjects with NAFLD, since fatty acid oxidation is a significant source of reactive oxygen species in obesity-related hepatic lipotoxicity. Finally, with appropriate modeling, PET like MRI can be used in the quantification of liver perfusion in humans[78,80].

In the oncologic field, FDG-PET scanning is the gold standard, due to the avidity of tumors for glucose. Moreover, as used in this context, serial SUV measurements is a reliable tool, with the individual patient serving as their control before and after treatment. In hepatocellular carcinoma, FDG-PET has shown its usefulness both for identifying more aggressive tumors predictive of lower survival rates, and for capturing early signs of response to treatment. In pre-clinical models, FDG-PET scanning showed capacity to quantify tumor response[81] with potential usefulness in tumor monitoring. In the clinical setting, FDG-PET has proven useful as survival predictor: patients with lower tumor to liver ratio (TLR) at time 0 showed three times longer survival than patients with high TLR[82] and the SUVmax parameter was an independent predictor of survival in HCC patients undergoing Sorafenib treatment[83]. Most interestingly, the discriminating capacity of FDG-PET associated with the clinic pathologic response to the anti-angiogenetic drug was shown to be detectable as soon as three weeks from the beginning of treatment[84], thus an ealry FDG-PET scan after starting anti-angiogenetic treatment seem to be a promising technique for monitoring early response[85].

Undoubtedly, PET imaging in the liver has provided new insight in quantifying different metabolic pathways in humans. Whereas measuring inflammation in the liver until now has been proven challenging, FDG-PET has a clear role in patients affected from hepatocellular carcinoma.

IMAGING MASS SPECTROMETRY

IMS combines the mass spectrometry with the Matrix-assisted laser desorption/ionization (MALDI) technique,

and allows the visualization of the spatial distribution of metabolites, drugs, peptides, proteins and lipids, directly on tissue sections without the need for extraction (biochemical analysis) or the use of specific markers (enzyme- or immune-histochemistry). The advantage of this technique is in mapping the subcellular localization and morphology on tissue sections and, at the same time, providing qualitative and quantitative evaluation of thousands of analytes. This bi-dimensional technique can also be adapted to fathom below the tissue surface to produce a three-dimensional map of the molecules (having molecular weights between 1000 to 70000 Da) present in the histological sections. The signals of ions obtained from the histological section/sample can be mapped in specific regions of the tissue and transformed into images on the basis of the density of the ions inside the tissue.

As an example of the use of IMS, accumulation of C14:1 acylcarnetine in a steatotic liver has suggested a deficiency of very long-chain acyl-CoA dehydrogenase permitting molecular genetics analysis to be addressed towards the detection of the underlying mutations[86].

There are in fact a growing number of techniques similar to IMS, including synchrotron infrared and ToF-SIMS (time of flight secondary ion mass spectrometry) micro-spectroscopies[87] capable of identifying molecular species of lipids concentrated in steatotic vacuoles or present in small lipid droplets that most probably correspond to the first step of lipid accretion. Likewise these techniques are of interest for tracking and understanding the effects (pharmacokinetics, toxicology, metabolomics) of drugs on cells in metabolic diseases and NAFLD. While not applicable *in vivo* they are likely to play an increasing role in the chemical imaging/analysis of pathology samples to provide high resolution targets, in both spatial and biochemical terms for the understanding of radiological findings of NAFLD.

RADIOMICS

The success of precision medicine requires a clear understanding of disease heterogeneity at the single patient level, yet despite the large radiological armamentarium available, most imaging-based clinical decision-making is still based solely on visual assessment. The combination of visual assessment and image-processing techniques that describe and quantify numerous image features, such as intensity-based and textural properties that are difficult to convey consistenty in verbal reports, can provide a comprehensive characterization of imaging data sets. This approach adds value to the individual quantitative measure or visual subjective report and the derived results can be combined with statistical modelling techniques to predict clinical end points. The field of research that examines the relationships between advanced quantitative imaging features from medical images and clincal data is called "Radiomics", while "Imaging Genomics" (or radiogenomics) refers to the study of imaging features in association with high-throughput genetic data[88] (Figure 2).

Radiomic features are derived from the information contained in the voxels of a segmented structure of an image, and are typically grouped into families having similar derivational or statistical roots. First-order statistical features are reflected in the histogram of voxel intensities, and include: the mean value, dispersion (standard deviation, mean absolute deviation), central moments (skewness and kurtosis describing asymmetry and sharpness, respectively) and randomness (entropy, uniformity). Texture or greyscale variation features, refer to higher-order statistical measures that describe the local spatial arrangement of intensities. This typically reduces to expressing how different parent matrices capture the spatial intensity distribution of an image, and is widely used as a low-level step in pattern recognition. In contrast to the pre-defined features associated with texture analysis, the rapidly growing approach to radiomics represented by deep learning draws on advanced optimization of self-generated patterns to create networks for recognition analogous to neurons. While originally developed for planar images, intensity-based and textural features measures and deep learning can generally be calculated for volumetric datasets.

Radiomics analysis, as a technique that objectively measures the structural and/or functional heterogeneity of the tissue by quantifying the spatial patterns of pixel intensities on cross-sectional imaging, can improve the clinical usefulness of multimodal biopsy data. Recent pilot studies employing radiomic approaches on multimodal and hybrid imaging (CT, optical, PET/MRI, PET/CT) have shown the usefullness of quantifying the overall tissue spatial complexity in identifying the regions which drive disease transformation, progression, and drug resistance in a variety of pathologies[17,90-93].

Both radiomics and imaging genomics are at very early stages in hepatology and many problems remain to be solved. To date, the vast majority of liver-related radiomic studies have focused on the analysis of malignant lesions for establishing prognostic or predictive models. For example, in 2007, Kuo *et al*[88] observed significant correlation between tumour margins in arterial phase images and doxorubicin-response gene expression. In 2015, Miura and coauthors[96] identified 53 up-regulated and 71 down-regulated subsets of genes in the highly aggressive HCC group compared with the low-grade HCC group as distinguished by gadolinium ethoxybenzyl-DTPA magnetic resonance imaging hyperintensity. They have also shown that clinico-pathological and global gene expression analyses revealed low-grade malignancies within high-grade HCCs compared with low-grade HCCs. Recently, Zhou *et al*[97] have extracted radiomics features from arterial and portal venous phase using CT images, identifying twenty-one radiomics features from a panel of 300 candidate features, to predict the early recurrence ($\leqslant$ 1 year) of hepatocellular carcinoma. A further example of the scope for synergy formed by the three-

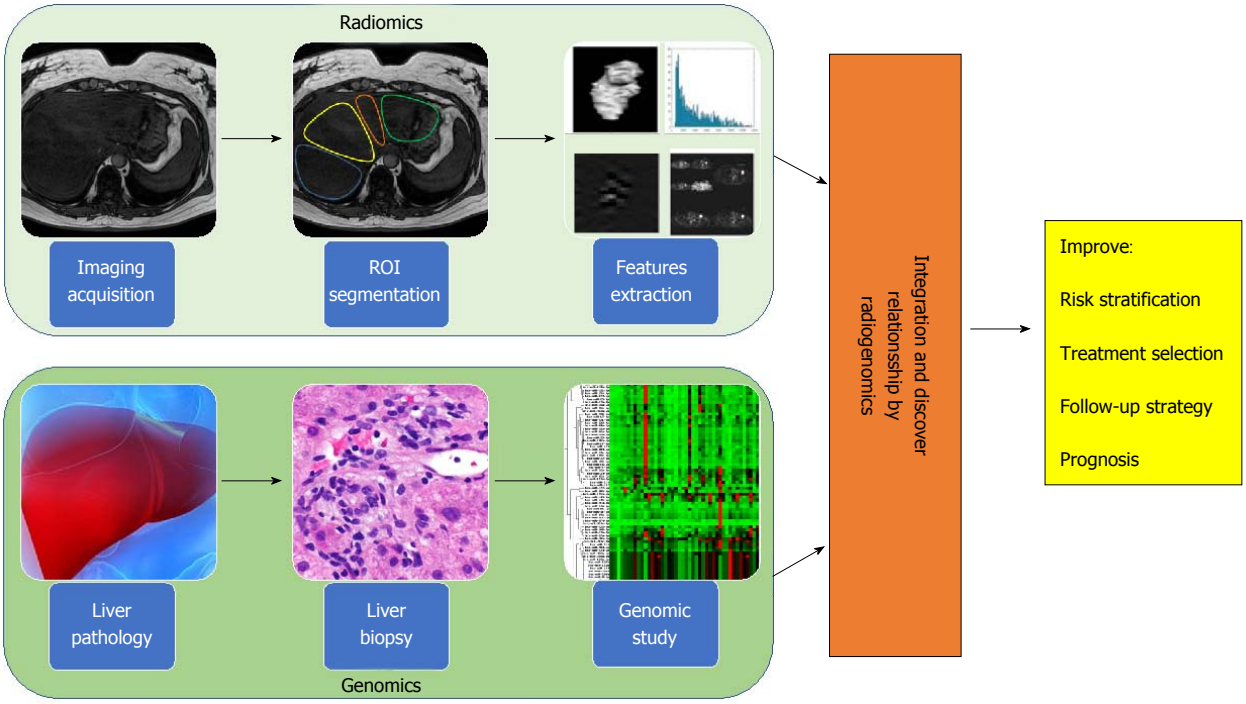

Figure 2 Radiogenomic approach to liver disease. Radiogenomics integrates radiomic data (upper panel), produced from the in silico extraction of features from bio-images, with genomic data (lower panel), coming from the study of bio-specimens with next generation sequencing technologies. Radiogenomics represents a powerful strategy to improve and personalize diagnostic accuracy, as well as measure response to therapy, leading to an overall improvement of patient management affected by liver disease.

dimensional vision of digital, liquid and phsyical biopsy, is the recognition of relationships between histologic and clinical phenotypes including microvascular invasion of hepatocellular carcinoma and prognosis of intrahepatic cholangiocarcinoma evidenced *via* MRI diffusion-weighted imaging and hepatobiliary phase after gadoxetic acid[94,95].

In one of the few reports applying the radiomic approach to the study of NAFLD, in 2015 Vanderbeck *et al*[90] developed an automated classifier that detected and quantified macrosteatosis with at least 95% precision. They applied an automatic quantifier of lobular inflammation and ballooning to digital images of hematoxylin and eosin stained slides of liver biopsy samples from 59 individuals with normal liver histology and varying severity of NAFLD. Their results demonstrated that automatic quantification of cardinal NAFLD histologic lesions is possible, and offer promise for further development of automatic quantification of NAFLD biopsies in clinical practice.

Genome-wide association studies of hepatic fat content as determined radiologically on the other hand, have already led to major breakthroughs in the field, with the identification of genetic variants influencing hepatic fat accumulation[98,99]. In addition, although fibrosis stage is considered the major prognostic determinant in patients with NAFLD/NASH[100], recent data suggest that the amount of hepatic fat accumulation plays a causal role in determining the development of NASH and its progression[101]. Thus, a reliable non-invasive measure of intrahepatic fat may represent an important outcome predictor in both clinical research and practice.

Radiogenomics findings in hepatic oncology illustrate the potential for further clinical insights and diagnostic pathways. For example, Banerjee *et al*[106] found an interesting correlation between a "Radiogenomic Venous Invasion" biomarker (a contrast-enhanced CT parameter) and a 91-gene HCC "venous invasion" gene expression signature. This biomarker may be able to predict histological microvascular venous invasion in HCC that may be useful for identifying patients less likely to a durable benefit from surgical treatment. Renzulli *et al*[107] also found a correlation between multiple imaging biomarkers, such as tumor dimension, non-smooth tumor margins, peri-tumoral enhancement, and gene expression called "two-trait predictor of venous invasion", as predictor of microvascular venous invasion.

Hepatology is a particularly interesting field of application for the new radiogenomic approach; patient management already requires the combination of information coming from *ex vivo* analyses of circulating biomarkers (serum proteins, transaminases, *etc.*), histology and direct *in vivo* multimodal imaging data (ultrasound, CT, MR, *etc.*) (Figure 3). Nowadays however, all these data remain locked within the various departments and isolated between institutions, creating an obstacle to the creation of the coherent program of collaborative research.

BIO-BANKING FOR LIVER DISEASE

Institutional biobanks are service units dedicated to the collection, processing, storage and distribution of

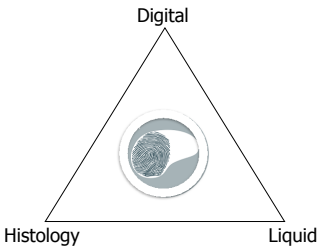

Figure 3 A new three-dimensional view of the liver biopsy. Digital biopsy, direct *in vivo* imaging of the whole liver, adds important pathophysiological and morphological context to liquid and invasive (percutaneous or surgical) liver biopsies that provide focal *ex vivo* analysis of circulating biomarkers and specimens of the liver respectively contributing to a three dimensional view for diagnosis and prognosis of liver disease.

biological materials and associated patient information. Biobanks are becoming more and more central to translational research in the fields of human health, biotechnologies and development in life science.

Generally, institutional biobanks can be classified in terms of the profile of material contained in the collection as: population-based, disease-specific or rare disease oriented. The biological material can be constituted of different types of samples such as solid tissues, plasma, serum, RBC, buffy coats, white cells, DNA, RNA, proteins, cell lines, saliva, urine and fecal samples. The widest European consortium of Biobanks is represented by the Bio-banking and Bio-Molecular Research Infrastructure (BBMRI-ERIC)[103]. Recently, an additional class of biobanks has emerged in the form of imaging biobanks. Specifically, the European Society of Radiology defines imaging biobanks as "organized databases of medical images and associated imaging biomarkers (radiology and beyond) shared among multiple researchers, and linked to other biorepositories"[104,105]. The primary role of imaging biobanks is the identification of novel imaging biomarkers to be used in research studies, as well as to support validation of novel biological or genetic biomarkers of disease. It is important to recognize the fundamental importance of integrating clinical, pathlogical and biological endpoint data (such as with genomic results coming from high-throughput sequence analysis of DNA or RNA) with *in vivo* imaging data derived from radiomic analysis of CT, MRI or PET images for the identification of biomarkers (both imaging and non-imaging) and their translation to clinical relevance. A major limitation of radiomics and radiogenomics research is that the large number of radiomic features and patholgical/biological variables that may or may not be involved necessitate the use of large datasets and condivision of data across disciplines. Thus, imaging biobanks should be embedded in wider biobank networks. This may be the product of an explicit program of data collection (such as the German national Cohort, and the United Kingdom Imaging Biobank projects) or through consistent practice across a number of individual clinical entities that interact as nodes in a network dedicated to a given pathology/ies. As an example of a product of the former, in a prospective study of 4.949 participants in the United Kingdom Imaging Biobank[105] in whom liver fat was measured using the PDFF MRI technique, Wilman *et al*[105] showed an association of increased liver fat with greater age, BMI, weight gain, high blood pressure and type 2 diabetes. An example of an individual clinical node for the latter form of network is the SDN Biobank, that is the institutional biobank of the IRCCS SDN. This structure is devoted to the collection of biological material (blood, urine, feces) as well as images (deriving from CT, PET, MRI) from patients affected by oncological, cardiological, neurological and metabolic diseases[21].

Due to the non-invasive nature of medical imaging and its ubiquitous use in clinical practice, the field of radiogenomics is rapidly evolving and initial results are encouraging. Biobanks will surely have a critical role for the development of the innovative radiogenomics protocols. Indeed, their collaboration across centres and disciplines is likely the only feasible approach to assembling the large numbers of images and samples, along with the clinical data required for testing associations in identifying and validating imaging and genomic biomarkers. To facilitate this collaboration, in the future it will be necessary to bring together images and biological samples from the same patients in innovative biobanks adapted to collect both kinds of material. The data coming from this kind of biobanks will have a critical role for the development of personalized protocols for diagnosis and patients care (Figure 4). In the context of fatty liver, well defined and correlated histologic and imaging targets from MRI and US (Table 1) already exist and they can form the basis of such a collection.

DIGITAL BIOPSY-THE ROAD AHEAD

Digital biopsies for NAFLD need to be fast and easy to obtain, and consistent among operators, technologies and methods, and most importantly provide clinically relevant results. To arrive at this level, there is a need for research, development and extensive collaboration.

A number of imaging techniques are already available for evaluation of fibrosis and steatosis. Wider adoption of these techniques and continued work to overcome their limitations can provide the basis for multi-centric collaboration on the optimization of imaging for fatty liver.

No individual centre has the breadth and depth of patients to go it alone in establishing a validated digital liver biopsy. Collaboration on imaging technique adoption should be performed in combination with establishing a cross-center imaging biobank linked to associated conventional biobanks.

An open need lays in the attraction of expertise and interest to carry out radiomics and radiogenomic studies and this follows the generation of data and the establishment of the bio-repository resources described above. Only at this point we will see the fruits of the labour, in terms of possible imaging biomarker signatures and associations with clinical and biological

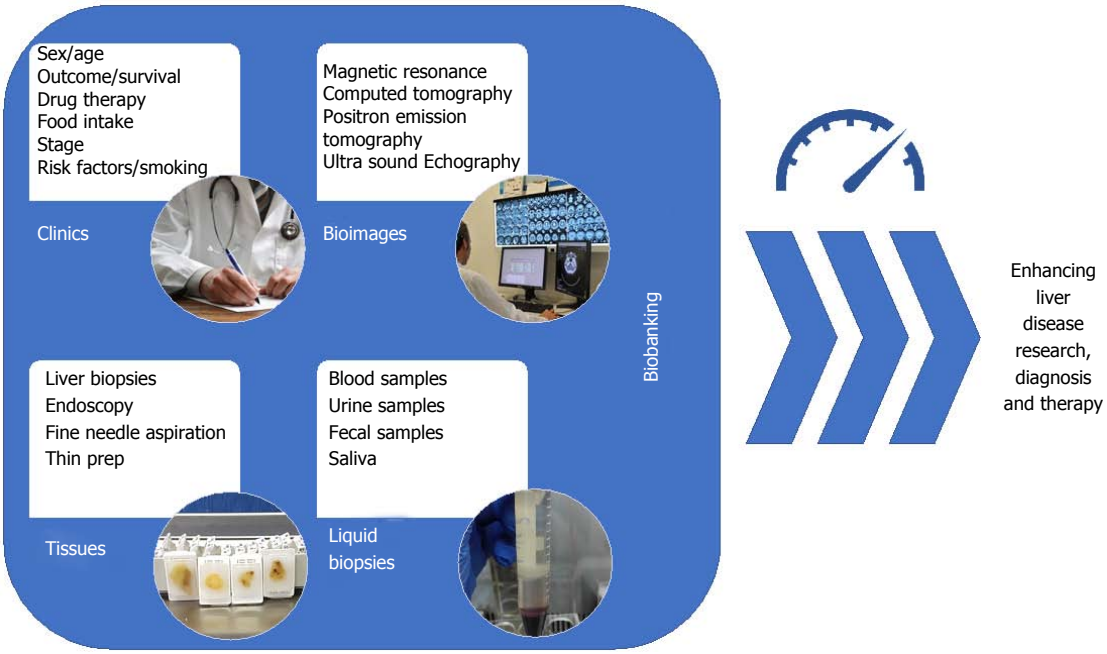

Figure 4 Modern vision of bio-banking. The collection of patient clinical data, tissue samples, liquid biopsies as well as bio-images, in organized datasets is defined as "bio-banking". With the advent of omics sciences (*i.e.*, proteomics and genomics) where a large number of biological specimens and associated data are needed for making a precision medicine approach to the patients collaborative studies across centers are essential to maximizing patient recruitment. Equally, accessible well-structures data stores permit re-use and re-examination of data reducing the cost of subsequent studies. In this context, the field of bio-banking has the possibility to enhance research on liver disease as well as improve diagnostics and therapeutics.

Table 1 Intrahepatic fat measurement

Ref.	Imaging modality	Classification	ROC-derived parameters		
			AUC	Sensitivity (%)	Specificity (%)
Mancini *et al*[60] 2009	US	^{1}H-MRS fat content > 5%	0.996	100	95
Xia *et al*[61] 2012	US	^{1}H-MRS fat content > 5.56%	NA	95.1	100
Edens *et al*[62] 2009	US	^{1}H-MRS fat content > 5.56%	NA	66.7	100
Di Lascio *et al*[65] 2018	US	^{1}H-MRS fat content > 5%	0.97	89	94
Sasso *et al*[108] 2012	CAP score (imaging derived)	Liver biopsy	S_0 *vs* $S_1S_2S_3$: 0.80	S_0 *vs* $S_1S_2S_3$: 76	S_0 *vs* $S_1S_2S_3$: 71
		S_0: < 10% of hepatocytes	S_0S_1 *vs* S_2S_3: 0.86	S_0S_1 *vs* S_2S_3: 87	S_0S_1 *vs* S_2S_3: 74
		S_1: 11%-33% of hepatocytes	$S_0S_1S_2$ *vs* S_3: 0.88	$S_0S_1S_2$ *vs* S_3: 78	$S_0S_1S_2$ *vs* S_3: 93
		S_2: 34%-66% of hepatocytes			
		S_3: 67%-100% of hepatocytes			

Both histologic and digital liver biopsy provided reliable measures of intrahepatic fat that are significantly correlated, but categorically different. Liver biopsy describes the histologic characteristics of the pathologic lesions and accounts for the percentage of hepatocytes with intracellular fat-derived vacuoles using categorical grading systems that are not directly representative of the hepatic triglyceride concentration[19-21]. On the other hand ^{1}H-MRS measures protons in acyl groups of liver tissue triglycerides and provides continuous quantitative values expressed as mg/g of hepatic tissue[109]. Moreover, ^{1}H-MRS uses a much larger volume of liver tissue than biopsy reducing sampling error and representing the most accurate measure of the overall liver triglyceride content. US: Ultrasound; ROC: Receiving operator characteristic; CAP: Controlled attenuation parameter.

endpoints. Prospective studies should then compare the results obtained using images acquired at different points in time by multiple technicians, and read by both radiologists and trained non-radiologists. The next step is studying and comparing quantitative image features to unravel the relationships between the digital biopsy features and histopathologic, metabolic and genetic characteristics and building models which link them to the disease outcomes. Validation studies with multiple users are required to show that intra- and inter-reader variations of the digital biopsy derived features are acceptable.

CONCLUSION

We foresee the combination of image-based digital liver biopsy with liver histology and liquid biopsy in a multimodal approach to the study of the fatty liver in clinical pathology as a key to personalizing patient care. To foster this approach in clinical and translational research there is a need to standardize the methods of acquisition, processing and storage of bio-images of the liver. A number of imaging techniques that offer a starting point for acquisition have been identified and their use in combination in prospective studies will

open a new venue of translational and clinical research. However, many of these tools are still limited to selected Centres and it is important to increase their accessibility by reducing costs.

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REVIEW

Alkaline sphingomyelinase (NPP7) in hepatobiliary diseases: A field that needs to be closely studied

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Abstract

Alkaline sphingomyelinase cleaves phosphocholine from sphingomyelin, platelet-activating factor, lysophosphatidylcholine, and less effectively phosphatidylcholine. The enzyme shares no structure similarities with acid or neutral sphingomyelinase but belongs to ecto-nucleotide pyrophosphatase/phosphodiesterase (NPP) family and therefore is also called NPP7 nowadays. The enzyme is expressed in the intestinal mucosa in many species and additionally in human liver. The enzyme in the intestinal tract has been extensively studied but not that in human liver. Studies on intestinal alkaline sphingomyelinase show that it inhibits colonic tumorigenesis and inflammation, hydrolyses dietary sphingomyelin, and stimulates cholesterol absorption. The review aims to summarize the current knowledge on liver alkaline sphingomyelinase in human and strengthen the necessity for close study on this unique human enzyme in hepatobiliary diseases.

Key words: Sphingomyelin; Alkaline sphingomyelinase; Nucleotide pyrophosphatase/phosphodiesterase 7; Autotaxin; Platelet-activating factor; Cholangiocarcinoma; Liver diseases; Gallstone

Core tip: Alkaline sphingomyelinase is an enzyme expressing in the intestinal tract and additionally human liver. It hydrolyzes sphingomyelin, platelet activating factor and lysophospholipase. In the intestinal tract, it digests dietary sphingomyelin, stimulates cholesterol absorption, and inhibits development of colon cancer. Less is known about the implications of the enzyme in liver diseases. The review summarizes the current knowledge of its roles in hepatobiliary disease and raised special topics for future investigations.

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ALK-SMASE IN NPP FAMILY

An enzyme that catalyzes sphingomyelin (SM) hydrolysis to ceramide at optimal alkaline pH was first identified in the intestinal tract by Nilsson in 1969[1]. The enzyme was thereafter named as alkaline sphingomyelinase (alk-SMase)[2] in line with acid and neutral SMases. However, after purification, characterization, and gene cloning[3-6], it was found that alk-SMase actually had no structural similarities with either acid or neutral SMase, but shared about 30% amino acid sequence similarities with enzymes in ecto nucleotide pyrophosphatase/phosphodiesterase (NPP) family. As a novel member in NPP family, alk-SMase is nowadays also called NPP7.

Comparing with other six NPP members, alk-SMase is distinctive in several aspects. Not like most NPP members, alk-SMase has no nucleotidase activity but a phospholipase C activity. It cleaves phosphocholine from phospholipids including SM, platelet activating factor (PAF)[7], lysophosphatidylcholine (lyso-PC), and phosphatidylcholine (PC) less effectively[5]. In NPP family, NPP2 (autotaxin) can also hydrolyze lyso-PC, but with a phospholipase D activity[8]. Another NPP member NPP6 can cleave phosphocholine from lysophospholipids mainly lyso-PC and lyso-PAF but not from SM[9]. Alk-SMase is so far the only one that has decent activities against SM and PAF in this family. In addition, while the activities of other NPP members could be identified in many organs and tissues, expression of alk-SMase is only restricted to intestinal tract in most species[10]. Western blot of rat tissues only shows positive band in intestinal mucosa and content but not in other organs including brain, heart, lung, liver, spleen, kidney and pancreas[4]. Interestingly, additional high activity was found in the bile of human[11], but not in bile of other species including rat, mouse, pig, cow, sheep, dog, guinea pig, and baboon[10] (and unpublished data). Furthermore, most NPP members are functioning as a proliferative and inflammatory factors that are important for cell survival[12], alk-SMase displays inhibitory effects on cell proliferation and inflammation[13,14]. Cell culture studies show that alk-SMase can inhibit cell proliferation by about 50%[15] and its activity *in vivo* is positively correlated to the activity of caspase 3, the key enzyme that triggers apoptosis[16-18]. Rectal administration of alk-SMase in rats suppresses colitis induced by dextran sulfate sodium (DSS)[19]. Recently the studies with alk-SMase knockout mice clearly showed that both initiation and malignant transformation of colon cancer induced by azoxymethane and DSS was enhanced by about 5 times in the knockout mice comparing with the wild type mice[20]. In agreement with the animal studies, clinical studies also found reduced alk-SMase activity in patients with inflammatory bowel diseases (IBD) and colon cancer, and the reduction is progressive from 25% in IBD to 75% in colonic carcinoma[21-23].

The anticancer effects of alk-SMase are thought to be achieved with a three armed mechanism[13]. First, it hydrolyzes SM to ceramide, which is a well-known antiproliferative and apoptotic molecule[18,24]. Second, it cleaves phosphocholine moiety from PAF and inactivates PAF[7], which is widely expressed in many inflammatory tissues promoting inflammation and tumorigenesis[25]. And finally NPP7 converts lyso-PC to monoacylglycerol[5], thus reducing the production of lysophosphatidic acid (LPA), which otherwise can be formed by NPP2[8]. LPA has emerged as an important messenger with potent inflammatory and carcinogenic effects mediated *via* several signaling transduction pathways after binding to G protein coupled receptors[26,27]. In supporting this three arm hypothesis, decreased ceramide and increased PAF[20] and LPA (Zhang P *et al* Abstract presented in AACR symposium, Shanghai, China, 2016) have been found in NPP7 knockout mice.

Similar to other NPP members, alk-SMase is anchored on the surface of the cell membrane with a short hydrophobic domain. The remaining part of the enzyme including the catalytic domain is exposed extracellularly[13]. The enzyme can be released by bile salt[28], and also by pancreatic trypsin, as there is a tryptic site just above the hydrophobic domain embedded inside the membrane[29].

ALK-SMASE IN HUMAN BILE

Alk-SMase in human bile was discovered by Nyberg *et al*[11] in bile collected from patients in our hospital. The activity in human bile is not derived from bacteria since it is similarly present in the samples with and without bacterial infection. Although gallbladder bile has higher activity than the hepatic bile, no activity was found in the homogenates of gallbladder mucosa, confirming that it is liver not gallbladder that expresses the enzyme. Because PCR experiment identifies alk-SMase mRNA in human HepG2 cells[30], the enzyme is believed to be expressed by hepatocytes, transported to the surface of the microvilli that extend into the bile canaliculi and released by bile salt into the lumen.

Alk-SMase in human bile shares similar characteristics as the one in the intestinal mucosa[31,32]. The enzyme becomes active at the pH around 7 and the maximal activity occurs at pH 9.0. Its activity requires the presence of bile salts[4,6]. The bile salt dependency is type specific, which differs from other lipases such as bile salt stimulated lipase[33,34]. Although different bile salts more or less increase alk-SMase activity with the maximal effects at their critical micelle concentrations, taurocholate (TC) and taurochenodeoxycholate (TCDC) are much more effective than other bile salts. On the other hand, the nonionic detergent Triton X100 and zwitterionic non-denaturing detergent CHAPS with similar structure as TCDC and TC have no stimulatory effect but inhibit alk-SMase activity in the presence of other bile salts[4,31,35]. The finding indicates that the bile salt induced activation is not a simple detergent effect on the physical state of the substrate SM in mixed micelles. Additional interaction between the enzyme

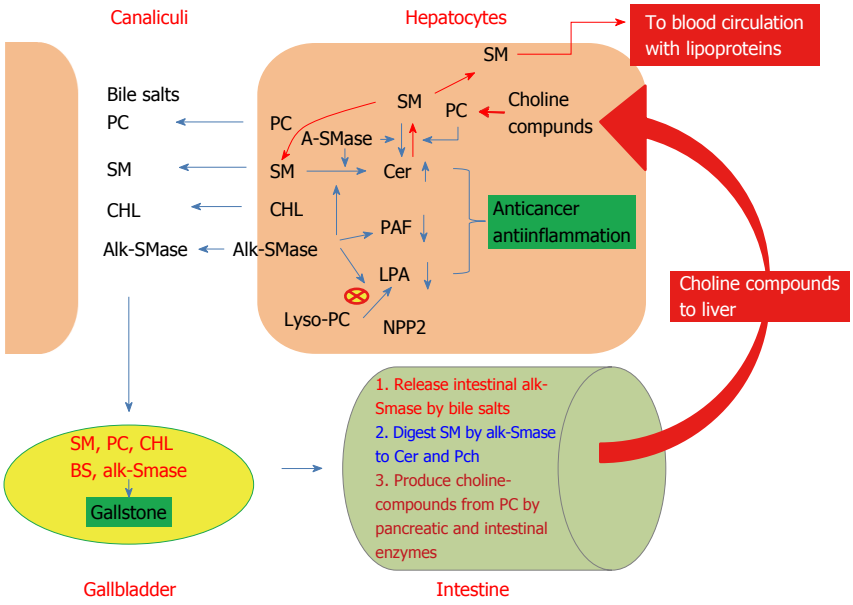

Figure 1 Metabolism of sphingomyelin in the liver and potential implications of alk-SMase in liver diseases. Liver alk-SMase is localized on the hepatocyte canaliculi membrane. It hydrolyzes SM, PAF, and lyso-PC, resulting in increased ceramide (Cer) and decreased PAF and LPA, thus having anticancer and anti-inflammatory effects. Together with PC, cholesterol, and bile salts, SM is released in canaliculi and transported to gallbladder, where the interactions of these compounds affect gallstone formation. When the bile is delivered into the intestinal tract, bile salt will release additional alk-SMase from intestinal mucosa, and digest intestinal SM to ceramide and phosphocholine (Pch). Meanwhile, PC will be hydrolyzed by enzymes from pancreas and intestinal mucosa to choline compounds such as free choline, lyso-PC, and Pch. These choline compounds will be transported to liver where to be used for synthesis of PC. Pch moiety in PC can be transferred to ceramide to form SM by SM synthases. Part of the SM formed will be released into blood together with lipoproteins, part to bile, and part to be degraded by alk-SMase and acid SMase (ASMase) in the liver.

protein and the bile salt is likely involved. Supporting this hypothesis, recent studies on crystal structure of human alk-SMase by Gorelik *et al*[36] showed that the enzyme forms a hydrophobic loop and a positively charged surface which can interact with bile salts. This needs to be proved in further investigations.

Similar also to intestinal alk-SMase, human bile alk-SMase is inhibited by PC, the most abundant phospholipid in the bile[31,37]. This might be related to a competition between PC and SM for the substrate binding site of the enzyme. As shown by both computer homology modelling studies[38] and crystal structural studies[36], alk-SMase forms a specific pocket and a long narrow groove that fits the phosphocholine head group, and the tails of these substrates, respectively. The binding affinity is stronger for SM than for PC[39,40].

Presence of alk-SMase in human bile enhances SM digestion in humans. Intestinal SM is derived from diet, shedding mucosal cells and bile. The enzyme responsible for digesting SM in the gut is alk-SMase and in alk-SMase knockout mice, about 90% of ingested SM cannot be digested but accumulated in the colon[41]. In many species except human, alk-SMase activity is absent in duodenum, increasing in the jejunum and declining in the colon[2]. SM digestion in the gut normally starts in the middle of the jejunum where alk-SMase is high and PC, the major inhibitor of alk-SMase, has been decreased due to the absorption[42]. The process of SM digestion is slow and incomplete in most species, resulting in about 40% ceramide and SM being identified in feces[43,44]. However, due to the presence of additional alk-SMase in the bile of human, human duodenum has considerable alk-SMase activity. Ohlsson *et al*[45] found that digestion of SM in human is more efficient than other species and about 81% of ingested SM can be digested. The more effective digestion of SM could be important for human health, as dietary SM stimulates the development of the gut of the new born and inhibits colonic tumorigenesis[46-48].

ROLE OF LIVER IN SPHINGOLIPID METABOLISM

It is well known that liver is an important organ for lipid metabolism such as fatty acid beta-oxidation, ketone body generation, cholesterol metabolism, lipoprotein synthesis, and phospholipid metabolism. For the phospholipid metabolism in liver, most previous studies focused on PC not SM, but the interest in SM was increasing in the latest decades. Liver is an organ with relatively high levels of SM. Comparing with subcutaneous and intra-abdominal adipose tissues, SM in human liver is 7-8 fold higher than in these adipose tissues[49].

The high levels of PC and SM in the liver are attributable to the fact that liver efficiently takes up choline containing compounds such as choline, lyso-PC and phosphocholine derived from digestion of phospholipids in the intestinal tract and uses them for synthesis of PC and SM[42,50] (Figure 1). As shown in animal studies, after feeding choline labeled SM, up to 30% of the

labeled choline is accumulated in the liver and more than 95% of them is utilized for PC synthesis[42]. This is important for SM synthesis, as at the last step of SM synthesis, phosphocholine is transferred from PC to ceramide, catalyzed by SM synthase, which is highly expressed in the liver[51,52]. For the hydrolysis of SM, liver has high acid SMase activity than most other organs in many species[41]. Acid SMase is an enzyme with two isoforms. One is the lysosome enzyme that breaks down internalized SM in lysosome and the other is a secretory form that can be secreted to the plasma membrane and hydrolyzes membrane bound SM[53]. That is why in Niemann Pick diseases with acid SMase deficiency, liver is one of the most affected organs with SM accumulation[54]. There are many other factors that influence SM levels in the liver, such as high fat diet[55], endotoxin infection[56], hepatitis B virus infection[57] and liver cancer[58].

SM synthesized in the liver can be released into plasma and bile (Figure 1). Comparing with other species, human plasma has at least two fold higher levels of SM than other species[59]. The plasma SM from liver is mainly transported with lipoproteins mainly VLDL and less with LDL and HDL[43]. SM in plasma is also derived from intestinal mucosa and other tissue cells, being about 1-1.5 g in total[43]. Most SM secreted from intestine is in chylomicron. SM in chylomicron is mainly not from the dietary products but from the membrane of enterocytes, because most sphingosine, the final digestion product of SM by alk-SMase and neutral ceramidase in the gut is not utilized to resynthesize SM after absorption in mucosal cells, but to convert to fatty acid and then to chyle triglyceride[44,60].

Secretion of phospholipids from hepatocyte canaliculi membrane is by a mechanism related to the interactions of bile salt and ABCB4[61]. Under physiological conditions, more than 95% of the phospholipids in bile is PC, and SM is accounted for about 3%[62]. The levels of SM in human bile is relatively low comparing with other species which don't have alk-SMase in the bile such as sheep[63]. But under pathological conditions, the level of SM in bile is subject to change. Barnwell *et al*[64] showed that *in vivo* perfusion of bile salts in rat significantly reduced PC content and meanwhile induced about 10 time increase of SM in the bile without obvious damage to the liver.

The implications of SM in plasma and bile are getting increasing interest. It has been known that high concentration of plasma SM is a risk factor for atherosclerosis[65]. Recent studies also found that plasma SM could be a biomarker for various liver diseases such as hepatitis, primary sclerosing cholangitis (PSC), and steatosis[57,66,67], indicating SM metabolism in liver significantly contributes to plasma SM levels. SM in the bile may have important implications, as it has stronger van der Waal interactions with cholesterol than PC[40] and its physical properties of SM is affected by bile salt which may affect gallstone formation in gallbladder[68].

ALK-SMASE IN HEPATOBILIARY DISEASES

Comparing the extensive studies on intestinal alk-SMase, which showed its important roles in SM digestion, colon cancer prevention, and cholesterol absorption[13,14,20], the progress of the research on human bile alk-SMase obviously lags behind. The main obstacle is lack of an animal model that expresses alk-SMase in the liver, and lack of a cell line that highly expresses alk-SMase, as the enzyme has already been downregulated in tumorigenesis. In a pilot study, we measured alk-SMase activity in 30 human liver biopsies and the results indicated a reduction of the enzyme activity in steatosis and PSC[30]. Recently we determined alk-SMase activity in 59 bile samples taken under ERCP and found significant reduction of alk-SMase activity in bile of patients with PSC and tumorigenic diseases, with the most remarkable reduction in cholangiocarcinoma[69].

Besides the reduction of activity, an abnormal transcript of alk-SMase was identified in both liver cancer HepG2 cells and colon cancer HT29 cells, which is caused by a shift of RNA splice site at transcriptional level, resulting in exon 4 deletion[30,70]. The enzyme translated from this transcript is totally inactive, as 73 amino acids coded by exon 4 were absent, of which a histidine is critical for formation of the substrate binding site[70]. According to the size of their mRNA, the wild type and the mutant isoform have been called 1.4 kb and 1.2 kb form, respectively. These two forms were found in the bile of many of the 59 patients with different hepatic diseases[69], but the activity of alk-SMase is positively correlated with the ratio of 1.4/1.2 kb form. Decrease in the 1.4 kb product and increase in the 1.2 kb product are likely associated with the development of cholangiocarcinoma. In the bile of one PSC and one cholangiocarcinoma patient, no alk-SMase activity and no 1.4 kb product but only high levels of 1.2 kb form were identified[69].

On the other hand, hepatobiliary diseases may also affect the levels of alk-SMase and SM digestion in the intestine because bile diversion strongly reduced alk-SMase activity in the small intestinal content by 85% and in the feces by 68% in rat[71]. The changes are believed to be related to bile salts, which release the alk-SMase from the intestinal mucosa to gut lumen[28].

HUMAN HEPATIC ALK-SMASE IN PERSPECTIVES

Considering the results from previous studies on alk-SMase, it is predictable that human liver alk-SMase may also have important implications for hepatobiliary diseases. The following questions are worth close investigation: (1) Can the remarkable reduction of alk-SMase activity and the increase in the aberrant isoform

in the bile be warning signals for carcinogenesis in the liver, particularly cholangiocarcinoma? It is well known that cholangiocarcinoma is a disease lacking an early biomarker, and most patients are not curable at the time when the disease is diagnosed[72]. Making the situation worsen is the fact that the incidence of cholangiocarcinoma is increasing[73]. To find an early biomarker for cholangiocarcinoma is therefore a challenge for clinical doctors and medical researchers. Alk-SMase activity in bile is significantly decreased in cholangiocarcinoma to an extent greater than in other hepatic diseases associated with increased expression of the 1.2 kb isoform[69]. The reduction of alk-SMase activity seems already occurring in both bile and liver biopsies in PSC patients[31]. PSC is a major risk factor for cholangiocarcinoma and about 15% of PSC patients may finally develop this type of cancer[74]. Future studies in a relatively large scale are necessary to evaluate whether the changed activity and 1.2 kb isoform expression in PSC patients can be biomarker for cholangiocarcinoma. To follow these changes might be helpful for identifying the early carcinogenesis. In addition, it is worthwhile to point out that about 70% of PSC patients may have IBD, particularly ulcerative colitis[75,76]. Reduction of alk-SMase activity in chronic ulcerative colitis has been reported[23]; (2) Are there a cross communication between NPP7 (alk-SMase) and NPP2 (autotaxin) in hepatobiliary diseases? NPP2 hydrolyzes lyso-PC with a phospholipase D activity and generating LPA, a potent inflammatory and proliferative factor[77]. Increased levels of NPP2 are a feature of many important hepatobiliary diseases such as PBC, PSC[78], steatosis[79], liver fibrosis[80], hepatitis C[81] and liver cancer[82]. Alk-SMase shares the same substrate lyso-PC with NPP2 but it cleaves phosphocholine instead of choline and thus generates monoacylglycerol not LPA[5]. Alk-SMase therefore may counteract NPP2 and thus reduce the formation of LPA. Recently we did find that in alk-SMase knockout mice treated with DSS, the levels of LPA in the colonic mucosa is higher in the knockout mice than in the wild type mice (Zhang P *et al*, Abstract presented in AACR symposium, Shanghai, China, 2016). Interestingly, a recent cohort study showed that primary biliary cirrhosis (PBC) patients who did not respond to ursodeoxycholic acid (UDCA) treatment display higher NPP2 levels than the responders[78]. Changes of alk-SMase may be implicated in the results, as alk-SMase activity can be increased by UDCA in liver cells[17]. No response to UDCA in these PBC patients may indicate a failure to upregulate alk-SMase by UDCA in these patients, leading to increased levels of NPP2; (3) Can alk-SMase protect liver against noxious effects of PAF? PAF is a type of bioactive lipid and can be synthesized rapidly in various inflammatory tissues. After binding to its G protein coupled receptors, PAF triggers several signal transduction pathways leading to activation of various phospholipases including C, D and A2 and to calcium mobilization, MAP kinase activation, and neutrophil mobilization[83,84]. In the liver PAF induces vasoconstriction[85] and may play a key role in several hepatic diseases such as CCl4 induced cirrhosis, ethanol and acetaminophen induced liver injury, viral induced hepatitis, and hepatocellular carcinoma[86-89]. All these diseases are associated with increased formation of PAF levels and PAF receptor expression. To inhibit the effects of PAF, previous studies were focused on PAF acetyl hydrolase and PAF receptor antagonists[90]. PAF is a substrate for alk-SMase which inactivates PAF by degrading it to phosphocholine and alkyl acetyl glycerol[7]. The activity is bile salt dependent with optimal pH at 7.5, which well fits the niche of the hepatobiliary system[7]. In alk-SMase knockout mice, PAF has been found to be significantly increased in the intestinal lumen[20]. It is therefore worthwhile examining the impact of alk-SMase on PAF action in these hepatic diseases and whether upregulation of alk-SMase may counteract the effects of PAF and benefit the patients; (4) What is the role of human bile alk-SMase in regulating SM levels in bile? Alk-SMase affects SM levels in the cell membrane. Overexpression of alk-SMase in COS7 cells[5] and incubation of the cells with purified alk-SMase result in reduced SM in the cell membrane[13]. Alk-SMase in human bile most likely can do the same things and thus affecting SM levels both in the hepatocyte canaliculi and bile. Phospholipids particularly PC and SM affect crystallization of cholesterol which is a key event involved in gallstone formation[68]. SM levels in the bile can be increased in the presence of high concentrations of bile salt[64] , and be decreased in the presence of high levels of alk-SMase released from canaliculi. Considering the influence of the membrane SM on cholesterol translocation and synthesis[40], and the more appreciable interaction of SM than PC with cholesterol[39,40], the impact of bile alk-SMase on gallstone formation through regulating SM levels both in the canalicular membrane and bile might be also worthwhile for close investigation.

CONCLUSION

Additional expression of alk-SMase in liver is unique for humans. As shown in the figure, by hydrolyzing its substrate SM, PAF, and Lyso-PC, alk-SMase generates anticancer and apoptotic molecule ceramide, reduces levels of PAF and LPA, which have been shown to be involved in a series of liver diseases including viral infection, steatosis, fibrosis, sclerosis, and tumorigenesis. Alk-SMase thus may play important roles in protecting the organ from these diseases. In addition, the enzyme is released into bile together with SM, PC, bile salt, and cholesterol and may interfere SM and PC levels and the physical-chemical interactions of these molecules in bile, thus affecting gallstone formation. Liver is an active organ for SM metabolism and for regulating plasma SM levels. Changed alk-SMase activity in bile and SM levels in plasma have been found in several hepatobiliary diseases, and such changes may have diagnostic and prognostic values. The contributions of alk-SMase, a unique human liver enzyme, for these changes need close investigation.

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MINIREVIEWS

Spontaneous bacterial and fungal peritonitis in patients with liver cirrhosis: A literature review

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Abstract

Spontaneous bacterial (SBP) and spontaneous fungal peritonitis (SFP) can be a life-threatening infection in patients with liver cirrhosis (LC) and ascites. One of the possible mechanisms of developing SBP is bacterial translocation. Although the number of polymorphonuclear cells in the culture of ascitic fluid is diagnostic for SBP, secondary bacterial peritonitis is necessary to exclude. The severity of underlying liver dysfunction is predictive of developing SBP; moreover, renal impairment and infections caused by multidrug-resistant (MDR) organism are associated with a fatal prognosis of SBP. SBP is treated by antimicrobials, but initial empirical treatment may not succeed because of the presence of MDR organisms, particularly in nosocomial infections. Antibiotic prophylaxis is recommended for patients with LC at a high risk of developing SBP, gastrointestinal bleeding, or a previous episode of SBP, but the increase in the risk of developing an infection caused by MDR organisms is a serious concern globally. Less is known about SFP in patients with LC, but the severity of underlying liver dysfunction may increase the hospital mortality. SFP mortality has been reported to be higher than that of SBP partially because the difficulty of early differentiation between SFP and SBP induces delayed antifungal therapy for SFP.

Key words: Liver cirrhosis; Spontaneous bacterial peritonitis; Spontaneous fungal peritonitis; Bacterial infections

Core tip: Spontaneous bacterial (SBP) and spontaneous fungal peritonitis (SFP) are infectious complications in patients with liver cirrhosis (LC). Renal impairment, severity of underlying liver dysfunction, and infections caused by multidrug-resistant (MDR) organisms are associated with a fatal prognosis in SBP. Antibiotic prophylaxis is recommended for patients with LC and with a high risk of developing SBP, gastrointestinal bleeding, or a previous episode of SBP, but the increase in the risk of infections caused by MDR organisms is of concern. Increased mortality of SFP compared with that of SBP may partially result from delayed diagnosis and

starting of antifungal therapy.

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INTRODUCTION

Patients with liver cirrhosis (LC) are at a high risk of developing bacterial infections because of hypoactivity of phagocytic cells in the hepatic reticuloendothelial system, decreased production of complement, and bacterial influx into the general circulation through portacaval shunts[1]. The most common are spontaneous bacterial peritonitis (SBP), urinary tract infections, respiratory infections (pneumonia), soft tissue infections, and bacteremia[2].

SBP can be a life-threatening LC complication, and the severity of underlying liver dysfunction and renal impairment such as hepatorenal syndrome (HRS), are associated with poor prognosis[3]. An increasing prevalence of multidrug-resistant (MDR) bacterial infections has been associated with failure of empirical antibiotic therapy, and the prognosis of SBP caused by MDR bacteria is poor[4]. Less is known about spontaneous fungal peritonitis (SFP) in patients with LC[5,6], but the available case reports indicate that the mortality of SFP is worse than that of SBP.

This article reviews the published data on the incidence, diagnosis, causative organisms, treatment, prognosis, and prognostic factors of SBP and SFP in patients with LC.

BACTERIAL INFECTIONS IN PATIENTS WITH LIVER CIRRHOSIS

Possible mechanisms underlying bacterial infections including SBP

In cirrhosis with portal hypertension, the microcirculation in the intestinal mucosa is disturbed, resulting in a reduction of mucosal blood flow, intestinal bacterial overgrowth, and impaired mucosal integrity[7,8]. Intestinal bacterial overgrowth, impairment in permeability of the intestinal mucosal barrier, and deficiencies in local host immune defenses are estimated to the major mechanisms to promote bacterial translocation (BT or pathological BT) in LC[7,9]. BT may be involved in the onset or aggravation of bacterial infections such as SBP and or HRS in patients with LC[7,8,10]. Pijls *et al*[11] reported increased large and small intestine permeability in patients with LC, and BT has been reported to be associated with bacterial infections caused by aerobic Gram-negative bacteria in patients with LC and was found to be more frequent in experimental LC models with than in those without ascites[7].

Studies of BT and bacterial DNA (bact DNA) translocation are limited in humans[7], but acute-phase proteins such as C-reactive protein (CRP) or procalcitonin have been used as BT biomarker[7], and detection of bact DNA by polymerase chain reaction (PCR) in biologic fluid has been proposed as a useful surrogate biomarker of BT in patients with advanced LC[7]. The presence of bact DNA in ascites increases the risk of developing SBP and HRS[12] and is an indicator of poor prognosis[7,13]. Assay of bact DNA by PCR has low diagnostic accuracy for SBP because of possible contamination and has poor sensitivity[14]. *In situ* hybridization of bact DNA in leukocytes recovered from the ascitic fluid in patients with LC has high sensitivity and specificity even in patients with culture-negative SBP[14,15]. Proinflammatory cytokines or oxidative stress may be involved in the pathogenesis of BT, and anti-tumor necrosis factor (TNF) has been reported to be an effective treatment in an animal model of LC[8]. In addition to the severity of liver dysfunction, the genetic diversity and virulence of causative bacteria also may influence the development of BT[16]. Genetic variation of superoxide dismutase 1 may involve the development of SBP[17]. Bacterial metabolites that reach the liver through portal system activate toll-like receptors (TLRs)[18], and the genetic polymorphism of TLR and nucleotide-binding oligomerization domain 2 genes may also be involved in the pathogenesis of BT, thus increasing the risk of infection in patients with LC by altering TLR binding to lipopolysaccharides or endotoxins[13,16]. Finally, intestinal colonization and translocation of drug-resistant bacteria may induce MDR SBP infections[19,20].

Bacterial infections in patients with liver cirrhosis

It is estimated that 30%-60% of inpatients with LC will develop a bacterial infection[1,21], and that the occurrence of these infections is four to five times higher in patients with LC than that in the general population[21]. Bacterial infections can trigger rapid deterioration of liver function and are a common precipitating factor of acute-on-chronic liver failure in cirrhosis patients[10,16].

Mortality and the incidence of LC-related complications are both significantly higher in patients with than without bacterial infections[22].

Karvellas *et al*[23] reported that septic shock secondary to SBP has a high mortality rate of approximately 80% in patients with LC, and bloodstream infections occur in 4%-21% of patients with LC, which is 10-fold higher than the rate in noncirrhotic patients[24]. The mortality of bloodstream infections in patients with LC ranges from 23% to 58%[25].

The incidence of bacterial infections in patients with LC is significantly correlated with the severity of the underlying liver dysfunction[10,22,26,27]. However, a recent study by Dionigi *et al*[27] found that patients with LC who become infected had an increased risk of death

independent of the severity of the underlying liver disease, even if they survived the acute infection. In patients with LC, infections caused by MDR bacteria are associated with a higher risk of mortality compared with those infections caused by susceptible bacteria[28,29]. An increased risk of MDR infections is a serious concern, is closely related to failure of antimicrobial therapy[29,30], and may induce deterioration of liver function[29]. MDR bacteria are resistant to at least three widely used antibiotic families[22]. These include the resistant bacteria that produce extended-spectrum β-lactamase (ESBL), such as *Pseudomonas aeruginosa,* and methicillin-resistant *Staphylococcus aureus* (MRSA)[31,32]. Recently, extensively drug-resistant (XDR) bacteria such as carbapenemase-producing *Klebsiella pneumoniae* and vancomycin-resistant *Enterococci* have been isolated from patients with LC[31]. The development of MDR infections is associated with suboptimal antibiotic use including antibiotic prophylaxis, nosocomial infection, recent infection with an MDR organism, current or recent hospitalization, exposure while receiving health care, and upper gastrointestinal bleeding in LC[33,34]. In a large prospective study, MDR bacteria were isolated in 4% of community-acquired infections, 14% of health care-associated infections, and 35% of nosocomial infections[33]. In two series of patients with LC evaluated in 2005-2007 and 2011-2012, Fernández *et al*[33] reported that MDR bacteria were more common in nosocomial infections and were associated with higher in-hospital mortality than in infections caused by antibiotic-susceptible bacteria. Pouriki *et al*[20] reported drug-resistant bacteria in intestinal cultures from 44% of uninfected patients with decompensated LC. Recent reports have found that asymptomatic intestinal colonization with MDR or XDR bacteria was an increased mortality risk in patients with LC in part because of BT of circulation of bacterial components[20].

SPONTANEOUS BACTERIAL PERITONITIS

Diagnosis

The development of ascites in patients with LC is a marker of poor prognosis and has been associated with high liver transplantation-free mortality ranging from 15% to 20% in 1 year and 40% to 60% in 5 year from the first onset[35].

SBP is as bacterial infection of the ascitic fluid with no apparent intraabdominal source of infection or malignancy[23]. A polymorphonuclear cell (PMN) count of $\geqslant$ 250 cells/mm^3 in the ascitic fluid, regardless of the isolation of bacteria from the fluid[36], is diagnostic for SBP. PMN cell count is routinely performed by using the manual laboratory counting which is time-consuming and costly[37]. Some studies have reported that the manual and the automated counting methods have a good agreement in the determination of PMN in the ascitic fluid, and automated methods have the potential to replace the manual counting method[37,38]. Culture-negative SBP, also known as culture-negative neutrocytic ascites (CNNA), should be treated in the same way as culture-positive SBP[39]. Estimates of the mortality of culture-positive SBP and CNNA are conflicting. Some report a lower mortality for CNNA compared with culture-positive SBP[40]; others report comparable rates[41]. Bacterascites has also been diagnosed in patients with positive microbiological culture of ascites and a PMN count < 250 cells/mm^3[36,42]. The incidence of bacterascites in inpatients with LC has been estimated at 3%-4%[42]. Most reports on bacterascites were published in the 1980s and early 1990s; very few are recent[43]. The diagnosis of SBP is necessary to distinguish from the cases with secondary bacterial peritonitis, partially because surgical treatments should be considered in secondary bacterial peritonitis but never in SBP[44,45]. Secondary bacterial peritonitis consists of ascitic fluid infection due to intraabdominal infections, for example, perforation of gastrointestinal tract or abscess[44,45]. It is much less frequent but has still high mortality rate compared with SBP in patients with LC[44,45].

Causative organisms

Ascitic fluid is culture positive in 35%-65% of patients with SBP[35,46-49]. The positive ascitic fluid bacterial cultures have been reported to increase by placement of the fluid directly into blood culture flasks at the bedside immediately after collection for a diagnosis of SBP[46,50]. BT from the intestinal tract is believed to be involved, as enterobacteria account for a relatively large percentage of the causative bacteria[8]. However, some reviews report a recent shift in the bacterial spectrum to include a high prevalence of Gram-positive bacteria (16.6%-68.3%) globally[32,51,52]. The change in etiology may have resulted from increases in the use of quinolones for bacterial prophylaxis and instrumentation in patients with LC[32]. The most frequently cultured organism in the ascitic fluid of patients with LC and SBP is *Escherichia coli*[18,30,52-56], followed by Gram-negative *Klebsiella* spp.. *Streptococcus* spp., *Staphylococcus* spp., and *Enterococci* are frequently isolated Gram-positive bacteria[30,53-56]. Kalvandi *et al*[57] reported *E. coli*, and Preto-Zamperlini *et al*[58] reported *Streptococcus pneumoniae* as the most frequent isolate in the ascitic fluid of children with SBP.

The incidence of recurrent SBP has decreased in parallel with the use of norfloxacin[59,60], but the increased prevalence of MDR bacteria in patients with SBP may be related to the use of long-term antibiotic prophylaxis or invasive procedures such as catheterization and ablation of hepatocellular carcinoma[61]. MDR bacteria are found frequently in nosocomial SBP (20%-35%)[61,62], but also occur in community-acquired SBP (4%-16%)[18]. Nosocomial SBP is also more likely to be antibiotic resistant. Balaraju *et al*[63] reported that up to 48% of the *E. coli* in patients with nosocomial SBP were resistant to third-generation cephalosporins. Li *et al*[62] found higher frequencies of ESBL-producing *E. coli* and *Klebsiella* spp. in cases of nosocomial compared with

non-nosocomial SBP.

Diagnostic markers

The gold standard for a diagnosis of SBP is the PMN count in the ascetic fluid[36], but paracentesis is not always possible. Laboratory markers are useful for early diagnosis of SBP and early prediction of the response to initial treatment because a lack of response is a predictor of SBP mortality[64-66]. TNF-α and interleukin-6 are significantly higher in the ascitic fluid of patients with SBP than in those with sterile ascites[67,68], and increases of those proinflammatory cytokines have been associated with renal impairment complicated by SBP and with mortality[67,69]. The lactoferrin concentration is also higher in patients with SBP than in those with sterile ascites[70-72], and the lactoferrin level in ascitic fluid has shown high sensitivity and specificity for the diagnosis of SBP[39]. The optimal timing of lactoferrin assays is not yet clear, and diagnostic assay kits are not commercially available[39].

Procalcitonin, a prohormone of calcitonin synthesized in the C cells of the thyroid gland[73,74], is an acute-phase reactant protein that has been studied in patients with SBP. Seven studies assayed serum procalcitonin[69,75-80]; three assayed procalcitonin in ascitic fluid[75,76,80]. Serum procalcitonin was significantly higher in SBP than in sterile ascites in six of the seven[69,75,76,78-80], which supports use of serum procalcitonin as an SBP marker. In a review by Yang *et al*[81] of the available data from 339 patients with LC accompanied by SBP, it was concluded that serum procalcitonin was a relatively sensitive and specific marker for the diagnosis of SBP. It has been reported that serum procalcitonin was significantly higher in cirrhotic patients with culture-positive SBP than in those with CNNA[77,82]. Two of the three evaluations of procalcitonin in ascitic fluid found no significant differences in procalcitonin levels in patients with SBP and those with sterile ascites[76,80]. The usefulness of ascitic fluid procalcitonin to distinguish between SBP and sterile ascites has not been demonstrated.

Calprotectin is a calcium- and zinc-binding protein with antimicrobial and antiproliferative functions. It is almost exclusively expressed in neutrophils, and its level in body fluids is proportional to the influx of neutrophils[69,83]. Burri *et al*[83] reported that ascitic fluid calprotectin level was correlated with the PMN count and that it reliably predicted a count of $\geqslant$ 250 cells/mm^3, which is the standard for a diagnosis of SBP. Subsequent studies found that ascites calprotectin is significantly higher in cirrhotic patients with SBP than in those without SBP[47,69]. Lutz *et al*[47] have shown that the ratio of calprotectin to total protein in ascitic fluid was a better diagnostic marker of SBP than calprotectin alone and that a high ratio was independently associated with 30-d mortality.

Leukocyte esterase activity, which can be assayed with commercially available reagent strips, may have diagnostic value[39]. Castellote *et al*[84] reported that the use of reagent strips is a rapid and inexpensive tool for the diagnosis of ascitic fluid infection and it had a high negative predictive value (99%), indicating that a negative result may be useful as screening to exclude SBP. Oey *et al*[85] reviewed 23 studies of leukocyte esterase in patients with SBP published between 2002 and 2015 and concluded that it had poor sensitivity and positive predictive value for the diagnosis of SBP. They found that the sensitivity of the reagent strips for diagnosing SBP was variable, and a negative test result strongly suggested the absence of SBP[85]. In another review of 26 studies published from 2002 to 2010, Koulaouzidis[86] confirmed the poor sensitivity and poor positive predictive value of leukocyte esterase activity as well as the high 93%-100% negative predictive value. A negative test result may thus indicate a high probability of the absence of SBP[84-86].

There is evidence for the diagnostic value of other markers including monocyte chemotactic protein-1 in serum[87] and ascitic fluid[88,89], lipopolysaccharide-binding protein in serum[90] and ascitic fluid[91], macrophage inflammatory protein type-1 beta in ascitic fluid[76], interferon-γ-induced protein-10 in serum[92] and ascitic fluid[92], triggering receptor expressed on myeloid cells-1 in ascitic fluid[93], high-sensitivity CRP in serum[94] and ascitic fluid[55], and neutrophil gelatinase-associated lipocalin in ascitic fluid[95]. Further study is needed to validate the diagnostic usefulness of these candidate markers.

Incidence

The incidence of SBP in LC with ascitic fluid has been estimated as 7%-30% in hospital inpatients[52,56] compared with 1.5%-3.5%[56,96,97] in outpatients with LC. The annual recurrence rate is approximately 70%[98], and the 1-year survival after recovery from the first episode of SBP is 30%-40%[99].

Prediction of incidence

Factors associated with the incidence of SBP in patients with LC and ascites include age, history of SBP[100], gastrointestinal bleeding[61,100], and endoscopic intervention for varix control[101]. Severity of liver dysfunction[42,54,56,61,102] including the Child-Pugh score or model for end-stage liver disease (MELD) score has been reported as a predictive factor, but few studies have not found an association of MELD score and the incidence of SBP[103] in patients with LC and refractory ascites. The MELD score does not include some clinical variables that are evaluated in the Child-Pugh score[104,105]. A PMN count[42,102] and a low protein concentration (< 1.5 g/dL) in the ascitic fluid, which may be related to decreased opsonic activity in the ascitic fluid[26,54,56], have also been reported as predictive factors, but the evidence for ascitic fluid protein is not conflicting[106]. The severity of liver dysfunction and serum and ascitic albumin levels have also been reported as risk factors for the recurrence of SBP[39].

Long-term use of proton pump inhibitors (PPIs) may increase the risk of SBP[107-110] because of facilitation of intestinal BT, but the association is controversial[26,111,112]. Multivariate analysis has identified the use of PPIs by patients with LC as an independent risk factor for the development of SBP[108,109], and Kwon *et al*[107] reported that PPI use was associated with the development of SBP and increased mortality, but other studies have not found a significant association of PPIs and development of SBP in patients with LC[112,113]. A recent meta-analysis by Yu *et al*[114] of 10 case-control and six cohort studies involving 8145 patients and published between 2008 and 2014 concluded that it is not possible to establish causality between PPI use and increased risk of SBP. A similar analysis by Khan *et al*[115] of six case-control and eight cohort studies concluded that there was a statistically significant association between PPI use and increased incidence of SBP, but that the difference in incidence was small. Kim *et al*[116] reported that PPI use was not associated with risk or SBP recurrence. PPI use may be associated with slightly increased risk for SBP[117], but the significance of the association has not been confirmed.

Mortality

SBP mortality has decreased in the four decades since it was first described as a result of early diagnosis and prompt treatment[56] and treatment by liver transplantation[103]. Hospital mortality is estimated as 10%-50% for the first episode and 31%-93% for the second or subsequent episodes[53,118]. Recent studies have estimated 1-mo mortality at > 20%[52,102], inpatient mortality at > 30%[61], and 1- and 2-year mortality following an SBP episode as 50%-70% and 70%-75%, respectively[23,39]. A recent nationwide cohort study in Taiwan reported that SBP mortality was 24.2% at 1 mo and 66.5% at 3 year[119].

Prediction of mortality

Predictors of mortality in SBP include severe underlying liver disease with a high Child-Pugh[48,52,64,96,100,102,104,105,120-122] or MELD score[47,48,52,104,121-123], renal impairment[16,52,54,64,65,96,100,124,125] such as HRS, and onset of severe sepsis[96]. Some studies did not find differences in the mortality of nosocomial and community-acquired SBP[63]; others have reported increased mortality in nosocomial compared with community-acquired SBP, which was correlated with the involvement of drug-resistant bacteria[4,21,63,65]. Other predictors of SBP mortality include old age[16,102], gastrointestinal bleeding[4,52], complications of shock[65], rapid deterioration of liver function[64], positive ascitic fluid culture[16,48], elevated blood leukocyte level[47,104,123], low serum sodium[63,102], complications of hepatic encephalopathy[52,63,122], presence of hepatocellular carcinoma[54], and complications of other infections, such as pneumonia[122]. Tandon *et al*[123] reported that peripheral blood leukocyte count $\geqslant 11 \times 10^9$ cells/L and MELD score $\geqslant 22$ were independent predictors of 30-d mortality in patients with LC and SBP.

Renal impairment develops in 30%-40% of patients with LC and SBP[16] and is a significant prognostic factor in patients with LC and SBP. The available evidence strongly associates renal impairment with SBP mortality[16,52,54,64,65,96,100,124,125], but Lim *et al*[97] exceptionally reported that renal impairment, including HRS, was not predictive of mortality possibly because of early and effective treatment of SBP. Tandon *et al*[121] reported 67% mortality in patients with SBP and renal failure, but only 11% in patients with SBP and normal renal function. Some clinical studies indicated the use of intravenous albumin in addition to antibiotics for treatment of SBP[70,126-130]. Possible mechanisms of albumin use for improvement of SBP include its oncotic properties, immunomodulatory and antioxidant effects, and its endothelium stabilization capacity[131]. Dosage and duration of intravenous albumin in previous studies were as follows: (1) 1.5 g/kg body weight at the time of diagnosis and 1 g/kg body weight on day 3[126-128,130]; and (2) 20% 50 mL every day for 3 d[70]. Moreover, some clinical studies indicated that the use of intravenous albumin and antibiotics can reduce the incidence of renal impairment and mortality in patients with LC and SBP[126,129,130]. The efficacy of albumin treatment in patients with LC and other types of bacterial infection remains unknown. Thévenot *et al*[132] reported that albumin did not improve renal function or survival at 3 mo in patients with LC and non-SBP bacterial infections.

A meta-analysis of studies published between 2002 and 2011 identified a four-fold increased risk of mortality of patients with LC and SBP caused by MDR bacteria[133], and a retrospective multicenter study in Korea found that SBP caused by ESBL-producing bacteria was an independent prognostic factor of high in-hospital mortality[54,108]. Chon *et al*[4] reported that both in-hospital mortality and follow-up mortality after recovery from SBP were significantly higher in nosocomial SBP than in community-acquired SBP. Alexopoulou *et al*[31] did not find significant differences in the 30-d mortality of infections caused by MDR bacteria and nonresistant bacteria in 60 cases of culture-positive SBP and 70 cases of bacteremia without SBP.

However, 30-d mortality from infections caused by XDR bacteria was significantly higher than that of infections caused by MDR or nonresistant bacteria[31]. In another study, an overall 20% 30-d survival of nosocomial SBP was related to inadequate empirical antibiotic treatment[32].

Antibiotic prophylaxis

Antibiotic prophylaxis is effective, but long-term antibiotic administration leads to the emergence of MDR, which is a serious concern worldwide[33,134-136]. The current practice guidelines of the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver recommendations[26] limit its recommended use to patients who are at the highest risk of developing a bacterial infection. However, some studies have reported significant decreases

in the incidence of SBP in patients with antibiotic prophylaxis[18,137]. Patients with LC and low ascites protein concentrations of < 1.5 g/dL are at risk of developing a first episode of SBP[26,138]. Primary antibiotic prophylaxis is recommended in patients with LC and no history of SBP and low ascites protein plus other predisposing factors, such as severe liver failure (Child-Pugh score > 9), impaired renal function (serum creatinine > 1.2 g/dL or blood urea nitrogen > 25 mg/dL), or hyponatremia (≤ 130 mEq/L), are present[23,26,102]. Primary prophylaxis is also recommended in patients with LC and acute gastrointestinal bleeding[138] or to reduce the incidence of SBP after gastrointestinal bleeding[134]. Antibiotic prophylaxis has also been associated with reduced rebleeding within 7 d and to lower 28-d mortality[18,134]. The standard therapy in patients with LC and acute gastrointestinal bleeding is oral norfloxacin[18], but Fernández *et al*[139] reported that intravenous ceftriaxone is more effective than oral norfloxacin. A randomized, controlled trial demonstrated that primary prophylaxis with trimethoprim/sulfamethoxazole also decreased the risk of SBP[140]. Recurrence rates as high as 70% have been reported in patients with LC who have recovered from a previous episode of SBP without secondary antibiotic prophylaxis[61]. Secondary prophylaxis is indicated for patients with a previous episode of SBP[26,53]. Norfloxacin was shown to decrease overall recurrence from 68% to 20%[26] and recurrence with a Gram-negative microorganism from 60% to 3% in the first year[61]. However, studies published between 2010 and 2016 reported that the frequency of recurrence with bacteria resistant to quinolones was 30%-33%[52]. The efficacy and safety of rifaximin for primary prophylaxis for SBP and for prophylaxis of recurrent SBP have been studied[141-145]. A study of primary prophylaxis by Assem *et al*[141] demonstrated that alternating norfloxacin and rifaximin as combination therapy was remarkably more effective compared with norfloxacin monotherapy in part because of an increased incidence of quinolone-resistant and Gram-positive SBP. Mostafa *et al*[142] showed that rifaximin prophylaxis was more effective than norfloxacin in patients with LC and at least one previous episode of SBP. A randomized, controlled trial of secondary prophylaxis by Elfert *et al*[143] demonstrated that SBP recurrence and mortality were significantly lower with rifaximin than with norfloxacin.

A systematic review by Goel *et al*[144] concluded that rifaximin was as effective for both primary and secondary prophylaxis as other systemically absorbed antibiotic. Rifaximin might be considered, particularly in cases involving quinolone-resistant bacteria[18]. The optimal duration of prophylaxis is currently unclear[26], but secondary prophylaxis should be continued until the ascites is resolved or liver transplantation can be performed[3].

Empirical antibiotic treatment for spontaneous bacterial peritonitis

Empirical antibiotic treatment must be initiated immediately after the diagnosis of SBP[53]. If the ascites neutrophil count decreases to < 25% of the pretreatment value after 2 d of antibiotic treatment, then there is an increased probability of failure to respond to any treatment[66]. Third-generation cephalosporins have been the most frequently used antibiotics in the treatment of SBP since 1985[4] and were highly effective until about 10 year ago[64]. They are still effective for community-acquired infections in patients with LC, with resolution rates of around 80%[33], but the development of resistance to third-generation cephalosporins is of great concern. Resistance can result in failure to respond to initial empiric therapy with a third-generation cephalosporin in 33%-75% of cases, and failure to respond is associated with reduced survival[48,49]. Recent studies indicate that third-generation cephalosporins are not appropriate for the treatment of nosocomial infections in patients with LC[34] because of effectiveness as low as 40% related to an increase in the prevalence of MDR bacteria in nosocomial infections[61,64]. SBP treatment recommendations distinguish between nosocomial and community-acquired infections[61] following evidence from studies like that by Lutz *et al*[146] who reported that approximately one third of health care-related and nosocomial SBP infections were resistant to third-generation cephalosporins. They also found that patients with health care-related SBP infections resistant to first-line treatment had worse survival than were those with infections susceptible to first-line treatment[146]. Moreover, Ariza *et al*[147] reported that resistance to third-generation cephalosporins occurred in 7.1% community-acquired SBP, 21.1% health care-related SBP, and 40.9% nosocomial SBP among 246 episodes in 200 patients with LC and SBP (2001-2009). Some studies recommend that third-generation cephalosporins not be used for empirical treatment of health care-related SBP[136,146]; Lutz *et al*[146] recommend piperacillin-tazobactam rather than third-generation cephalosporins. The most recent recommendations restrict third-generation cephalosporins to selected patients, primarily those with community-acquired SBP[21,32].

Daptomycin is effective against Gram-positive *Enterococci* resistant to vancomycin and against MRSA. A recent randomized, controlled study reported that empirical treatment of nosocomial SBP with meropenem plus daptomycin was more effective in resolving SBP and had better 3-mo survival than did third-generation ceftazidime[31,64]. Piano *et al*[64] and European expert opinion[49] both recommend a combination regimen of meropenem and daptomycin for the management of nosocomial SBP. A randomized trial by Jindal *et al*[122] reported that cefepime, a fourth-generation cephalosporin with good activity against most nosocomial Gram-negative bacteria and Gram-positive cocci, was as effective as imipenem for resolution of SBP. Following evaluation of antibiotic susceptibility in 575 SBP cases, Shi *et al*[30] recommended cefoperazone/sulbactam or piperacillin/tazobactam for the empirical treatment of SBP.

SPONTANEOUS FUNGAL PERITONITIS

Patients with LC are at an increased risk of fungal infection[138] because the antibiotics used for prevention of SBP can select for excessive growth of fungi in the intestinal flora with subsequent fungal translocation into peritoneal cavity and development of SFP[6]. Fungi are much larger than bacteria, which makes fungal translocation across the gut mucosa more difficult than BT, and may require higher intestinal permeability, which is more common in patients with advanced LC[148,149]. Fungal translocation may be facilitated by upper gastrointestinal bleeding, which is also common in patients with advanced LC[150]. Immunosuppression and malnutrition in LC patients also promote this fungal translocation[6]. Direct percutaneous inoculation of fungi is the proposed route of fungal infection in patients with refractory ascites and a history of paracentesis[150].

There are few data on the characteristics of SFP in LC patients[5,6], but case studies are available. Fungal colonization in ascitic fluid is not a rare complication in end-stage liver disease[5], and studies of the clinical characteristics of SFP are becoming more frequent. SFP is defined as a fungal infection of ascitic fluid with no apparent intraabdominal source of infection or malignancy[148]. A PMN count of $\geqslant$ 250 cells/mm^3 in the ascitic fluid with a positive fungal culture regardless of co-colonization of bacteria is diagnostic of SFP[151]. A positive fungal culture with a PMN count of < 250 cells/mm^3 is diagnosed as fungiascites or fungal ascites[151]. Fungal ascites has a higher mortality rate than does bacterascites[151]. Of spontaneous peritonitis cases, 0%-7.2% are culture positive for fungus[6,30,32,62,82,148,152-154], and the most frequent isolate is *Candida albicans*[5,6,148,149,153-156]. Other causative fungi include *Candida glabrata*[30], *Candida krusei*[153], *Cryptococcus* spp.[148,157], *Aspergillus* spp.[150,154], and *Penicillium* spp.[154]. Polymicrobial infections, i.e., bacterial co-colonization, occurs in 32%-74% of SFP cases[148,149,153,154,156], but early diagnosis by conventional microbial culture is difficult because of the time required for growth[155], and the efficacy of PCR or assay of the fungal biomarker 1,3-beta-D-glucan in ascitic fluid has not been established[149,155].

High Child-Pugh or MELD scores increase the risk of SFP in patients with LC[5,6,148], and a retrospective case-control study by Gravito-Soares *et al*[153] found no significant difference in the Child-Pugh or MELD scores of patients with SFP and SBP. The risk of SFP is increased in patients with LC who undergo invasive procedures[153] and increases with the length of the hospital stay[153,156]. Some studies have reported that a significantly greater proportion of SFP infections than SBP infections were nosocomial[148,156], but the data are conflicting[6]. In previous studies of spontaneous peritonitis with ascitic culture-positive diagnosed between 2003 and 2016, nosocomial SFP was confirmed in 7.7% (53/689) of nosocomial spontaneous peritonitis, and non-nosocomial SFP was confirmed in 1.7% (17/1018) of non-nosocomial spontaneous peritonitis[156].

Risk factors associated with hospital mortality in SFP include severe underlying liver disease[148], Child-Pugh score[155], MELD score[149], antibacterial prophylaxis[6], incidence of HRS[6], low ascites protein concentration[6], Acute Physiology And Chronic Health Evaluation Ⅱ score[149], and sepsis shock[153]. SFP mortality is estimated to be 56%-90%[5,148,149,151,153], and 1-mo mortality may[148,153] or may not be significantly higher than SBP mortality[149,157]. Hwang *et al*[148] reported a high SFP mortality that was related to unresponsiveness to initial empirical treatment for suspected SBP. The condition of nearly all the patients with SFP worsened after initial empirical treatment, and they died during the early stage of peritonitis regardless of undergoing antifungal treatment[148]. Some patients with SFP improved after receiving the initial empirical treatment without any antifungal agents[148]. Those patients may have had SBP and colonization by innocent fungi[148], as it was not possible to distinguish fungal colonization from true SFP in the clinical setting[148]. SFP is usually diagnosed after the identification of fungi in cultures of ascitic fluid. The mortality is high because of delayed diagnosis, lack of clinical signs, lack of suspicion of SFP, and delay in treatment with antifungal therapy[5,6,148,155,156,158]. Fungal resistance to empirical specific antifungal therapy together with delayed diagnosis and treatment is related to poor prognosis of SFP[153].

Recent treatment guidelines on management of infections in LC do not include antifungals for prophylaxis or optimum treatment but do include recommendations for fungal infections[138]. Echinocandins are recommended as first-line treatment for patients with LC and nosocomial SFP or critically ill patients with LC and community-acquired SFP[148]. Fluconazole is recommended for less severe infections[6,138,152]. De-escalation from echinocandins to fluconazole is advised in critically ill patients with LC and SFP when their condition is stable and sensitivity tests are available[6,152]. However, directed antifungal therapy may not improve the outcomes of some patients with SFP[149,156] because of lack of response to the administration of empirical treatment and delay in starting antifungal therapy[156].

Although early differentiation between SFP and SBP may be difficult partially because identification of fungi in cultures of ascitic fluid is time-consuming, clinician is able to suspect SFP or SBP due to MDR if spontaneous peritonitis is not improved after 48 h empirical antibiotic treatment[151]. Therefore, new cultures in ascitic fluid and blood may be performed in these patients[151]. Moreover, additional administration of antifungal agents or administration of antifungal agents and alternation of antibiotics may be considered in these patients[151,159].

CONCLUSION

The evidence of previous studies confirms that SBP and SFP in patients with LC is often life-threatening. The severity of liver dysfunction, the presence of renal impairment, and the emergence of MDR bacteria have

a clinically significant influence on the prognosis of SBP. The efficacy of the recommended antibiotic therapy for SBP may be decreased in nosocomial infections because of increases in the prevalence of MDR bacteria. In SFP, mortality is associated not only with the severity of the underlying liver disease but also with delay in diagnosis and initiation of antifungal therapy. Further research is needed to better our understanding of the nature of SFP and improve the response to treatment with the available antifungal agents.

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MINIREVIEWS

Impact of direct acting antivirals on occurrence and recurrence of hepatocellular carcinoma: Biologically plausible or an epiphenomenon?

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Abstract

Hepatocellular carcinoma (HCC) is a major cause of morbidity and mortality worldwide. Chronic hepatitis C virus infection (HCV) is the most common cause of HCC in many European countries, Japan and Pakistan. Introduction of the new direct acting antivirals (DAAs) has revolutionized the management of HCV worldwide, with high rates of sustained virologic response in patients who could not have tolerated the previous interferon based treatments. However, recently there have been reports raising caution about the long term effects of DAAs, particularly a possible increased risk of HCC. Therefore this review explores the current molecular studies as well as clinical data that investigate the impact of DAAs on occurrence and recurrence of HCC.

Key words: Hepatocellular carcinoma; Direct acting antivirals; Hepatitis C

Core tip: Our aim is to consolidate the existing literature as well as to identify whether there is a particular subset of the population in which this phenomenon was witnessed. The ground-breaking discovery of the new group of direct acting antiviral agents (DAAs) had led to a paradigm shift in the management of chronic hepatitis C (CHC). Wide variations have been observed in the studies assessing the long-term role of DAA based therapy on occurrence and recurrence of HCC. There is a need to differentiate weather the reported higher occurrence and recurrence rates are due to DAA or host and disease related factors and to identify subset of individuals particularly at risk. Also, future investigations should be directed towards

assessing the long-term effects of DAAs on group of patients that have not been studied thus far. Some important Centers in Europe and United States have been delaying antiviral treatment for 6 mo or more after the recent treatment for HCC. Hence, until more robust data is available, clinical practices should continue as per current guidelines in those patient groups who can benefit from DAA therapy with close surveillance of patients with advance fibrosis.

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INTRODUCTION

The ground breaking discovery of the new group of direct acting antiviral agents (DAAs) had led to a paradigm shift in the management of chronic hepatitis C (CHC) which is the most common cause of hepatocellular carcinoma (HCC) in Japan, Pakistan, United States and many European countries[1,2]. With annual incidence of HCC ranging from 1% to 7% in patients with HCV related cirrhosis, (HCC) is a leading cause of morbidity and the second most common cause of cancer related deaths worldwide[3,4]. Besides contribution of several host and viral factors in the pathogenesis of disease progression, achieving sustained virologic response (SVR) has been found as the single most important factor in reducing HCV associated HCC incidence[5].

The novel DAAs not only provided a potent, oral alternative to injectable interferons, but also had a shorter duration of treatment, better efficacy with over 90% achievement of SVR and a more favorable side effect profile[6]. However, since 2016, concerns were raised regarding the effect of DAAs on progression to HCC[7]. In addition, their long-term benefits including impact on HCC have been questioned in the context of specific populations and subgroups which were not included in the landmark trials investigating DAA based therapy[3].

Therefore, this review aims to explore existing molecular studies as well as clinical observations in order to determine whether there is an association between the use of DAAs and the occurrence or recurrence of HCC among patients with HCV related liver disease. We also aim to evaluate whether there is a subset of the population in which this phenomenon has been observed.

DAA AND CARCINOGENESIS: BIOLOGICALLY PLAUSIBLE OR NOT?

The carcinogenesis of HCC is a chronic process with several steps that may serve as potential targets for drug therapy. Unlike the hepatitis B virus, HCV is an RNA virus which is unable to integrate into the host genome and thus it is unlikely to have direct carcinogenic activity[8]. While the mechanism of carcinogenesis due to HCV is not completely understood, observations from transgenic mice models suggest that liver cancer occurs because of rapid hepatocyte turnover, dysregulation of apoptosis and generation of reactive oxygen species, arising in the setting of a chronic inflammatory state induced by HCV[9,10]. This indirect mechanism of cirrhosis driven carcinogenesis is supported by clinical data which shows a greater risk of HCC with chronic HCV infection and worsening liver fibrosis[8].

One of the mechanisms proposed regarding the risk of DAAs towards development of HCC is that DAAs downregulate interferon genes, disrupting the innate immunosurveillance of the body[8]. In the chronic phase of HCV, an estimated 10^{12} virions are produced per day by infected hepatocytes[11]. These trigger an immune response mediated primarily by natural killer (NK) cells which release cytokines such as interferon (IFN)-γ. IFNs upregulate interferon stimulated genes which have an anti-proliferative response by prolonging all phases of the cell cycle and decreasing viral replication. When HCV infection becomes persistent, NK cells become dysfunctional due to continuous antigenic stimulation by the high load of virions, resulting in impaired production of IFNs[12]. In mice models, it was observed that decreased levels of IFN-γ independently control tumorigenesis[13]. Thus the downregulation of these genes in IFN free therapy could contribute to development of HCC.

Analysis of the peripheral blood mononuclear cells (PBMCs) of CHC patients treated with DAAs has shown that in comparison to healthy controls, these patients have attenuated activity of both NK cells and monocytes, reflected by a decreased level of inflammatory cytokines in patients who had achieved rapid virological response (RVR), *i.e.*, undetectable HCV RNA at the end of 4 wk of DAA treatment[14]. Natural Killer cell group 2D (NKG2D) is an activating receptor of immune responses which has been studied in the context of HCV associated HCC. It has been found that in HCV patients treated with IFN free DAA therapy, an on treatment decrease in the expression of NKG2D correlated to the early occurrence and recurrence of clinically evident HCC within the 6-mo surveillance period following treatment[15].

With DAA based therapy, HCV RNA becomes undetectable in days to weeks, a much more rapid response than that observed with IFN based regimens. It is hypothesized that rapid eradication of HCV and subsequent abrupt resolution of the chronic inflammatory state disrupts the natural immune response of the body, possibly favoring the proliferation of neoplastic cells[16]. Since clinical studies have shown no difference among different DAA regimens and development of HCC, it is hypothesized that if this effect does exist then it would have to be a class effect of the DAAs[17,18].

Table 1 Possible factors contributing to hepatocellular carcinoma after hepatitis C virus eradication by direct acting antivirals

Down regulation of *IFN* genes
Presence of fibrosis
Sudden disruption of chronic inflammatory state
Impaired immune response by NK cells
T cell dysfunction
Decreased microRNA-122

IFN: Interferon; NK: Natural killer.

An experimental study analyzing the soluble inflammatory milieu from plasma cells of cirrhotic HCV patients found that HCV specific CD8+ T cells failed to recover from the baseline after DAA therapy. These cells play a role in HCC surveillance; therefore, their reduced activity in IFN free therapy could potentially affect development of HCC[19]. Furthermore, serum levels of microRNA (miRNA) 122 were found to be reduced in DAA treated HCV patients after achieving SVR[20]. Previous evidence shows that miRNA-122, which is the most abundant miRNA in the liver, functions as a tumor suppressor against HCC[21]. Thus it is hypothesized that decreased miRNA-122 in DAA treated patients could contribute to an increased risk of HCC recurrence[22]. Table 1 summarizes the possible factors that could lead to HCC in DAA treated HCV patients.

DAA AND CARCINOGENESIS: REVIEW OF EXISTING EVIDENCE/DATA

Studies that observed increased incidence of HCC

While DAAs represented a major breakthrough in the treatment of CHC, one of the first reports questioning their long-term role in development of HCC came from Reig *et al*[8] in 2016. The authors retrospectively assessed a cohort of 58 patients who had a history of HCC secondary to HCV and had received different regimens of DAA based therapy. After a median follow up of 5.7 mo (range 0.4-14.6 mo), 16 out of their 58 patients (27.6%) showed radiographic evidence of HCC. This study alerted the scientific community regarding the potential risks associated with use of DAAs and the authors called for a large-scale assessment to confirm their findings[8]. However, their method of statistical analysis was questioned by Camma and colleagues who felt that reporting the crude rate of recurrence was a weakness of the study because of the variation in time elapsed from treatment of HCC and starting DAAs (median 11.2 mo, range 1.2-87.7 mo)[23]. Cammà *et al*[23] used the data presented by Reig *et al*[24] to calculate the actuarial probability of HCC recurrence by plotting a Kaplan Meier curve. For their analysis, Cammà *et al*[23] used the time of HCC treatment as the initiation point, not the time of initiating DAA treatment as used by the original authors. With this method they found a much lower recurrence rate than that reported in the original study (7% and 13% at 6 and 12 mo respectively).

Reig *et al*[24] later went on to present a follow up of their original cohort. In 2017, they reported that not only did they observe a higher recurrence rate of HCC among the DAA treated patients in their study; they also found a more aggressive pattern of recurrence in terms of tumor staging and subsequent treatment options. Renzulli *et al*[25] have also reported a rapid development of HCC following DAA treatment with a more aggressive pattern of microvascular invasion. The median duration between completion of DAA treatment and diagnosis of HCC in their patients was 82 d (range 0-318).

A retrospective study from Italy by Conti *et al*[18] reported HCC occurrence and recurrence rates of 3.16% (95%CI: 1.45-5.90) and 28.81% (95%CI: 17.76-42.07) respectively in a cohort of 344 CHC patients treated with different DAA regimens over a follow up of 24 wk. Approximately 69% of this patient population had HCV genotype 1 and 91% had achieved SVR. However, the lack of a control group makes the interpretation of these findings difficult. The authors attempted to account for this limitation by comparing their findings to those of a historic cohort of untreated cirrhotic patients at their center. They found an HCC occurrence rate of 3.2%, which was similar to their current study. Conti *et al*[18] have interpreted their results with caution. The authors claim that while DAA treatment of HCV does not seem to reduce the occurrence or recurrence of HCC, anitiviral treatment should be started as early as possible to prevent the development of cirrhosis and recommend active surveillance of all cirrhotic patients, during and after DAA therapy.

In Portugal, Cardoso *et al*[26] found HCC incidence to be 7.4% within a one year follow up of cirrhotic patients that had achieved SVR after being treated with sofosbuvir and ledipasvir. These *de novo* HCC patients had been asymptomatic, and were detected on radiological screening. This emphasizes the recommendation of Conti *et al*[18] that there should be close monitoring of CHC patients for development of HCC, despite achieving SVR[25]. In a larger study from Belgium, it was found that there was no difference in the early occurrence of HCC among patients treated with DAAs with or without Peg-IFN. However, this study reported HCC recurrence of 15% in patients treated with DAAs alone as compared to 0% in those who received a combination of Peg-IFN and DAAs. This study had a predominantly HCV Genotype 1 population. In these patients the authors also noted that those who developed HCC had a higher baseline risk of HCC which is a potential confounder[27].

The most recent study on this research question is by Ida *et al*[28], published in October 2017. The study population comprised 100 patients from Japan with HCV genotype 1, treated with Daclastavir and Asunaprevir, who were followed for 15 mo. In this group, there were 5 new cases and 12 recurrences of HCC. The authors

have hypothesized that the high rate of HCC seen in this study could be related to a history of HCC, as these patients already had advanced fibrosis which is known to be implicated in the process of hepatocarcinogenesis. Table 2 summarizes the studies that report an increased incidence of HCC in DAA treated HCV patients.

Studies that did not note any significant effect

In 2016 and 2017 there have been several other similar reports, fueling the debate regarding the role of DAAs in HCC. Two large retrospective cohort studies have been conducted in the United States to investigate this matter; one by Ioannou *et al*[33] with a sample size of 62354 and the other by Kanwal *et al*[34] with 22500 study participants. Both studies concluded that DAAs are not associated with a significant risk of HCC as compared to IFN based treatment. Ioannou *et al*[33] found that DAA induced SVR reduced the risk of HCC by 71%. This effect was similar in groups who had received DAAs alone, DAAs in combination with IFN or IFN alone regimens, thus suggesting that achieving SVR could be the crucial factor for risk reduction of HCC, regardless of the therapeutic agents used[33]. Kanwal *et al*[34] found that while there was a relative risk reduction in HCC, the absolute risk of HCC still persisted in those patients who had DAA induced SVR. In both studies the risk of HCC was greater in cirrhotic patients. Additionally, Kanwal *et al*[34] found that diabetes mellitus, alcohol use and a higher Fib-4 Index for assessment of fibrosis were risk factors for occurrence of HCC in patients who had achieved SVR. However, both study populations were restricted to United States veterans and were mostly patients with HCV genotype 1. The specific population included in these studies might limit the generalization of their findings.

The ANRS Collaborative Study Group assessed HCC recurrence rates among 3 French multicenter prospective cohorts who had received DAA based therapy after HCC curative treatment[35]. Their study included a diverse patient population with cirrhotics, non-cirrhotics and liver transplant recipients. They did not find any evidence that DAAs increases the risk of HCC recurrence. The strength of this study was that analysis of data from 3 distinct patient cohorts yielded fairly consistent results. Secondly, they included patients who had received curative therapy for HCC as opposed to non-curative therapies such as chemoembolization, leading to speculation that some of the earlier studies that had reported a higher recurrence rate might have included patients in whom the initial tumor staging was incorrect or was incompletely treated[35]. Table 3 summarizes the studies that do not show an increased risk of HCC in DAA treated patients.

Geographical variation of incidence of HCC in DAA treated patients

Many of the studies investigating the link between DAAs and HCC have been from Japan or European countries. Racial differences are known to be implicated in the progression to HCC among HCV infected patients[52]. In a large cohort of United States veterans with HCV, it was found that Hispanics were at greater risk of developing cirrhosis and HCC. Black race and Hispanic ethnicity have also been identified as independent predictors of treatment failure[53]. Indeed the first report raising caution about the possible association of DAAs with recurrence of HCC in an aggressive form did come from the Spanish cohort followed by Reig *et al*[8].

A systematic review and meta-analysis by Waziry *et al*[54] found that there was no difference in HCC occurrence and recurrence in CHC patients who received IFN based or DAA based regimens. However, the authors acknowledge that in their analysis they were unable to account for geographical variations. Due to the heterogeneity in the studies included in this meta-analysis, the results should be interpreted with caution. In this meta-analysis most of the IFN based studies were from Japan whereas DAA based studies were from Europe. Hence, due to baseline difference in between two populations it's hard to draw an accurate conclusion regarding occurrence or recurrence of HCC among DAA and IFN based therapy. Furthermore, they had to exclude several studies which had incomplete data regarding BCLC staging of HCC.

In our literature review, the populations that we have found to be under-represented in terms of the current research question include Indians, Arabs and Africans. Additionally, while the 2 studies with the largest sample size are from the United States, these were limited to veterans only[33,34].

Genotype based variation of HCC in DAA treated patients

HCV genotype is an important consideration when considering progression to HCC. HCV genotype 3 is generally more aggressive and is associated with a higher risk of progression to cirrhosis and HCC[55]. To the best of our knowledge, the studies published so far suggesting a greater risk of HCC with DAA treatment have not identified a particular genotype of HCV that is significantly associated with this disease progression. However, as most of these reports are from Japan or European countries such as France and Italy, therefore the major disease burden studied has been of HCV genotype 1[35,37]. Table 4 lists the factors predisposing to development of HCC in DAA treated HCV patients.

WAY FORWARD

There is wide variation in the studies assessing the long-term role of DAA based therapy on occurrence and recurrence of HCC, both in terms of the baseline characteristics of study population and the different DAA regimens used. At present, most of the studies have been reported from regions where HCV genotype 1 is the most prevalent one[34,35]. Data is very limited from Asian populations where HCV Genotype 3 is the most common genotype. This is an important consideration as the different genotypes of HCV have a unique

Table 2 Studies showing an increased incidence of hepatocellular carcinoma in direct acting antivirals treated patients

Ref.	Treatment groups	Country/sample size	Male gender *n* (%)	Age (yr)	Genotype *n* (%)	Duration of follow up	Cirrhosis *n* (%)	History of HCC *n* (%)	HCC occurrence	HCC recurrence
Reig *et al*[8]	All DAA treated	Spain *n* = 58	40 (69)	Median 66.3 (45-83)	G1a = 8 (13.8%), G1b = 45 (77.6%), G3 = 2 (3.4%), G4 = 3 (5.2%)	median 5.7 mo	55 (94.8)	58 (100)	Not applicable	16 (27.6%)
Conti *et al*[18]	All DAA treated	Italy *n* = 344	207 (60.2)	Median = 63 (29-85)	G1= 237 (69), G2= 40 (11.6), G3= 38 (11), G4= 29 (8.4)	24 wk	All cirrhotic	59 (17)	9 of 285 patients (3.16%, 95%CI: 1.45–5.90)	17 of 59 patients (28.81%, 95%CI: 17.76–42.07)
Cardoso *et al*[26]	All DAA treated	Portugal *n* = 54	> 70%	No HCC: 59 yr (41-81). Patients with HCC = 58 yr (55-72)	No HCC: G1 = 78%, G3 = 18%. Patients who developed HCC: G1 = 75%, G3 = 25%	Median = 12.0 mo (9.4-12.5 mo)	All cirrhotic	0 (0)	4 (7.4%)	Not applicable
Ida *et al*[28]	All DAA treated	Japan *n* = 100	46 (46)	Median 72.5 (26-87)	G1 = 100 (100)	15 mo		26 (26)	5 (5)	12 (12)
Kozbial et al[29]	All DAA treated	Austria	-	56-74 yr	G1a = 3 (15.8), G1b = 13 (68.4), G3a = 2 (10.5), G4 = 1 (5.2)			3 (15.8)	6.60%	3 patients
Issachar *et al*[30]	All DAA treated	Israel *n* = 273	-	-	-	15 mo	-	-	6 (2.1)	3 (1.05)
Kwong *et al*[31]	patients divided into 3 eras: IFN era (2003-2010), protease inhibitor era (2011-2013), and DAA era (2014-2015)	United States *n* = 48158	29858 (62)	Median 55 (49-60) yr		Median 493 d	All cirrhotic		Incidence Rate of HCC was 49% higher in the DAA era (IR 6.6/100 person-years [py], 95%CI: 5.6-7.9) *vs* the IFN era (4.5/100 py, 95%CI: 4.2-4.7; IRR 1.49, 95%CI: 1.24-1.79, $P < 0.001$).	Not applicable
Strazulla *et al*[32]	daclatasvir and simeprevir for 24 weeks to reach SVR	Italy *n* = 1 case report	1 male	53	G1	24 wk	Decompe-nsated cirrhosis	Treated	Not applicable	1 recurrence
Bielen *et al*[27]	PEG-IFN+ DAA = 77 (13.5), DAAs only = 490 (86.4)	Belgium *n* = 567	PEG IFN + DAA group: 55 (71.4) DAA only group: 307 (62.7)	PEG IFN + DAA group age : 52 ± 9, DAA only group: 59 ± 12	PEG IFN + DAA group: all genotype 1. DAA only: G1 = 69%, G4 = 14.7%	6 mo	Metavir fibrosis score F3/F4 included only	PEG IFN + DAA = 1/77 (1.3%),DAA alone: 41/490 (8.4%)	Early occurrence rate of HCC = 1.7% and 1.1% in patients treated with DAA with and without PEG-IFN, respectively	Early recurrence rate was 0% in patients treated with PEG-IFN+DAA and 15.0% in patients treated with DAA without PEG-IFN

DAAs: Direct acting antivirals; IFN: Interferon; HCC: Hepatocellular carcinoma.

Table 3 Studies that do not report an increased risk of hepatocellular carcinoma with direct acting antivirals

Ref.	Treatment N /sample size	Country	Male gender *n* (%)	Age (yr)	Genotype *n* (%)	Duration of follow up	Cirrhosis *n* (%)	History of HCC *n* (%)	HCC occurrence	HCC recurrence
Ioannou *et al*[33]	IFN only = 35871 (58%), DAA + IFN = 4535 (7.2%), DAA only = 21948 (35%) *n* = 62354	United States	96.60%	Mean 55.8 ± 7.6	G1 = 77.4 %, G2 = 13.5%, G3 = 8.3%, G4 = 0	Mean follow-up DAA only group = 1.53 years, DAA+IFN group= 3.6 yr, IFN only group = 9.1 yr	Cirrhosis: 16.8%, decompe-nsated cirrhosis: 4.7%	None	Total 3271 incident cases. IFN group = 0.81/100 person years, DAA + IFN = 1.06 /100 py, DAA only = 1.32/100 py	Not applicable
Kanwal *et al*[34]	All DAA treated *n* = 22500	United States	21761 (96.7%)	Mean 61.6 ± 6.1	G1 = 19531 (86.8%), G2 = 1422 (6.3%), G3 = 940 (4.2%), G 4-6 = 217 (1%)	22963 person years of follow-up	8766 (39.0%)	None	271 (1.2)	Not applicable
ANRS CO22 HEP ATH-ER *et al*[35]	DAA group = 189, no DAA = 78 *n* =267	France	DAA group = 147 (78%)	DAA group = 62 ± 9 yr, no DAAs = 66 ± 10 yr	65 % genotype 1	Median: 20.2 mo after DAA initiation and 26.1 mo for untreated patients	Cirrhosis: DAA group = 152 (80%), no DAAs = 55 (72 %)	All treated	Not Applicable	DAA group = 24 (12.7%), no DAAs = 16 (20.5%)
ANRS CO12 CIRVIR *et al*[35]	DAA group = 13, no DAA = 66 *n* = 79	France	DAA group = 11 (85%), no DAA = 39 (59%)	DAA group = 61 ± 10 yr, no DAA = 65 ± 9 yr	Genotype 1: DAA group = 11 (85%), no DAA group = 53/63 (84 %)		All cirrhotic	All treated	Not Applicable	DAA group = 1 (7.7%), no DAAs = 31 (47%)
ANRS CO23 CUPILT *et al*[35]	All DAA treated *n* = 314	France	257 (82%)	61 ± 8 yr	212 (67.5%) genotype 1		49 (15.6%)	Treated	Not Applicable	7 (2.2%)
Cabibbo *et al*[36]	All DAA treated *n* = 143	Italy	80 (60.1)	Mean 70.4 ± 8.9	G1a: 9 (6.3), 1b: 114 (79.7), G2: 9 (6.3), G3: 7 (4.9), G4: 4 (2.8)	6, 12 and 18 mo	All cirrhotic	All treated	Not applicable	6-, 12- and 18-mo HCC recurrence rates were 12%, 26.6% and 29.1%, respectively
Nagata *et al*[37]	IFN-based: 1145. IFN-free DAA group: 752 *n* = 1897	Tokyo	IFN group: 621 (54), IFN free: 340 (45)	Median: IFN group: 59 (19-79); IFN free: 69 (24-87)	IFN group: G1a = 8 (7), G1b = 833 (73), G2a = 182 (16), G2b = 105 (9), G3 = 1 (0)	Median for IFN group: 6.8 (0.2-22.0); IFN free: 1.8 (0.1–7.7)		5% of IFN group, 11% of IFN free group	IFN group: 18 (2.5%). IFN-free group: 7 (1.1%)	IFN group: 18 (53%). IFN-free group: 22 (29%)
Ikeda *et al*[38]	All DAA treated *n* = 177	Japan	M:F = 52: 37 in each group	DAA group: 71 (39-85)		Median 20.7 mo		All treated	Not applicable	HCC recurrence rates at 1st and 2nd year were 18.1 and 25.0% in pts with DAA therapy and 21.8 and 46.5% in those without DAAs, (P = 0.003)
Zanetto *et al*[39]	DAA treated= 23, control = 23 *n* = 46	Italy		DAA group = 59 (49-69), controls= 58 (46 -70)	DAA group: G1a = 5 (22), Gb = 9 (39), G2 = 1 (4), G3 = 5 (22), G4 = 3 (13)	Median= DAA group = 10 mo, Control group = 7 mo	All cirrhotic	All treated	Not applicable	12.5% of DAA-treated patients and 8.3% of control group had HCC recurrence (P = 0.60)

Zavaglia *et al*[40]	All DAA treated *n* = 31	Italy	20 (64.5)	Mean 65 ± 8	G1a = 4 (13), G1b = 23(74), G2 = 2 (6.5), G4 = 2 (6.5)	Median 8 mo	All cirrhotic	All treated	Not applicable	1 (3.2)
Ogata *et al*[41]	All DAA treated *n* = 1170	Japan	493 (42)	Median = 67 (21-88)	All genotype 1	Time from the end of DAA therapy until last visit: 1.3 yr		None	22 cases (1.8%)	Not applicable
Minami *et al*[42]	DAA group = 27, IFN group = 38, Controls = 861 *n* = 926	Japan	DAA group: 18 (67), IFN group: 27 (71), Controls: 489 (57)	Median age: DAA group = 71 (48-82) IFN group = 66 (49-79), Control = 71 (44–91)	Genotype 1: DAA = 21 (78), IFN = 29 (76), Controls = 633 (74). Genotype 2: DAA= 6 (22), IFN= 9 (24), Control= 147 (17)	1 and 2 yr		All treated	Not applicable	Cumulative recurrence rates at 1 and 2 yr were 21.1% and 29.8%, respectively, in the DAA group, 26.3% and 52.9%, respectively, in the IFN group, and 30.5% and 61.0%, respectively, in the control group
Deterding *et al*[43]	*n* = 974	Germany			G1 = 743 (76.2)		All had advanced cirrhosis		12 (1.2)	
Degasperi *et al*[44]	*n* = 565	Italy	60%	Median age = 65 (30-87) yr	G1a = 15%, G1b = 49%, G2 = 13%, G3 = 11%, G4 = 12%, G5 = 1%	Median 42 wk for occu-rrence, 39 wk for recurrence	All cirrhotic	48 (8%)	20 (4%) estimated annual incidence of 1.6%	9 (19%), annual incidence of 7 .7%
Bourliere *et al*[45]	DAA + RBV ± PEG IFN = 21%. IFN free DAA therapy = 79% *n* = 1393			47 (19-79) yr		2.5 (0.6-4.3) yr			0 (0)	0 (0)
Nagaoki *et al*[46]	PEG-IFN/ RBV = 244, DCV/ASV = 154 *n* = 398	Japan			All genotype 1	Median for PEG-IFN/RBV = 96 (10–196) and DCV/ASV group = 23 (4–78) mo			PEG-IFN/RBV = 13 (5.3%), DCV/ASV group = 7 (4.5%)	
Cheung *et al*[47]	All DAA	United Kingdom			198 (48.8) 171 (42.1)	15 mo	All decompensated cirrhosis	29 (71.4%)	17 (5%)	2
Mettke *et al*[48]	158 DAA treated, 184 controls	Germany				Median = 440 (91–908) and 592 (90-1000) d	All cirrhotic		6 and 14 patients during follow-up, resulting in an HCC incidence of 2.9 (AVT) and 4.48 (Con) per 100 py, respectively	
Ji *et al*[49]		China	51%	Mean age 51	82.2% genotype 1b	Median 14 (3-35) mo	48% cirrhotic	None		Not applicable
Innes *et al*[50]		Scotland				1.8 yr		None	44 (5.1%)	Not applicable
Torres *et al*[51]	All DAA treated	United States	7 (87.5%)	Median 64 (57-87) years	G1 = 6 (75%), mixed genotype = 2 (25%)	12 mo	7 (87.5%)	All treated	Not applicable	0 (0)

DAAs: Direct acting antivirals; IFN: Interferon; HCC: Hepatocellular carcinoma.

Table 4 Factors predisposing to hepatocellular carcinoma in hepatitis C virus patients treated with direct acting antivirals after sustained virologic response

Past history of hepatocellular carcinoma[18,28,36]
Male gender[28]
Cirrhosis[34]
Hypoalbuminemia[41]
Thrombocytopenia[41]
Raised AFP levels[41]

response to DAA based therapy and are associated with a unique burden of HCC[56]. Beside male patients, group of individuals with prior history of HCC, cirrhosis and elevated AFP at baseline were found with greater risk of HCC if treated with DAAs. Currently, the phenomenon appears to be a class effect rather than an individual drug effect. Hence, focusing on these HCV patients and measuring the impact of DAAs on progression or development of HCC will help to estimate the more accurate risk. In certain studies higher rates of HCC recurrence was found. There is a need to differentiate whether the reported higher recurrence rate and more aggressive pattern of recurrence are due to DAA or host or disease related factors including presence of fibrosis, gaps in initial tumor staging and receiving non-curative therapies such as chemoembolization. Also, future investigations should be directed towards assessing the long-term effects of DAAs on these populations that have not been studied thus far[3]. In the meantime, a consensus recommendation seen in most of the studies at present is that even after achieving SVR, there should be close surveillance of patients with CHC especially with advance fibrosis and those who received a recent treatment for HCC in order to detect HCC at an early stage[18,19,26,32]. Some important Centers in Europe and United States have been delaying antiviral treatment for 6 mo or more after recent treatment for HCC in these patients. Moreover, until more robust data is available to investigate the role of DAAs in HCV related HCC cases, clinical practice should continue as per current guidelines in those patient groups who can benefit from DAA therapy[51].

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ORIGINAL ARTICLE

Basic Study

Homologous recombination mediates stable *Fah* gene integration and phenotypic correction in tyrosinaemia mouse-model

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Author contributions: Junge N was involved in conception and design of the research, performed the majority of the experiments, analysed the data and wrote the manuscript; Yuan QG performed mouse surgery and immunostaining and revised the work critically for important intellectual content; Huong Vu T had substantial contributions to the experiments, animal care and analysis and interpretation of data for the work; Krooss S, Bednarski C, Balakrishnan A and Cathomen T helped with the experiments and design of the research and revised the work critically for important intellectual content; Manns MP and Baumann U revised the work critically for important intellectual content; Sharma AD and Ott M were initiator and supervisor of the work, the developed initial concept and design of the research and conducted important preliminary studies.

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Abstract

AIM

To stably correct tyrosinaemia in proliferating livers of fumarylacetoacetate-hydrolase knockout (*Fah*$^{-/-}$) mice by homologous-recombination-mediated targeted addition of the *Fah* gene.

METHODS

C57BL/6 *Fah*$^{\Delta exon5}$ mice served as an animal model for human tyrosinaemia type 1 in our study. The vector was created by amplifying human Fah cDNA including the TTR promoter from a lentivirus plasmid as described. The *Fah* expression cassette was flanked by homologous arms (620 bp and 749 bp long) of the *Rosa26* gene locus. Mice were injected with 2.1 × 10^8 VP of this vector (*rAAV8-ROSA26.HAL-TTR.Fah-ROSA26.HAR*) *via* the tail vein. Mice in the control group were injected with 2.1 × 10^8 VP of a similar vector but missing the homologous arms (*rAAV8-TTR.Fah*). Primary hepatocytes from *Fah*$^{-/-}$ recipient mice, treated with our vectors, were isolated and 1 × 10^6 hepatocytes were transplanted into secondary *Fah*$^{-/-}$ recipient mice by injection into the spleen. Upon either vector application or hepatocyte transplantation NTBC treatment was stopped in recipient mice.

RESULTS

Here, we report successful HR-mediated genome editing by integration of a *Fah* gene expression cassette into the "safe harbour locus" *Rosa26* by recombinant *AAV8*. Both groups of mice showed long-term survival, weight gain and FAH positive clusters as determined by immunohistochemistry analysis of liver sections in the absence of NTBC treatment. In the group of C57BL/6 *Fah*$^{\Delta exon5}$ mice, which have been transplanted with hepatocytes from a mouse injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26.HAR* 156 d before, 6 out of 6 mice showed long-term survival, weight gain and FAH positive clusters without need for NTBC treatment. In contrast only 1 out 5 mice, who received hepatocytes from *rAAV8-TTR.Fah* treated mice, survived and showed few and smaller FAH positive clusters. These results demonstrate that homologous recombination-mediated *Fah* gene transfer corrects the phenotype in a mouse model of human tyrosinaemia type 1 (*Fah*$^{-/-}$ mice) and is long lasting in a proliferating state of the liver as shown by withdrawal of NTBC treatment and serial transplantation of isolated hepatocytes from primary *Fah*$^{-/-}$ recipient mice into secondary *Fah*$^{-/-}$ recipient mice. This long term therapeutic efficacy is clearly superior to our control mice treated with episomal *rAAV8* gene therapy approach.

CONCLUSION

HR-mediated *rAAV8* gene therapy provides targeted transgene integration and phenotypic correction in *Fah*$^{-/-}$ mice with superior long-term efficacy compared to episomal *rAAV8* therapy in proliferating livers.

Key words: Gene therapy; *AAV8*; Liver based metabolic disease; Targeted integration; *ROSA26*; Paediatric liver disease

Core tip: Recombinant adeno-associated virus (rAAV) has been explored for gene delivery in various murine models of hereditary liver disease, but in young children transgene expression from *AAV*-epigenomes diminishes over time. We thus explored, whether homologous recombination-mediated targeted gene addition of the fumarylacetoacetate hydrolase (*Fah*) gene would stably correct tyrosinaemia in rapidly proliferating livers of *Fah*$^{-/-}$ mice. Here, we report successful homologous recombination-mediated genome editing of a *Fah* gene expression cassette at the *Rosa26* locus by *rAAV8*. We demonstrate that this approach corrects the phenotype and is long lasting in a proliferating state of the liver, as shown by serial transplantation.

Junge N, Yuan Q, Huong Vu T, Krooss S, Bednarski C, Balakrishnan A, Cathomen T, Manns MP, Baumann U, Sharma AD, Ott M. Homologous recombination mediates stable *Fah* gene integration and phenotypic correction in tyrosinaemia mouse-model. *World J Hepatol* 2018; 10(2): 277-286 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/277.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.277

INTRODUCTION

Therapy for many liver-based metabolic diseases (LBMD) is limited to supportive measures and may entail significant side effects, such as organ failure, metabolic crisis, malignancy and impairment of quality of life. Until now, the only established curative treatment is liver organ transplantation (LTX). Although LTX for LBMDs has excellent long-term outcomes, the procedure is associated with significant morbidity and mortality and dependent on limited donor organ availability. Gene therapy could provide a minimally invasive therapeutic alternative to whole organ transplantation.

Recombinant adeno-associated viruses (rAAV) have evolved as promising vehicles for gene therapy to date and shown to produce long-term therapeutic effects in many mouse models of inherited liver diseases as well as in patients with haemophilia B[1-3]. AAV of serotype 8 has been shown to target mainly hepatocytes in the liver and is considered to be safe for clinical application[3-6]. Recombinant AAVs express the transgenes from epige-

nomic circular DNA with only rare genomic integration events[7]. Insertional mutagenesis resulting from random vector integrations has been observed in only one study[8] and these results remain to be confirmed by other studies[9]. Notably, AAV gene therapy in 77 dogs did not cause tumour formation during an observation period of up to 10 years[10]. Nathwani *et al*[11] presented a study in non-human primates with no signs of insertional mutagenesis 5 years after AAV application. Further, serotype 8 shows lower seroprevalence of preformed antibodies in humans than other AAV serotypes[3,12] thus minimizing risk of significant immune response.

Epigenomic expression of the therapeutic transgene from rAAV is thought to gradually decline in tissues with high cell turnover. Therapeutic efficacy of AAV-mediated gene transfer would thus decrease in growing livers of newborns or in diseases with intrinsic stimuli causing hepatocyte turnover. In some studies, gene correction by homologous recombination of rAAV transduced therapeutic genes was shown to result in long-term cellular persistence. Although the feasibility of *in vivo* gene correction in mice has been demonstrated in several models, superior therapeutic efficacy of gene therapy by gene addition mediated by homologous recombination remains to be demonstrated. Therefore, we examined whether the application of a *Fah* expression cassette flanked by homologous arms for the ROSA 26 Locus improves the efficacy and persistence of *Fah* gene delivery by integration at the *Rosa26* gene locus through homologous recombination in a mouse model of human tyrosinaemia type 1. We used C57BL/6 $Fah^{\Delta exon5}$ mice, which served as an animal model for human tyrosinaemia type 1[13]. Liver physiology and function in these animals can be maintained by providing water that is supplemented with the drug NTBC [2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione]. Control mice die 20-45 d after deprivation of NTBC due to liver failure. In the absence of NTBC, gene corrected hepatocytes proliferate and repopulate the liver.

MATERIALS AND METHODS

Animal model

All mouse experiments were granted permission and were performed according to the guidelines of the Hannover Medical School, Germany and the local government. Mice were kept on standard laboratory chow and free access to drinking water. They were housed in a restricted access room with controlled temperature and a light/dark cycle. We used C57BL/6 $Fah^{\Delta exon5}$ mice, which served as an animal model for human tyrosinaemia type 1[13]. Tyrosinaemia type 1 is caused by genetic alterations of the gene coding for FAH. The mutated *Fah* gene produces an unstable protein, which results in deficiency of fumarylacetoacetate hydrolase activity. The mice were provided with water supplemented with 1 mg/100 mL of NTBC [2-(2-nitro-4-(fluoromethyl) benzoyl) cyclohexane-1,3-dione] before performing experiments. Surgery was done under general anaesthesia with 2% isoflurane and 2 litres/min oxygen flow.

Cloning of AAV plasmids

For cloning of the *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26.HAR* plasmid, 620 and 749 bp Rosa26 gene locus homologous arms flanking the *Fah* expression cassette were subcloned into a pBlue-Script II plasmid. The entire transgene was further subcloned into the AAV backbone plasmid for virus generation. For the *Fah* expression cassette, we amplified hFah cDNA, including the TTR promoter, from a lentivirus plasmid described earlier from our group[14] by PCR (Phusion® High-Fidelity PCR Kit, Thermo scientific).

For cloning the *rAAV8-TTR.Fah expression cassette,* we created a similar plasmid with the same transgene cassette but not flanked by the homologous arms.

Preparation of adeno-associated virus serotype 8 vector

The adeno-associated virus serotype 8 (AAV8) vectors (Figure 1A), *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and *rAAV8-TTR.Fah*, were prepared as described previously[15]. The titre was determined by qRT-PCR using primers spanning the region of the TTR promoter, as published before[16].

AAV8 vector administration into $Fah^{-/-}$ mice

Mice were injected with 2.1×10^8 VP *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR via* the tail vein. Mice in the control group were injected with 2.1×10^8 VP *rAAV8-TTR.Fah*. Viruses were diluted in sorbitol to a total volume of 220 μL for injection. Non-treated control mice were injected with 0.9% sodium chloride. We used one control mouse group (n = 3) for the first generation experiment. Subsequently, the mice were monitored and weighed daily until they reached stable conditions or gained body weight. After 45 to 47 d, a 1/3 hepatectomy was conducted to analyse the presence of FAH protein-positive cell clusters. Tissues were fixed in 4% paraformaldehyde or snap frozen for subsequent analyses.

Serial transplantation of hepatocytes from virus-injected mice

Primary hepatocytes from primary $Fah^{-/-}$ recipient mice were isolated with the two-step collagenase (Roche) perfusion method, as described previously[4]. Hepatocytes (1×10^6) were transplanted into secondary $Fah^{-/-}$ recipient mice by injection into the spleen. Control mice were injected with sodium chloride into the spleen. We used one control mouse group (n = 3) for the second generation experiment.

Immunohistochemistry

Tissues were embedded in paraffin (ROTH) and cut in 2-μm-thick slices. Immunohistochemistry was carried out as described previously[17]. Briefly, after deparaffinization and blocking for endogenous H_2O_2, the slides were incubated in 1 x target retrieval solution

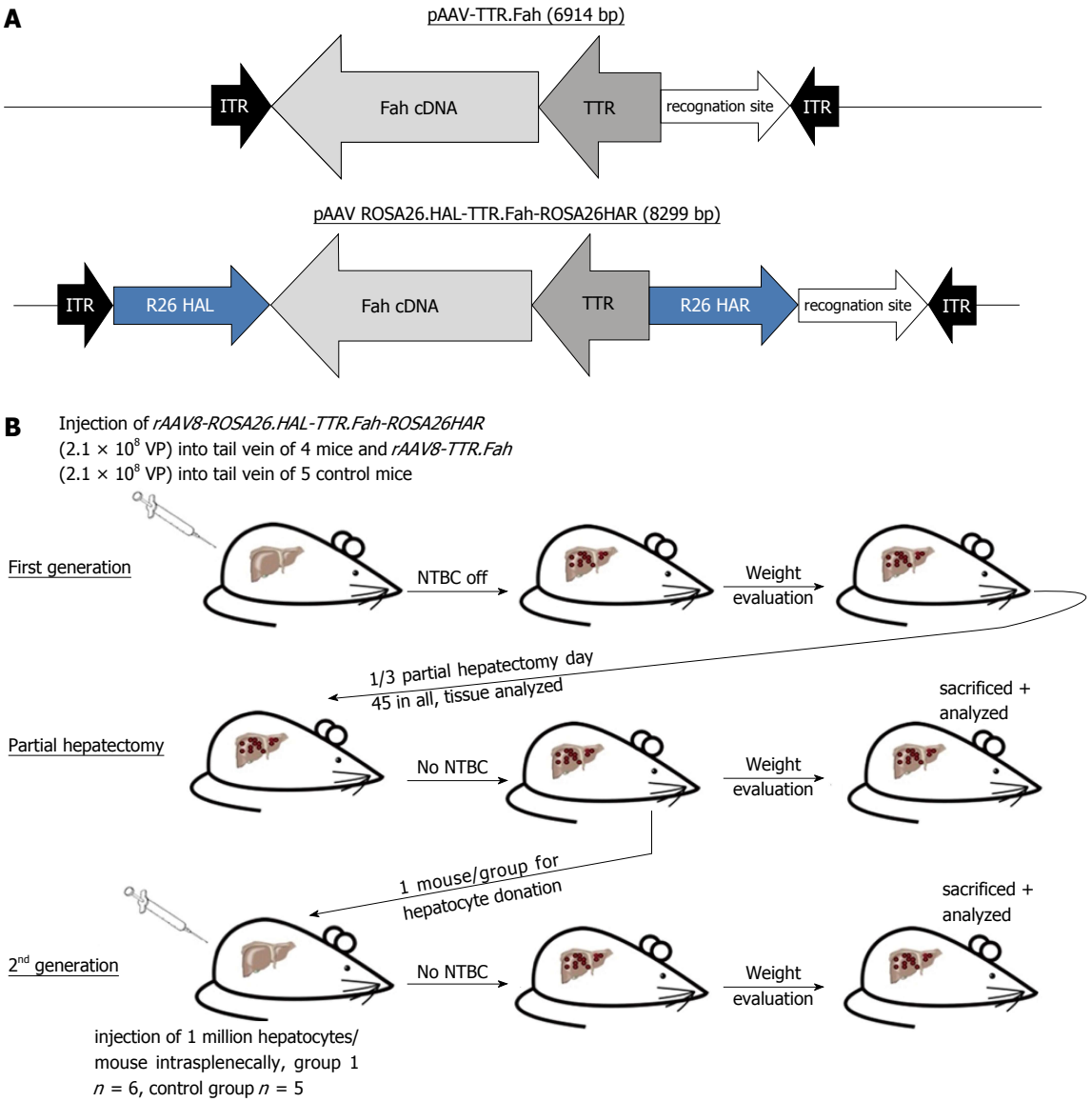

Figure 1 Flowchart of treating mice. A: Vector map for *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and for *rAAV8-TTR.Fah*. *Fah* cDNA is driven by the TTR promotor and for *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* located between the homologous arms of the Rosa26 locus. The vector was cloned into an AAV backbone; B: Scheme for the *in vivo* experiments. First-generation mice (*C57BL/6 FAH*$^{\Delta exon5}$ strain) were injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (group 1, $n = 4$) or *rAAV8-TTR.Fah* (control group, $n = 5$). The NTBC treatment was stopped, and after 45 d, a partial hepatectomy was performed. In each group, one mouse was used as the donor for hepatocyte transplantation into *C57BL/6 Fah*$^{\Delta exon5}$ mice. These recipients were the second generation of mice in our study. NTBC treatment was discontinued after hepatocyte transplantation. TTR: Transthyretin promoter (liver specific); R26 HAL: Homologous arm left for target locus in *Rosa26*; HAR: Homologous arm right for target locus in *Rosa26*; ITR: Inverted terminal repeat.

(Dako) at 98 ℃ for 20 min. For FAH (primary antibody, Abcam, ab81087) staining, tissues were blocked with the Avidin/Biotin blocking kit (Vector laboratories). Goat serum (Abcam) or rabbit serum (Abcam) was then used for blocking. Biotinylated goat anti-rabbit and rabbit anti-goat secondary antibodies (Vectastain, Vector laboratories) were used. Colour development was conducted using AEC substrate chromogen (Dako). Counterstaining was performed using haematoxylin (Merck Millipore, Germany).

Integration PCR

Genomic liver DNA was extracted from snap-frozen liver tissue with the DNeasy Blood and Tissue Kit (Qiagen) according to the protocol of the vendor. Two primers were designed, A and B. A was located in the *Rosa26* locus of recipient mouse 5′ to the donor gene. B was located in the *Fah* sequence of the donor DNA. Primer sequences were A: 5′-GGAGAGAGGCATTCAT GGGAGTGGAAAGTTAAGC-3′ and B: 5′-GCAGCATGG TCCAGTACATGTGCTTAAAGTTAGACC-3′. The expected length of the PCR amplicon was 1107 bp. PCR amplification was conducted with the Phusion® PCR Kit (New England BioLabs), and 200 ng of liver genomic DNA was used. The amplification was carried out under the following conditions: one cycle for 190 s at 98 ℃, followed by 50 cycles for 10 s at 98 ℃ and 90 s at 72 ℃, finished by one cycle for 10 min at 72 ℃. The

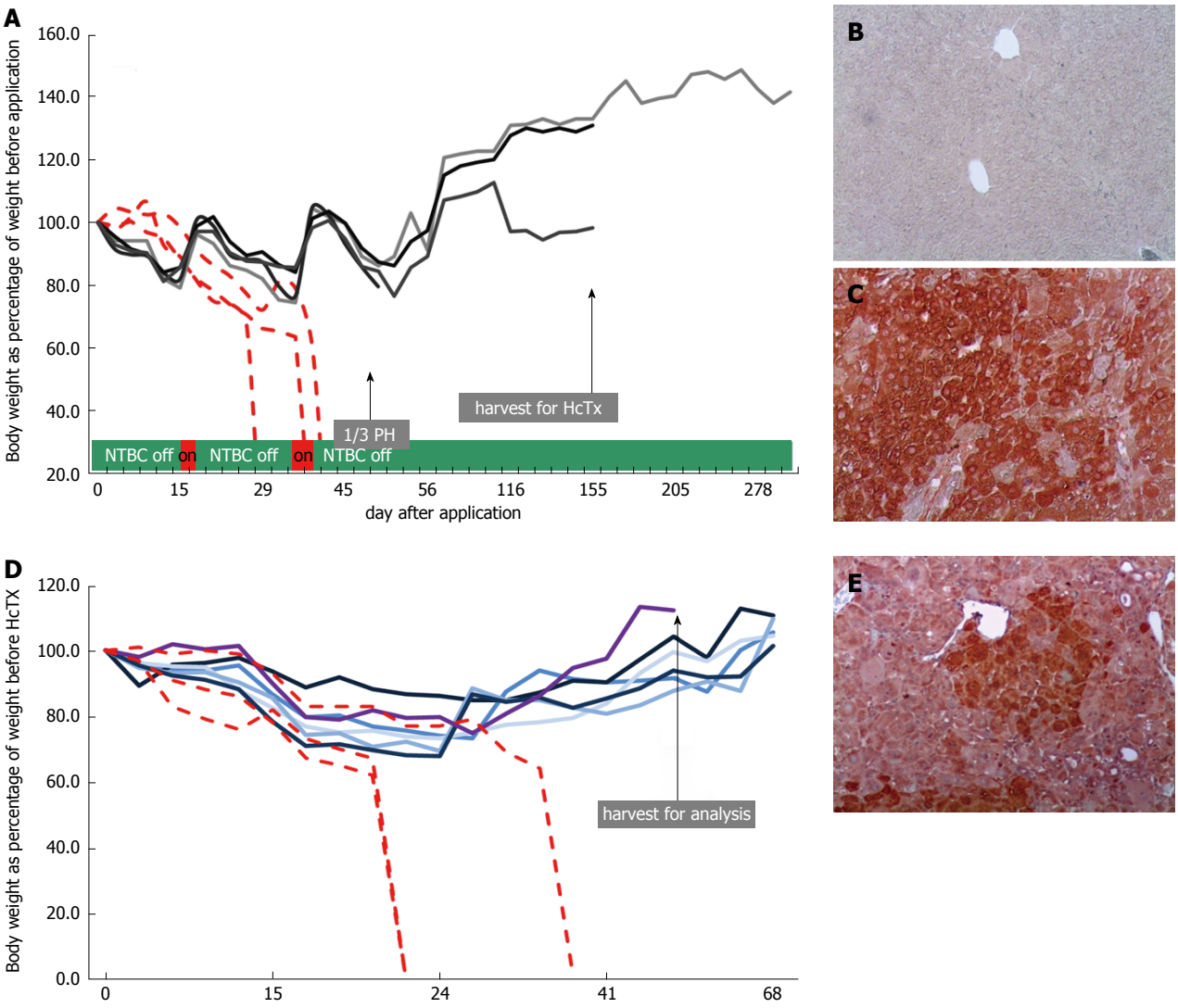

Figure 2 Mice treated with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR*. A: Weight graph and survival for first-generation mice (n = 4) injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and 3 untreated controls (injected with sodium chloride). Continuous line = *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* mice, broken line = controls (same control mice as displayed at Figure 3A). Body weight is displayed as percentage of body weight at the time of virus injection or sodium chloride injection (controls). The timeline (x-axis) is displayed in days beginning with the day of virus/sodium chloride injection as day zero; B: FAH staining of liver tissue from controls (mice with sodium chloride injection) after death (100 × magnification); C: FAH staining of liver tissue from partial hepatectomy in mouse injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (100 × magnification); D: Weight graph and survival for second-generation mice (continuous line), which were transplanted with one million hepatocytes from mice primarily injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and controls (same control mice as displayed at Figure 3D) without hepatocyte transplantation (broken line). Body weight is displayed as percentage of body weight at time of hepatocyte transplantation. The timeline (x-axis) is displayed in days, beginning with the day of hepatocyte transplantation as day zero; E: FAH staining of liver tissue from a partial hepatectomy from a second-generation mouse, which received one million hepatocytes from mice primarily injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (100 × magnification). 1/3PH: One third partial hepatectomy; HcTx: Hepatocyte transplantation.

PCR product was analysed utilizing gel electrophoresis on a 1% agarose gel (Biozym) for 50 min at 90 V.

qRT-PCR for FAH expression

RNA was isolated from snap frozen liver tissue of sacrificed mice. RNA was isolated with RNeasy® mini Kit (Qiagen) and QIAshredder® according to manufacturer instructions. After DNase treatment cDNA writing was performed (iScript™ reverse transcriptase supermix, BIO-RAD). SYBR green qRT-PCR (Qiagen QuantiTect Sybr green®) was performed at Stratagene Mx3000P (Aligent) with following primer (forward primer AGAATGCGCTGTTGCCAAA, reverse primer GGAAGCTCGGCCATGGTAT) spanning exon 5-6 and beta actin as housekeeping gene.

RESULTS

Long-term functional correction of the Fah gene defect by homologous recombination at the ROSA26 Locus in mice

We confirmed the correct design of our plasmids (Figure 1A) by sequencing and by evaluating FAH-Expression in Hepa1.6 cells by RT qPCR. For our experiments we used *Fah*$^{-/-}$ mice that contain a disruptive insertion in exon 5 of the *Fah* gene[13].

We prepared a high titre AAV8 vector suspension using the aforementioned AAV vector plasmids.

Next, we injected 4 mice with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR via* the tail vein (Figure 1B). To stimulate the proliferation of FAH-expressing hepatocytes, protective NTBC-treatment was discontinued immediately after injection. Whereas control mice (injected with saline) died before 45 d, all mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* survived beyond 45 d after injection (Figure 2A). On the 45th-47th days, 1/3 of the liver was removed and analysed for the presence of FAH cell clusters by immunohistochemistry. All animals injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* showed robust repopulation of the liver as indicated by survival, weight gain (Figure 2A) and multiple large FAH protein positive cell clusters in immunohistochemistry analyses (Figure 2C). Importantly, these mice survived without NTBC until the end of the study (day 288; Figure 2A).

Due to high selection pressure for gene corrected hepatocytes in the *Fah*$^{-/-}$ model, phenotypic correction of the enzyme deficiency as result of diluted, but still sufficient, FAH protein expression from epigenomic AAV DNA could not be excluded in the first generation. To test whether homologous sequences facilitated targeted integration and increased therapeutic efficacy, we isolated primary hepatocytes from one recipient mouse after recovery from partial hepatectomy and transplanted 1 x 10^6 cells each into the spleens of the secondary *Fah*$^{-/-}$ recipient mice (Figure 1B). All recipient animals (6/6) that were transplanted with hepatocytes from repopulated *Fah*$^{-/-}$ mouse showed liver repopulation and survived long-term in the absence of NTBC (Figure 2D and E).

Missing long-term in vivo correction of Fah in the absence of homologous sequences after hepatocyte transplantation.

To establish unequivocally that homologous recombination is indeed capable of long-term stable correction of *Fah* deficiency and superior to non-homologous, episomal gene therapy, we generated a control group with five mice, who were injected with *rAAV8-TTR.Fah*. All five primary recipient mice survived with weight gain (Figure 3A) and showed clusters of FAH-positive cells at partial hepatectomy on day 45 (Figure 3C). To show inferiority of this episomal approach we further increased the proliferation conditions by transplanting hepatocytes (1 × 10^6 cells for each recipient) from one first generation recipient mouse into 5 secondary *Fah*$^{-/-}$ recipient mice in this group also. Only one of the five secondary recipient mice (hepatocyte recipients) survived NTBC withdrawal and showed few and small FAH-positive cell clusters (Figure 3D and E). Hence, these results suggest that in the absence of homologous arms, the observed FAH-positive clusters in the primary recipient *Fah*$^{-/-}$ mice mostly resulted from epigenomic AAVs or an unexplained mechanism of integration/anchorage on cellular DNA, which was lost upon transplantation into secondary *Fah*$^{-/-}$ recipient mice.

Successful targeted integration of Fah cDNA at the Rosa26 locus

So far, our results revealed that mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* had robust liver repopulation and improved survival after secondary transplantation. However, it is important to prove that homologous arms facilitated targeted integration/gene addition of *Fah* cDNA into the *Rosa26* locus. We therefore examined targeted integration by genomic PCR amplifying portions of the *Rosa26* gene locus and the *Fah* transgene cassette. Indeed, we found an expected band of 1071 bp in mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (Figure 4) but not in mice injected with *rAAV8-TTR.Fah*. Our data thus indicate that homologous arms facilitated targeted integration at a frequency sufficient for increased therapeutic outcome and phenotypic correction in *Fah*$^{-/-}$ mice. This is further confirmed by Sybr green qRT-PCR results. These showed a clearly higher expression of FAH in mice treated with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* compared to mice treated with *rAAV8-TTR.Fah* alone (Figure 5).

In summary, we can conclude that in the first generation we could not detect a difference for survival, weight gain and FAH positive cell cluster between mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* or *rAAV8-TTR.Fah* but in secondary generation (recipients of 1 x 10^6 hepatocytes from first generation) we could detect a clear improved survival for the group with homologous arms in the vector. In this group 6 out of 6 mice survived and in the other group 1 out 5 mice survived. Furthermore the detection FAH positive cell clusters showed the same distribution.

DISCUSSION

In *in vitro* and *in vivo* studies[18,19], the AAV vector is used as the vector of choice for gene correction approaches by homologous recombination; one important reason is its single-stranded nature. Reports on gene correction or gene addition by homologous recombination for liver-based metabolic diseases are rare and have shown correction frequencies[20] too low for phenotypic correction, except for the study of Paulk *et al*[19]. However, they used a mouse model with a point mutation for *Fah* gene; therefore, their approach was a gene correction. Here, we provide proof of concept for *in vivo* targeted gene addition mediated by homologous recombination in a liver-based metabolic disease. Our findings demonstrate that in a state of extensive hepatocyte proliferation, targeted integration by homologous recombination was superior to gene therapy based on episomal AAV gene therapy.

Primary recipient mice that were injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* or *rAAV8-TTR.Fah* survived and showed phenotypic rescue after NTBC withdrawal. Notably, livers of mice from

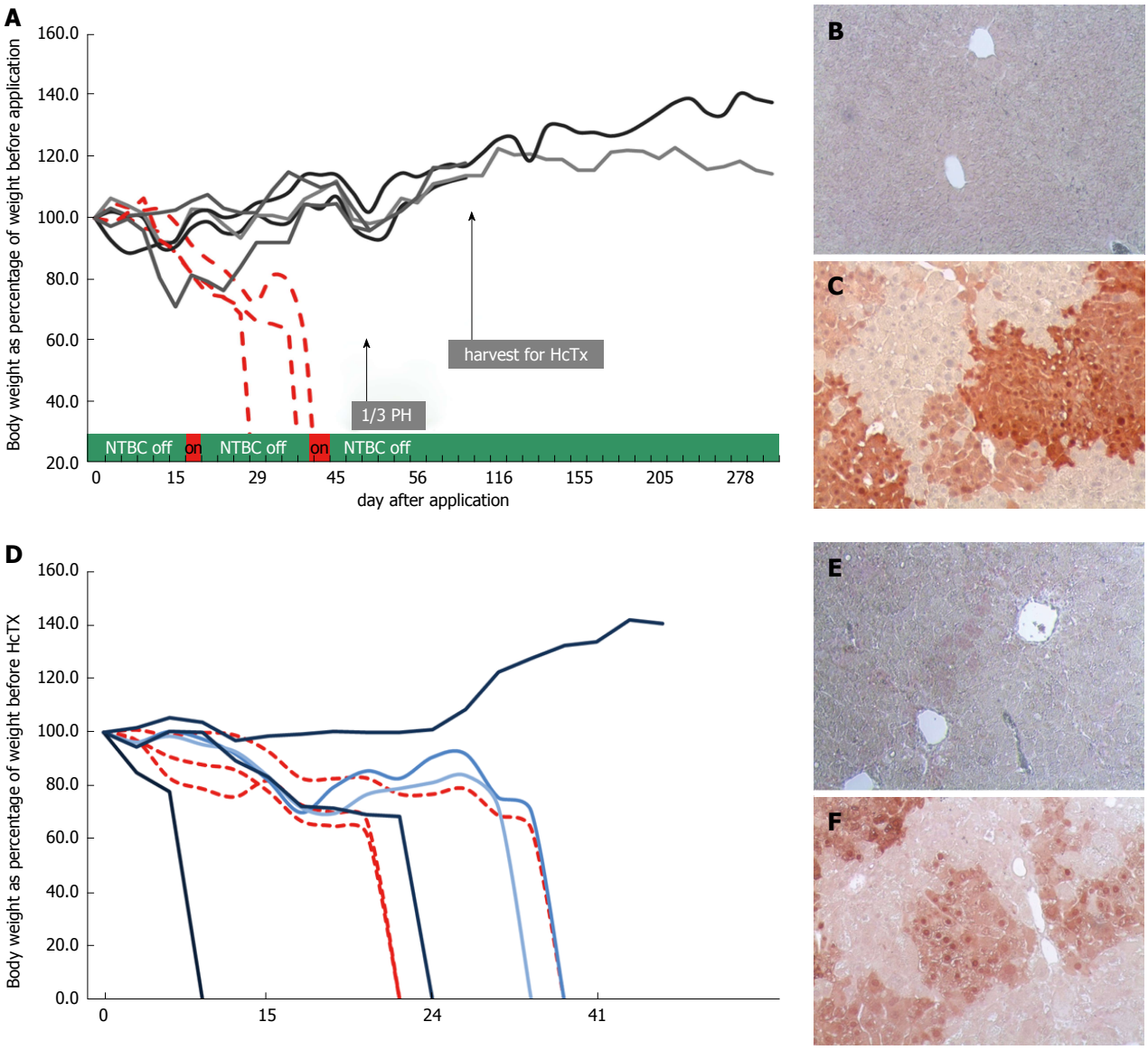

Figure 3 Mice treated with *rAAV8-TTR.Fah*. A: Weight graph and survival for first-generation mice injected with *rAAV8-TTR.Fah* (*n* = 5) and 3 untreated controls (injected with sodium chloride). Continuous line = *rAAV8-TTR.Fah* mice, broken line = controls (same control mice as displayed at Figure 2A). Body weight is displayed as percentage of body weight at the time of virus injection or sodium chloride injection (controls). The timeline (x-axis) is displayed in days beginning with the day of virus/sodium chloride injection as day zero; B: FAH staining of liver tissue from controls (mouse with sodium chloride injection) after death (100 × magnification); C: FAH staining of liver tissue from a partial hepatectomy from a mouse injected with *rAAV8-TTR.Fah* (100 × magnification); D: Weight graph and survival for second-generation mice (continuous line), which were transplanted with one million hepatocytes from mice primarily injected with *rAAV8-TTR.Fah* and controls (same control mice as displayed at Figure 2D) without hepatocyte transplantation (broken line). Body weight is displayed as percentage of body weight at time of hepatocyte transplantation. The timeline (x-axis) is displayed in days, beginning with the day of hepatocyte transplantation as day zero; E: FAH staining of liver tissue from a partial hepatectomy in a second-generation mouse, which received one million hepatocytes from mice primarily injected with *rAAV8-TTR.Fah* (100 × magnification); F: FAH staining of liver tissue from a partial hepatectomy from the single second-generation mouse that showed cluster and weight gain.

both groups showed clear FAH-positive cell clusters in immunohistochemistry. We determined the presence of FAH positive areas in the both groups of primary recipients. We did not find significant differences in FAH positivity indicating similar number of FAH positive hepatocytes in both groups of mice. So far, cell clusters have always been explained by clonal expansion of corrected hepatocytes, which would implicate the necessity of vector integration. In the tyrosinemia mouse model *Fah* corrected hepatocytes have a strong selective advantage so they grow clonally, form nodules and can repopulate the entire liver at least[21,22]. Therefore, it is reasonable that a small number of hepatocytes with random integrations or another unexplained mechanism such as of integration/anchorage on cellular DNA proliferate preferentially and repopulate the diseased liver, leading to FAH-positive cell clusters. A human liver contains approximately 300 billion hepatocytes, which means, in case of 10% transduction efficiency with an integration rate of 0.1%, a single individual will have approximately 30 million hepatocytes with at least one integration event[23]. Therefore, one can assume that the phenotypic correction in these mice can be explained by the selective proliferation advantage of a small

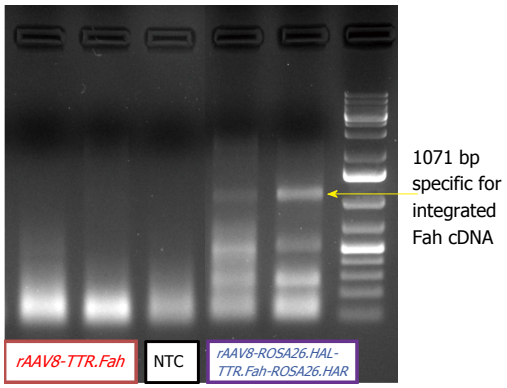

Figure 4 Integration PCR gel electrophoresis. A representative gel picture from the analyses of genomic liver DNA, that was extracted from snap-frozen liver tissue harvested between 60-70 d after hepatocyte transplantation. Primers were located in the *Rosa26* locus and in the FAH sequence of the donor DNA. Product could only be amplified if targeted integration occurred. The expected length of the PCR amplicon was 1107 bp. The PCR product was analysed utilizing agarose gel electrophoresis.

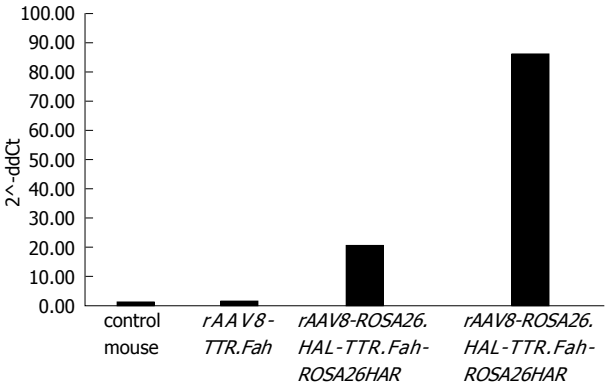

Figure 5 Sybr green qRT-PCR. Shown are the 2^-ddct values of two mice treated with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and one mouse treated with *rAAV8-TTR.Fah* calculated on an untreated control mouse.

number of hepatocytes with successfully integrated *Fah* cassettes. A spontaneous reversion of the genetic defect, as the underlying cause for phenotypic correction and FAH-positive cell clusters, as described in humans[24], is not possible in the *Fah*^{exon5} mouse model[25].

Therefore we increased the proliferation conditions by hepatocyte transplantation from one first generation recipient per group into secondary *Fah*$^{-/-}$ recipient mice (1 x 10^6 hepatocytes for each secondary recipient mouse). In this experiment, the advantage of homologous recombination became clearly visible, since phenotypic correction could be achieved in all mice (6/6). In the *rAAV8-TTR.Fah* group, only 1/5 mice survived. In accordance with these results, 6/6 mice co-injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* showed clear FAH-positive cell clusters in livers, whereas only 1/5 mice injected with *rAAV8-R26.Fah* had FAH-positive clusters. Furthermore Sybr green qRT-PCR showed higher FAH expression in liver tissue of *ROSA26.HAL-TTR.Fah-ROSA26HAR-mice* than in *rAAV8-R26.Fah-mice.*

Partial hepatectomy and serial transplantation together are supposed to have triggered at least 30 rounds of cell doubling for the hepatocytes[26], nevertheless we could not find any tumour formation in any of our mice. This is in line with other studies showing a good safety profile for *rAAV8* gene therapy[5]. Our proof of concept approach demonstrated that the targeted integration/addition of a therapeutic gene allows for safer (compared to random integration) and more efficient (compared to epigenomic) gene therapy, especially for gene therapy of liver-based metabolic diseases in paediatric patients, since the *Rosa26* locus exists in mice[27,28] as well as in humans[29]. In contrast to the assumption that homologous recombination alone is not sufficient for a long-lasting phenotypic correction of a liver-based metabolic disease, we could show the opposite with this study, at least for diseases with selection advantage for corrected hepatocytes, like tyrosinaemia type 1. Further potential target diseases with selection advantage could be Wilson disease or bile-acid transporter defects. Continuing studies should evaluate the efficiency of this approach in liver-based metabolic diseases without selection advantage such as Crigler Najjar Syndrome.

In summary, we demonstrate that targeted *in vivo* integration of a *Fah* expression cassette mediated by homologous arms is a highly efficient approach to stably correct a metabolic liver disease in an FAH mouse model with extensive hepatocyte proliferation. Since many metabolic disorders must already be treated in children with fast-dividing hepatocytes, targeted transgene integration is an important step to safe and long-lasting gene therapy in the developing liver.

ARTICLE HIGHLIGHTS

Research background

We describe an important proof of concept in the field of AAV gene therapy for liver based metabolic diseases (LBMD). First gene therapy studies in humans are done (Hemophilia B) or very ready to start (Crigler-Najjar Syndrome); even an EMA approved drug for AAV gene therapy (Glybera) exists already. But all these approaches have a major weakness, the missing permanence of the gene therapy effect, especially in young children. But they are the main target group for gene therapy in LBMD, since early therapy could avoid irreversible damage to the organs of the patient. In these patients the advantage of recombinant AAV gene therapy, the almost missing integration into the host genome turns into a disadvantage since donor cDNA will be lost during cell turn over.

Research motivation

Targeted integration into safe harbors like the ROSA26 locus could overcome the problem of diminishing donor-cDNA in rAAV gene therapy. There are studies, showing proof of concept for targeted integration with nucleases like zinc fingers or CRISP/CAS9, but these approaches contain also new potential sources of side effects. However in our study only natural appearing cellular repair mechanism has been used to generate a targeted integration.

Research objectives

Up to know it was assumed that the efficiency of gene addition by targeted integration into a safe harbor mediated by homologous recombination would be to low for phenotypic correction of liver based metabolic diseases (LBMD) in growing livers. But we could show in a disease model for LBMD with selection advantage of corrected hepatocytes that this is not the case. This could be

transferred to other diseases like the group of familial intrahepatic cholestasis or Wilson disease or even to diseases with less selection advantage.

Research methods

C57BL/6 *Fah*$^{\Delta exon5}$ mice served as an animal model for human tyrosinaemia type 1 in our study. We treated these mice with a rAAV Vector containing human *Fah* cDNA, a liver specific promotor (TTR) and homologous arms for ROSA26 locus. We compared this group to mice treated with a vector without homologous arms. Hepatocyte proliferation was induced by partial hepatectomy and serial hepatocyte transplantation. Survival of mice without NTBC and existence of FAH positive cell cluster at immunohistochemistry staining on liver tissue of the mice were the main endpoints.

Research results

We could show for the first time proof of concept for phenotypic correction of a LBMD in a mouse model under conditions of extensive hepatocyte proliferation with rAAV mediated gene addition by targeted integration at a safe harbor without the use of nucleases or gene repair. Further studies have to show if this concept is transferable to LBMD with less section advantage of corrected hepatocytes.

Research conclusions

Our study shows that phenotypic correction of a LBMD by rAAV gene therapy under conditions of extensive hepatocyte proliferation is possible with homologous recombination (HR) alone and does not necessarily have the need for nucleases. In conclusion we showed that HR-mediated rAAV8 gene therapy provides targeted transgene integration and phenotypic correction in *Fah*$^{-/-}$ mice with superior long-term efficacy compared to episomal rAAV8 therapy in proliferating livers. In opposite to approaches with the aim of point mutation repair on genes of LBMD our system with gene addition into a safe harbour can be easily transferred to other LBMDs and is not mutation specific.

Research perspectives

Our results are an important step into the solution of a main clinical problem for gene therapy of LBMD, since mostly this therapy is mandatory in growing children, where episomal gene therapy is not lasting. In opposite to studies with nucleases our study focus on a natural mechanism for targeted integration which avoids potential side effects of nucleases. A very important question for following studies would be if these results could also be observed in LBMD with less selection advantage for corrected hepatocytes (*e.g.,* Crigler-Najjar Syndrom).

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ORIGINAL ARTICLE

Basic Study

Multipotent stromal cells stimulate liver regeneration by influencing the macrophage polarization in rat

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Abstract

AIM

To investigate the influence of the umbilical cord-derived multipotent stromal cells (MSCs) on recovery of the liver after the subtotal resection, that is, removal of 80% of the organ mass, a renowned model of the small-for-size liver remnant syndrome.

METHODS

The MSCs were obtained from the intervascular tissue of umbilical cords, dissected from rat fetuses, by the explant culture technique. The vital labeling of MSCs with PKH26 was carried out on the 3rd passage. The subtotal resection was performed on male Sprague-Dawley rats. The experimental group animals received a transplant 10^6 MSCs infused into the spleen. Hepatocyte proliferation was assessed by counting of either mitotic figures or Ki67-positive cells in microscopic images. MSC differentiation was assessed with antibodies to hepatocyte-specific marker cytokeratin 18 (CK18), cholangiocyte-specific protein CK19, smooth muscle cell-specific protein α-SMA, the endothelial cell marker CD31, or the active fibroblast marker FAPα. Total macrophages of the liver were selectively stained in cryosections incubated with anti-CD68 antibodies (1:100, Abcam), while the M2a and M2c macrophage populations were selectively stained with anti-CD206 antibodies. Expression of interleukin and growth factor genes was evaluated with PCR-RT.

RESULTS

Intrasplenic allogeneic transplantation of the umbilical cord-derived multipotent stromal cells stimulates reparative processes within the residual liver tissue after subtotal resection (removal of 80% of the organ mass), as indicated by increased rates of hepatocyte proliferation and accelerated organ mass recovery. These effects may result from paracrine influence of the transplanted cells on the resident macrophage population of the liver. The transplantation favors polarization of macrophages to M2 phenotype (the M2-polarized macrophages specifically express CD206; they are known to suppress inflammation and support tissue repair). No differentiation of the transplanted cells into any of the liver cell types have been observed in the study.

CONCLUSION

We found no direct evidence for the paracrine effect of MSCs on liver regeneration after the subtotal liver resection in rats. However, the paracrine mechanism of the therapeutic activity of transplanted MSC is indirectly indicated by a decrease in the total number of CD68 + macrophages and an increase in the proportion of M2 pro-repair macrophages in the regenerating liver as compared to animals in which the transplantation was only mimicked.

Key words: Liver; Regeneration; Multipotent stromal cells; Macrophages

Core tip: Umbilical cord-derived multipotent stromal cells stimulate reparative processes within the liver after subtotal resection (removal of 80% of the organ mass). Multipotent stromal cells stimulate hepatocyte proliferation in rats after subtotal resection and favor polarization of macrophages to M2 phenotype. The transplanted multipotent stromal cells do not differentiate into any of the liver cell types under these conditions.

Elchaninov A, Fatkhudinov T, Usman N, Arutyunyan I, Makarov A, Lokhonina A, Eremina I, Surovtsev V, Goldshtein D, Bolshakova G, Glinkina V, Sukhikh G. Multipotent stromal cells stimulate liver regeneration by influencing the macrophage polarization in rat. *World J Hepatol* 2018; 10(2): 287-296 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/287.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.287

INTRODUCTION

Multipotent stromal cells (MSCs) are found to be a helpful supplement in various repair processes in mammals, particularly in solid organs[1]. These cells are considered as a promising tool of regenerative medicine[2]. Their ability to stimulate reparative regeneration of damaged liver have been confirmed[3], but the mechanisms remain uncertain.

The experimentally proven enhancement of liver regeneration by MSCs must be essentially paracrine, because their transplantation to residual livers causes an increase in concentrations of HGF and several other growth factors within the regenerating tissues[4]. Therapeutic activity of MSCs is apparently related to their anti-inflammatory properties, which also represent a sort of paracrine regulation, as manifested by a local increase in IL-4, IL-13, and TSG-6 production paralleled by relative shortage of TNFα and IL-6[5]. Modulation of inflammatory reactions may be implemented *via* influence of these, or similar, paracrine factors on immune cells, especially on macrophages[6,7]. Several studies, however, confirm the ability of MSCs to differentiate into hepatocytes, which may support quite a different explanation for the positive influence of MSCs on liver repair[3,8].

In a number of clinical cases, a residual portion of the liver left after an extended resection, *e.g.*, due to a tumor or metastasis, or even a transplanted portion of donor liver tissue supposed to replace the bulk of liver tissue in the host, is too small to effectively support the body homeostasis[9]. The resulting post-hepatectomy liver failure is a major manifestation of the small-for-size liver remnant syndrome, when the critically reduced liver mass is not only insufficient to maintain normal liver function, but also incapable of compensatory growth. The

problem could be solved by some specific controllable stimulation of this growth combined with intensive liver care and management for acute hepatic failure.

This study deals with influence of the umbilical cord-derived MSCs on the reparative regeneration of the liver after the subtotal resection, that is, removal of the 80% organ mass, a renowned model of the small-for-size liver remnant syndrome.

MATERIALS AND METHODS

Outbred male Sprague-Dawley rats, body weight 250-270 g, were obtained from the Institute for Bioorganic Chemistry branch animal facilities (Pushchino, Moscow region, Russia). All experimental work involving animals was carried out according to the standards of laboratory practice (National Guidelines No. 267 by Ministry of Healthcare of the Russia, June 1, 2003), and all efforts were made to minimize suffering. The study was approved by the Ethical Review Board at the Scientific Research Institute of Human Morphology (Protocol No. 16, November 19, 2015).

The MSCs were obtained from the intervascular tissue of umbilical cords, dissected from rat fetuses, by the explant culture technique. Their identity as MSCs was verified by their capacity of clonogenic growth on the untreated plastic, expression of specific set of surface antigens, and their ability to differentiate into mesodermal derivatives (Arutyunyan *et al*[2], 2016). The vital labeling of MSCs with PKH26 (Sigma-Aldrich Co LLC, United States) was carried out on the 3rd passage. The labeled cells were washed twice with saline (PanEco, Russia) and transferred to culture dishes for the labeling quality assessment or into syringes for transplantation to experimental animals.

The animals (n = 83) were operated as described earlier[15]. Hepatic tissue from sham-operated animals (n = 43) served as additional control in the assessment of hepatocyte proliferation, immunostaining and genes expression.

Animal survival was calculated as (the total number of operated animals minus the number of spontaneous deaths divided by the total number of operated animals. The animals were drawn from the experiment in CO_2-chamber at 3 h, 6 h, 24 h, 48 h, 3 d, 7 d, or 10 d after the surgery (5-6 animals for each term) between 9 am and 11 am. The regenerating livers were promptly dissected, weighed (along with the whole animal, to determine the liver-to-total mass ratio, with intact animals of similar age (n = 10) being used for comparison), and preserved for analysis. A part of the material was fixed in 10% buffered formalin for a routine histological procedure (dehydration, paraffin sectioning, HE staining, mounting, and microscopy). Another part of the material was frozen in liquid nitrogen for *in vivo* cell tracing and/or immunochemistry (see below).

Hepatocyte proliferation assessment

Hepatocyte proliferation was assessed by counting of either mitotic figures or Ki67-positive cells in microscopic images.

Mitotic index of hepatocytes was calculated for each animal individually as a number of mitoses per 6×10^3 hepatocytes, expressed in promille (‰).

Ki67-positive cells were counted on cryosections immunostained with corresponding primary antibodies followed by FITC-conjugated secondary antibodies, both supplied by Abcam, United Kingdom, and used in 1:100 and 1:200 dilutions, respectively; cell nuclei were counterstained with DAPI (Sigma-Aldrich Co LLC). The Ki67 proliferation index was calculated for each animal individually as a number of Ki67-positive hepatocytes per 3×10^3 hepatocytes.

MSC differentiation assessment

The cryosections were stained with antibodies to hepatocyte-specific marker cytokeratin 18 (CK18), cholangiocyte-specific protein CK19, smooth muscle cell-specific protein α-SMA, the endothelial cell marker CD31, or the active fibroblast marker FAPα. All of the primary antibodies were obtained from Abcam and used in 1:100 dilutions as recommended by the manufacturer and followed by FITC-conjugated secondary antibodies (1:200, Abcam); cell nuclei were counterstained with DAPI (Sigma-Aldrich Co LLC). Expression of the cell type-specific proteins was meticulously sought out in MSCs identified within regenerating liver tissue by PKH26 label. The observations were done using Leica DM 4000 B fluorescent microscope with LAS AF v.3.1.0 build 8587 software (Leica Microsystems CMS GmbH, Germany).

Selective immunostaining of macrophages

Total macrophages of the liver were selectively stained in cryosections incubated with anti-CD68 antibodies (1:100, Abcam), while the M2a and M2c macrophage populations were selectively stained with anti-CD206 antibodies (Santa-Cruz, United States) used in 1:100 dilution as recommended by the manufacturer. The signal was visualized by using FITC-conjugated secondary antibodies (1:200, Abcam) combined with DAPI-counterstaining of the nuclei. The CD68+ and CD206+ cells were counted in microscopic images and related to the total cell counts to obtain corresponding indexes of macrophage content.

Real-time PCR assay

Total RNA was isolated with RNeasy Plus Mini Kit (QIAGEN). Estimated concentration of RNA in the eluate was 0.1 g/L; the quality was controlled by electrophoresis. To remove traces of genomic DNA, the samples were treated with RNase-free DNase I (Thermo Scientific, Waltham, MA, United States; 1 U per μg of RNA); the efficacy of DNA elimination was confirmed by PCR with nontranscribed genomic region-specific primers. Reverse transcription reactions were set up using MMLV RT Kit (Evrogen CJSC, Moscow, Russia) and held at 39 ℃ for 1 h.

Table 1 Oligonucleotide sequences

Target designation	5'-end primer	3'-end primer
Il1β	CTGTCTGACCCATGTGAGCT	ACTCCACTTTGGTCTTGACTT
Il6	TACATATGTTCTCAGGGA GAT	GGTAGAAACGGAACTCCAG
Il10	GCCCAGAAATCAAGGAGCAT	TGAGTGTCACGTAGGCTT CTA
Tnfα	CCACCACGCTCTTCTGTCTA	GCTACGGGCTTGTCACTCG
Hgf	GGCCATGGTGCTACACTCTT	TTGTGGGGGTACTGCGAATC
Tgfβ1	CCGCAACAACGCAATCTATG	AGCCCTGTATTCCGTCTCCTT
Actβ	GAGATTACTGCCCTGGCTCC	GCTCAGTAACAGTCCGCCTA
B2m	CTCGCTCGGTGACCGTGAT	GGACAGATCTGACATCTCGA
Gapdh	GCGAGATCCCGCTAACATCA	CCCTTCCACGATGCCAAAGT

Table 2 Liver-to-body weight ratio dynamics (Mean ± SD, %)

Time after operation, d	Multipotent stromal cells transplantation group	Comparison group
Intact controls	4.5 ± 0.9	
3	1.8 ± 0.3	1.4 ± 0.1[a]
7	2.4 ± 0.7	2.7 ± 0.3[a]
10	3.1 ± 0.2	2.6 ± 0.2[a]

[a]$P > 0.05$.

PCR mixtures based on qPCRmix-HS SYBR system (Evrogen CJSC) containing oligonucleotide primers (custom made by SYNTOL, Moscow, Russia) in 0.2-0.4 μmol/L final concentrations were set up in duplicates. Structures of the oligonucleotides with corresponding target symbols and descriptions are given in Table 1. Real-time PCR was carried out in DT-96 Real-Time PCR Cycler (DNA-Technology JSC, Moscow, Russia) at 95 ℃ 15 s, 62 ℃ for 10 s + reading, 72 ℃ for 20 s. The relative expression values were calculated by approach originally introduced (Pfaffl MW[10], 2001) with modifications (Vandesompele J *et al*[11], 2002) using *actb*, *b2m*, and *gapdh* (Table 1) as reference targets.

Statistical analysis

The data were analyzed using SigmaStat 3.5 (Systat Software Inc., Chicago, IL, United States). Proportion values were compared by 2-sample *z*-test; relative gene expression values were compared by the Mann-Whitney *U* test; more-than-two-groups comparisons were done using ANOVA on ranks; *P*-values < 0.05 were considered significant.

RESULTS

Animal survival

Some of the animals died within 3 d after the surgery, but none of them died after the 3 d timepoint, animal survival in the comparison group (72.1% ± 6.8%, $n = 43$) was significantly higher than in the MSC transplantation group (47.5% ± 7.8%, $n = 40$).

Liver mass recovery

MSC transplantation stimulated compensatory growth of residual hepatic tissues, which was expressed in more rapid liver mass recovery (Table 2). In the MSC transplantation group, the liver-to-body ratio returned to normal values by day 10 after the surgery, while in the comparison group it still did not reach the initial level (as measured for the intact control animals).

Hepatocyte proliferation dynamics

Mitotic index of hepatocytes was significantly higher in the MSC transplantation group than in the comparison group on day 3 as well as on day 10 after the surgery (Figure 1A, C, D, F and G). On day 7 after the surgery, however, this index was significantly lower in the MSC transplantation group than in the comparison group (Figure 1B, E and G).

A similar tendency was revealed by using the Ki67 immunostaining (Figure 2). Although no significant differences in the Ki67 proliferation index between the groups were observed on day 3 after the surgery (Figure 2A, D and G), this index for the MSC transplantation group on day 7 was significantly lower (Figure 2B, E and G), and on day 10 significantly higher, than for the comparison group (Figure 2C, F and G).

Differentiation of transplanted MSCs

The transplanted cells expressed neither CK18 or CK19 (Figure 3A and B), nor α-SMA (Figure 3D). Solitary CD31-expressing PKH26-labeled cells were observed at the site of injection on days 3 and 10 after the surgery (Figure 3C).

Macrophage polarization profiles of regenerating liver

It turned out, that on day 7 after the surgery the animals of the MSC transplantation group had significantly decreased numbers of total liver macrophages defined as CD68+ cells (Figure 4A, B and E). At the same time, calculated numbers of M2 macrophages (defined as CD206+ cells) in regenerating livers on day 7 after the surgery were higher in the MSC transplantation group than in the comparison group (Figure 4C, D and F).

Transplantation-dependent changes in gene expression

No transplantation-dependent changes in gene expression at the site of transplantation have been revealed in this study, despite that such changes were extensively sought for. We analyzed expression dynamics for a set of genes, which reportedly participate in regulation of mammalian liver recovery, and found no changes that could be related

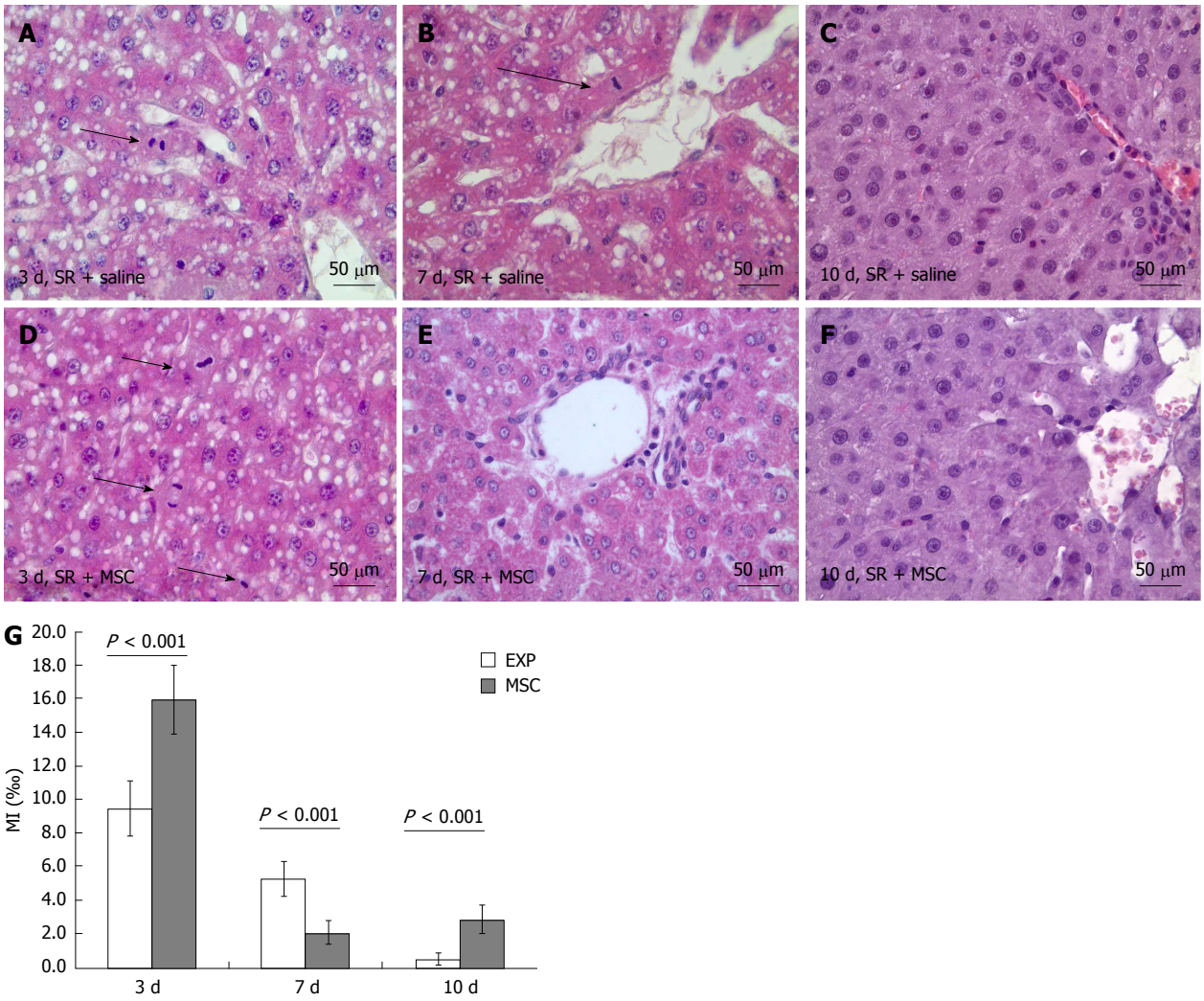

Figure 1 Influence of the multipotent stromal cells transplantation on mitotic activity of hepatocytes; Mitotic activity of hepatocytes in the residual livers at 3 (A, D), 7 (B, E) and 10 d (C, F) after the surgery; Mitotic index of hepatocytes is plotted against time after the surgery (G); HE staining. Arrowheads indicate mitotic figures in hepatocytes (A, B, D); Mitotic index of hepatocytes: Horizontal axis represents time elapsed after the surgery, vertical axis represents mitotic index of hepatocytes, ‰; White columns represent hepatocyte proliferation in the comparison group, gray columns represent hepatocyte proliferation in the multipotent stromal cells transplantation group; The data is presented as mean values with confidence intervals. SR: Subtotal resection; MSC: Multipotent stromal cells.

to the MSC transplantation (Figure 5).

DISCUSSION

In this study we observed a stimulating effect of the umbilical cord-derived MSCs on liver regeneration after subtotal resection in rats, manifested as increased survival of the operated animals, increased rates of liver mass recovery, and increased proliferation activity of hepatocytes.

The obtained evidence of the effect of MSCs on hepatocyte proliferation is consistent with the results of other studies[4]. It should be noted that the stimulating effect MSCs on hepatocyte proliferation is, apparently, due to accelerated passage of the cell cycle phases. This is indicated by the fact that on day 3 after the subtotal liver resection combined with MSC administration (mimicked by sham injection of saline in the comparison group), the Ki67 index did not differ between the groups indicating similar extent of hepatocyte engagement in cell cycle for both groups. At the same time, the proportion of hepatocytes undergoing mitosis per second was significantly higher in animals which received the MSCs, that is, their hepatocytes passed G1, S, and G2 phases more rapidly, to be able to divide by day 3 after the surgery. Such acceleration of cell cycles apparently led to their synchronization resulting in waves of hepatocyte proliferation with a temporary decrease in the mitotic activity of in between[12]. It is possible, that the increased rates of cell cycling in the early postoperative period were the cause of significantly better survival of animals in the MSC transplantation group.

Despite the clearly demonstrated regeneration-stimulating effect of the umbilical cord-derived MSC transplantation, exact mechanisms of this stimulation remained obscure.

However, the obtained data indicate that the replacement mechanism of stimulation of regeneration in this case is not the leading one.

The transplanted MSCs did not differentiate into

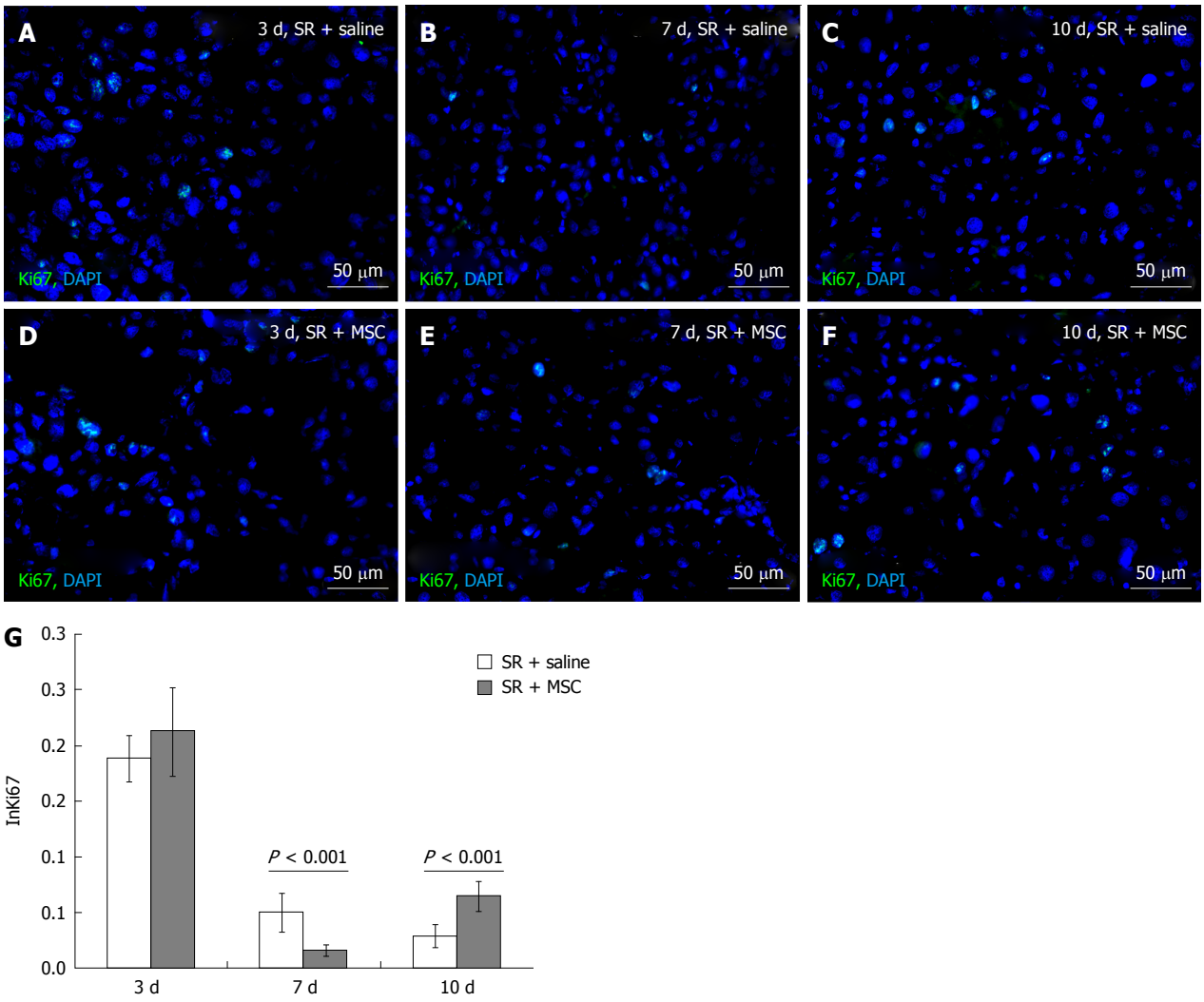

Figure 2 Influence of the multipotent stromal cells transplantation on Ki67 index of hepatocytes. Ki67 expression in the residual livers at 3 (A, D), 7 (B, E) and 10 d (C, F) after the surgery; cell nuclei are counterstained DAPI (blue); Index of Ki67+ hepatocytes is plotted against time after the surgery (G): Horizontal axis represents time elapsed after the surgery, vertical axis represents Ki67 proliferation index (lnKi67) of hepatocytes; White columns represent hepatocyte proliferation in the comparison group, gray columns represent hepatocyte proliferation in the multipotent stromal cells transplantation group; The data is presented as mean values with confidence intervals. SR: Subtotal resection; MSC: Multipotent stromal cells.

hepatocytes, cholangiocytes, smooth muscle cells, active fibroblasts, or myofibroblasts. Solitary PKH26-labeled MSCs at the site of transplantation were positive for the endothelial cell marker CD31. It is known that endothelial differentiation of MSCs is stimulated by VEGF and supported the endothelium-derived extracellular matrix; in combination they make MSCs to express the endothelium-specific markers[13]. It is also known, that the umbilical cord-derived MSCs give a stronger response to endothelial induction than the bone marrow-derived MSCs[14]. Despite that, only a minor fraction of transplanted cells could possibly differentiate into endothelial cells upon transplantation. This is consistent with our previously reported data on rapid elimination of transplanted MSCs by host macrophages. In particular, about 90% of transplanted cells in spleen and regenerating liver were destroyed by association with CD68+ cells[15].

We found no direct evidence for the paracrine effect of MSCs on liver regeneration after the subtotal liver resection in rats. Although MSCs show the ability to synthesize a rich set of biologically active molecules[2], the paracrine effect provided by MSCs is dose-dependent and typically short-term, and apparently difficult to study *in vivo*. Besides, several other studies demonstrating the positive effect of MSCs on liver regeneration show that repeated administration of MSCs, as well as an "in advance" transplantation of the cells before damage, gives a stronger effect, which may have a stronger paracrine component[16,17].

However, the paracrine mechanism of the therapeutic activity of transplanted MSC is indirectly indicated by a decrease in the total number of CD68 + macrophages and an increase in the proportion of M2 pro-repair macrophages in the regenerating liver as compared to animals in which the transplantation was only mimicked. It is known that the effect of MSC on inflammation is mediated by a local increase in IL-4, IL-13, TSG-6 and a decrease in TNFα, IL-6[5] influencing the immune system cells, especially macrophages[6,7]. It has been shown

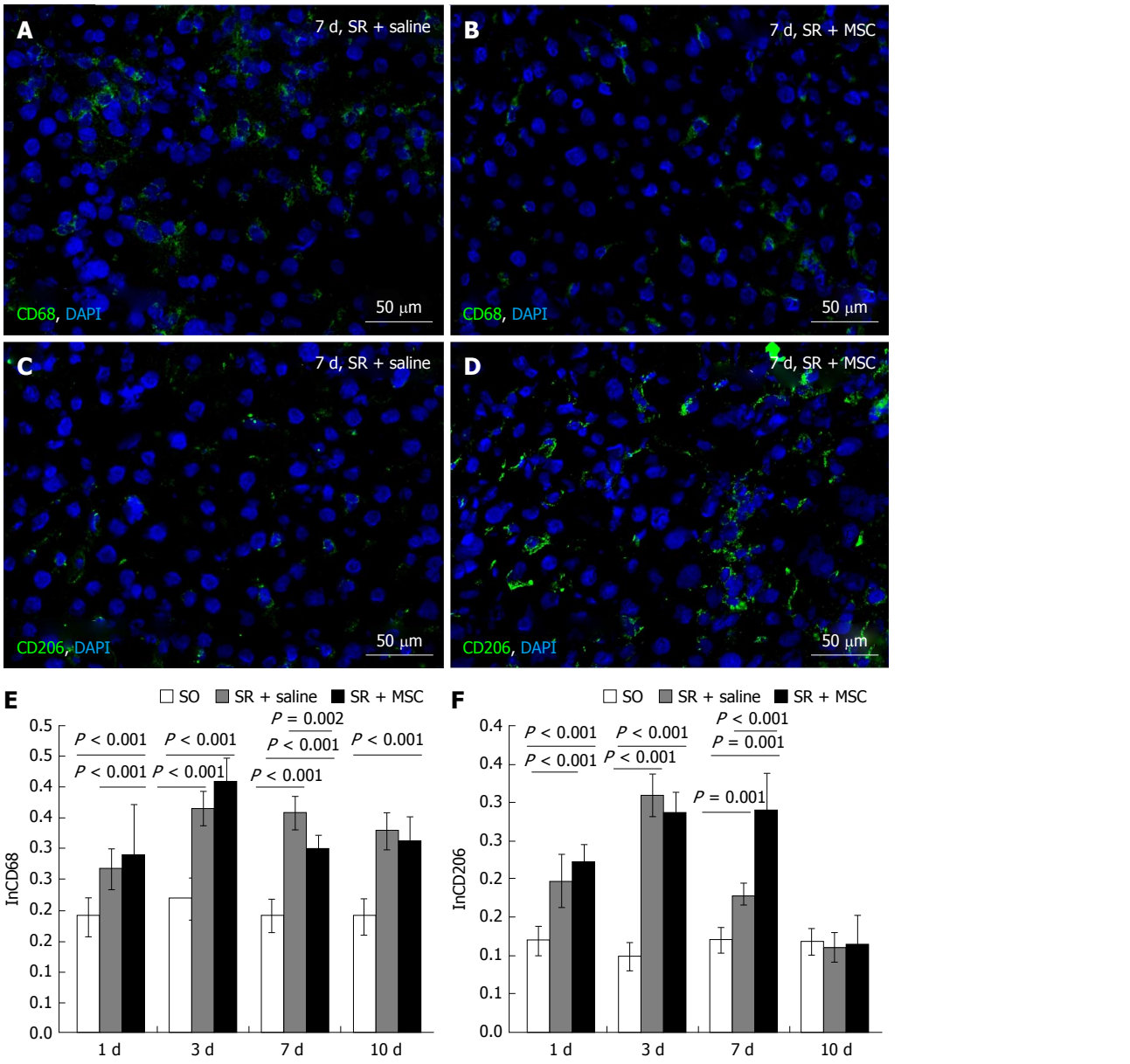

Figure 3 Influence of the transplantation on macrophage polarization profiles. CD68+ cells (green) in the liver in the comparison group (A) and in the residual liver in the MSC transplantation group (B) on day 7 after the surgery. The diagram shows dynamic changes in the CD68+ cell content liver in the comparison group and in the multipotent stromal cells (MSC) transplantation group (E); Relative quantities of CD206+ cells (green) in the liver in the comparison group (C) and in the residual liver in the MSC transplantation group (D) on day 7 after the surgery; The diagram shows dynamic changes in the CD206+ cell content in the course of regeneration; cell nuclei are counterstained with 4',6-Diamidino-2-phenylindole (blue). In E and F, white columns represent the sham-operated animals, gray columns represent the comparison group, black columns represent the MSC transplantation group, horizontal axis represents time elapsed after the surgery. SR: Subtotal resection; SO: Sham-operated animals; MSC: Multipotent stromal cells.

in many studies on various models that after MSC transplantation the number of activated macrophages of the alternative M2 phenotype (which have pro-repair and anti-inflammatory properties) increases, and the number of pro-inflammatory M1 macrophages decreases[18,19]. This is consistent with the data obtained by us on the model of liver regeneration after its subtotal resection in rats.

Exact molecular mechanisms of the paracrine effect of MSC transplantation on hepatocyte proliferation and liver macrophage behavior in the aftermath of subtotal liver resection remain unclear. Further studies in this direction are very desirable, since any possibility of controlling hepatocyte proliferation and/or liver macrophage polarization could be of great therapeutic importance, especially in severe liver injuries.

ARTICLE HIGHLIGHTS

Research background

The resulting post-hepatectomy liver failure is a major manifestation of the small-for-size liver remnant syndrome, when the critically reduced liver mass is insufficient to maintain normal liver function. The problem can be solved by some specific controllable stimulation of compensatory growth combined with intensive liver care and management for acute hepatic failure. Multipotent stromal cells may therefore represent a reasonable choice not only in cases of extensive hepatectomy but also for-other types of severe liver damage (*e.g.*

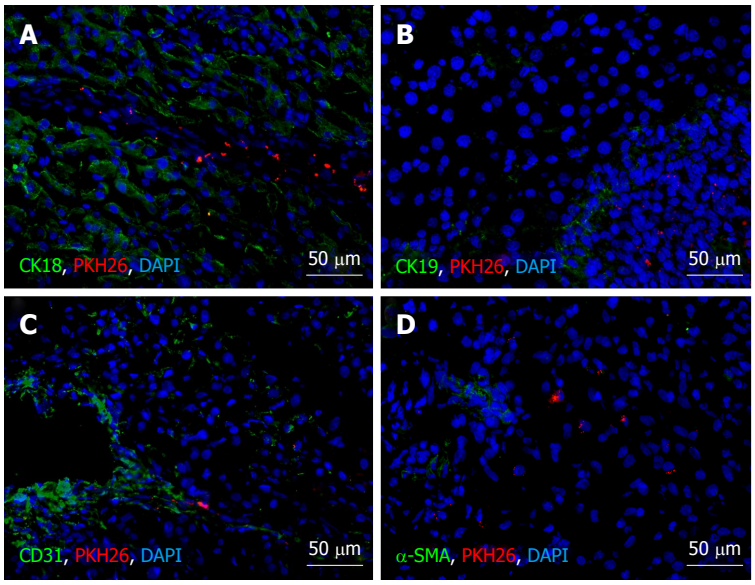

Figure 4 Immunochemical analysis of differentiation of the transplanted multipotent stromal cells on day 10 after the surgery. Immunostaining of cell type-specific proteins, A: CK18 (hepatocytes); B: CK19 (cholangiocytes); C: CD31 (endotheliocytes); D: αSMA (smooth muscle cells). Fluorescent microscopy images display immunostaining (green), PKH26 label (red), and cell nuclei counterstained with 4', 6-Diamidino-2-phenylindole (blue).

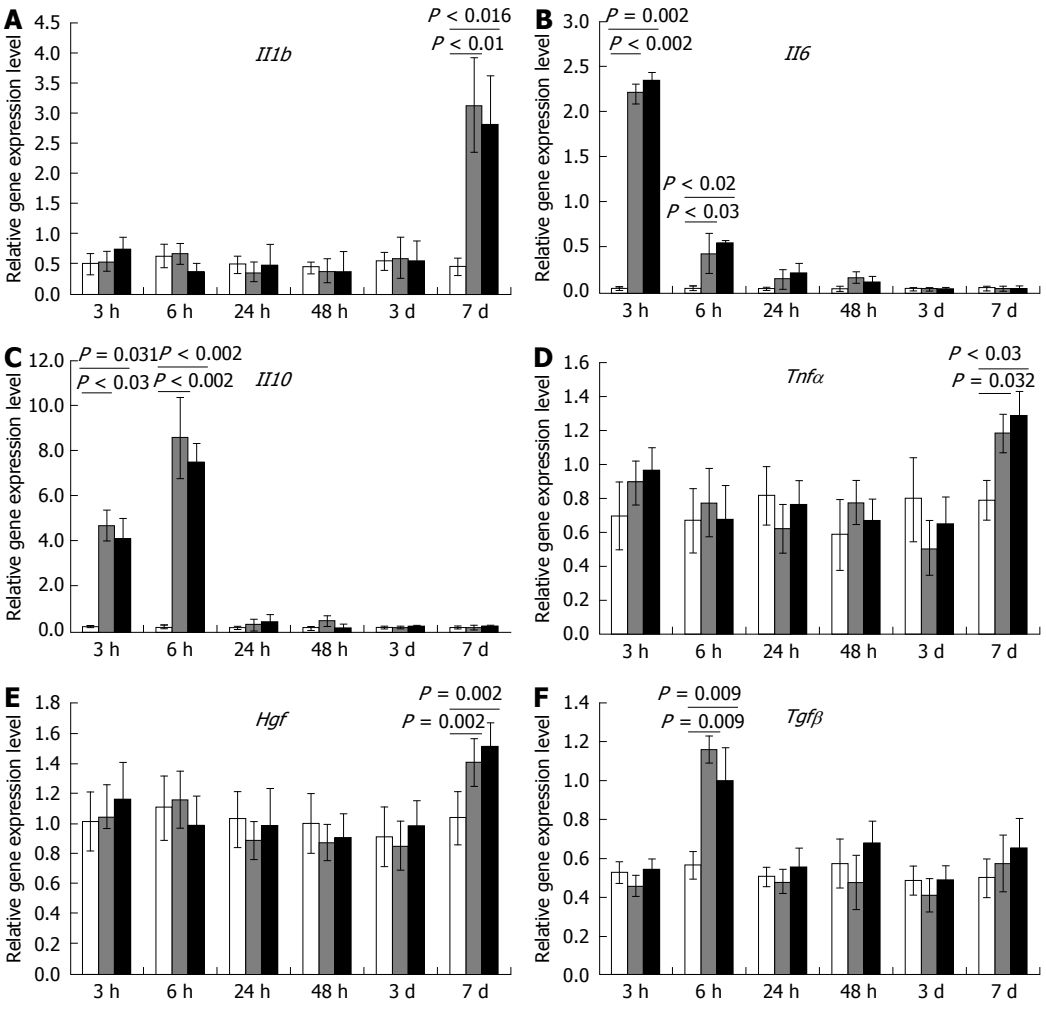

Figure 5 Influence of the transplantation on cytokine and growth factor gene expression within regenerating livers. A: *Il1b*; B: *Il6*; C: *Il10*; D: *Tnfα*; E: *Hgf*; F: *Tgfβ*. White columns correspond to gene expression values (in relative units) for the sham-operated animals, gray columns represent the values for the comparison group, black columns represent the values for the Multipotent stromal cells transplantation group, horizontal axis represents time elapsed after the surgery; The data is presented as mean values with confidence intervals. SR: Subtotal resection; SO: Sham-operated animals; MSC: Multipotent stromal cells.

cirrhosis). Multipotent stromal cells are found to be a helpful supplement in various repair processes in mammals, particularly in solid organs. These cells are considered as a promising tool of regenerative medicine. Their ability to stimulate reparative regeneration of damaged liver has been confirmed, but the mechanisms remain uncertain.

Research motivation

The experimentally proven enhancement of liver regeneration by multipotent stromal cells (MSCs) must be essentially paracrine, because their transplantation to residual livers causes an increase in concentrations of HGF and several other growth factors within the regenerating tissues. Therapeutic activity of MSCs is apparently related to their anti-inflammatory properties, which also represent a sort of paracrine regulation, as manifested by a local increase in IL-4, IL-13, and TSG-6 production paralleled by relative shortage of TNFα and IL-6. Modulation of inflammatory reactions may be implemented *via* influence of these, or similar, paracrine factors on immune cells, especially on macrophages. Several studies, however, confirm the ability of MSCs to differentiate into hepatocytes, which leads to quite a different explanation for the positive influence of MSCs on liver repair.

Research objectives

In our experiments we found that intrasplenic allogeneic transplantation of the umbilical cord-derived multipotent stromal cells stimulated hepatocyte proliferation and organ mass recovery after subtotal resection. These effects may result from positive paracrine influence of the transplanted cells on polarization of the liver resident macrophages to M2 phenotype.

Research methods

The MSCs were obtained from the intervascular tissue of umbilical cords, dissected from rat fetuses, by the explant culture technique. The vital labeling of MSCs with PKH26 was carried out on the 3rd passage. The subtotal resection was performed on male Sprague-Dawley rats. The experimental group animals received a transplant 106 MSCs infused into the spleen. Hepatocyte proliferation was assessed by counting of either mitotic figures or Ki67-positive cells in microscopic images. MSC differentiation was assessed with antibodies to hepatocyte-specific marker cytokeratin 18 (CK18), cholangiocyte-specific protein CK19, smooth muscle cell-specific protein α-SMA, the endothelial cell marker CD31, or the active fibroblast marker FAPα. Total macrophages of the liver were selectively stained in cryosections incubated with anti-CD68 antibodies (1:100, Abcam), while the M2a and M2c macrophage populations were selectively stained with anti-CD206 antibodies. Expression of interleukin and growth factor genes was evaluated with PCR-RT.

Research results

Intrasplenic allogeneic transplantation of the umbilical cord-derived multipotent stromal cells stimulates reparative processes within the residual liver tissue after subtotal resection (removal of 80% of the organ mass), as indicated by increased rates of hepatocyte proliferation and accelerated organ mass recovery. These effects may result from paracrine influence of the transplanted cells on the resident macrophage population of the liver. The transplantation favors polarization of macrophages to M2 phenotype (the M2-polarized macrophages specifically express CD206; they are known to suppress inflammation and support tissue repair). No differentiation of the transplanted cells into any of the liver cell types have been observed in the study.

Research conclusions

In this study we observed a stimulating effect of the umbilical cord-derived MSCs on liver regeneration after subtotal resection in rats, manifested as increased survival of the operated animals, increased rates of liver mass recovery, and increased proliferation activity of hepatocytes. We found no direct evidence for the paracrine effect of MSCs on liver regeneration after the subtotal liver resection in rats. However, the paracrine mechanism of the therapeutic activity of transplanted MSC is indirectly indicated by a decrease in the total number of CD68 + macrophages and an increase in the proportion of M2 pro-repair macrophages in the regenerating liver as compared to animals in which the transplantation was only mimicked.

Research perspectives

Exact molecular mechanisms of the paracrine effect of MSC transplantation on hepatocyte proliferation and liver macrophage behavior in the aftermath of subtotal liver resection remain unclear. Further studies in this direction are very desirable, since any possibility of controlling hepatocyte proliferation and/or liver macrophage polarization could be of great therapeutic importance, especially in severe liver injuries.

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Basic Study

Morphological and biochemical effects of weekend alcohol consumption in rats: Role of concentration and gender

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Abstract

AIM

To examine the association between weekend alcohol consumption and the biochemical and histological alterations at two different concentrations of alcohol in both genders in rats.

METHODS

Wistar rats weighing 170-200 g were divided into groups as follows: (1) Control groups; and (2) weekend alcohol-consumption group: 2 d/weekly per 12 wk, at two different concentrations: (1) Group of males or females with a consumption of a solution of alcohol at 40%; and (2) group of males or females with a consumption of a solution of alcohol at 5%. At the end of the experiment, serum and liver samples were obtained. The following enzymes and metabolites were determined in serum: Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Lactate Dehydrogenase, and Gamma-Glutamyltransferase, and glucose, triglycerides, cholesterol, bilirubin, and albumin. Liver samples from each group were employed to analyze morphological abnormalities by light microscopy.

RESULTS

In all of the weekend alcohol-consumption groups, AST activity presented a significant, 10-fold rise. Regarding ALT activity, the groups with weekend alcohol consumption presented a significant increase that was six times greater. Bilirubin levels increased significantly in both groups of females. We observed a significant increase in the parameters of fatty change and inflammation due to weekend alcohol consumption. Only the group of females that consumed alcohol at 40% presented slight hepatocellular disorganization

CONCLUSION

The results obtained herein provide solid evidence that weekend alcohol consumption gives rise to liver damage, demonstrated by biochemical and histological alterations, first manifested acutely, and prolonged weekend alcohol consumption can cause greater, irreversible damage.

Key words: Weekend alcohol consumption; Liver morphology; Transaminases; Damage

Core tip: At present, it is considered that the main weekend alcohol consumers comprises the young population, due to the gratifying effect of alcohol, this being a very important social and health problem. Our findings demonstrate an effect of the damage that is caused by weekend alcohol consumption, regardless of gender or the concentration of alcohol. Even more so, greater damage can be observed in females, and the metabolism of ethanol probably participates, specifically due to its first-pass metabolism, which is carried out in the stomach.

Morales-González JA, Sernas-Morales ML, Morales-González Á, González-López LL, Madrigal-Santillán EO, Vargas-Mendoza N, Fregoso-Aguilar TA, Anguiano-Robledo L, Madrigal-Bujaidar E, Álvarez-González I, Chamorro-Cevallos G. Morphological and biochemical effects of weekend alcohol consumption in rats: Role of concentration and gender. *World J Hepatol* 2018; 10(2): 297-307 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/297.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.297

INTRODUCTION

Various reports have demonstrated the adverse effects to health caused by the consumption of alcohol[1-3]. Similarly, it is well known that in Mexico, the main alcoholic beverages consumed are beer and distilled beverages (brandy, tequila, rum, whisky, cognac, vodka, *etc*.)[3], which contain approximately 5% and 36%-40% of alcohol, respectively[1]. It has been reported that alcohol consumption presents various patterns that are associated with gender, age, socioeconomic situation, consumption type (regular drinkers, intense or weekend drinkers), and, the alcoholic-beverage type (wine, mixed drinks)[4]. On the other hand, according to what has been reported in ENCODAT 2016, young people exhibit the tendency toward alcohol consumption in total population in Mexico (12-65 years of age), with daily alcohol consumption at 2.9% and habitual consumption (weekend) at 8.5%. Likewise, it was found that, although males consume more alcohol, women present an important index of alcohol consumption[3].

Weekend alcohol consumption for young people is becoming an important social and familial problem, but also a considerable health problem[5], and it can be due to the increase of Allopregnanolone (the testosterone metabolite that participates in the gratifying effect of alcohol)[6]. Various reports have associated the harmful effect to health engendered by weekend alcohol consumption, such as the following: reports on and the increase in deaths during Fridays,

Saturdays, and Sundays[7]; the greater neurocognitive and neurobehavioral deterioration, which is similar in many aspects to that observed in chronic alcohol drinkers[5]; deaths caused by ischemic heart disease[8,9], or the idiopathic arrhythmias that initiate during weekends, a risk factor for producing visual alterations (dyschromatopsia)[10].

On the other hand, some biochemical alterations have been reported as being caused by weekend alcohol consumption. Stranges *et al*[11] reported that there are modifications at the level of the enzymes released by the liver into the blood Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), and Gamma-Glutamyltransferase (GGT), and these authors concluded that these modifications were caused by alcohol consumption, finding that the highest elevated enzyme is GGT, identifying differences by gender: In males, daily alcohol drinkers exhibited highest GGT levels, while in females, highest GGT was observed in weekend alcohol drinkers. The authors concluded that, in addition to the amount of alcohol consumed, the pattern of consumption can affect liver function and that there are gender differences with respect to liver function and possible damage to this organ[11]. Hojnacki *et al*[12] compared the effect of the daily alcohol-consumption pattern (moderate, 12%) and weekend alcohol consumption (concentrated, 20%) utilizing squirrel monkeys. These authors found that moderate-daily alcohol consumption causes a moderate diminution in Body Weight (BW) and produces increases in the profile of coronary protective lipoproteins [HDL2/HDL3 cholesterol increases and low density lipoprotein (LDL) cholesterol decreases]; in contrast, in concentrated alcohol consumption, unfavorable alterations are produced in the lipoproteins (LDL cholesterol increases and Apolipoprotein B increases), together with weight loss and body fat depletion[12]. Rocha *et al*[13], on employing a weekend alcohol-consumption model in rat (alcohol in the drinking fountain at 30% during 3 d/wk per 70 d), reported an average alcohol consumption of 4.56 g per day, which gave rise to a dyslipedemic profile, an increase in energy expenditure, and hepatic metabolic changes similar to those associated with chronic ethanol consumption[13]. Finally, Liu *et al*[14] compared the effect of the damage that two alcohol-consumption patterns cause: Moderate-daily consumption 0.8 g/kg/7 d/wk *vs* weekend alcohol consumption 2.8 g/kg/2 d/wk, with a weekly consumption in both groups of 5.6 g/kg. The authors found an increase in the levels of total cholesterol and of HDL-C in both groups; on the other hand, a diminution was observed in the LDL-C levels of the moderate-daily consumption pattern, the latter significantly raised in weekend drinkers. Blood concentrations of alcohol as well as BW gain were greater in weekend alcohol consumers. These findings of weekend alcohol consumption (4 wk) favored the development of atherosclerotic plaque (increase in the plaque, diminution in the lux lumen, *etc.*), while precisely the contrary took place with moderate-daily alcohol consumption[14]. Some reports describe the histologic damage that alcohol causes to the liver[15-17] but, to our knowledge, there are no reports on the histologic changes caused by weekend alcohol consumption to the liver.

There are numerous studies on the effects of chronic weekend alcohol consumption; however, few reports exist, to our knowledge, on the association between weekend alcohol consumption and the damage produced in the liver. The objective of this study was to examine the association between weekend alcohol consumption and the biochemical and histological alterations at two different concentrations of alcohol in both genders in Wistar rats.

MATERIALS AND METHODS

Reagents

All chemical reagents were obtained from Merck (Merck de México, S.A.) and were of the best quality available.

Animals

We utilized male Wistar rats with an initial Body Weight (BW) of 170-200 g that were obtained from the Escuela Superior de Medicina (ESM) Bioterium of the Instituto Politécnico Nacional (IPN) in Mexico City. The rats were housed in cages at the ESM Bioterium. They were maintained at a temperature of 22 ℃ with 12-h/12-h light-dark cycles and received standard rat-pellet food (Purina de México, S.A.) and water *ad libitum* prior to the treatments. After 14 d of adaptation, the procedure was initiated. The protocol and the experimental procedures were conducted according to the Mexican Official Norm for the Use and Care of Laboratory Animals (NOM-062-ZOO-1999, México)[18]. The protocol was authorized by the Comité Interno del Cuidado y Uso de los Animales de Laboratorio (CICUAL), with registry number ESM.CICUAL-12/23-06-2017, and by the Research Committee with registry number ESM. CI-01/13-06-2017, both committees of the ESM of the IPN.

Experimental design

The animals were randomly divided into six groups in the following manner: (1) Control groups (males and females); and (2) weekend alcohol-consumption group: 2 d/weekly per 12 wk at two different concentrations: (1) Group of males with consumption of a solution of alcohol at 40%; (2) group of females with consumption of a solution of alcohol at 40%; (3) group of males with a consumption of a solution of alcohol at 5%; and (4) group of females with a consumption of a solution of alcohol at 5%. The control groups (females and males) had access to food and water *ad libitum* at all times. Daily alcohol consumption was quantified, reported in g/kg of weight/day, and BW gain weekly was reported in g.

Serum samples

At the end of the experiment, the animals were sacrificed by decapitation after being previously

anesthetized with pentobarbital sodium (at an overdose of 40 mg/kg BW). Blood samples were obtained and centrifuged in a clinical centrifuge to obtain the sera, which were frozen at -70 ℃ for later use.

Liver histology

Hepatic samples from each group Sere employed for light microscopy. Samples were fixed with formaldehyde (10% in isotonic solution), embedded in paraffin, and stained with Hematoxylin and Eosin. Biopsy specimens were coded and read blindly without knowledge of the other data by independent observers at two different laboratories (MLS-M and JB-R). The criteria utilized to analyze the morphological abnormalities were the same as those reported by Morales-González *et al*[15] as follows: Fatty infiltration (+, mild; ++, moderate; +++, severe, and ++++, very severe); inflammation (+, zonal localization, focal inflammatory cells; ++, moderate, not restricted to any one zone of the acinus, and +++, diffuse), and hepatocellular disorganization (+, isolated foci in zone 3 of the liver acinus; ++, more widespread, and +++, definitively diffused in the hepatic acini); apoptosis (+, mild; ++ moderate; +++ severe, and ++++ very severe), and mitosis(+ mild; ++ moderate; +++ severe, and ++++ very severe).

Determination of enzymes and metabolites in serum

The activities of serum Alanine Aminotransferase [ALT; Expansion Coefficient (EC) 2.6.1.2], Aspartate Aminotransferase (AST, EC 2.6.1.1), Lactate Dehydrogenase (LDH, EC 1.1.1.27), and Gamma-Glutamyltransferase (GGT, EC 2.3.2.2) were measured colorimetrically utilizing diagnostic kits (Spinreact de México, SA de CV), following the manufacturer's instructions; the results are reported in units/L.

Serum concentrations of glucose, triglycerides, cholesterol, bilirubin, and albumin were determined by spectrophotometric techniques using diagnostic kits (Spinreact de México, SA de CV) following the instructions provided by the manufacturer; the results are reported in mg/dL, except for albumin, which is reported in g/dL.

Statistical analysis

The results were analyzed using the SigmaPlot ver. 12.3 statistical software program. The results are expressed as the mean ± SE of the mean (SEM), as required. We carried out a statistical analysis using Student *t* test and/or Analysis of Variance (ANOVA) and the Student-Newman-Keuls method as *post-hoc* evaluation for multiple comparisons. We considered differences among the groups to be statistically significant when $P < 0.05$.

RESULTS

Effect of weekend alcohol consumption on weight gain

Average alcohol consumption per group was as follows: In females, at 5% of 0.83 g/kg per day; in the male group at 5% of 1.63 g/kg per day, and, in groups of females and males, at 40%, this was 5.52 and 2.26 g/kg per day, respectively (Table 1).

All of the weekend alcohol-consumption groups presented a significant weight gain in comparison with the control group. The group of females as well as in that of males with 40% alcohol consumption presented an increase in weight gain similar to that of 121.3 and 127.8 g, respectively. Surprisingly, the group of males at 5% exhibited a very important weight gain of 214 g; however, this was not so in the group of females with 5% consumption (98 g) (Table 1).

Activity of ALT, AST, LDH and GGT in serum after weekend alcohol consumption

The effect of weekend alcohol consumption was evaluated by determining the activity of ALT, AST, LDH, and GGT, because these enzymes classically reflect liver function, which is dependent on morphofunctional integrity.

In Figure 1, we observe the AST activity (upper panel) in the diverse experimental groups. In all of the weekend alcohol-consumption groups, AST activity presented a significant rise of 10 times in comparison with the control (23.8 U/L). Regarding ALT activity, the control presented ALT activity of 36.5 U/L, and the remaining groups with weekend alcohol consumption presented a significant increase that was 6-fold greater, independently of the alcohol concentration (Figure 1, lower panel).

The activity of the LDH enzymes (upper panel) and of the GGT enzymes (lower panel) can be observed in Figure 2. LDH activity in the control group was 147 U/L, noting that weekend alcohol consumption favored an increase in all groups of between 18 and 20 times greater in comparison with the control group, not finding differences among these in terms of the weekend alcohol-consumption groups with regard to of the activity of this (LDH) enzyme. On the other hand, with respect to the activity of the GGT enzyme, differences were not found in any weekend alcohol-consumption group in comparison with the control (22.6 U/L), or among the alcohol-treated groups.

Effects of weekend alcohol consumption on serum concentrations of glucose, triglycerides, and cholesterol

In the weekend alcohol-consumption groups (Table 2), we quantified serum metabolite modifications, which depend on the hepatic metabolism, such as glucose, cholesterol, and triglycerides. Regarding glucose levels, it may be observed in Table 2 that weekend alcohol consumption in all groups favored the increase of the serum levels of this metabolite significantly with a $P < 0.05$ *vs* control. The serum concentration of cholesterol in the control group was 58 mg/dL, while in the groups with weekend alcohol consumption, the following was found: in the group of females at 5% (71.83 mg/dL); males at 5% (70.67 mg/dL), and in females at 40% (68.33 mg/dL), this was statistically significant ($P < 0.05$) in all alcohol-consumption groups *vs* control. The

Table 1 General characteristics of rats: weight gain and alcohol consumption

Group	Initial body weight (g)	Final body weight (g)	Body weight gain (g) (%)	Average alcohol consumption (g/kg/d)
Control	176.50 ± 3.3	255.00 ± 23	78.5 ± 22 (43)	0
Females 5%	171.00 ± 10.57	269.00 ± 15.16	98.0 ± 20.71[a] (57)	0.83 ± 0.04
Females 40%	172.92 ± 4.61	294.25 ± 13.39	121.3 ± 13.91[a] (70)	5.52 ± 1.85
Males 5%	198.00 ± 3.21	412.00 ± 8.74	214.0 ± 11.53[a] (108)	1.63 ± 0.01
Males 40%	172.80 ± 9.17	300.00 ± 85.43	127.8 ± 41.29[a] (73)	2.26 ± 0.61

Values are expressed as the mean ± SE in each experimental group (n = 3-6). [a]P < 0.05 *vs* control group.

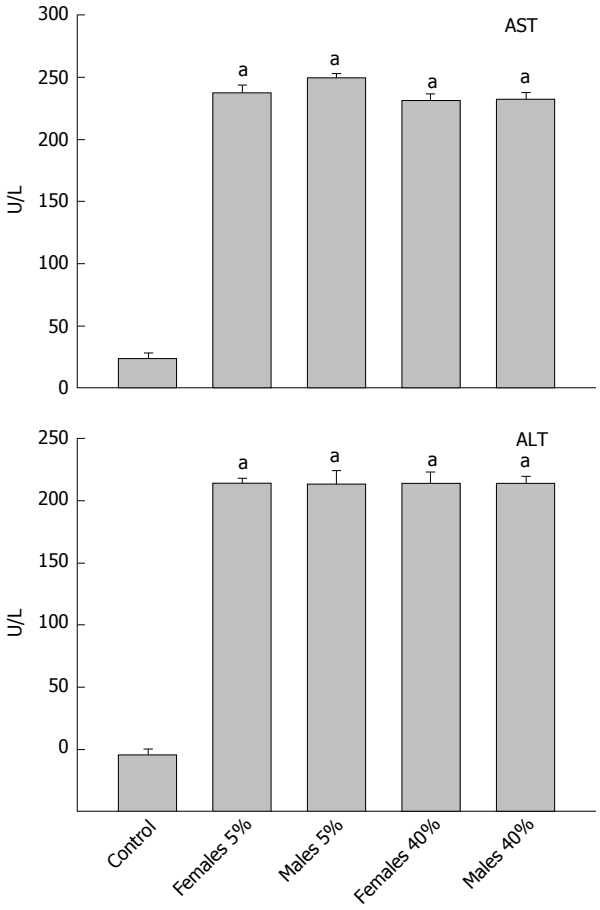

Figure 1 Activities of serum Aspartate and Alanine Aminotransferases after weekend alcohol consumption. Values are expressed as the mean ± SEM in each experimental group (n = 3-6). [a]P < 0.05 *vs* the control group. AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase.

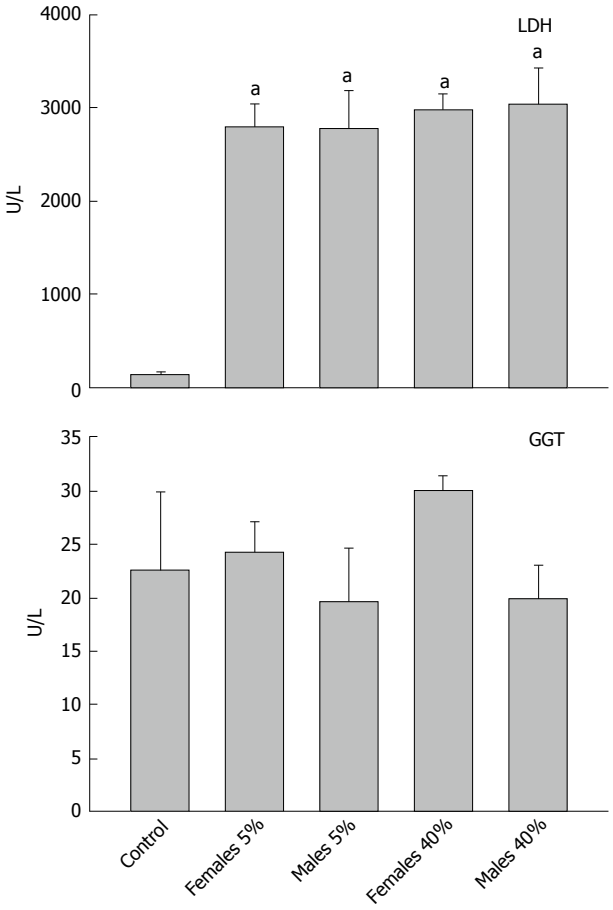

Figure 2 Activities of serum Lactate Dehydrogenase and Gamma-Glutamyltransferase after weekend alcohol consumption. Values are expressed as the mean ± SEM in each experimental group (n = 3-6). [a]P < 0.05 *vs* the control group. LDH: Lactate Dehydrogenase; GGT: Gamma-Glutamyltransferase.

serum levels of triglycerides were raised due to alcohol consumption (Table 2).

Effects of treatment with weekend alcohol consumption on serum concentrations of albumin and bilirubin

The results of the metabolic integrity of the liver are presented in Table 3. Bilirubin levels increased significantly in both groups of females (0.26 mg/dL), in comparison with the control group (0.15 mg/dL; P < 0.05). Regarding the groups of males with weekend alcohol consumption at 5% and 40%, their bilirubin levels were found to be 0.15 and 0.18 mg/dL, respectively, the latter not significant in comparison with the control group (0.15 mg/dL). Surprisingly, the levels of albumin in serum were not affected in any weekend alcohol-consumption group in comparison with the control group (Table 3).

Effect of weekend alcohol consumption on histological indicators (fatty change, inflammation, hepatocellular disorganization, necrosis, and apoptosis)

In Table 4, we can observe the effect exerted by weekend alcohol consumption on histological changes. We noted a significant increase in the parameters of fatty change and inflammation due to weekend alcohol consumption compared with the control group (Table 4). In Figure 3, we are able to observe representative images of the female group with alcohol consumption

Table 2 Effects weekend alcohol consumption on serum glucose, cholesterol, and triacylglycerols

Group	Glucose (mg/dL)	Cholesterol (mg/dL)	Triacylglycerols (mg/dL)
Control	85 ± 3.89	58 ± 1.5	90 ± 2.4
Females 5%	157.33 ± 6.88[a]	71.83 ± 2.79[a]	104.5 ± 8.70
Males 5%	166.33 ± 9.13[a]	70.67 ± 4.37[a]	111.33 ± 5.69[a]
Females 40%	145.5 ± 11.04[a]	68.33 ± 2.455[a]	104.167 ± 3.5[a]
Males 40%	153 ± 7.09[a]	66 ± 2.98	105.8 ± 4.92[a]

Metabolites are expressed as mean ± SE (n = 3-6). [a]$P < 0.05$ *vs* control.

Table 3 Effects weekend alcohol consumption on serum albumin and bilirubin

Group	Bilirubin (mg/dL)	Albumin (g/dL)
Control	0.15 ± 0.01	2.70 ± 0.11
Females 5%	0.26 ± 0.05[a]	2.87 ± 0.09
Males 5%	0.15 ± 0.02	2.90 ± 0.20
Females 40%	0.26 ± 0.03[a]	2.82 ± 0.08
Males 40%	0.18 ± 0.02	2.80 ± 0.21

Metabolites are expressed as mean ± SE (n = 3-6). [a]$P < 0.05$ *vs* control.

at 5%, where inflammation and steatosis are observed, as well as slight periportal fibrosis. In Figure 4, we may observe slight inflammation, steatosis, and slight periportal fibrosis in the group of males with alcohol consumption at 5%, while the groups of males and females with alcohol consumption at 40% were those with greatest histological changes in terms of the parameters of fatty change and inflammation, respectively (Table 4). Only the group of females that consumed alcohol at 40% presented slight hepatocellular disorganization (Table 4). Figure 5 presents the images of the group of females with alcohol consumption of 40%, where steatosis is observed, and inflammation, although this was more marked in comparison with the group of females with alcohol consumption at 5%. The latter can be noted, because it surpasses the limiting plaque and the periportal fibrosis is more evident (which can be observed as the loss of the amount of the periportal cell, replaced by fibrotic tissue). In Figure 6, moderate inflammation with leukocytes is observed that surpasses the limiting plaque, and also, the degree of periportal fibrosis is very important; likewise, apoptosis was only present in the group of males with alcohol consumption at 40% (Table 4). Last, the necrosis parameter is present in all of the groups that consumed alcohol on weekends, this being greater in the groups that consumed alcohol on weekends at 40% (females and males) (Table 4).

DISCUSSION

It is known that chronic alcohol consumption is a risk factor for various diseases, such as diabetes, cancer, gastritis, and gastric ulcers, and that it is especially related to liver damage[1]. In recent years, attention has been focused on weekend alcohol consumption as a cause of cognitive-intellectual alterations[5], a risk factor for homicides[7], cardiovascular diseases such as arrythmias or infarct[8,9], and even of visual alterations[10]. At present, it is considered that main weekend alcohol consumers comprise the young population, due to the gratifying effect of alcohol[6], this being a very important social and health problem.

Rocha *et al*[13], on utilizing the weekend alcohol consumption model for 10 wk in male rats and an alcohol concentration in the drinking fountain of 30%, reported an average alcohol consumption of 4.56 g/d, and that alcohol favors the increase of BW by approximately 30%, diminution in glucose levels, the rise of triglycerides and of ALT, and normal levels of total protein and cholesterol. In our study, alcohol consumption at a concentration similar to that reported by Rocha was 40%, finding similarity in average daily consumption of alcohol, in addition to an increase in BW in females (70%) as well as in males (73%) in comparison with the control (43%) (Table 1), the latter analogous to that reported previously by Rocha. In the same manner, we, like Rocha, found neither changes in albumin levels nor in those of total proteins, respectively (Table 3), which is probably due to that the damage is not yet chronic and severe. The weight gained is probably due to the increase in lipids, which may be observed in their elevation in blood (Table 2), as well as in hepatic tissue (Table 4) (Figures 3-6), caused by weekend alcohol consumption. Other reports demonstrate that weekend alcohol consumption gives rise to alterations in BW that are greater than those compared with moderate-daily alcohol consumption, and that this moderate consumption produces an increase in the protective lipoprotein profile. In contrast, concentrated alcohol consumption produces unfavorable alterations in the lipoproteins[12,14].

Surprisingly, alcohol consumption at 5% caused the greatest BW increase (108%) (Table 1). This can be explained by the metabolism of ethanol, in that, at different concentrations, the metabolism of the first pass of ethanol is modified, which is predominantly gastric[19] and is supplied mainly by the activity of the gastric ADH[20]. Previously, Roine *et al*[21] demonstrated that consumption of a concentrated solution of alcohol (40%) results in low levels of alcohol in blood in comparison with a diluted solution (4%), and that this effect is associated with first-pass metabolism and with lesser bioavailability with high concentrations of ethanol. Dohmen *et al*[22] demonstrated that, on administering a

Table 4 Histopathological changes induced by the consumption of weekend ethanol at two concentrations

Group	Fatty change	Inflammation	Hepatocellular disorganization	Necrosis	Apoptosis
Control	0	0	0	0	0
Females 5%	+/++	+/++	0	0/+	0
Males 5%	+/++	0/++	0	0/+	0
Females 40%	+/+++	+	0/+	0/++	0
Males 40%	+/++	+/+++	0	0/++	0/++

Histopathological parameters were evaluated as described in the Material and Methods.

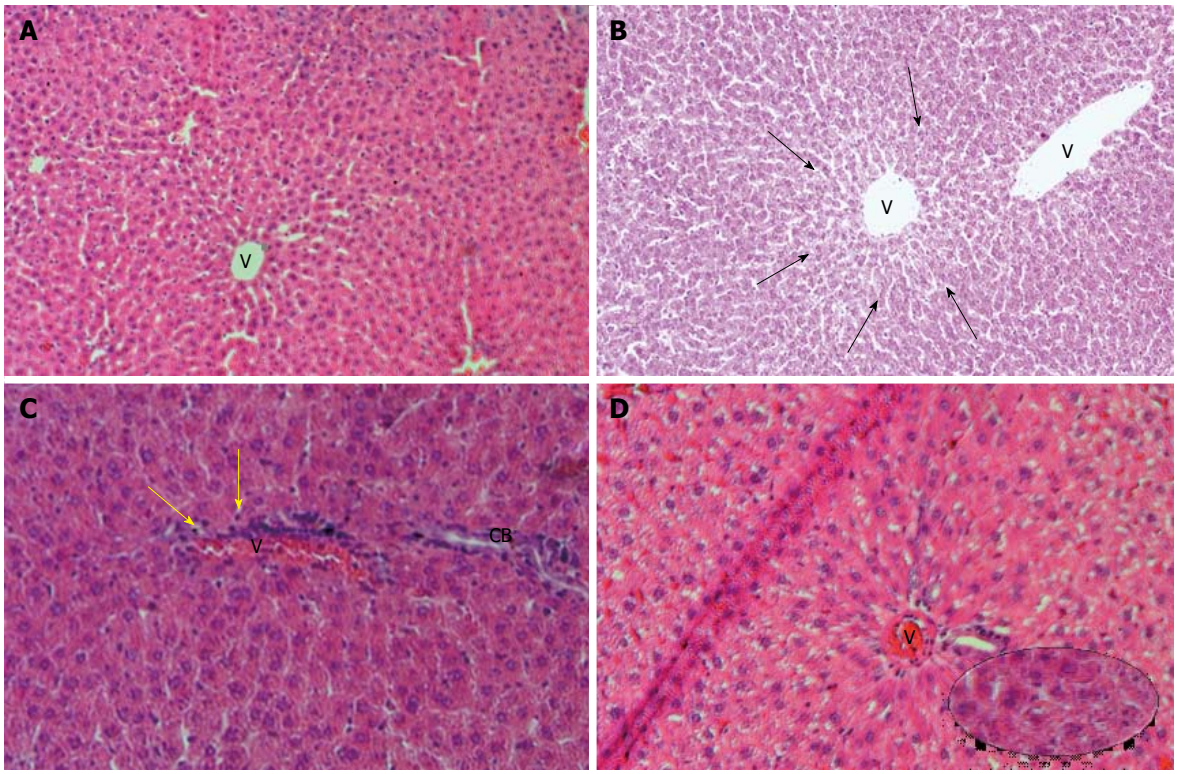

Figure 3 Hepatic histology of the group of females with weekend alcohol consumption at 5%. A: Image of the control group; B-D: Images of the group of females with alcohol consumption at 5%. A: Hepatocytes were observed as formed in a line (40 ×); B: A zone of less pigmentation is observed, marked with black arrows, corresponding to periportal necrosis (40 ×); C: Slight inflammation with blue-colored cells (leukocytes) around the portal vein, which do not surpass the limiting plaque (yellow arrows) (60 ×); D: In the lower left part, steatosis is observed (60 ×). V: Portal vein; BC: Bile canaliculus. Hematoxylin and Eosin stain.

solution of alcohol at 5%, first-pass metabolism is low, while with a 40% ethanol solution, first-pass metabolism is high and is furnished by the activity of the gastric dehydrogenase alcohol. It is probable that, in the groups of weekend alcohol consumption with a concentration of 40%, the subjects had initial alcohol metabolism in the stomach, and low ethanol concentration. In contrast, the group of males with a consumption of 5% had highest ethanol concentration, which probably modified the metabolites involved in the accumulation of body fat, such as glucose, triglycerides, and cholesterol, as may be observed in Table 2, where these metabolites exhibit a slight increase in comparison with the remaining groups. Our data are in agreement with those reported by Rocha *et al*[13] where, after 10 wk of alcohol consumption (3 d per week at 30% ad libitum), the authors found that food consumption was very similar to that exhibited between the control group and the group with alcohol consumption. Nonetheless, the group that consumed alcohol had a greater weight gain than the control. Thus, we think that it is the alcohol and its metabolism that is the cause of the weight gain, because it alters the fat level in the liver (Table 4), which we have previously reported[2,15,16]. Baraona *et al*[23] found that the first metabolic pass of the alcohol is found to be diminished in women due to low activity of gastric ADH; the authors concluded that the latter can increase the vulnerability of women to the effects of ethanol. In our study, we found that the levels of bilirubin were higher in both groups of females, regardless of the concentration of alcohol administered (Table 3). Likewise, greater histological changes in fatty change and inflammation (Table 4) and in alcohol consumption levels were lower in the group of females at 5% (0.83 ± 0.04) (Table 1).

On the other hand, the transaminase enzymes are indicators for the diagnosis of liver diseases. For

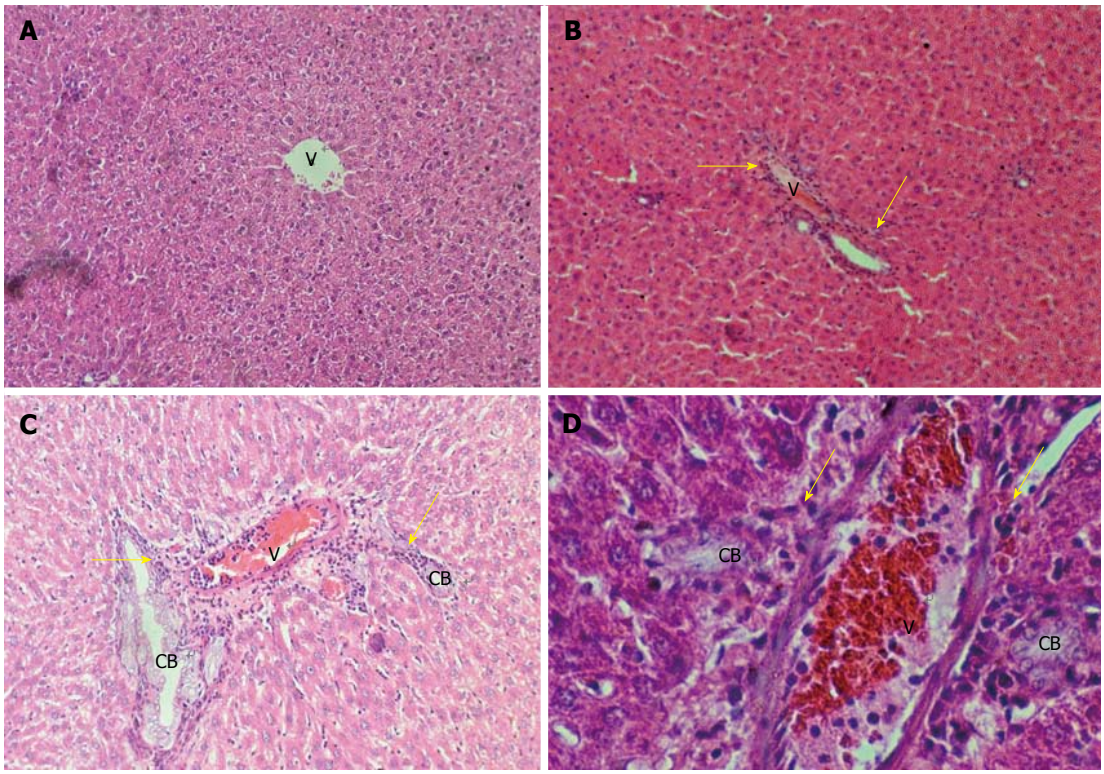

Figure 4 Hepatic histology of the group of males with weekend alcohol consumption at 5%. A: Image of the control group; B-D: Images of the group of males with alcohol consumption at 5%. A: The uniformity of the structures is observed to be conserved (40 ×); B: Presence of fine and thick steatosis (pigmentation-diminution zone in the cytoplasm), inflammation present with leukocytes in single file around the portal vein (yellow arrows) (40 ×); C: Important inflammation is observed, represented by leukocytes around the portal vein and in the Bile Canaliculus (BC) (yellow arrows) (40 ×); D: Fine as well as thick steatosis is observed (pigmentation-diminution zone in the cytoplasm), as well as leukocyte infiltrate (yellow arrow) (100 ×). V: Portal Vein; BC: Bile canaliculus. Hematoxylin and Eosin stain.

example, ALT and AST are released into the bloodstream at high concentrations, in which there is a membrane alteration in the hepatocyte; however, hepatocellular necrosis is not a requirement for the release of these enzymes, which gives rise to low correlation between the level of aminotransferases and liver damage[15]. This could be explained by the early release of hepatocytes, which initiate a proliferative process such as a sign, due to that it has been reported that liver regeneration is linked by selective enzyme release[15]. We previously reported that these comprise an alteration in the levels of transaminases depending on time of exposure to alcohol. Parra-Vizuet *et al*[24] reported a diminution in the serum levels of AST and ALT 24 h after the administration of a unique dose of alcohol of 1.5 g/kg. On the other hand, Morales-González *et al*[15] and Ramírez-Farías *et al*[25] reported an increase in the serum levels of the transaminases (ALT, AST, LDH, GDH and OTC) 7 d after the administration of ethanol. Similarly, Morales-González *et al*[26] reported a diminution in the activity of transaminase enzymes in hepatic tissue 24 h after ethanol administration (5 g/kg). Rocha *et al*[13] reported a rise in ALT during weekend alcohol consumption (4.5 g/d). Our results demonstrate that weekend alcohol consumption produces an increase in the serum of transaminases (AST, ALT and LDH) regardless of gender or of the concentration of alcohol (Figures 1 and 2). Nonetheless, surprisingly, alcohol consumption does not affect the serum levels of GGT (Figure 2). On the other hand, Stranges *et al*[11] reported that the principal transaminase affected by alcohol consumption is GGT. We consider that, in our study, there was no elevation of GGT, due to that weekend alcohol consumption was of 12 wk, and probably, sufficiently severe damage did not exist for it to be reflected by alteration of GGT, or by the levels of albumin (Table 3). This coincides with that reported by Rocha *et al*[13] in terms of protein levels in blood not being affected on consuming alcohol.

It has been reported that ethanol favors hepatic steatosis, probably because of the increase of lipogenesis, diminution of lipid transport of the liver, alteration of oxygenation of fatty acids[27], and even infiltration of monocytes, macrophages, *etc*., which are fundamental for pathogenic activity after acute or chronic hepatic injury[27]. The latter can explain in part the histological findings encountered in our study, where fatty change as well as inflammation comprised the most important changes in weekend alcohol consumption in all of the groups (Table 4), probably due to that they are related with the acute damage caused by ethanol to the liver[16-24]. Likewise, it was reported that acute treatment with ethanol in rats induced hepatic steatosis accompanied by an increase in the production of neutral fats (triglycerides). Thus, the serum levels of the triglycerides may not only reflect the production of the liver, but also the equilibrium between the production

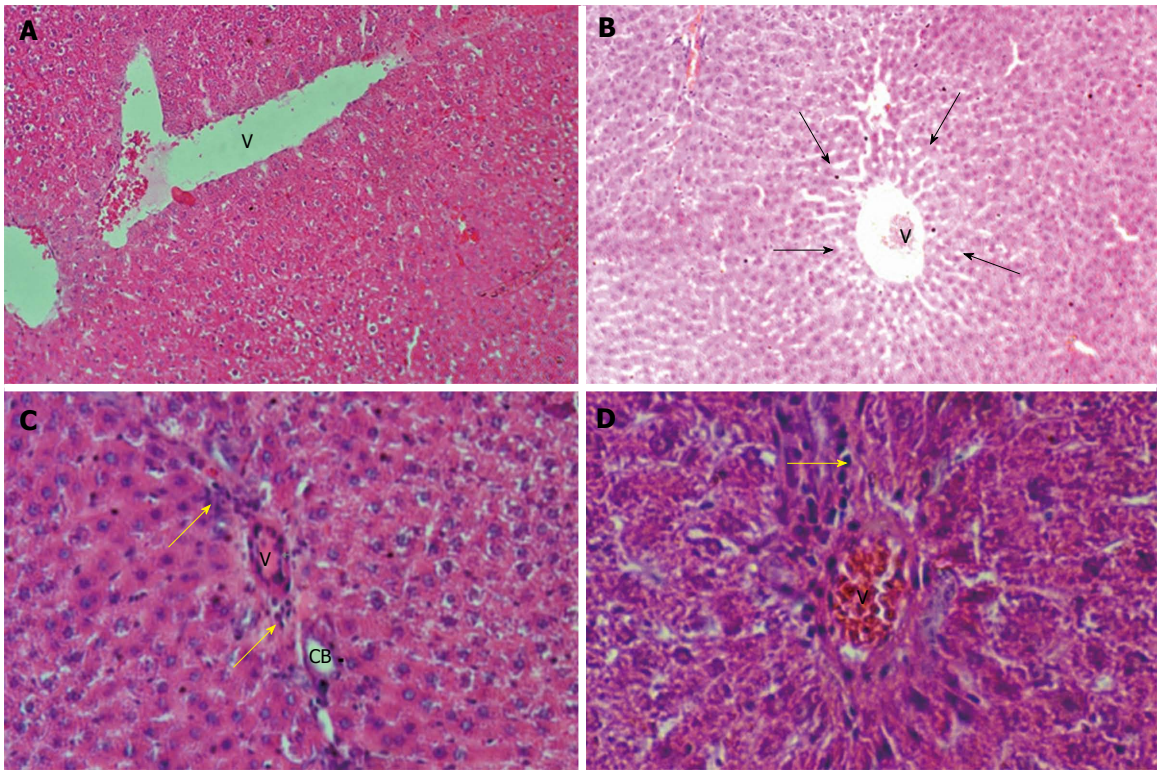

Figure 5 Hepatic histology of the group of females with weekend alcohol consumption at 40%. A: Image of the control group; B-D: Images of the group of females with alcohol consumption at 40%. A: The uniformity of the structures is observed to be conserved (40 ×); B: Periportal fibrosis, loss of cells around the portal vein (black arrows) (40 ×); C: Fine and thick steatosis and leukocytes in single file around the portal vein and some that emerge from the limiting plaque (yellow arrows) (60 ×); D: Both fine and thick steatosis and single-file leukocytes are observed (100 ×). V: Portal Vein; BC: Bile canaliculus. Hematoxylin and Eosin stain.

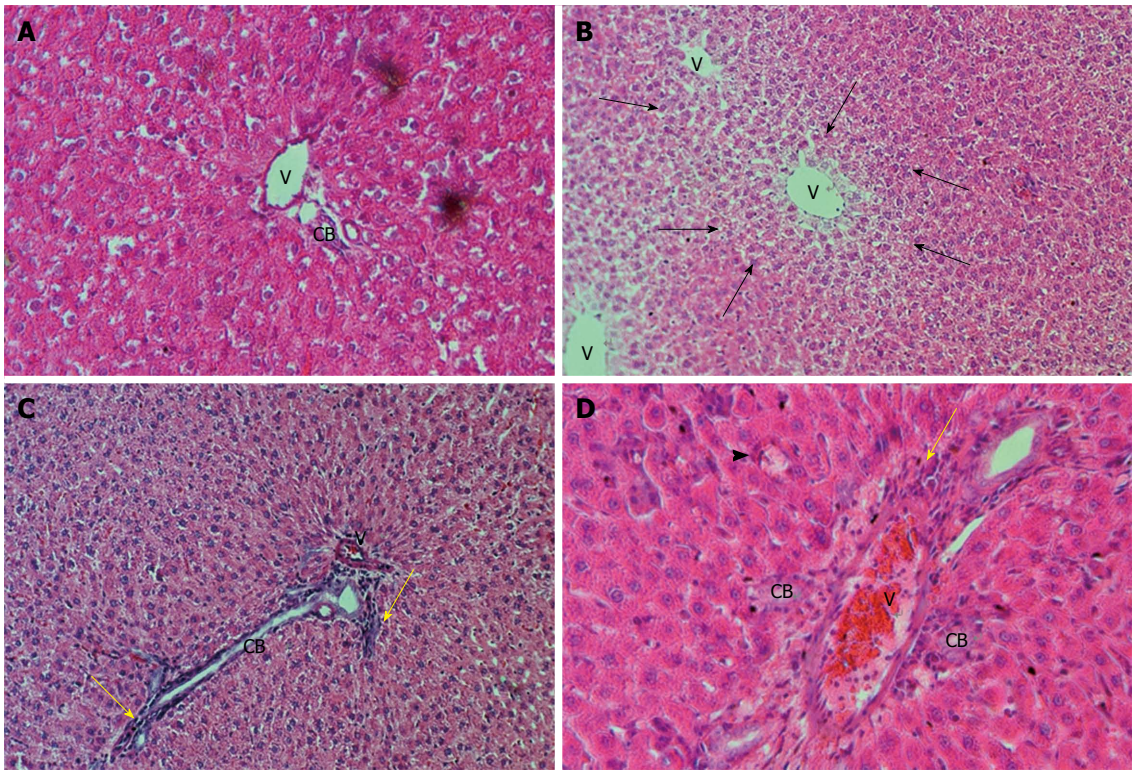

Figure 6 Hepatic histology of the group of males of 40%. A: Image of the control group; B-D: Images of the group of males with alcohol consumption at 40%. A: The conserved, uniformized structures of the form and size of the hepatocytes are observed (40 ×); B: Periportal fibrosis is observed (black arrows) (40 ×); C: Leukocytes are observed in a cited single file (yellow arrows) (40 ×); D: Fine and thick steatosis is observed (zone with less pigment inside the cellular cytoplasm of the hepatocyte), leukocytes (yellow arrow), and the apoptotic cell (point of the black arrow) (60 ×). V: Portal Vein; BC: Bile canaliculus. Hematoxylin and Eosin stain.

and utilization of neutral fats[28]. Our data reveal that weekend alcohol consumption produces fatty liver (Table 4) and the mobilization of neutral fats (Table 2), which is more evident when alcohol is consumed at 40%. Notwithstanding this, the group of males that consumed alcohol at 5%, a lesser amount than the groups at 40%, exhibited a pattern that was very similar in terms of levels of triglycerides and steatosis.

In conclusions, our findings demonstrate an effect of the damage that is caused by weekend alcohol consumption, regardless of gender or the concentration of the alcohol. Even more so, greater damage can be observed in females, and the metabolism of ethanol probably participates, specifically due to its first-pass metabolism, which is carried out in the stomach. Finally, the results obtained herein provide solid evidence that weekend alcohol consumption gives rise to liver damage, demonstrated by the biochemical and histological alterations, first manifested acutely, and their prolonged consumption can cause greater, irreversible damage.

ARTICLE HIGHLIGHTS

Research background

It is known that alcohol consumption is a risk factor for various diseases, specifically liver diseases. Due to that alcohol consumption, especially weekend alcohol consumption, has Increased, in recent years the effect has been studied of the damage that this pattern of alcohol consumption can cause to various organs, for example, to the heart and the liver. This study contributes, to our knowledge for the first time, solid evidences of the damage caused by weekend alcohol consumption to the liver, as well as of the relationship that gender and the concentration of alcohol maintain in generating liver damage.

Research motivation

The consumption of alcohol is a problem of prime magnitude at the worldwide level that gives rise to a great number of medical (gastritis, cirrhosis, cancer, infarcts, *etc.*), as well as non-medical problems (automobile accidents, homicides, absenteeism from work, *etc.*). Therefore, carrying out investigations to understand the mechanisms of cellular damage and identifying patterns of alcohol consumption that are on the rise, such as weekend alcohol consumption, and how these patterns come to damage the liver at different magnitudes, are of utmost importance.

Research objectives

The majority of studies that investigate alcohol-associated liver damage address chronic or acute alcohol consumption. Few experimental studies inquire into the liver damage that is caused by weekend alcohol consumption. To our knowledge, this is the first report that describes the histological alterations caused by weekend alcohol consumption. It is of utmost importance to ascertain the mechanisms of liver damage given rise to by weekend alcohol consumption, due to the growing number of young people who acquire this consumption pattern, and to find a therapeutic window.

Research methods

While the histological study is well known worldwide and has been employed in an infinite number of investigations, it can newly be a very important tool to find mechanisms of cellular damage caused by alcohol that are complemented with biochemical assays, in this manner taking the first steps in utilizing other investigative tools, such as electronic microscopy, molecular biology assays, *etc.* The most important and novel in this is the experimental design.

Research results

Histological and biochemical findings demonstrate that weekend alcohol consumption causes liver damage, irrespective of gender or the concentration of the alcohol. The contribution of this work resides in that a probable mechanism of damage to the liver due to weekend alcohol consumption comprises the metabolism of the first pass of the alcohol, which is carried out in the stomach. Lacking is the study in this model of variables such as age, food consumption, doses of alcohol, the consumption of antioxidants, *etc.*

Research conclusions

As conclusions of the investigation, this is the first report, to our knowledge, that describes the histological alterations caused by weekend alcohol consumption that, in addition to the biochemical assays, provides solid evidence on the damage caused by weekend alcohol consumption, which initially can be acute and reversible, but that probably can become irreversible. We employed a known technique, histology, but with the experimental design being novel. This type of study, in which the mechanisms of damage are investigated, can open a therapeutic window in future clinical practice.

Research perspectives

These perspectives include, in the first place, considering weekend alcohol consumption as a health problem of utmost importance, and even the same as chronic alcohol consumption. In second place, we conducted the investigation of the mechanisms of liver damage in terms of weekend alcohol consumption, and third, we found that this would be novel in the design of experimental models, in this manner utilizing techniques such as histology and biochemical assays, which comprise the first step in terms of orientation to the mechanisms of damage caused by alcohol, and these can be confirmed with molecular biology techniques. The questions to solve include knowing the following in terms of this model: How is the activity in the hepatic and gastric dehydrogenase alcohol enzyme found? Does the Nrf2 factor participate as cytoprotector?, and can the consumption of antioxidants prevent the alterations that weekend alcohol consumption cause?

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ORIGINAL ARTICLE

Retrospective Cohort Study

Survival outcomes of liver transplantation for hepatocellular carcinoma in patients with normal, high and very high preoperative alpha-fetoprotein levels

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Informed consent statement: Patients' consent to this retrospective study was not required since treatments given to patients were not influenced by the study and no individual patients would be identified as the clinical data used in the study are anonymous.

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Abstract

AIM

To investigate the impact of alpha-fetoprotein (AFP) on long-term recurrence rate and overall survival and we also aimed to define the level of AFP leading to a higher risk of disease recurrence and affecting patient survival.

METHODS

Data of adult patients who received liver transplant (LT) for hepatocellular carcinoma (HCC) at our hospital from January 2000 to December 2013 were reviewed. Reviewed data included demographic characteristics, preoperative AFP level, operative details, follow-up details, and survival outcomes. Patients were mostly listed for LT based on Milan or UCSF criteria. For the purpose of this study, normal AFP level was defined as AFP value < 10 ng/mL, high AFP level was defined as AFP value ⩾ 10 to < 400 ng/mL, and very high

AFP level was defined as AFP ⩾ 400 ng/mL. The patients were divided into these 3 groups accordingly. Survival rates were plotted as Kaplan-Meier curves and compared by log-rank analysis. Continuous variables were expressed as median (interquartile range). Categorical variables were compared by Spearman's test. Discriminative analysis was used to define the lowest value of AFP that could affect the overall survival in study population. Statistical significance was defined by a *P* value of < 0.05.

RESULTS

Totally 250 adult patients underwent LT for HCC in the study period. Eight-four of them received deceased-donor LT and 166 had living-donor LT. The patients were divided into 3 groups: Group A, AFP < 10 ng/mL (*n* = 83); Group B, AFP ⩾ 10 to < 400 ng/mL (*n* = 131); Group C, AFP ⩾ 400 ng/mL (*n* = 36). The commonest etiology was hepatitis-B-related cirrhosis. The Model for End-stage Liver Disease scores in these groups were similar (median, 13 *vs* 13 *vs* 12; *P* = 0.745). The time to operation in Group A was longer (median, 94 *vs* 31 *vs* 35 d; *P* = 0.001). The groups were similar in hospital mortality (*P* = 0.626) and postoperative complication (*P* = 0.702). Pathology of explants showed that the 3 groups had similar numbers of tumor nodules, but the tumors in Group C were larger (A: 2.5 cm, B: 3.0 cm, C: 4.0 cm; *P* = 0.003). Group C had a bigger proportion of patients who were beyond Milan criteria (*P* = 0.010). Poor differentiation and vascular permeation were also more common in this group (*P* = 0.017 and *P* = 0.003 respectively). It also had poorer 5-year survival (A: 85.5%, B: 82.4%, C: 66%; *P* = 0.029). The 5-year disease-free survival was 84.3% in Group A, 80.1% in Group B, and 61.1% in Group C. Receiver operating characteristic area under the curve for AFP in predicting tumor recurrence was 0.685. The selected cut-off value was 54 ng/mL for AFP (C-index 0.685; 95%CI: 0.592-0.779; sensitivity 0.595; specificity 0.687). On discriminative analysis, AFP value of 105 ng/mL was shown to affect the overall survival of the patients.

CONCLUSION

HCC patients with a high preoperative AFP level had inferior survival after LT. AFP level of 54 ng/mL was associated with disease recurrence, and AFP level of 105 ng/mL was found to be the cut-off value for overall survival difference.

Key words: Alpha-fetoprotein; Liver transplantation; Recurrence; Survival

Core tip: Various established criteria have been used to identify patients with hepatocellular carcinoma who would benefit from liver transplant with reasonable survival. Alpha-fetoprotein (AFP) level has been identified as an important factor associated with suboptimal survival with high recurrence rate. This study demonstrated that AFP level correlated well with the pathological findings of tumor differentiation and microvascular invasion, which are usually confirmed in explant pathology. In this set of data, AFP level of 54 ng/mL was associated with disease recurrence, and AFP level of 105 ng/mL was found to be the cut-off value for overall survival difference.

She WH, Chan ACY, Cheung TT, Lo CM, Chok KSH. Survival outcomes of liver transplantation for hepatocellular carcinoma in patients with normal, high and very high preoperative alpha-fetoprotein levels. *World J Hepatol* 2018; 10(2): 308-318 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/308.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.308

INTRODUCTION

Liver transplant (LT) is the best treatment option for hepatocellular carcinoma (HCC) as it removes both the tumor and the cirrhotic liver. The Milan criteria have been well adopted worldwide as a set of guidelines for listing patients for LT. Patients within the Milan criteria have a 5-year post-LT survival of 65%-80%, with a recurrence risk of 8%-15%[1]. However, the Milan criteria are criticized for being too stringent, since many patients beyond the criteria could still have reasonable post-LT survival[2-8]. Therefore, in additional to morphological consideration of tumor, the adoption of biological markers such as alpha-fetoprotein (AFP), response to therapy and evolution after therapy[9] is advocated.

AFP has been used as a tumor marker for HCC, and a high AFP level has been shown to be associated with poorer outcomes[10,11]. In previous studies, suboptimal results with high recurrence rates were seen in patients who had received LT with an AFP level of > 1000 ng/mL[12-14]. Such a level is considered a contraindication to LT. This level is applied not only to extended criteria but also to patients within the Milan criteria. Unfortunately, the exact consensual cut-off value remains undefined.

In this study, we investigated the impact of AFP on long-term recurrence rate and overall survival. We also aimed to define the level of AFP leading to a higher risk of disease recurrence and affecting patient survival.

MATERIALS AND METHODS

Prospectively collected data of adult patients who received deceased-donor LT (DDLT) or living-donor LT (LDLT) for HCC at our hospital in the period from January 2000 to December 2013 were reviewed and analyzed. These data included demographic characteristics, preoperative AFP level, operative details, follow-up details, and survival outcomes. Institutional review board approval was not required for this study because it was a retrospective analysis of anonymous data. Patient treatments were not affected by this study.

Patient selection for LT

The strategies adopted for selection of patients with known HCC for LT have been described elsewhere[15,16]. In brief, tumor evaluation was done with computed tomography of the abdomen and thorax, in addition to radionuclide bone scan at initial diagnosis. In recent years, dual-tracer (11C-acetate and 18 F-fluoro-deoxyglucose) positron emission tomography (PET) was performed to exclude extrahepatic metastasis. Patients who were 65 years old or younger and not eligible for partial hepatectomy or local ablation were considered for LT. The age limit as a selection criterion was getting relatively loose as long as the patient was physically fit. The Milan criteria[1] and the UCSF criteria[14] were used for selection of patients for listing. Patients who had recurrent HCC after hepatectomy would still be considered for LT if their disease was still within selection criteria. There was no mandatory waiting period prior to LT, and bridging therapy with transarterial chemoembolization was offered to LT candidates with reasonable liver function. From October 2009 onwards, an arbitrary Model for end-stage liver disease (MELD) score of 18 points were given to DDLT candidates with HCC remaining at stage 2 six months after radiological confirmation of their stage-2 disease. Two MELD points were added every three months as long as their disease remained at stage 2 or below[17].

Patients who were beyond the Milan and UCSF criteria because they had slightly larger tumors or slightly more tumors were not eligible for DDLT but could be considered for LDLT if they had no portal or hepatic vein invasion.

Treatments

Surgery was performed using standard techniques. Cell-saver device was not used. Explants were examined by pathologists for tumor size and number, differentiation, and presence of microscopic vascular invasion. Tumors found on explant examination were regarded as incidental tumors. Neither medical nor radiation adjuvant treatment was given to any patient after LT. The patients were monitored regularly by measurement of serum AFP level, chest radiography and abdominal and chest computed tomography every 3 mo. Recurrences suspected on clinical grounds were confirmed by histological examination as far as possible.

Donor and recipient operations were performed as described elsewhere[18]. The decision to use left-lobe graft *vs* right-lobe graft was based on a number of donor and recipient factors, the most important of which were the ratio of graft weight to standard liver volume, the ratio of graft weight to recipient weight, MELD score, and donor liver anatomy. The Urata formula [Liver volume (mL) = Body surface area (m^2) × 706.2 + 2.4] was used to calculate standard liver volume[19]. Implantation process and techniques were similar for left and right lobe grafts[18,20]. The immunosuppression and prophylaxis regimens prescribed have been described earlier[21].

Statistical analysis

For the purpose of this study, normal AFP level was defined as AFP value < 10 ng/mL, high AFP level was defined as AFP value ⩾ 10 to < 400 ng/mL, and very high AFP level was defined as AFP ⩾ 400 ng/mL. The patients were divided into these 3 groups accordingly.

Receiver operating characteristic (ROC) analysis was used to evaluate the ability of AFP to predict postoperative recurrence and to choose the optimal cut-off value for subsequent analysis. For indication for LDLT, high specificity was essential for avoiding excluding a large number of patients who would not develop recurrence.

Clinical profiles and outcomes of patients were compared on the basis of AFP level. Comparisons were made on short- and long-term outcomes, including graft function, graft survival, patient survival, and incidence of biliary complication. Continuous variables were expressed as median (range), and the Mann-Whitney *U* test was used for subgroup comparison. Categorical variables were compared by χ^2 test or Fisher's exact test. The cumulative probability of recurrence and survival was estimated by the life-table method and compared by the log-rank test. Deaths from all causes were included in the calculation of survival. Patients without recurrence were regarded as censored observations in the calculation of cumulative recurrence rates. Variables related to graft, tumor and tumor treatment before LT were analyzed for prognostic significance. The Kaplan-Meier method was used for survival analysis and the log-rank test was used for survival comparison. Discriminative analysis was used to define the lowest value of AFP that could affect the overall survival in the study population. Statistical significance was defined by a *P* value of < 0.05. The computer software SPSS, version 20.0 (SPSS Inc., Chicago, IL, United States), was used for all statistical calculations.

RESULTS

From January 2000 to December 2013, 250 adult patients underwent LT for HCC. Eight-four of them received DDLT and 166 had LDLT. The patients were divided into 3 main groups according to their preoperative AFP level: Group A, AFP < 10 ng/mL (normal); Group B, AFP ⩾ 10 to < 400 ng/mL (high); Group C, AFP ⩾ 400 ng/mL (very high) (Table 1). Patients in Group C were significantly younger (*P* = 0.037). The 3 groups has similar distribution of sex (*P* = 0.492). The commonest etiology was hepatitis-B-related cirrhosis. The median MELD scores in the 3 groups were similar (*P* = 0.745). The median time to operation in Group A was significantly longer (*P* = 0.001).

There were no differences in terms of blood transfusion amount, operation time, cold ischemic time or warm ischemic time among the groups, suggesting that the operative procedures were similar in the groups. Moreover, no differences were found in intensive care

Table 1 Comparison of Group A, Group B and Group C

	Group A (n = 83)	Group B (n = 131)	Group C (n = 36)	P value
Age (yr)	56 (38-65)	55 (3-72)	51.5 (11-66)	0.037
Male/Female	67/16	113/18	29/7	0.492
Diagnosis:				
Cirrhosis				
Cryptogenic	2	2	1	
Hepatitis B	67	89	25	
Hepatitis C	4	22	3	
Alcoholic	0	1	1	
Hepatitis B + C	1	2	0	
Alcoholic + hepatitis C	1	0	1	
Alcoholic + hepatitis B	0	1	0	
Autoimmune	1	0	0	
Wilson's disease	0	0	0	
Preoperative MELD score	13 (6-35)	12 (6-35)	12 (8-43)	0.745
Waiting time (d)	94 (1-2735)	31 (1-1874)	35 (1-1473)	0.001
Blood transfusion (units)	4.2 (0-32)	2 (0-56)	4 (0-31)	0.128
Fresh frozen plasma transfusion (units)	8 (0-24)	6 (0-30)	6 (0-22)	0.609
Platelet transfusion (units)	8 (0-26)	6 (0-32)	8 (0-22)	0.978
Operation time (min)	650 (370-1105)	678 (333-1110)	707 (300-1273)	0.598
Cold ischemic time (min)	182 (62-652)	125 (60-633)	133 (70-500)	0.206
Warm ischemic time (min)	49.5 (25-102)	52 (26-108)	55.5 (30-93)	0.209
Hospital stay (d)	1.7 (8-132)	15 (0-83)	15 (7-47)	0.251
Intensive care unit stay (d)	3 (1-42)	3 (0-30)	3 (2-16)	0.283
Follow-up (mo)	82.4 (0.59-204.9)	89.1 (0-210.82)	68.2 (5.95-204.24)	0.242
Hospital mortality	2 (2.4%)	2 (1.5%)	0	0.626
LDLT:DDLT	45:38	93:38	28:8	0.012
Explant Milan Within:Beyond	56:23	84:46	15:21	0.010
Explant UCSF Within:Beyond	63:16	98:32	23:13	0.188
No. of tumor in explant	1 (1-multiple)	2 (1-multiple)	1 (1-20)	0.272
Largest size of tumor in explant (cm)	2.5 (0.90-7.00)	3.0 (0.25-9.00)	4.0 (1.5-19.5)	0.003
Differentiation:				0.017
Well	26	41	3	
Moderate	41	66	25	
Poor	2	8	6	
Undifferentiated	0	2	0	
Unknown	10	13	2	
Vascular permeation:				0.003
No	60	85	14	
Yes	18	40	21	
Unknown	1	5	1	
Graft loss	18 (21.7%)	28 (21.4%)	15 (41.7%)	0.033
Patient status Alive:Dead	65:18	104:27	21:15	0.027
Graft survival, yr				0.038
1	96.40%	93.10%	97.20%	
3	89.20%	84.70%	80.60%	
5	85.50%	81.60%	66.00%	
Patient survival, yr				0.029
1	96.40%	94.70%	97.20%	
3	89.20%	85.50%	80.60%	
5	85.50%	82.40%	66.00%	
Disease-free survival, yr				0.007
1	92.80%	89.30%	80.60%	
3	88.00%	81.70%	72.20%	
5	84.30%	80.10%	61.10%	
Postoperative early complication by Clavien grading:				0.702
No	40	68	20	
Ⅰ	19	24	9	
Ⅱ	5	13	2	
ⅢA	11	11	3	
ⅢB	6	6	2	
ⅣA	1	7	0	
ⅣB	0	0	0	
Ⅴ	1	2	0	

Group A-AFP < 10 ng/mL; Group B-AFP ⩾ 10 to < 400 ng/mL; Group C, AFP ⩾ 400 ng/mL. MELD: Model for end-stage liver disease; DDLT: Deceased-donor liver transplant; LDLT: Living-donor liver transplant.

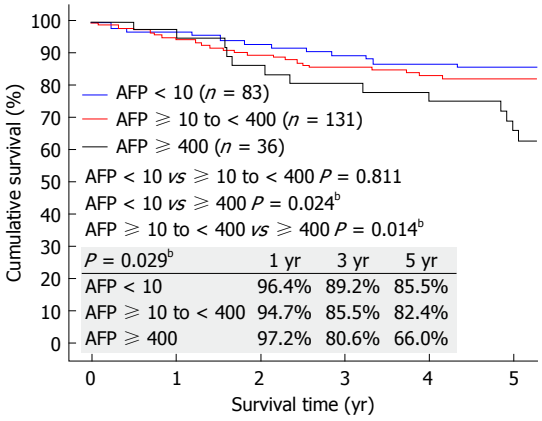

Figure 1 Overall survival of patients with preoperative AFP < 10 ng/mL, ⩾ 10 to < 400 ng/mL, and ⩾ 400 ng/mL. AFP: Alpha-fetoprotein. [b]*P* < 0.01.

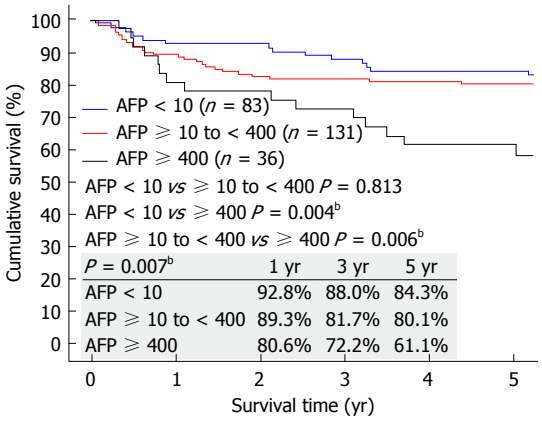

Figure 2 Disease-free survival of patients with preoperative AFP < 10 ng/mL, ⩾ 10 to < 400 ng/mL, and ⩾ 400 ng/mL. AFP: Alpha-fetoprotein. [b]*P* < 0.01.

unit stay (*P* = 0.283), hospital stay (*P* = 0.251), hospital mortality (*P* = 0.626), or postoperative complication (*P* = 0.702). Pathology of explants showed that the 3 groups had similar numbers of tumor nodules but the tumors in Group C were larger (*P* = 0.003), and thus more patients in Group C were beyond the Milan criteria (*P* = 0.010). Furthermore, Group C had more cases of poor differentiation (*P* = 0.017) and vascular permeation (*P* = 0.003).

No patients were lost to follow-up in the study period. The 3 groups had similar follow-up period (*P* = 0.242). More patients in Group C had graft loss (*P* = 0.033), and hence this group had poorer 5-year graft survival (*P* = 0.038) and 5-year patient survival (*P* = 0.029) (Figure 1). Most patients died of recurrent HCC. The 5 year-disease-free survival was 84.3% in Group A, 80.1% in Group B, and 61.1% in Group C. Disease-free survival was similar in Groups A and B (*P* = 0.813) but significantly different between Groups A and C (*P* = 0.004) and between Groups B and C (*P* = 0.006) (Figure 2).

Disease recurrence and ROC curve analysis

Recurrence of HCC was identified in 42 patients (42/250 = 16.8%). The ability of preoperative AFP to predict

Area	Standard error	Asymptotic 95% confidence interval	
		Lower bound	Upper bound
0.685	0.048	0.592	0.779

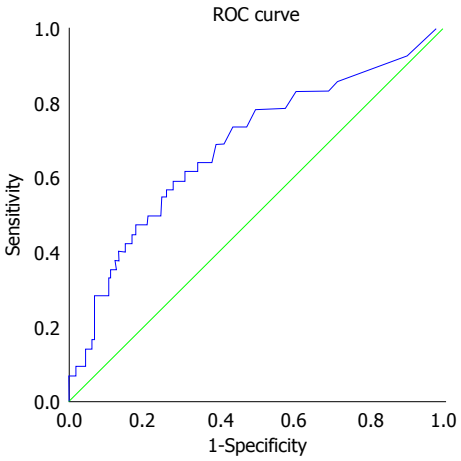

Figure 3 Receiver operating characteristic curve for alpha-fetoprotein in predicting hepatocellular carcinoma recurrence after liver transplant.

HCC recurrence was analyzed by ROC curve. Area under the curve for AFP was 0.685. Among the cut-off values with sufficient specificity, the cut-off point with the highest C-index was chosen as the optimal cut-off value for subsequent analysis. The selected cut-off value was 54 ng/mL for AFP (C-index 0.685; 95% confidence interval 0.592-0.779; sensitivity 0.595; specificity 0.687) (Figure 3).

Further analysis was performed to identify the lowest AFP level that could affect patient survival. On discriminative analysis, AFP value of 105 ng/mL was identified as the level that could affect the overall survival of the patients. Patients with AFP ⩽ 105 ng/mL (Group D) were compared with patients with AFP >105 ng/mL (Group E) (Table 2). Patients in Group E were younger (*P* = 0.017). When it comes to preoperative comorbidity, underlying cause of cirrhosis, MELD score, operative details, postoperative complication and hospital stay, no significant differences were seen. However, Group E had poorer 5-year graft survival (*P* = 0.024), patient survival (*P* = 0.045) (Figure 4), and disease-free survival (*P* = 0.006) (Figure 5). Looking into the details of the pathological results of the 2 groups, it was clear that the tumors in Group E had worse pathology. Group E had larger tumors (*P* = 0.017) and fewer cases of well differentiation (15.71% *vs* 32.78%; *P* = 0.001), while vascular permeation was more common in this group (44.29% *vs* 26.67%; *P* = 0.014) (Table 2).

DISCUSSION

AFP has been used as a tumor marker for HCC. Its elevation depends on pathological characteristics, including tumor size and degree of differentiation of tumor cells. It is a well-established surrogate of tumor biology, as it correlates with histological grading and

Table 2 Comparison of the two subgroups, Group D and Group E

	Group D (n = 180)	Group E (n = 70)	P value
Age (yr)	56 (38-67)	53.5 (3-72)	0.017
Male:Female	150:30	59:11	0.855
Diagnosis:			
Cirrhosis			
Cryptogenic	4	1	
Hepatitis B	136	45	
Hepatitis C	19	10	
Alcoholic	1	1	
Hepatitis B + C	1	2	
Alcoholic + hepatitis C	1	1	
Alcoholic + hepatitis B	1	0	
Autoimmune	1	0	
Wilson's disease	0	0	
Preoperative MELD score	12 (6-35)	12 (6-43)	0.972
Waiting time (d)	58.5 (1-2735)	32 (1-1874)	0.183
Blood transfusion (units)	3 (0-56)	4(0-32)	0.988
Fresh frozen plasma transfusion (units)	6 (0-30)	6 (0-22)	0.798
Platelet transfusion (units)	6 (0-30)	8 (0-32)	0.708
Operation time (min)	654 (333-1110)	716.5 (300-1273)	0.151
Cold ischemic time (min)	137.5 (60-652)	127.5 (66-633)	0.195
Warm ischemic time (min)	51 (25-108)	53 (28-93)	0.308
Hospital stay (d)	15.5 (0-132)	16 (7-48)	0.497
Intensive care unit stay (d)	3 (0-42)	3 (2-30)	0.806
Follow-up (mo)	87.2 (0.0-210.8)	75.4 (3.8-206.8)	0.173
Hospital mortality	4 (2.2%)	0	0.486
LDLT: DDLT	112:68	54:16	0.025
Explant Milan Within:Beyond	116:60	39:30	0.170
Explant UCSF Within:Beyond	135:41	49:20	0.354
No. of tumor in explant	1.5 (1-multiple)	1 (1-20)	0.551
Largest size of tumor in explant (cm)	2.85 (0.90-7.00)	3.5 (0.25-19.5)	0.017
Differentiation			0.0001
Well	59	11	
Moderate	91	41	
Poor	6	10	
Undifferentiated	0	2	
Unknown	20	5	
Vascular permeation			0.014
No	124	35	
Yes	48	31	
Unknown	4	3	
Graft loss	37 (20.6%)	24 (34.3%)	0.023
Patient status Alive:Dead	143:37	47:23	0.041
Graft survival, yr			0.024
1	95.00%	94.30%	
3	87.20%	81.40%	
5	83.80%	72.30%	
Patient survival, yr			0.045
1	95.60%	95.70%	
3	87.20%	82.90%	
5	83.80%	73.80%	
Disease-free survival, yr			0.006
1	91.10%	84.30%	
3	85.00%	75.70%	
5	82.20%	70.00%	
Postoperative early complication by Clavien grading			0.798
No	90	38	
Ⅰ	35	17	
Ⅱ	16	4	
ⅢA	19	6	
ⅢB	11	3	
ⅣA	6	2	
ⅣB	0	0	
Ⅴ	3	0	

Group D-AFP ⩽ 105 ng/mL; Group E-AFP >105 ng/mL. MELD: Model for end-stage liver disease; DDLT: Deceased-donor liver transplant; LDLT: Living-donor liver transplant.

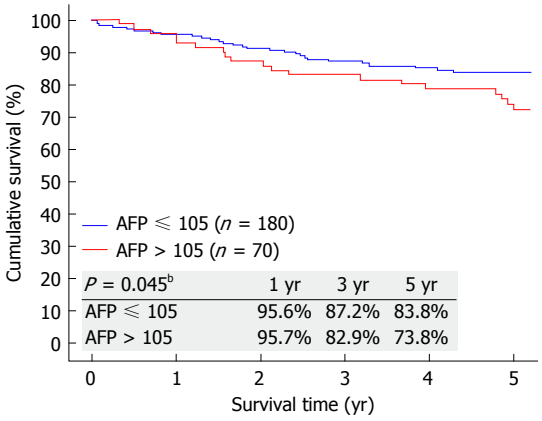

Figure 4 Overall survival of patients with preoperative alpha-fetoprotein ⩽ 105 ng/mL and > 105 ng/mL. AFP: Alpha-fetoprotein. [b]*P* < 0.01.

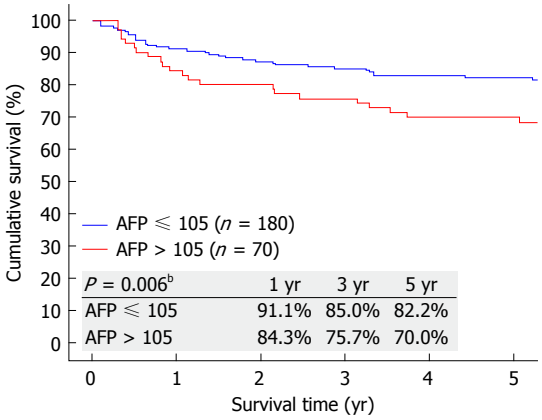

Figure 5 Disease-free survival of patients with preoperative alpha-fetoprotein ⩽ 105 ng/mL and > 105 ng/mL. AFP: Alpha-fetoprotein. [b]*P* < 0.01.

vascular invasion[12,13,22,23]. The presence of microvascular invasion and poor differentiation of tumor cells are associated with recurrence in patients within and beyond various transplant criteria[24-29]. However, most of the time, preoperative histological results are not available, and therefore prediction of disease recurrence and overall survival cannot be made preoperatively.

AFP is an oncogene protein produced by HCC. Currently, an AFP level of > 400 ng/mL together with a liver mass with characteristic features is a diagnostic feature of HCC. Serum AFP is a well-established prognostic marker of increased tumor virulence in HCC[12,13,30-35]. It has also been shown to be associated with increased risk of waitlist dropout[13,36,37] and post-LT recurrence[12,30-35,38-40]. AFP level has been integrated into a number of transplant criteria, including the Hangzhou criteria[41], the extended Toronto criteria[42], the "total tumor volume"[43], and the Kyoto criteria[44].

Regarding LT for HCC, disease recurrence is a major concern. Extrahepatic metastasis is a clear contraindication to LT as it represents systemic disease, which cannot be cured by LT. The presence of macrovascular invasion has been shown to be an independent risk factor for recurrence and associated with worsened survival[25,45], and therefore is considered a contraindication to LT in the Milan, UCSF and "up-to-seven" criteria[1,5,14]. Poor tumor biology (poor cellular differentiation and presence of microvascular invasion) is associated with an increased risk of tumor recurrence. However, most of the time, the tumor biology cannot be known before operation. Liver biopsy of the target lesion can be an important tool for identifying tumor differentiation and microvascular invasion. It has been proposed that liver biopsy should be included into the Toronto criteria for any number and any size of HCC lesion[42]. While AFP is known to be a well-established surrogate of tumor biology for its correlation with histological grading and vascular invasion[12,13,22,23], it has been used for risk stratification, with elevation of AFP associated with a higher incidence of disease recurrence. Unfortunately, there is no exact cut-off value as an absolute value for contraindication to LT.

AFP is associated with vascular invasion and intrahepatic metastasis is not expressed in well-differentiated HCC[46]. AFP is considered an independent prognostic factor which correlates with histological differentiation[12,47]. This was also reflected in our patients. In Group A, the median time to operation was longer and the outcome was better, suggesting that this group had more favorable tumor biology. Microvascular invasion has been proven to be a strong predictor of outcome (liver resection or LT) for HCC patients[48-51]. Unfortunately, tumor differentiation and microvascular status require histological proof, and most of the time they can only be known after operation. Our patients were divided into 3 main groups, with normal, high and very high preoperative AFP levels. Results showed that higher AFP level was associated with tumor recurrence and correlated with tumor differentiation and microvascular invasion. It was apparent that when the AFP value was lower, the chance of poor differentiation and vascular permeation was also lower. However, this could have been affected by the fact that Group C had more patients beyond the Milan criteria. ROC analysis was performed to assess the ability of preoperative AFP in predicting HCC recurrence after LT. According to C-index analysis based on ROC, the optimal cut-off value was set at 54 ng/mL. However, on further discriminative analysis, 105 ng/mL was set as the cut-off value and it demonstrated significant survival difference despite same amounts of patients who were within the Milan and UCSF criteria. This suggested the importance of using AFP as one of the preoperative surrogate markers to evaluate LT candidates, as it represents additional information on identifying high-risk patients preoperatively so as to predict the risk of recurrence and to let patients have realistic anticipation regarding their long-term outcomes.

HCC patients within the Milan criteria consistently have a 10%-15% risk of disease recurrence after LT[1]. However, strictly following the rules would turn down a substantial number of patients who could benefit from LT and get a cure, even with reasonable disease-

free and overall survival. Therefore, allowance is given mainly based on tumor number and size[5,35,52]. It is hoped that further developed selection tools with expanded criteria can identify patients with low risk of recurrence. Applying AFP cut-off could have resulted in the exclusion of certain patients who could have reasonable survival but were at higher risk of tumor recurrence as compared with patients with a lower AFP level. However, using AFP alone to predict the subsequent disease course would result in bias in selection of patients for LT, since patients with the same AFP level can have similar disease-free survival as well as overall survival. Therefore, in listing patients for LT, other factors should also be considered, such as established criteria and tumor status assessed by PET. Fluorine-18-fluorodeoxyglucose PET is able to pick up poorly differentiated HCC[53], while 11C-choline PET has strong avidity for HCC, particularly well differentiated and moderately differentiated tumors[54-56]. Both ways provide preoperative information without the need for biopsy of the liver tumor. For patients who have poor liver function that precludes liver resection, ablative therapy and transarterial chemoembolization, palliative treatment would be the only option if they are denied LT. For these patients, LT should not be ruled out if a relatively inferior survival outcome is acceptable to them. Since LDLT is almost exclusively performed among family members, oftentimes they would accept a relatively inferior survival outcome of the recipient, given a reasonable donor operative outcome.

The findings of this study may not be universally applicable, as AFP 105 ng/mL is a value calculated from our cohort of 250 patients. Moreover, this is a retrospective cohort study with inevitable selection bias. Furthermore, the different levels of AFP (normal, high and very high) were arbitrarily defined. Before adopting AFP as a decision-making tool based on current selection criteria, we have to balance the risk of disease recurrence (hence overall survival) and the patients' expectation. Still, it is hoped that this study can shed some light on the importance of adding AFP to the armamentarium of assessment tools for LT listing.

HCC patients with a high preoperative AFP level had inferior survival after LT. AFP level of 54 ng/mL was associated with disease recurrence, and AFP level of 105 ng/mL was found to be the cut-off value for overall survival difference.

ARTICLE HIGHLIGHTS

Research background

Liver transplantation is the best treatment option for hepatocellular carcinoma. However, only patients' tumor criteria should fit the current adopted selection criteria. Most of the criteria are morphological descriptions, including size and number, with the recently added alpha-fetoprotein in some of the updated criteria.

Research motivation

We hoped to identify the cutoff value of alpha-fetoprotein in predicting disease recurrence and overall survival. Apart from using size and number as the selection criteria for liver transplantation, the additional use of alpha-fetoprotein might be able to give practical prediction of disease recurrence.

Research objectives

The objective of this study is to investigate the impact of alpha-fetoprotein on the long-term recurrence rate and overall survival of recipients of liver transplantation for hepatocellular carcinoma.

Research methods

Data of adult patients who received liver transplantation for hepatocellular carcinoma at our hospital from January 2000 to December 2013 were reviewed. Data of included patients were analyzed. We defined the different levels of alpha-fetoprotein as normal (< 10 ng/mL), high (≥ 10 to < 400 ng/mL) and very high (≥ 400 ng/mL). The patients were divided into these 3 groups accordingly. Group comparison was then made.

Research results

Alpha-fetoprotein level was normal in 83 patients, high in 131 patients, and very high in 36 patients. The commonest etiology was hepatitis-B-related cirrhosis. The Model for End-stage Liver Disease scores in these groups were similar (median, 13 *vs* 13 *vs* 12; $P = 0.745$). Patients with normal alpha-fetoprotein level had longer time to operation (median, 94 *vs* 31 *vs* 35 d; $P = 0.001$). The groups were similar in hospital mortality ($P = 0.626$) and postoperative complication ($P = 0.702$). Pathology of explants showed that the 3 groups had similar numbers of tumor nodules, but patients with very high alpha-fetoprotein level had bigger tumors ($P = 0.003$). This group also had a bigger proportion of patients who were beyond Milan criteria ($P = 0.010$). Poor differentiation and vascular permeation were commoner in this group ($P = 0.017$ and $P = 0.003$ respectively). It also had poorer 5-year overall survival ($P = 0.029$) and disease-free survival ($P = 0.007$). Receiver operating characteristic area under the curve for alpha-fetoprotein in predicting tumor recurrence was 0.685. The selected cut-off value was 54 ng/mL (C-index 0.685; 95%CI: 0.592-0.779; sensitivity 0.595; specificity 0.687). On discriminative analysis, alpha-fetoprotein value of 105 ng/mL was shown to affect the overall survival of the patients.

Research conclusions

This study showed that patients with high preoperative alpha-fetoprotein levels had poorer post-transplant survival. An alpha-fetoprotein level of 54 ng/mL was associated with disease recurrence, and 105 ng/mL was found to be the cutoff value for overall survival difference. These findings would be useful when considering liver transplantation for patients with a high alpha-fetoprotein level. Currently, there is no definite cutoff value of alpha-fetoprotein for ideal oncological outcomes in hepatocellular carcinoma. With the above alpha-fetoprotein values, superior long-term disease-free and overall survival will be achievable if liver transplantation is offered to patients with a lower preoperative alpha-fetoprotein level. The additional use of alpha-fetoprotein will allow better prediction of the long-term survival outcome, and hence affect the future practice in selection of patients for liver transplantation.

Research perspectives

Selection of patients for liver transplantation should not be based on morphological criteria alone. Other biomarkers such as alpha-fetoprotein should be added to the criteria currently used.

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ORIGINAL ARTICLE

Clinical Practice Study

Hepatitis C virus knowledge improves hepatitis C virus screening practices among primary care physicians

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Institutional review board statement: The Health Sciences Institutional Review Board (HSIRB) at the University at Buffalo reviewed the study protocol and deemed the study met exempt criteria 45 CFR 46.101(b)(2). The permissible exempt category was "Research involving the use of educational tests, survey procedures, interview procedures or observation of public behavior (anonymous survey)".

Informed consent statement: The HSIRB considered the return of the anonymous survey as deemed voluntary consent to participate in the study and therefore did not require an individual consent form from each participant.

Conflict-of-interest statement: Martinez AD has served as a speaker, a consultant and an advisory board member for Gilead, Intercept, Salix, Bayer, BMS, and Abbvie, and has received research funding from Abbvie, Gilead, Merck, Tobira, and Intercept; Talal AH has served as a speaker, a consultant and an advisory board member for Abbott Laboratories, Merck, and Abbvie, and has received research funding from Merck, Gilead, Abbott Laboratories, Abbvie, Intercept and Conatus; None of the other authors have any financial disclosures.

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Abstract

AIM

To understand the role of knowledge as a promoter of hepatitis C virus (HCV) screening among primary care physicians (PCP).

METHODS

A 45-item online questionnaire assessing knowledge of HCV natural history, risk factors, and treatment was distributed to 163 PCP. Logistic regression, adjusted for survey responses, assessed associations between PCP knowledge of HCV natural history and treatment and birth cohort (*i.e.*, birth between 1945 and 1965) screening. Response stratification and weighting were used to account for nonresponse and to permit extension of responses to the entire survey population. Associations between various predictors including demographic characteristics, level of training, and HCV treatment experience and HCV knowledge were assessed.

RESULTS

Ninety-one individuals (55.8%) responded. Abnormal liver enzymes (49.4%), assessment of HCV-related risk factors (30.6%), and birth cohort membership (20%) were the leading HCV screening indications. Most PCP (64.7%) felt that the combination of risk-factor and birth cohort screening utilizing a self-administered survey while awaiting the physician (55.3%) were the most efficient screening practices. Implementation of birth cohort screening was associated with awareness of the recommendations (*P*-value = 0.01), knowledge of HCV natural history (*P*-value < 0.01), and prior management of HCV patients (*P*-value < 0.01). PCP with knowledge of HCV treatment was also knowledgeable about HCV natural history (*P*-value < 0.01). Similarly, awareness of age-based screening recommendations was associated with HCV treatment knowledge (*P*-value = 0.03).

CONCLUSION

Comprehensive knowledge of HCV is critical to motivate HCV screening. PCP-targeted educational interventions are required to expand the HCV workforce and linkage-to-care opportunities as we seek global HCV eradication.

Key words: Viral hepatitis; Hepatitis C virus global eradication; Hepatitis C virus diagnosis; Hepatitis C virus surveillance; Knowledge of hepatitis C virus

Core tip: Many hepatitis C virus (HCV)-infected patients worldwide are unaware of their infection status. The key to increasing HCV detection and linkage-to-care is augmentation of virus screening by primary care physicians (PCP). Understanding factors that promote HCV screening among PCP is crucial to its eradication. We assessed PCP knowledge of HCV natural history and treatment and awareness of screening recommendations. PCP knowledge of HCV natural history and prior management of HCV patients were important predictors of implementation of HCV screening. Comprehensive HCV education targeted to PCP, including screening recommendations, is critical to increase HCV detection and linkage-to-care to obtain global eradication.

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INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of cirrhosis that can ultimately result in end-stage liver disease, hepatocellular carcinoma[1], and liver transplantation[2,3]. An estimated 5.2 million individuals in the United States are HCV-infected[4]. HCV mortality continues to increase, and it now surpasses the combined mortality of 60 notifiable infectious diseases[5]. Simultaneously, direct acting antivirals (DAAs) have improved HCV treatment tremendously; all oral, highly efficacious agents with minimal side effects and short treatment duration. HCV elimination in the United States, an often cited goal, is substantially hampered since 40% of HCV-infected patients are unaware of their infection status[6], and the 20000 hepatologists and infectious diseases physicians[7] in the United States are insufficient to care for up to 5 million HCV-infected individuals[4].

Historically, primary care physicians (PCP) had a role in HCV detection and counseling, but HCV treatment was considered beyond their practice scope[8], a notion that has recently been challenged[9,10]. Many PCP, the gatekeepers of the healthcare system, have limited HCV knowledge[11,12]. A systematic review identified significant knowledge gaps among PCP related to HCV natural history, diagnostic approaches, and treatment[12]. The rapid change in the HCV therapeutic landscape has only magnified the need for HCV education. Indeed, a recent survey indicated that the vast majority (84%) of PCP desired additional HCV training[13]. Delivering HCV education to resident physicians may effectively increase HCV knowledge among PCP. Indeed, most Family Medicine residency training program directors believe that chronic HCV is a significant primary care problem and PCP should be involved in building capacity for HCV management[14]. PCP education on HCV natural history and treatment has also been shown to expedite HCV treatment, adherence, and viral eradication[15,16].

Strategies have also been implemented to increase identification of HCV-infected individuals who remain undiagnosed. In 2012, the Centers for Disease Control and Prevention (CDC) and the US Preventive Services Task Force (USPSTF) promoted the recommendation that all individuals born between 1945 and 1965 (*i.e.,* birth cohort) should have a one-time HCV screening test[17,18], since 75% of undiagnosed HCV–infected individuals are birth cohort members[19]. Unfortunately, however, limited implementation of birth cohort

screening has diminished its originally anticipated impact with screening rates that are substantially reduced compared to those originally proposed and that vary widely from institution to institution[20,21]. Limited PCP knowledge of birth cohort screening recommendations may partially account for diminished impact. Indeed, systematic reviews have established the pre-eminent role of provider education in successful implementation of quality of care clinical guidelines[22]. Education should target uncertainties in physician knowledge and should be modified over time in order to ensure continued guideline application[23].

In consideration of PCP's expanding role in HCV treatment[9,10,14] combined with the need for knowledge on HCV natural history, screening, and treatment[12], we assessed knowledge of HCV natural history and treatment on implementation of birth cohort screening recommendations. We surveyed 91 PCP affiliated with an academic medical center. We sought to provide insight into topics for provider education, particularly to physicians in training, as these are important considerations in order to expand the HCV workforce.

MATERIALS AND METHODS

Study population and eligibility

The University at Buffalo (UB)-affiliated primary care clinics is a network of 9 clinics catering to urban and suburban patients in and around the City of Buffalo, New York. Eligible participants were PCP working in UB-affiliated clinics as supervising physicians or residents and who had experience with HCV treatment. Participant's scope of practice was General Internal Medicine, Family Medicine or a related combination of the two in such disciplines as Pediatrics or Social and Preventive Medicine. Medical students and physician extenders (*i.e.*, nurse practitioners or physician assistants) were excluded from the study. The study was deemed exempt from review by UB's Health Sciences Institutional Review Board, and it considered the return of the anonymous survey as deemed voluntary consent.

Hypothesis, questionnaire development and administration

The primary objective of this study was to evaluate the association of PCP knowledge of natural history and treatment of HCV on the implementation of birth cohort-based HCV screening. As a secondary objective, we sought to evaluate PCP related factors that could influence implementation of birth cohort-based HCV screening. We hypothesized that PCP with greater knowledge of HCV natural history and treatment would be more likely to implement birth cohort-based screening for HCV. The study design was a prospective questionnaire-based single-site study. Over a six month period, eligible PCP were distributed an anonymous, web-based 45 question survey that contained 18 knowledge questions that assessed HCV natural history and 19 that assessed knowledge of HCV treatment. Survey completion took approximately 30 min. Physicians who did not respond to the initial request or who partially completed the initial survey were sent follow up completion reminders weekly. No gifts or incentives were offered for survey completion. The survey instrument also inquired about general information concerning PCP practice locations and specialties.

Testing for internal validity and data analysis

After initial questionnaire development by subject matter experts, the survey was pretested among 5 providers. Based upon responses received, changes were made to the survey lay-out and format. The final version of the questionnaire was then distributed for completion. The internal validity of the survey was evaluated by including questions with similar meaning and by checking for agreement in the responses. We found that agreement between questions was moderate (kappa statistic estimate: 0.536; $P < 0.01$).

Statistical analysis was performed using R (http://www.r-project.org/). Categorical variables are summarized as counts and/or percentages, while continuous variables are summarized by their mean/median and standard deviation/interquartile range, as appropriate. Kappa statistic was used to evaluate agreement between paired dichotomous data. Knowledge of HCV natural history and treatment were evaluated as the number of correctly answered questions. To estimate the density of the scores, we used kernel density estimation methods with a Gaussian kernel and the corresponding bandwidth parameter was automatically selected *via* the R function "density". Logistic regression was used to assess the effect of patients' characteristics on the birth cohort based screening. Linear regression was used to evaluate the effect of patient characteristics on the knowledge of HCV natural history and treatment. Post-stratification was used to compensate for the fact that physicians with certain characteristics are not as likely to respond to the survey. We use weighting to adjust the regression results with weights being the percentages of the levels of the variable "primary care location" (the response rates of the three levels of this variable are significantly different) thereby extending the results from the responders to the entire population. In linear regression, Box-Cox transformation was used to achieve normality of knowledge of HCV natural history and treatment. The significance level in all tests (2-sided) was set to = 0.05. Predictors that were evaluated for HCV knowledge (both for natural history and treatment) were gender, prior experience in evaluating patients with HCV infection (*i.e.*, at least one HCV-infected patient evaluated in the past two years), clinical practice locations and level of medical training among those currently in medical training.

RESULTS

Study participants

A total of 163 surveys were distributed to PCP who were randomly selected from the population satisfying the

Table 1 Information about the entire population invited to complete the survey (*n* = 163)

Variable	Total	Level	*n*	Percent
Completed survey	163	No	72	44.2
		Yes	91	55.8
Gender	163	Female	80	49.1
		Male	83	50.9
Primary practice location	163	Buffalo general medical center	33	20.3
		Erie county medical center	56	34.4
		Others	74	45.4
Role in primary care clinic	163	Resident in training	134	82.2
		Supervising physician/attending	29	17.8
Level of training[1]	143	Resident PGY1	48	33.6
		Resident PGY2	44	30.8
		Resident PGY3	40	28.0
		Resident PGY4 and above	11	7.7

[1]Twenty subjects had missing values the variable "level of training". PGY: Post-graduate year.

Table 2 Baseline characteristics of those individuals who responded to the survey (*n* = 91) from among the entire population invited to complete the survey (*n* = 163)

Variable	Total[1]	Level	Count/Mean	Percent/SD
Gender	91	Male	50	55.0
		Female	41	45.1
Specialty of practice	91	Family medicine	9	9.9
		Others	82	90.1
Primary practice location	91	Erie county medical center	36	39.6
		Buffalo general medical center	29	31.9
		Others	26	28.6
Evaluated at least one HCV patient in past 2 yr	90	Yes	47	52.2
		No	31	34.4
		Not Sure	12	13.3
Role in primary care clinic	91	Supervising physician/attending	15	16.5
		Resident in training	76	83.5
Level of training	85	Resident PGY1	25	29.4
		Resident PGY2	30	35.3
		Resident PGY3 or above	30	35.3
Awareness of age-based rule for screening	85	Yes	49	57.6
		No	36	42.4
Implementation of age-based rule for screening	85	Yes	34	40.0
		No	51	60.0
Knowledge of HCV natural history	85	Scores from 0 to 18	10.6	4.7
Knowledge of HCV treatment	82	Scores from 0 to 19	11.0	2.9

[1]Missing values account for difference between number of responses recorded and the total number of survey respondents (*n* = 91). HCV: Hepatitis C virus; PGY: Post-graduate year.

eligibility criteria, and 91 (55.8%) responded. Baseline characteristics extrapolated to the entire population to whom the survey was distributed and those of the responders are illustrated (Tables 1 and 2, respectively). The survey was distributed to an approximately equal percentage of males and females, most of whom were in training (82.2%), and who had their primary practice location at one of the two principal UB-affiliated hospitals. Among the respondents, 54.9% were male, 45.1% female and 90.1% practiced internal medicine or its combined tracks. Residents in training comprised of 83.5% of the respondents, and the remaining were attending/supervising physicians. Practice location was predominantly a university-affiliated county hospital-based primary care clinic (39.6%). Electronic medical charting was used by 80.2% of the respondents.

PCP knowledge of HCV natural history and treatment

The distribution of scores indicating the number of correctly answered questions associated with the 18 items that assessed PCP knowledge of HCV natural history is illustrated in Supplementary Figure 1. Figure 1A illustrates the corresponding kernel density of the scores computed using a Gaussian kernel and a bandwidth parameter equal to 1.405. The density plot indicates that knowledge of HCV natural history is spread among three groups: (1) A group with "low" knowledge of HCV natural history; (2) a group constituting the majority of respondents has "moderate" knowledge, and (3) a smaller group of PCP with high knowledge.

The distribution of the scores associated with the 19 questions that assessed PCP knowledge of HCV

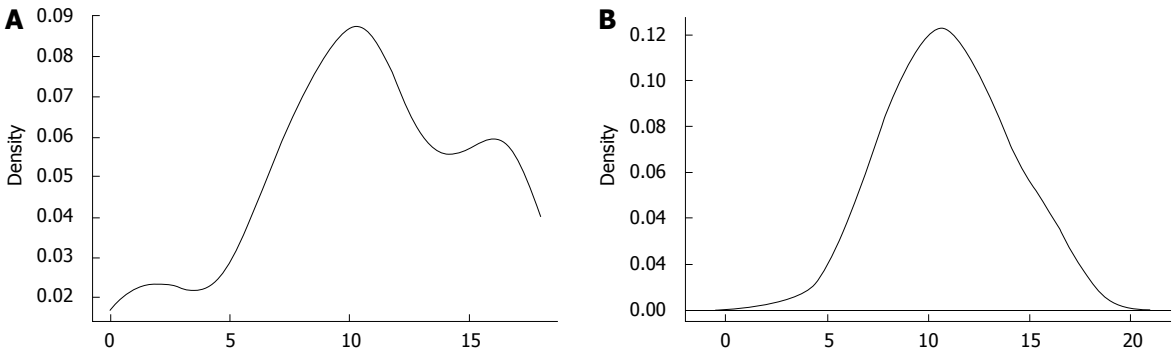

Figure 1 Distribution of responses for knowledge of hepatitis C virus natural history and treatment. A: Plot of the density of scores for the variable "Knowledge of hepatitis C virus (HCV) Natural History". The density estimation uses a Gaussian kernel with bandwidth 1.405. The data illustrate that HCV natural history knowledge is spread among three separate groups: those with low knowledge, the majority that has moderate knowledge, and a smaller group with vast knowledge; B: Plot of the density of scores for the variable "Knowledge of HCV Treatment". The density estimation uses a Gaussian Kernel with bandwidth 1.394. The plot illustrates the distribution of scores for primary care physicians (PCP) knowledge of HCV treatment. Out of a total of 19 possible points, most PCP knowledge scores were greater than 10 with knowledge symmetrically distributed around a score of 11.

Table 3 Regression analysis for Box-Cox transformed knowledge of hepatitis C virus natural history

Variable	Level	Estimation	SD	*P* value
Intercept		-24.90	1.39	0.001
Knowledge of HCV treatment		5.93	0.10	0.001
Gender	Male		Reference level	
	Female	0.04	0.54	0.94
Primary practice location	Erie county medical center		Reference level	
	Buffalo general medical center	0.54	0.77	0.49
	Others	-0.58	0.61	0.35
At least one HCV patient in past 2 yr	Yes		Reference level	
	No/not Sure	-0.54	0.65	0.41
Level of training	Resident PGY 1		Reference level	
	Resident PGY 2	-0.98	0.83	0.24
	Resident PGY 3 and above	0.06	0.78	0.94
Awareness of age-based rule for screening	Yes		Reference level	
	No	-0.92	0.65	0.17
Implementation of age-based rule for screening	Yes		Reference level	
	No	-0.07	0.65	0.91

The table illustrates those factors significantly associated with knowledge of HCV natural history. HCV: Hepatitis C virus; PGY: Post-graduate year.

treatment is illustrated in Supplementary Figure 2. Scores are calculated as previously, that is, each question that is answered correctly receives 1 point, for a total of 19 points. In contrast to the results obtained for HCV natural history knowledge, using a Gaussian kernel with bandwidth parameter 1.394, the corresponding density plot (Figure 1B) illustrates that most PCP have knowledge scores greater than 10 with general knowledge symmetrically distributed around a score of 11.

Predictors of PCP knowledge of HCV natural history and treatment: We next utilized linear regression to evaluate the association between various predictors and two principle outcome measures, knowledge of HCV natural history and of HCV treatment. Both outcome variables are represented by the total scores obtained in answering the relevant questions and are of interval scale. To satisfy the assumption of normality, we use the Box-Cox transformation with $\lambda = 1.116$ for the scores corresponding to knowledge of HCV treatment and with $\lambda = 1.75$ for the scores corresponding to knowledge of HCV natural history. Further, the percentages from the primary care location variable corresponding to the population surveyed (20.25%, 34.35%, and 45.40%) are treated as weights for the observations. In this way, we adjust the regression results to also include those who did not respond to the survey.

We found that knowledge of HCV natural history is significantly associated with knowledge of HCV treatment (*P*-value < 0.01), after adjusting for all relevant predictors (Table 3). That is, PCP with higher knowledge of HCV treatment tended to have higher knowledge of HCV natural history. Also, we found that knowledge of HCV treatment is significantly associated with awareness of the age-based screening recommendation (*P*-value = 0.03, Table 4), indicating that PCP who are aware of the age-based screening recommendation for HCV tend to have higher knowledge of HCV treatment. These findings suggest that knowledge of HCV natural history is a prerequisite for knowledge about HCV treatment. Similarly, those who were aware of age-based HCV screening recommendations also

Table 4 Regression analysis for Box-Cox transformed knowledge of hepatitis C virus treatment

Variable	Level	Estimation	SD	*P* value
Intercept		11.98	2.12	0.001
Knowledge of HCV natural history	score < 6		Reference level	
	6 ⩽ score < 15	-0.68	1.38	0.62
	15 ⩽ score	-2.42	1.64	0.14
Gender	Male		Reference level	
	Female	0.31	0.86	0.72
Primary practice location	Erie county medical center		Reference level	
	Buffalo general medical center	0.27	1.20	0.82
	Others	-1.44	0.95	0.13
At least one HCV patient in past 2 yr	Yes		Reference level	
	No/not sure	0.88	1.02	0.39
Level of training	Resident PGY 1		Reference level	
	Resident PGY 2	1.51	1.30	0.25
	Resident PGY 3 and above	1.76	1.25	0.16
Awareness of age-based rule for screening	Yes		Reference level	
	No	-2.21	1.00	0.03
Implementation of age-based rule for screening	Yes		Reference level	
	No	1.01	1.01	0.32

The table illustrates those factors significantly associated with knowledge of HCV treatment. HCV: Hepatitis C virus; PGY: Post-graduate year.

Table 5 Regression analysis for implementation of birth cohort screening recommendations

Variable	Level	Estimate	SD	*P* value
Intercept		-4.48	2.19	0.04
Knowledge of HCV treatment		-0.02	0.11	0.86
Knowledge of HCV natural history		0.27	0.08	0.002
Gender	Male		Reference level	
	Female	-0.94	0.68	0.17
Primary practice location	Erie county medical center		Reference level	
	Buffalo general medical center	0.77	0.81	0.34
	Others	-0.36	0.72	0.62
At least one HCV patient in past 2 yr	Yes		Reference level	
	No/not sure	2.43	0.90	0.001
Level of training	Resident PGY1		Reference level	
	Resident PGY2	1.57	1.18	0.19
	Resident PGY3 or above	1.62	1.15	0.16
Awareness of age-based rule for screening	Yes		Reference level	
	No	-2.32	0.88	0.01

The table illustrates those factors significantly associated with implementation of birth cohort screening recommendations. HCV: Hepatitis C virus; PGY: Post-graduate year.

scored better on the HCV knowledge assessment.

Predictors for implementation of birth cohort-based screening: Next we focused on implementation of the age-based screening recommendation. As this is the primary outcome variable of interest, we used logistic regression to identify potential significant associations between this variable and a number of predictor variables. As above, we treat the percentages from the primary care location variable as weights for the observations to adjust the results for those invited but who did not complete the survey. We found that the implementation of age-based screening for HCV is significantly associated with knowledge of HCV natural history (*P*-value < 0.01), with awareness of birth cohort based screening (*P*-value = 0.01), and with whether the PCP had seen HCV patients previously (*P*-value < 0.01) (Table 5). Therefore, PCP who have higher HCV natural history knowledge levels, who have not seen HCV patients in the past two years, and who are aware of birth cohort recommendations for HCV are more likely to implement age-based screening in their practices.

Analysis of reasons for and appropriateness of obtaining an HCV screening test: At the time of the survey, the majority of the PCP had ordered an HCV screening test in the recent past (88.2%) (Table 6). Of the reasons that PCP decided to pursue HCV screening, the leading reason (49.4%) was abnormal liver function or other biochemical tests while 30.6% of the PCP cited HCV risk factors, and 20% cited membership in the "birth cohort" as the leading reasons to screen for HCV. In terms of PCP ordering the appropriate screening test, 55.3% of the respondents identified the correct initial screening test as the HCV antibody. In addition, 28.2% of the PCP indicated that they do not follow any society

Table 6 Primary care physician screening practices for hepatitis C virus infection

Question	Total	Option	Count	Percent
In the past 2 yr, have you ordered a test with an intention to screen for HCV?	85	Yes	75	88.2
		No or Not Sure	10	11.8
What is the strongest indication to screen for HCV?	85	Risk factor identified on patient encounter	26	30.6
		Patients born between 1945-1965	17	20.0
		Abnormal liver enzymes	42	49.4
How have you screened for hepatitis C?	85	HCV antibody	47	55.3
		Anti HCV antibody and HCV RNA PCR	11	12.9
		Other combinations of Anti HCV antibody, HCV RNA, liver function tests, and "let the lab choose"	27	31.8
Do you follow professional society guidelines for HCV screening?	85	Yes	61	71.8
		No	24	28.2

HCV: Hepatitis C virus.

Table 7 Primary care physician practice patterns for hepatitis C virus screening

Question	Total	Option	Count	Percent
How often are HCV risk factors assessed during a clinic visit?	85	Always	14	16.5
		Often	30	35.3
		Sometimes	25	29.4
		Rarely or never	16	18.8
Do you order an HCV screening test after identifying at least one risk factor?	85	Always	28	32.9
		Often	30	35.3
		Sometimes or rarely	27	31.8
Do you document HCV screening discussion/risk factor assessment in the health maintenance section of the patient's chart?	85	Always or often	20	23.5
		Sometimes	24	28.2
		Rarely	29	34.1
		Never	12	14.1

HCV: Hepatitis C virus.

guidelines when ordering an HCV screening test.

We also assessed PCP HCV screening practice patterns including how often PCP assessed HCV-related risk factors (Table 7). A total of 81.2% of providers assessed for HCV risk factors at least sometimes. In terms of how the knowledge of the presence of HCV risk factors was utilized, 68.2% of PCP frequently or always ordered an HCV screening test after identification of at least one HCV risk factor. Documentation of a discussion with the patient concerning screening and risk assessment was performed always or often by 23.5% of PCP, whereas 14.1% never documented the screening discussions and risk assessment in the patient's chart.

PCP perceptions to screening for HCV: With regards to HCV screening, only 30.6% of PCP was satisfied with the existing screening approaches utilized at their practice site (Table 8). The most effective strategy to screen patients in the clinic was incorporation of both risk-based and birth cohort-based screening. We also evaluated the PCP's perception as to the most effective way to initiate HCV screening, to which 55.3% suggested that having the patient complete a screening questionnaire during the waiting period prior to the evaluation was the most effective strategy.

PCP barriers to screening for HCV: We next evaluated PCP-identified barriers in their practice location for effective screening for HCV (Table 9). Constraints in the allotted time with the patient to obtain all risk factors were cited as a barrier by 14.1% of participants. An additional 14.1% mentioned that unawareness of screening guidelines among PCPs was a barrier to HCV screening. Lack of a pre-set health maintenance evaluation protocol for the clinic location, such as automatic stop prompts and screening alerts, in combination with the above two reasons were cited as barriers by 16.4% of participants. Other reasons were mentioned by 55.3% of respondents and are described in the last column of Table 9.

DISCUSSION

The development of DAAs has resulted in the need for significant expansion in the number of providers who can treat HCV. While PCP have always had a role in detection and counseling for HCV[8], their scope of practice is expanding to include HCV treatment. Several recent studies have documented equivalent HCV eradication rates whether patients were treated by PCP, hepatologists, or infectious diseases physicians[9,10]. Recent investigation has also documented that family medicine training program directors believe that HCV is a significant problem for family physicians and that HCV

Table 8 Primary care physician perceptions toward screening for hepatitis C virus

Question	Total	Option	Count	Percent
Satisfied with the screening approach in the clinic	85	Yes	26	30.6
		No	25	29.4
		Not Sure	34	40.0
What is the most effective strategy in screening HCV in your clinic	85	Incorporate risk based screening	19	22.4
		Incorporate birth cohort based screening	11	12.9
		Incorporate both risk based and birth cohort screening	55	64.7
Most effect way to initiate screening during a clinic visit	85	Have patient fill out a screening questionnaire during wait period	47	55.3
		Incorporate mandatory screening questions into EMR	19	22.4
		Facilitate screening by use of posters in patient rooms	9	10.6
		Printed patient handout about screening	10	11.7

HCV: Hepatitis C virus; EMR: Electronic medical record.

Table 9 Primary care physician identified barriers to screening for hepatitis C virus (n = 85)

Option	Count	Percent
Inconsistency in offering HCV screening as a part of pre-set health maintenance protocol, time constraints in obtaining all HCV risk factors, unawareness of screening guidelines	14	16.5
Time constraints in obtaining all HCV risk factors	12	14.1
Unawareness of screening guidelines	12	14.1
Other combinations of inconsistency in offering HCV screening as a part of pre-set health maintenance protocol, time constraints in obtaining all HCV risk factors, taboo in asking confidential and personal information as outlined in the screening questionnaire, and unawareness of screening guidelines	47	55.3

HCV: Hepatitis C virus.

education should be part of their training curriculum[14]. As limited data exist on PCP knowledge of HCV natural history, screening and treatment and the effect of this knowledge on implementation of age-based screening, we performed this investigation primarily to understand the factors associated with implementation of HCV screening recommendations and to identify gaps in HCV-related knowledge among PCP.

Broadly, we found that knowledge of HCV natural history is a prerequisite for implementation of HCV screening. More specifically, we found that knowledge of particular recommendations, in this case those associated with birth cohort screening, were necessary for their implementation. We also observed that knowledge of HCV treatment was significantly associated with knowledge of HCV natural history, indicating the need to educate PCP about the entire HCV disease process. Among our survey respondents, laboratory abnormalities were the single most important indication to screen for HCV as opposed to screening based upon guideline implementation, and most PCP endorsed the combination of risk-factor and birth cohort screening utilizing a self-administered survey. These findings indicate that HCV educational interventions targeted toward PCP should be comprehensive covering all aspects of the infection. Furthermore, they should emphasize guideline-based screening recommendations.

We found that PCP knowledge of the natural history of HCV positively impacts the implementation of birth cohort based screening by PCP. Increasing HCV screening is required to identify the 40% of HCV-infected individuals who are unaware of their infection status[6], so that the stated national goal of HCV elimination can be realized[24]. Substantial HCV-related knowledge and familiarity with HCV screening may enable PCP to offer HCV treatment thereby providing additional linkage-to-care opportunities[25]. A recent study reported that only 22% of PCP believed they should treat HCV. PCP who managed a high proportion of HCV-infected patients and practices that actively managed a variety of related conditions (HIV, mental health and substance use disorders) were factors significantly associated with a higher likelihood of offering HCV treatment[13]. HCV education targeted to PCP is likely to play an important role in increasing the number of treating physicians.

Ongoing HCV education to PCP is also required given the rapid progress in our understanding of HCV natural history and recent therapeutic advances. For example, recent data have confirmed all-cause and liver-specific mortality reductions as a result of HCV eradication[26-30]. Similarly, further PCP education is needed not only on when to screen, *i.e.,* basing screening on guidelines instead of on clinical abnormalities, but how to screen. Indeed, we would encourage pharmaceutical companies to continue to invest in PCP education through sponsorship of educational programs targeted to PCP. Our survey revealed that many PCP continue inappropriate and ineffective HCV screening strategies, such as obtaining aminotransferase or HCV RNA levels, instead of obtaining an initial HCV antibody assessment.

Physician engagement is crucial to successful guideline implementation, and an initial crucial step is guideline awareness[31]. Our study illustrates that physician knowledge of HCV natural history, as well as awareness of birth cohort screening recommendations, were associated with age-based screening implementation. PCP knowledge of HCV treatment did not predict implementation of birth cohort screening, but was associated with knowledge of natural history of HCV. PCP knowledge gaps have been cited as obstacles to liver disease screening for hepatocellular carcinoma surveillance among cirrhotic patients[32] and in hepatitis B[33] infection. Education targeted to patients may also improve screening and linkage to care. Consequently, education to both providers and to patients is extremely important toward achieving HCV elimination. Linking HCV screening with treatment programs will also be tremendously important toward achieving the goal of eradication.

Study strengths include a reasonable number of survey responses obtained from physicians in training, an important population for HCV treatment workforce expansion. Furthermore, we obtained a reasonable response rate obtaining responses from one half of those to whom the survey was distributed. Additionally, weighting PCP responses permitted inference to those physicians who did not complete the survey, and we also assessed the instrument's internal validity. Study limitations include responses largely from PCP in training at an academic medical center, which may affect generalizability to community-based PCP. Additional limitations include a relatively small sample size and responses obtained *via* a self-administered online questionnaire. Future investigation should endeavor to include additional respondents at other academic centers.

HCV-related knowledge gaps among PCP must be addressed in order to increase the HCV workforce leading to increased opportunities for HCV screening and engagement into care. Our study illustrates how PCP knowledge of HCV natural history and treatment can influence birth cohort-based screening practices. It also provides insights into PCP attitudes and barriers toward HCV screening in the primary care setting. As PCP engagement is paramount to successful intervention implementation, our study highlights topics needed for provider-based educational interventions designed to optimize HCV screening in clinical practice.

ARTICLE HIGHLIGHTS

Research background

In order to achieve global hepatitis C virus (HCV) eradication, it is crucial to increase HCV diagnosis and linkage-to-care.

Research motivation

In many countries, primary care physicians (PCP) care for those who are HCV-infected yet undiagnosed. Increasing PCP willingness to screen and to treat HCV is crucial to its global eradication. Understanding promotors or barriers to HCV screening and linkage-to-care among PCP, especially the role of knowledge of the infection and screening guidelines, is crucial to expansion of the HCV workforce.

Research objectives

We sought to assess PCP knowledge about HCV natural history and treatment as well as with regard to implementation of birth cohort screening recommendations.

Research methods

We administered a 45-item survey to 163 PCP, 82% of whom were in training in internal or family medicine.

Research results

PCP knowledge of HCV natural history and prior management of HCV patients were important predictors of implementation of HCV screening. Clinical abnormalities remained the leading indication for ordering an HCV screening test.

Research conclusions

Comprehensive HCV education targeted to PCP, including screening recommendations, is critical to increase HCV detection and linkage-to-care to obtain global eradication. Familiarity with HCV management increased the likelihood that PCP would care for HCV-infected patients.

Research perspectives

Increasing physician education should lead to increased HCV screening. Linking HCV screening to treatment is crucial to obtain global HCV eradication.

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ORIGINAL ARTICLE

Observational Study

Outcomes assessment of hepatitis C virus-positive psoriatic patients treated using pegylated interferon in combination with ribavirin compared to new Direct-Acting Antiviral agents

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Author contributions: Damiani G and Pigatto P designed the research; Franchi C, Pigatto P and Pacifico A performed the research; Damiani G analyzed the data; Altomare A and Pace MC supervised the manuscript; Petrou S revised the manuscript in order to improve and polish language; Damiani G, Leone S and Fiore M wrote the paper.

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Abstract

AIM

To evaluate the outcomes in biological treatment and quality of life of psoriatic patients with chronic hepatitis C (CHC) treated with new Direct-Acting Antiviral agents (DAAs) compared to pegylated interferon-2α plus ribavirin (P/R) therapy.

METHODS

This is a retrospective study involving psoriatic patients in biological therapy who underwent anti-hepatitis C virus (HCV) treatment at the Department of Dermatology Galeazzi Orthopaedic Institute Milan, Italy from January 2010 to November 2017. The patients were divided into two groups: patients that underwent therapy with DAAs and patients that underwent HCV treatment with P/R. Patients were assessed by a dermatologist for psoriasis symptoms, collecting Psoriasis Area Severity Index (PASI) scores and the Dermatology Quality of Life Index (DLQI). PASI and DLQI scores were evaluated 24 wk after the end of HCV treatment and were assumed as an outcome of the progression of psoriasis. Switching to a different bDMARD was considered as an inadequate response to biological therapy. The dropout of HCV therapy and sustained virological response (SVR) were considered as outcomes of HCV therapy.

RESULTS

Fifty-nine psoriatic patients in biological therapy underwent antiviral therapy for CHC. Of this, 27 patients were treated with DAAs and 32 with P/R. After 24 wk post treatment, the DLQI and the PASI scores were significantly lower ($P < 0.001$ and $P < 0.005$, respectively) in the DAAs group compared with P/R group. None of the patients in the DAAs group (0/27) compared to 8 patients of the P/R group (8/32) needed a shift in biological treatment.

CONCLUSION

DAAs seem to be more effective and safe than P/R in HCV-positive psoriatic patients on biological treatment. Fewer dermatological adverse events may be due to interferon-free therapy.

Key words: Hepatitis C virus; New Direct-Acting Antiviral agents; Psoriasis; Biological disease modifying drugs

Core tip: Psoriasis is a chronic inflammatory disease affecting approximately the 2% of population in Europe and North America. The hepatitis C virus (HCV) infection affects approximately the 3% of the world population with an estimated prevalence of 5 million people in the United States. Up to 0.06% of people in the United States suffer from both psoriasis and HCV. Psoriatic patients with HCV are excluded by randomized controlled clinical trials. Therefore, no data is currently available concerning the concomitant administration of biological disease modifying drugs and the new Direct-Acting Antiviral agents (DAAs) medications approved for the treatment of HCV infection. The aim of this study is to evaluate the outcomes in biological treatment and quality of life of psoriatic patients with HCV infection treated with DAAs compared to the previous standard therapy of Pegylated Interferon plus Ribavirin.

Damiani G, Franchi C, Pigatto P, Altomare A, Pacifico A, Petrou S, Leone S, Pace MC, Fiore M. Outcomes assessment of hepatitis C virus-positive psoriatic patients treated using pegylated interferon in combination with ribavirin compared to new Direct-Acting Antiviral agents. *World J Hepatol* 2018; 10(2): 329-336 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/329.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.329

INTRODUCTION

Psoriasis is a chronic systemic inflammatory disease affecting approximately 2% of the population in Europe and North America with approximately 7.5 million people in the United States[1,2]. Hepatitis C virus (HCV) is an infection affecting approximately 71 million people worldwide; the most affected regions being WHO Eastern Mediterranean and European Regions with a prevalence of 2.3% and 1.5%[3]. Prevalence of HCV infection in other WHO regions varies from 0.5% to 1.0%[3]. According to the Centers for Disease Control and Prevention, there are currently about 3.3 (2.7-3.9) million people with chronic hepatitis C (CHC) in the United States[4]. The estimated prevalence of both psoriasis and HCV in the United States is 0.02%-0.06% (55000-150000 persons)[5].

The pathogenesis of psoriasis remains unclear, however Th1/Th17 autoimmune involvement has been hypothesized[6]. Its association with HCV is still a matter of debate. Some evidence suggests that psoriasis may be initiated and maintained by both HCV and interferon-α[7]. In a large case-control study, including 12502 psoriatic patients and 24287 controls, the prevalence of HCV in psoriatic patients was increased compared to controls (1.03% *vs* 0.56%; $P < 0.001$)[8].

The therapeutic armamentarium available for psoriasis encompasses the conventional disease modifying drugs (cDMARDs) and biological DMARDs (bDMARDs). cDMARDS represent the first line therapy in high-need psoriatic patients, while bDMARDs are for those subjects in whom cDMARDs have either failed, were not tolerated, or were contraindicated[9]. While bDMARDs are generally safer than cDMARDs, there is one concern emerging from registries or long-term studies which is an increased risk of infection. When patients lose an adequate response to bDMARDs the possible options include increasing the dose, shortening the dosing interval, combination therapy with a topical or another systemic treatment, or switching to a different drug[10].

Recently, the standard of care for CHC has changed. The new Direct-Acting Antiviral agents (DAAs) such as Sofosbuvir, Daclatasvir and the Sofosbuvir/Ledipasvir

combination are now part of the preferred regimens in the WHO guidelines and can achieve cure rates above 95%[3]. These agents are much more effective, safer and better-tolerated than older therapies involving pegylated interferon and ribavirin (P/R) which nowadays play a very limited role for specific scenarios[3]. Of the 71 million persons living with CHC globally in 2015, 20% (14 million) knew their diagnosis and 7.4% of those diagnosed (1.1 million) were started on treatment[3].

Psoriatic patients with CHC are excluded by randomized controlled clinical trials. Data on psoriatic patients with HCV infection treated with bDMARDs is available solely on the reports of single cases or analyses of small groups of patients. Therefore, no data is currently available concerning the concomitant administration of bDMARDs for psoriasis and the new DAAs approved for the treatment of CHC[9].

The aim of this study is to evaluate the disease progression and outcomes in biological treatment and quality of life of HCV psoriatic patients on bDMARDs also treated with new DAAs compared to P/R therapy.

MATERIALS AND METHODS

Study population

This is an observational analysis performed by checking all psoriatic patients admitted to the Department of Dermatology and Venereology of I.R.C.C.S. Istituto Ortopedico Galeazzi (Milan, Italy) after 2007. Furthermore, in the present study we collected only HCV positive psoriatic patients admitted in our department from 2010 until now undergoing biological therapy. The study complies with the Declaration of Helsinki and all patients signed a standard consent regarding sensitive personal data treatment.

Study design

The present study is a spin-off observational retrospective study on HCV psoriatic patients, resulted as re-analysis of a previous study towards the re-circulation of CD4+ memory T-cells in psoriatic patients, approved by the Institutional Review Board (Comitato Etico dell'Ospedale San Raffaele, Milano)[11]. This previous study had as exclusion criterion the absence of acute and chronic systemic or cutaneous infections during sample collections[11], conversely we enrolled the previously discarded HCV psoriatic patients, focusing on their HCV eradication management. The subjects were divided into two groups: (1) Patients that underwent therapy with DAAs (DAA-group) (2) patients that underwent HCV treatment with P/R (P/R-group). Patients were assessed by a dermatologist for psoriasis symptoms, collecting Psoriasis Area Severity Index (PASI) and Dermatology Quality of Life Index (DLQI). They were also assessed by a hepatologist to determine liver function while collecting biochemical and clinical data in two times: Baseline (before HCV treatment) (T0) and 24 wk after the end of HCV treatment (T1). The data was extracted to monitor psoriasis biological therapy and HCV therapy[12,13].

Inclusion criteria

Inclusion criteria were age > 18 years old, signed consent forms, no previous transplantation, no pregnancy, no hereditary hepatic diseases, no drug addiction, and no alcohol abusers [Alcohol Use Disorders Identification Test (AUDIT) score < 7], negative results at screening tuberculosis, absence of acute and chronic systemic or cutaneous infections during sample collection. Psoriasis diagnosis was performed by a Dermatologist following the Psoriasis Italian Guidelines[10], and the diagnosis of CHC according the WHO guidelines[14].

Exclusion criteria

Exclusion criteria were age < 18 years old, pregnancy, drug addiction, alcohol abusers [Alcohol Use Disorders Identification Test (AUDIT) score > 7], HIV infection.

Outcomes of the study

PASI, DLQI were evaluated 24 wk after the end of HCV treatment (T1) and were assumed as an outcome of the progression of psoriasis. Switching to a different bDMARD was considered as inadequate responses to biological therapy. The dropout of HCV therapy, and sustained virological response (SVR) were considered as outcomes of HCV therapy.

Data collection and variables definitions

Baseline clinical characteristics, medical history, biochemical variables, and pharmacologic treatments employed during hospitalization were retrospectively collected and recorded on a computer database. SVR was defined as a confirmed undetectable serum HCV-RNA level 24 wk after the discontinuation of HCV therapy. Patients not fulfilling the SVR definition criteria were classified as non-SVR.

Statistical analysis

Continuous variables are presented as mean ± SD and categorical variables are presented as absolute values and percentages. Comparisons between continuous variables were performed using Mann–Whitney or Kruskall–Wallis tests and comparisons between categorical variables were performed using the χ^2 or Fisher's exact test. Statistical significance was defined as $P < 0.05$. Data analysis was performed using statistical software R-version 3.2.4.

RESULTS

A total of 59 psoriatic patients met the inclusion criteria (27 in the DAA-group and 32 in the P/R-group) and were included in this analysis. The patients' main characteristics are summarized in Table 1. All the patients in the P/R-group were treated with pegylated interferon-2α plus ribavirin; in the DAA-group 25 patients (92.6%) were treated with Sofosbuvir plus Daclatasvir, 1 patient was treated with Sofosbuvir + ribavirin: 1 (3.7%) and 1 patient (3.7%) was treated with Sofosbuvir plus simeprevir plus ribavirin. The median age was 56.7 ±

Table 1 Levels of sIL-2R, ALT, and HBV DNA in the sera of patients with chronic hepatitis B virus infection (mean ± SD)

	DAA-group	P/R-group
Age, yr, mean ± SD	56.7 ± 8.9	58.2 ± 6.7
Male/female, *n* (%)	18/9 (66.7/33.3)	24/8 (75/25)
BMI, mean ± SD, kg/m^2	24.3 ± 2.41	25 ± 1.86
Psoriasis duration, yr, mean ± SD	21.5 ± 8.6	18 ± 6.7
Current biological therapy, *n* (%)		
Etanercept	12 (44.4)	15 (46.9)
Adalimumab	9 (33.3)	10 (31.3)
Infliximab	-	-
Ustekinumab	5 (18.5)	7 (21.9)
Secukinumab	1(3.7)	-
Shifted biological drugs, mean ± SD	1.2 ± 0.3	1.5 ± 0.5
Psoriatic arthritis, *n* (%)	2 (7.4)	3 (9.4)
HCV		
Genotype, *n* (%)		
1	21 (77.8)	29 (90.6)
2	5 (18.5)	1 (3.1)
3	-	2 (6.3)
4	1 (3.7)	-
5/6	-	-
MELD score	9.3 ± 2.5	6,5 ± 0.8
HCV viral load (T0), IU/mL, mean ± SD	6.2 log10 ± 5.7 log10	6.1 log10 ± 6.0 log10
SVR, *n* (%)	27/27 (100)	12/32 (37.5)
Autoimmune comorbidities, *n* (%)		
Rheumatic arthritis	1 (3.7)	-
Ankylosing spondylitis	-	1 (3.1)
Systemic erythematosus lupus	1 (3.7)	-
Other comorbidities, *n* (%)		
Cardiovascular disease	6 (22.2)	3 (9.4)
Metabolic syndrome	2 (7.4)	1 (3.1)
COPD	5 (18.5)	2 (6.3)
Renal insufficiency	0 (0)	0 (0)
HBV	1 (3.7)	1 (3.1)
	0 (0)	0 (0)

Categorical variables are expressed as *n* (%). Numeric variables are expressed as median and SD. BMI: Body mass index; COPD: Chronic obstructive pulmonary disease; DAA: New Direct-Acting Antiviral agent; HBV: Hepatitis B virus; HCV: Hepatitis C virus; P/R: Pegylated interferon-2α plus ribavirin; MELD: Model for end-stage liver disease; SVR: Sustained virological response.

8.9 years in the DAA-group and 58.2 ± 6.7 years in the P/R-group, respectively; 66.7% patients were men in the DAA-group and 75% patients were men in the P/R-group, respectively. The median body mass index (BMI) was 24.3 ± 2.41 in the DAA-group and 25 ± 1.86 in the P/R-group, respectively. The median psoriasis duration was 21.5 ± 8.6 years in the DAA-group and 18 ± 6.7 years in the P/R-group, respectively. In the DAA-group the bDMARD used, was Etanercept, Adalimumab, Ustekinumab and Secukinumab in 12 (44.4%), 9 (33.3%), 5 (18.5%), and 1 (0.04%) patients, respectively; in the P/R-group the bDMARD used, was Etanercept, Adalimumab and Ustekinumab in 15 (46.9%), 10 (31.3%) and 7 (21.9%) patients, respectively. Switching among biologic therapies, before hepatitis C treatment, was 1.2 ± 0.3 in DAA-group and 1.5 ± 0.5 in P/R-group. Two patients (7.4%) of the DAA-group and 3 patients (9.4%) of the P/R-group presented psoriatic arthritis, respectively. In the DAA-group the HCV genotypes were 1, 2 and 4 in 21 (77.8%), 5 (33.3%) and 1 (3.7%) patients, respectively; in the P/R-group the HCV genotypes were 1, 2 and 3 in 29 (90.6%), 1 (3.1%) and 2 (6.3%) patients, respectively. The MELD score was 9.3 ± 2.5 in DAA-group and 6.5 ± 0.8 in the P/R-group, respectively. The viral load at the baseline was 6.2 $\log_{10}$ ± 5.7 $\log_{10}$ UI/mL in the DAA-group and 6,1 $\log_{10}$ ± 6.0 $\log_{10}$ UI/mL in the P/R-group, respectively. All the 27 patients (100%) in the DAA-group obtained SVR, whereas only 37.5% (12/32) of patients in the P/R-group achieved a SVR. Two out of 27 patients of DAA-group had autoimmune comorbidities: 1 (3.7%) rheumatic arthritis and 1 (3.7%) systemic erythematosus lupus; 1 (3.1%) out of 32 patients of P/R-group had autoimmune comorbidity: ankylosing spondylitis. In the DAA-group other comorbidities were cardiovascular diseases, metabolic syndrome, chronic obstructive disease, and HBV in 6 (22.2%), 2 (7.4%), 5 (18.5), 1 (3.7%) patients, respectively. In the P/R-group other comorbidities were cardiovascular diseases, metabolic syndrome, chronic obstructive disease, and HBV in 3 (9.4%), 1 (3.1%), 2 (6.3%), 1 (3.1%) patients, respectively. None of the 59 psoriatic patients enrolled in the study had renal insufficiency.

The comparison between the patient scores and laboratories findings according to HCV eradication treatment is shown in Table 2: There were no differences in PASI scores between the 2 HCV treatment groups; after 24 wk to the end of the HCV treatment there

Table 2 Levels of sIL-2R, ALT, and HBV DNA in the sera of patients with chronic hepatitis B virus infection (mean ± SD)

	DAA-group	P/R-group	*P* value
PASI (T0), mean ± SD	11.6 ± 5.2	9.4 ± 3.5	-
PASI (T1), mean ± SD	5.2 ± 1.6	8.3 ± 4.5	< 0.005
Biological treatment shifts, *n* (%)	-	8 (25)	< 0.001
Topical treatments (T0), *n* (%)	27 (100)	32 (100)	-
Topical treatments (T1), *n* (%)	27 (100)	32 (100)	-
DLQI (T0), mean ± SD	13 ± 2.3	12 ± 3.1	-
DLQI (T1), mean ± SD	4.2 ± 2.3	11 ± 2.3	< 0.001
HCV treatment details, *n* (%)	Sofosbuvir + daclatasvir: 25 (92.6) Sofosbuvir + simeprevir + ribavirin: 1 (3.7) Sofosbuvir + ribavirin: 1 (3.7)	Pegylated interferon-2α + ribavirin 32 (100)	-
Laboratory tests, mean ± SD			
T0			
ALT	45.6 ± 16.5	43 ± 9.2	-
AST	54.6 ± 5.51	52.4 ± 12.5	-
GGT	42.0 ± 13.59	43.3 ± 14.6	-
T1			
ALT	42.21 ± 12.4	43 ± 8.5	-
AST	51.3 ± 4.2	51.9 ± 8.9	-
GGT	40.8 ± 12.2	42.9 ± 14.1	-
Dropout HCV-treatment, *n* (%)	0/27 (0)	9/32 (28.1)	< 0.005
SVR, *n* (%)	27/27 (100)	12/32 (37.5)	< 0.005

Categorical variables are expressed as *n* (%), and compared by χ^2 or Fisher's exact test. Numeric variables are expressed as median and SD, and compared by Mann–Whitney or Kruskall–Wallis tests. Statistical significance was considered as a *P*-value of < 0.05. DAA: New Direct-Acting Antiviral Agent; P/R: Pegylated interferon-2α plus ribavirin; PASI: Psoriasis Area Severity Index; T0: Baseline time (before starting the hepatitis C eradication treatment); T1: Six months after the end of hepatitis C eradication treatment; DLQI: Dermatology Quality of Life Index; HCV: Hepatitis C virus; SVR: Sustained virological response; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transferase.

was a significant reduction of PASI score in DAA-group when compared to P/R-group patients ($P < 0.005$). Patients in the P/R-group (8 patients, 25%) need a significantly ($P < 0.001$) frequent change of biological treatment compared to DAA-group (no patients). There was no difference in the use of topical treatments in the 2 groups at baseline (100% in both groups) and after 24 wk to the end of the HCV treatment (100% in both groups). Although there was no difference in the DLQI at the beginning of the treatment in the 2 groups, after 24 wk the DLQI was significantly improved in the DAA-group when compared to P/R-group patients ($P < 0.001$). HCV treatment was significantly better tolerated ($P < 0.005$) by DAA-group patients with no dropout; 9 out of 32 P/R-group patients (28.1%) dropped out of HCV treatment. HCV eradication was significantly higher ($P < 0.005$) in DAA-group compared to P/R-group: SVR was obtained in 100% (27/27) of patients treated with DAAs and in 37.5% (12/32) of patients treated with P/R. Three Adverse Drug Events (ADEs) were observed in DAA-group patients during HCV treatment. Two of the patients experienced fatigue and one experienced insomnia. Fifteen of the thirty-two patients of the P/R-group experienced ADEs during HCV treatment. Ten experienced fatigue with nausea, three diarrhea and two cefalea.

DISCUSSION

In our study, psoriatic patients of the DAA-group had a better prognosis compared to the P/R-group, both in progression of psoriasis (and consequent worsening of quality of life) and in eradication of HCV. Patients with a worse prognosis treated with P/R could be attributed to two possible causes: (1) Interferon "itself" has dermatological side effects[15], worsening psoriasis and (2) DAAs are more effective than interferon-based therapy increasing the rates of SVR (even up to 100%)[16], suggesting that HCV could promote psoriatic disease.

Eradication treatment of HCV with interferon has been described as the drug that can induce *de novo* psoriasis or flares in psoriatic patients[17-19]. The flare usually occurs one to 6 wk after starting interferon-α and may lead to its discontinuation[20].

There appears to be an intricate relationship between psoriasis and HCV infection as seen in previous reports. HCV in predisposed individuals upregulates cathelecidin, Toll like receptor 9 (TLR-9) and interferon (IFN)-γ which are all involved in the development of psoriasis plaques. Pegylated interferon may also initiate and maintain psoriasis inflammation by activating Th1 and Th17 *via* myeloid DCs[7,21]. Cathelecidin bonds self-DNA. It is released after various injury stimuli *via* TLR-9 plasmacytoid and DCs to produce type I interferons (α/β), which drives T-cell polarization towards Th1 and Th17 *via* myeloid DCs. This initiates the trigger and further maintenance of psoriasis[7]. Likewise, pegylated interferon may act directly *via* myeloid resident DCs on Th1 cells and also on Th17 cells at the same time by increasing their activation and release of IL-17. IL-17 is a chemo-attractant for neutrophils to the skin and IL-22 causes keratinocyte hyperproliferation[21]. Despite these preliminary hypotheses, data regarding the association of HCV and psoriasis remain unclear and a matter of

debate. Some studies show no association[22] while others show an increased prevalence of psoriasis among HCV-patients as reported by Cohen[8]. Furthermore, limited data is present in literature regarding psoriasis induction and exacerbation due to interferon in HCV-psoriatic patients[21].

Experimental studies have shown that an intradermal injection of IFN-γ, both on the non-lesional psoriatic and healthy skin, causes an elevation of inflammatory products such as TNF, IL-23 and inducible nitric oxide synthase which are characteristic of psoriatic plaques[23]. An association between HCV infection and psoriasis has been suggested. The prevalence of HCV in psoriatic patients was increased compared to controls (1.03% *vs* 0.56%; $P < 0.001$)[8]. In a single center cross-sectional study conducted in a Japanese university hospital, the frequency of HCV infection was significantly higher in psoriatic (7.5%) than in non psoriatic patients (3.3%) in overall ages[24]. Interestingly, when stratified by age at the first visit, HCV infection frequency was significantly higher in patients with psoriasis than in controls aged over 60 years (11.8% *vs* 6.6%, respectively, $P = 0.0215$) and 70 s (19.5% *vs* 7.3%, $P < 0.0001$)[24]. Psoriatic patients with CHC were significantly older at onset than non psoriatic CHC patients (median, 54 *vs* 39 years)[24]. There was also a stronger male predominance (male/female ratio, 4.4:1), similar family history of psoriasis, higher association of diabetes mellitus and hypertension, and significantly lower body mass index, in an age-stratified (≥ 40 years) analysis[24]. Psoriatic patients with CHC were less obese, but still had a higher frequency of diabetes mellitus and hypertension[24]. The authors hypothesized that psoriasis and HCV have pathophysiological factors in common with both mediated by proinflammatory cytokine tumor necrosis factor (TNF-α)[24]. In HCV infection, continuous inflammation mediated by TNF-α leads to liver cirrhosis and diabetes mellitus[24]. The link between psoriasis and HCV infection has been shown experimentally in a recent study of Chun *et al*[7]; the authors performed two 2 mm punch biopsies of lesional and nonlesional skin in 10 patients who were HCV-negative psoriatic and 7 HCV-positive psoriatic patients. The biopsies were used to measure cathelicidin, TLR9 and IFNc mRNA expression by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)[7]. The mRNA expression was calculated relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and demonstrated that the cutaneous levels of inflammatory genes in HCV positive psoriatic patients are higher than the levels in patients with only psoriasis[7]. The increased cutaneous levels of cathelicidin, TLR9 and IFNc of HCV-positive psoriatic patients as compared to HCV-negative psoriatics suggest that HCV infection may predispose patients to developing psoriasis[7]. These findings seem to be confirmed clinically due to the worse prognosis of psoriasis in HCV-positive patients[7,25]. The mean PASI score is significantly higher in cohorts of patients affected with hepatitis C than those with psoriasis alone[7,25].

In line with previous studies, our study confirmed that bDMARDs are safe in psoriatic patients with HCV infection[26-29]. In literature bDMARDs are safe either in psoriatic patients with HCV infection and past HBV infection[30]. Patients in the DAA-group had a significantly better response to bDMARDs compared to P/R-group, requiring a smaller transition to different bDMARDs[10]. This may be due to a more favorable outcome in psoriasis (due to interferon-free therapy and greater HCV eradication), but also due to intrinsic DAAs action[31]; Immune reconstitution occurs in patients with whom HCV was successfully eradicated *via* DAAs therapy[31]. Restoration of the CD4+ T-cell compartment in the peripheral blood and a re-differentiation of the T lymphocyte memory compartment resulted in a more effector memory T cell population and a reduction in expression of the co-inhibitory molecule TIGIT in bulk T lymphocytes[31]. Burchill *et al*[31] observed a partial reversal of the exhausted phenotype in HCV-specific CD8+ T cells and a dampening of the activation state in peripheral NK cells. Spaan *et al*[32] showed that viral load decline, as a consequence of DAAs therapy in patients with chronic hepatitis C infection, reduces serum levels of NK cell-stimulating cytokines and causes correction of the altered NK cell phenotype observed in chronic HCV patients. CHC is characterised by innate immune activation with increased interferon-stimulated gene expression and by an altered phenotype of interferon-responsive natural NK cells[33]. DAAs treatment could improve the pro-inflammatory status due both to psoriasis and to the HCV infection making bDMARDs actions more effective.

The current study contains some limitations that need to be taken into account. This is a retrospective observational study conducted in a single Italian centre, thus our conclusions should be interpreted with caution and cannot be generalized to all HCV-positive psoriatic patients. In particular, a general under-reporting of toxicity, as with all observational studies, is possible. Another limitation is the small sample size and the relatively short follow-up. This is the first study to compare DAAs to P/R for the management of HCV-positive psoriatic patients and unmeasured confounding variables could influence our findings. Thus, larger series with long-term follow-up are required to confirm this preliminary data.

In conclusion, new DAAs are more effective than P/R in the eradication of HCV and the control of symptoms in psoriatic patients with CHC. Future studies are needed to evaluate the effects of DAAs in this clinical setting, which may further aid in elucidating the etiologic and pathogenetic mechanism of psoriasis.

ARTICLE HIGHLIGHTS

Research background

Up to 0.06% of people suffer from both psoriasis and hepatitis C virus (HCV). Psoriatic patients with HCV are excluded by randomized controlled clinical trials.

Research motivation
No data is currently available concerning the concomitant administration of biological drugs and the medications approved for the treatment of HCV infection, as new Direct-Acting Antiviral agents (DAAs).

Research objectives
Evaluate the outcomes in biological treatment and quality of life of psoriatic patients with chronic hepatitis C (CHC) treated with new DAAs compared to pegylated interferon-2α plus ribavirin (P/R) therapy.

Research methods
Psoriatic patients, in biological therapy, who underwent anti-HCV treatment were retrospectively reviewed. The patients were divided into two groups: patients that underwent therapy with DAAs and patients that underwent HCV treatment with P/R. Patients were assessed for Psoriasis Area Severity Index (PASI) scores and the Dermatology Quality of Life Index (DLQI) switching to a different bDMARD, dropout of HCV therapy and sustained virological response (SVR).

Research results
Twenty-seven patients were treated with DAAs and thirty-two with P/R. At three months, after completion of antiviral therapy, the DLQI and the PASI scores were significantly lower ($P < 0.001$ and $P < 0.005$, respectively) in DAAs group compared with P/R group. None of the patients in the DAAs group compared to the eight patients of the P/R group needed a change in biological treatment.

Research conclusions
DAAs seem to be more effective and safe than P/R in HCV-positive psoriatic patients on biological treatment.

Research perspectives
This is the first study which evaluated the HCV treatment of psoriatic patients on biological agents. Future studies are needed to evaluate the effects of DAAs in this clinical setting, which may further aid in elucidating the etiologic and pathogenetic mechanism of psoriasis.

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META-ANALYSIS

Outcomes of kidney transplantation in patients with hepatitis B virus infection: A systematic review and meta-analysis

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Abstract

AIM

To assess outcomes of kidney transplantation including patient and allograft outcomes in recipients with hepatitis B virus (HBV) infection, and the trends of patient's outcomes overtime.

METHODS

A literature search was conducted using MEDLINE, EMBASE and Cochrane Database from inception through October 2017. Studies that reported odds ratios (OR) of mortality or renal allograft failure after

kidney transplantation in patients with HBV [defined as hepatitis B surface antigen (HBsAg) positive] were included. The comparison group consisted of HBsAg-negative kidney transplant recipients. Effect estimates from the individual study were extracted and combined using random-effect, generic inverse variance method of DerSimonian and Laird. The protocol for this meta-analysis is registered with PROSPERO (International Prospective Register of Systematic Reviews; no. CRD42017080657).

RESULTS

Ten observational studies with a total of 87623 kidney transplant patients were enrolled. Compared to HBsAg-negative recipients, HBsAg-positive status was significantly associated with increased risk of mortality after kidney transplantation (pooled OR = 2.48; 95%CI: 1.61-3.83). Meta-regression showed significant negative correlations between mortality risk after kidney transplantation in HBsAg-positive recipients and year of study (slopes = -0.062, *P* = 0.001). HBsAg-positive status was also associated with increased risk of renal allograft failure with pooled OR of 1.46 (95%CI: 1.08-1.96). There was also a significant negative correlation between year of study and risk of allograft failure (slopes = -0.018, *P* = 0.002). These associations existed in overall analysis as well as in limited cohort of hepatitis C virus-negative patients. We found no publication bias as assessed by the funnel plots and Egger's regression asymmetry test with *P* = 0.18 and 0.13 for the risks of mortality and allograft failure after kidney transplantation in HBsAg-positive recipients, respectively.

CONCLUSION

Among kidney transplant patients, there are significant associations between HBsAg-positive status and poor outcomes including mortality and allograft failure. However, there are potential improvements in patient and graft survivals in HBsAg-positive recipients overtime.

Key words: Hepatitis B; Kidney transplant; Kidney; Renal transplantation; Transplantation; Meta-analysis

Core tip: Hepatitis B is one of the most common infectious diseases worldwide. Despite advances in medicine, chronic hepatitis B virus (HBV) infection is currently incurable. In addition, clinical outcomes of kidney transplantation in HBV infected patients are still unclear. To further assess these outcomes, we conducted this systematic review and meta-analysis to assess patient and allograft outcomes after kidney transplantation in patients with HBV Infection. We found significant associations between HBV positive status and poor outcomes including 2.5-fold increased risk of mortality and 1.5-fold increased risk of allograft loss.

Thongprayoon C, Kaewput W, Sharma K, Wijarnpreecha K, Leeaphorn N, Ungprasert P, Sakhuja A, Cabeza Rivera FH, Cheungpasitporn W. Outcomes of kidney transplantation in patients with hepatitis B virus infection: A systematic review and meta-analysis. *World J Hepatol* 2018; 10(2): 337-346 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/337.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.337

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most common infectious diseases and major health problems worldwide[1-3]. In 2017, approximately 257 million people have chronic hepatitis B virus infection[1]. Despite advances in medicine which have resulted in a cure for hepatitis C infection in recent years[4], chronic HBV infection is still currently considered as an incurable disease[2,3,5], leading to significant mortality (887000 death in 2015) and morbidities including cirrhosis and hepatocellular carcinoma[1,2].

Advances in immunosuppression and kidney transplant techniques have led to significant improvements in short-term survival of the renal allograft[6]. Long-term graft survival, however, has remained relatively lagged behind and has now become one of the main problems in kidney transplantation[7-9]. Although HBV is preventable disease by HBV vaccine, HBV infection remains a challenge issue in patients with end-stage renal disease on dialysis, affecting from 1.3% up to 14.6% of chronic dialysis patients [hepatitis B surface antigen (HBsAg) – positive] depending on geographical regions[10-12], and, consequently leading to chronic HBV infection kidney transplant patients[13-31]. Among renal transplant patients with HBV (HBsAg positive), there have been reported cases of HBV reactivation[32], massive liver necrosis due to fulminant hepatitis, and severe cholestatic hepatitis after kidney transplantation[10,16,33-36]. In addition, chronic HBV infection may result in HBV-related membranous nephropathy after kidney transplantation[37-43].

Even though HBV is incurable, current more available antiviral agents against HBV effectively suppress viral replication[10]. Thus, these agents can prevent hepatic fibrosis[10] and potentially reduce significant hepatic and extra-hepatic complications related to chronic HBV. In spite of improvement of HBV care, outcomes of kidney transplantation including patient and allograft outcomes in recipients with HBV infection remain unclear. Thus, we conducted this meta-analysis to (1) Assess the risks of mortality and allograft failure in kidney transplant recipients with HBsAg-positive status; and (2) evaluate trends of patient's outcomes overtime.

MATERIALS AND METHODS

Literature review and search strategy

The protocol for this meta-analysis is registered

with PROSPERO (International Prospective Register of Systematic Reviews; no. CRD42017080657). A systematic literature search of MEDLINE, EMBASE, and the Cochrane Database of Systematic Reviews from database inception to October 2017 was conducted to identify studies assessing outcomes of kidney transplantation including patient and allograft outcomes in patients with HBV. The systematic literature review was undertaken independently by two investigators (C.T. and W.C.) applying the search approach that incorporated the terms of "hepatitis B" or "HBV", or "viral hepatitis" and "kidney transplantation" which is provided in online supplementary data 1. No language limitation was applied. A manual search for conceivably relevant studies using references of the included articles was also performed. This study was conducted by the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) statement[44] and previously published guidelines[45,46].

Selection criteria

Eligible studies must be randomized controlled trials or observational studies including cohort studies, case-control, or cross-sectional that assessed the risks of mortality and/or allograft loss after kidney transplantation in patients with HBV (HBsAg-positive). They must provide the effect estimates odds ratios (OR), relative risks (RR), or hazard ratios (HR) with 95%CI. The comparison group consisted of HBsAg-negative kidney transplant recipients. Retrieved articles were individually reviewed for their eligibility by the two investigators (C.T. and W.C.) noted previously. Discrepancies were discussed and resolved by mutual consensus. Newcastle-Ottawa quality assessment scale was used to appraise the quality of study for case-control study and outcome of interest for cohort study[47]. The modified Newcastle-Ottawa scale was used for cross-sectional study[48], as shown in Table 1.

Data abstraction

A structured data collecting form was used to derive the following information from each study including title, year of the study, name of the first author, publication year, country where the study was conducted, demographic and characteristic data, mean age, patient's sex, donor type, % of patients with HBeAg, % of patients with coexist patients infected with hepatitis C virus, and adjusted effect estimates with 95%CI and covariates that were adjusted in the multivariable analysis.

Statistical analysis

Comprehensive Meta-analysis (version 3; Biostat Inc) was used to analyze the data. Adjusted point estimates from each study were consolidated by the generic inverse variance approach of DerSimonian and Laird, which designated the weight of each study based on its variance[49]. Given the likelihood of increased inter-observation variance; a random-effect model was utilized to assess the pooled prevalence and pooled OR with 95%CI for the risks of atrial fibrillation with sleep duration, insomnia, and frequent awakening. Cochran's Q test and I^2 statistic were applied to determine the between-study heterogeneity. A value of I^2 of 0-25% represents insignificant heterogeneity, 26%-50% represents low heterogeneity, 51%-75% represents moderate heterogeneity, and > 75% represents high heterogeneity[50]. The presence of publication bias was evaluated *via* the Egger test[51].

RESULTS

A total of 1673 potentially eligible articles were identified using our search strategy. After the exclusion of 1639 articles based on the title and abstract for clearly not fulfilling inclusion criteria on the basis of the type of article, study design, population or outcome of interest, leaving 34 articles for full-length review. Eighteen of them were excluded from the full-length review as they did not report the outcome of interest while six articles were excluded because they were descriptive studies without comparative analysis. Thus, the final analysis included 10 cohort studies[18,21,31,36,52-57] with 87623 kidney transplant patients. The literature retrieval, review, and selection process are demonstrated in Figure 1. The characteristics and quality assessment of the included studies are presented in Table 1. Patients in most included studies used calcineurin inhibitor-based immunosuppression. Mean age was between the ages of 32-49.

Mortality after kidney transplantation in patients with HBsAg-positive vs HBsAg-negative status

Ten studies assessed the mortality risk after kidney transplantation in patients with HBV as shown in Table 1. Compared to *HBsAg-negative* patients, HBsAg-positive status was significantly associated with increased risk of mortality after kidney transplantation (pooled OR = 2.48; 95%CI: 1.61-3.83, I^2 = 82, Figure 2). When meta-analysis was limited only to non-HCV patient population, the pooled OR of mortality was 2.98 (95%CI: 1.47-6.07, I^2 = 89). When meta-analysis was limited only to studies with adjusted analysis for confounders[36,53,54], the pooled OR of mortality was 1.27 (95% CI: 1.06-1.51, I^2 = 85). Meta-regression showed significant negative correlations between mortality risk after kidney transplantation in HBsAg-positive patients and year of study (slopes = -0.062, P = 0.001, Figure 3). Meta-regression showed no significant impact of donor type on the association between HBsAg-positive status and increased risk of mortality after kidney transplantation (P = 0.11).

Impact of antiviral treatments on patient survival after kidney transplantation in patients with HBsAg-positive

Of 10 studies[18,21,31,36,52-57], 3 studies[18,21,54] provided data on prophylactic antiviral treatment for HBV. When meta-analysis was limited only to studies with HBsAg-positive

Table 1 Main characteristics of the studies assessing outcomes of kidney transplantation in patients with hepatitis B virus

Study	Lee *et al*[55]	Breitenfeldt *et al*[56]	Chan *et al*[21]	Morales *et al*[57]	Ridruejo *et al*[52]	Aroldi *et al*[53]	Yap *et al*[18]	Reddy *et al*[36]	Grenha *et al*[31]	Lee *et al*[54]
Country	Taiwan	Germany	Hong Kong	Spain	Argentina	Italy	Hong Kong	USA	Portugal	Korea
Study design	Cohort study	Cohort study	Cohort study	Cohort study	Cohort study	Cohort study	Cohort study	Cohort	Cohort study	Cohort study
Year	2001	2002	2002	2004	2004	2005	2010	2011	2015	2016
Total number	477	927	509	3365	231	541	126	75681	2284	3482
Age (yr)	38.6 ± 11.5	41.7	N/A	45.6 ± 13.0	38	31.7	49.2	N/A	44.3	40.6 ± 12.9
Male	280 (58.7%)	595 (64.2%)	N/A	2119 (63.0%)	136 (58.9%)	322 (59.5%)	90 (71.4%)	45249 (59.8%)	1524 (66.7%)	2084 (59.9%)
Living donor	N/A	19 (2.0%)	N/A	N/A	N/A	62 (11.5%)	41 (32.5%)	32096 (42.4%)	80 (3.5%)	2571 (73.8%)
HBsAg	62 (13.0%)	37 (4.0%)	67 (13.2%)	76 (2.2%)	17 (7.3%)	77 (14.2%)	63 (50%)	1346 (1.8%)	76 (3.3%)	160 (4.6%)
HBeAg in HbsAg (+) patients	N/A	11/37 (29.7%)	29/67 (43.3%)	N/A	N/A	34/77 (44.2%)	16/63 (25.4%)	N/A	N/A	N/A
HBV treatment	N/A	N/A	Lamivudine 26/67 (39%)	N/A	N/A	N/A	Lamivudine 38/63 (60%)	N/A	N/A	Not specified 129/160 (81%)
Anti-HCV	151 (31.7%)	130 (14.0%)	0 (0%)	513 (15.2%)	106 (45.9%)	244 (45.1%)	0 (0%)	0 (0%)	113 (4.9%)	55/3482 (1.6%)
Immunosuppression	Cyclosporine, steroid, azathioprine, MMF	N/A	Cyclosporine, steroid, azathioprine	Cyclosporine, steroid, azathioprine, MMF, FK506	Cyclosporine, steroid, azathioprine	Cyclosporine, steroid, azathioprine	Cyclosporine/ tacrolimus, steroid, MMF	Cyclosporine/ tacrolimus, steroid, azathioprine, MMF, mTOR	N/A	Cyclosporine/ tacrolimus, steroid, azathioprine/MMF
Follow-up after KTx	6.0 ± 7.0 yr	9.2 ± 4.4 yr	82 ± 58 mo	N/A	39.9 (1-10.4.2) mo	11 yr	140.1 mo	1098 d	10 yr	89.1 ± 54.1 mo
Mortality	Overall 2.72 (1.48-4.99) No HCV 4.61 (2.41-8.84)	Overall 4.08 (2.10-7.93) No HCV 3.60 (1.72-7.54)	No HCV 8.07 (3.65-17.86)	Overall 2.06 (1.24-3.40) No HCV 2.97 (1.66-5.33)	Overall 2.20 (0.57-8.34)	Overall 2.36 (1.50-3.70) No HCV 4.40 (2.06-9.41)	No HCV 11.70 (1.45-94.40)	Overall 1.07 (0.88-1.31) Living donor 0.98 (0.59-1.63) Deceased donor 1.09 (0.88-1.36)	Overall 1.33 (0.78-2.29) No HCV 1.02 (0.54-1.94)	2.37 (1.16-4.87)
Graft failure	Overall 1.84 (1.08-3.15) No HCV 3.56 (1.89-6.71)	Overall 2.07 (1.06-4.05) No HCV 1.48 (0.71-3.08)	No HCV 1.61 (0.86-3.03)	Overall 0.62 (0.37-1.02) No HCV 0.59 (0.32-1.09)	5.45 (1.95-15.23)	Overall 1.55 (1.12-2.14) No HCV 0.65 (0.27-1.54)	N/A	Overall 1.02 (0.81-1.28) Living donor 0.90 (0.62-1.30) Deceased donor 1.10 (0.82-1.47)	Overall 1.57 (0.99-2.49) No HCV 1.63 (0.98-2.71)	1.38 (0.55-3.50)
Confounder adjustment	None	None	None	None	None	Age, Hepatitis C status	none	Recipient age, gender, BMI, race, comorbid, dialysis duration, donor HBcAb, expanded criteria donor, HLA DR mismatch, cold ischemia time, induction therapy, immunosuppressants	none	Age, sex, DM, BMI, primary renal disease, donor type, hypertension, ischemic heart disease, immunosuppressive agents
New Castle-Ottawa score	S 4 C 0 O 3	S 4 C 0 O 3	S 4 C 0 O 3	S 4 C 0 O 3	S 4 C 0 O 3	S 4 C 1 O 3	S 4 C 0 O 3	S 4 C 2 O 3	S 4 C 0 O 3	S 4 C 2 O 3

FK506: Tacrolimus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HCV: Hepatitis C virus; KTx: Kidney transplantation; MMF: Mycophenolate mofetil; S, C, O: Selection, comparability, and outcome.

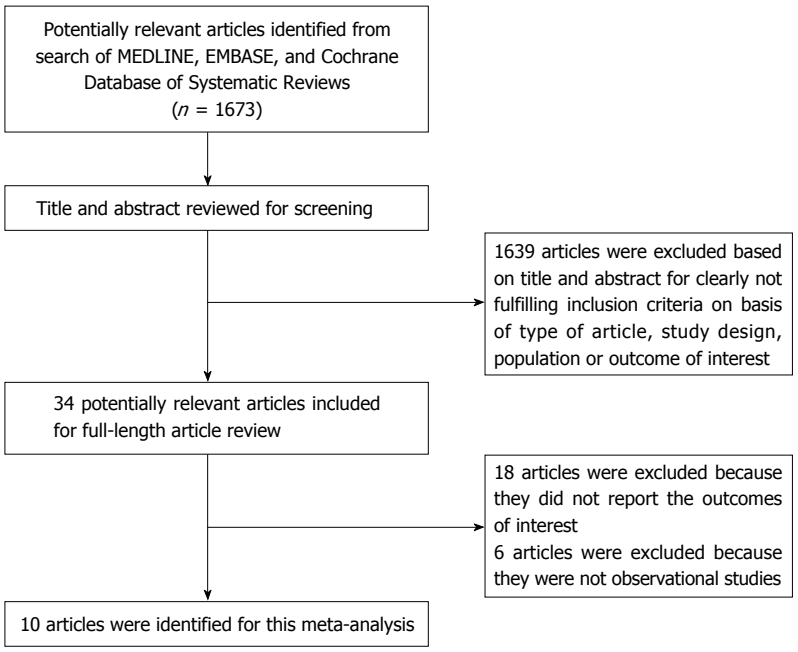

Figure 1 Literature review process.

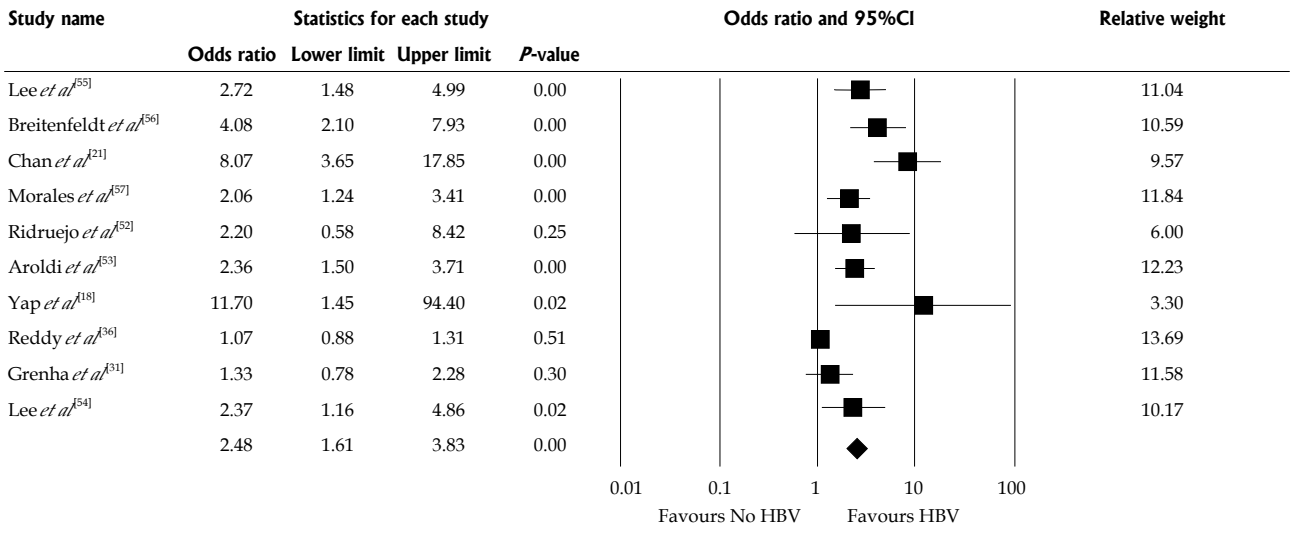

Figure 2 Forest plots of included studies evaluating mortality after kidney transplantation in patients with hepatitis B surface antigen-positive *vs* hepatitis B surface antige -negative.

recipients (> 50%) treated with prophylactic antiviral treatment for HBV, the pooled OR of mortality was 3.85 (95%CI: 0.91-16.23, I^2 = 50%). In a recent study by Lee *et al*[54], which 81% of HBs Ag-positive recipients were treated with prophylactic antiviral treatment, HBsAg-positive status was significantly associated with increased risk of mortality after kidney transplantation with adjusted HR of 2.37 (95%CI: 1.16-4.87).

Yap *et al*[18] demonstrated that recipients treated with nucleoside/nucleotide analogues had significantly better patient survival, when compared to those who were not on treatment (83% *vs* 34% at 20 years, *P* = 0.006). In patients who had lamivudine-resistant HBV, the investigators showed that treatment with adefovir or entecavir was effective with a three-log reduction in HBV DNA by 6 mo. When compared to patients who were treated with lamivudine or adefovir, Lee *et al*[54] demonstrated that those treated with new generation antiviral agent entecavir had better patient survival (log-rank, *P* = 0.050).

Renal allograft failure in patients with HBsAg-positive vs HBsAg-negative

There were 9 studies assessed renal allograft outcomes in HBsAg-positive patients (Table 1). HBsAg-positive status was significantly associated with increased risk of renal allograft loss with pooled OR of 1.46 (95%CI: 1.08-1.96, I^2 = 69, Figure 4). When meta-analysis was

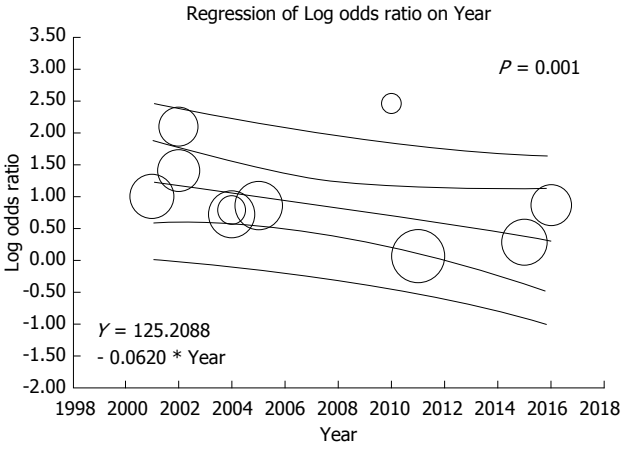

Figure 3 Graphical display of significant negative correlations between mortality risk after kidney transplantation in hepatitis B surface antigen-positive patients and year of study.

limited only to non-HCV patient population, the pooled OR of allograft failure was 1.33 (95%CI: 1.00-1.77, I^2 = 80). When meta-analysis was limited only to studies with adjusted analysis for confounders[36,53,54], the pooled OR of allograft failure was 1.25 (95%CI: 0.90-1.73, I^2 = 54). There was also a significant negative correlation between year of study and risk of allograft failure (slopes = -0.018, P =0.002, Figure 5). Meta-regression showed no significant impact of donor type on the association between HBsAg-positive status and increased risk of renal allograft loss (P =0.52).

Evaluation for publication bias

We found no publication bias as assessed by the funnel plots (Supplementary Figures 1 and 2) and Egger's regression asymmetry test with P = 0.18 and 0.13 for the risks of mortality and allograft failure after kidney transplantation in HBV infected patients, respectively.

DISCUSSION

In this systematic review, we demonstrated that HBsAg-positive status in kidney transplant recipients was significantly associated with poor outcomes after transplantation including a 2.5-fold increased risk of mortality and 1.5-fold increased risk of allograft loss. Theses associations existed in overall analysis as well as in limited cohort of hepatitis C virus-negative patients.

Chronic HBV infection can negatively impact the clinical outcomes of kidney allograft recipients. Compared to the HBsAg-negative recipients, HBsAg-positive recipients carry a higher risk of hepatic complications including chronic hepatitis, liver failure, fibrosing cholestatic hepatitis, and hepatocellular carcinoma[34,36,58]. In addition, some immunosuppressive agents after kidney transplantation may also put patients at higher risks of HBV reactivation[23,40]. HBV genome contains glucocorticoid responsive element that activates transcription of HBV genes[35]. Moreover, cyclosporine may also enhance HBV replication, leading to higher risks of HBV-related complications in kidney transplant recipients[37]. Previously, in 2005, Fabrizi *et al*[27] conducted a meta-analysis of six observational studies and demonstrated a significant association between HBsAg seropositive status and increased mortality after kidney transplantation. Since then, although hepatitis B is still incurable, there have been significant advancements in antiviral agents including the United States Food and Drug Administration approvals of entecavir in 2005 and telbivudine in 2006[59] resulting in reasonably sustained suppression of HBV replication after kidney transplantation[10]. Our meta-analysis with a new era of medicine also demonstrated a 2.7-fold increased risk of mortality in kidney transplant recipients with HBsAg positivity, when compared to HBsAg-negative recipients. In addition, our meta-analysis is the first to demonstrate a significant negative correlation between the mortality risk and year of study, which potentially represents improvements in patient care and management for chronic HBV in kidney transplant patients[60]. Although antiviral treatment has been shown to reduce mortality after kidney transplantation due to decrease in liver complications[18,21,54], in the era of antiviral therapies, Lee *et al*[54] recently showed that deaths from liver complications remained a significant problem accounting for 40% of deaths in HBsAg patients and 22.2% of all mortalities that occurred in recipients treated with antiviral agents[54].

There are several plausible explanations for the increased risk of renal allograft failure in recipients with HBsAg-positivity. Firstly, it is known that chronic HBV infection can result in HBV-related membranous nephropathy, not only in patients with native kidneys but also in kidney transplant recipients[37-43]. Secondly, due to a concern of HBV reactivation, physicians may avoid or limit the use particular immunosuppression or Rituximab in HBsAg-positive patients when it is indicated such as for recurrent glomerulonephritis post-transplantation[61,62]. Lastly, treatment of chronic HBV infection itself such as tenofovir could affect renal function[63]. Thus, the findings from our meta-analysis confirm an increased risk of allograft failure in HBsAg seropositive patients, when compared to HBsAg-negative recipients. Also, we found a significant negative correlation between the risk of allograft failure in HBsAg positive patients and year of study. Recently, data analysis from the Organ Procurement Transplant Network/United Network for Organ Sharing database (OPTN/UNOS) suggested no increased risk for allograft failure or death in HBV-infected kidney transplant patients in a recent era (between 2001 and 2007)[36]. Although follow-up time was limited to only 3 years post-transplant, these data along with the findings from our study suggest potential improvements in patient and graft survivals in HBsAg-positive recipients overtime.

There are several limitations in this meta-analysis that bear mentioning. First, there was low to moderate statistical heterogeneity between studies in meta-

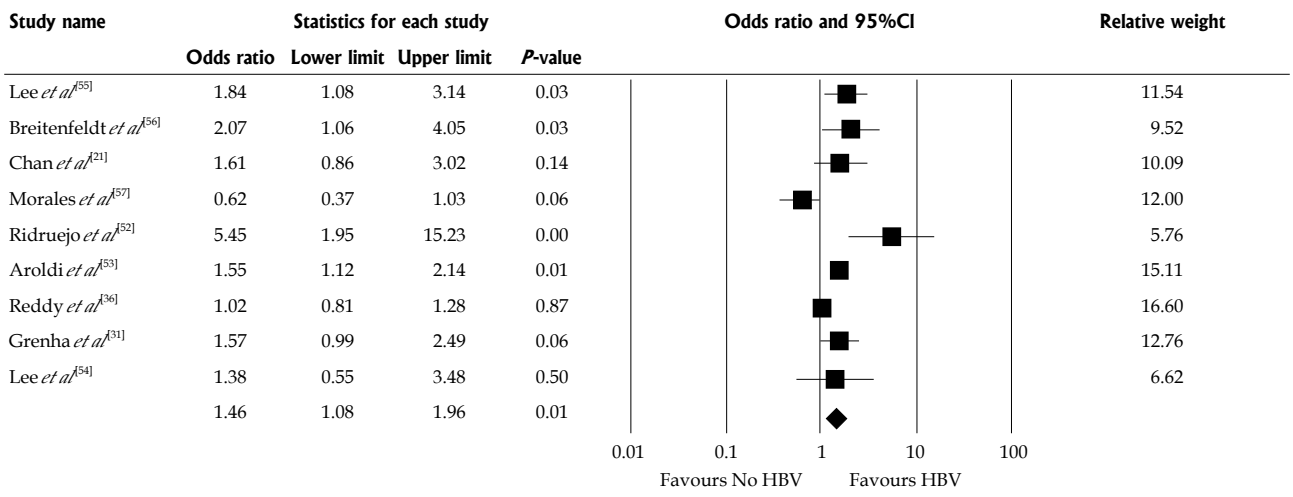

Figure 4 Forest plots of included studies evaluating renal allograft failure in patients with hepatitis B surface antigen-positive *vs* hepatitis B surface antigen-negative.

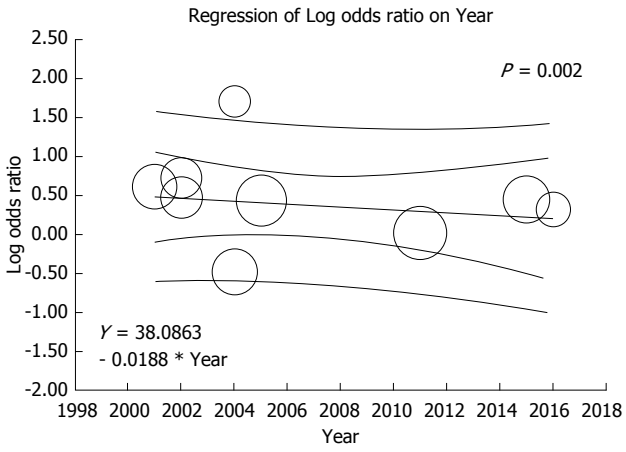

Figure 5 Graphical display of a significant negative correlation between year of study and risk of allograft failure.

analysis assessing the risks of mortality and allograft failure in HBsAg-positive recipients. The possible source of this heterogeneity includes the difference in population, type of donor, number of patients with positive HBeAg, immunosuppression regimens, and difference in confounder adjustments. In addition, the data on the graft quality (*e.g.*, Kidney Donor Profile Index) and surgical technique in HBsAg-positive recipients were limited. Second, despite the associations of HBsAg-positive status with poor kidney transplant outcomes, there is limited evidence whether the treatment with antiviral drugs for chronic HBV helps improve patient and allograft survival. However, with potential improvements in patient and graft survivals overtime demonstrated in our meta-analysis, future studies are required to evaluate if advancement in patient care for chronic HBV plays an important role. Also, additional studies are required to identify optimal antiviral treatment regimens and duration of suppressive therapy for HBV after kidney transplantation, since outcomes after withdrawal of antiviral treatment in kidney transplant recipients with chronic HBV infection remain unknown. While cautious withdrawal of antiviral therapy post kidney transplantation has been described especially in those with stable renal allograft function, low immunological risk for rejection and no evidence for HBV activity[10,23], fatal hepatitis flares in several kidney transplant recipients have been reported after withdrawal of antiviral therapy[64]. Lastly, this is a meta-analysis of observational studies. Thus, it can at best identify only associations of HBsAg-positive status with poor kidney transplant outcomes, but not a causal relationship.

In summary, our study reveals an association between HBsAg-positive status in kidney transplant recipients and higher risks of mortality and allograft failure after kidney transplantation. However, there are also significant negative correlations between the risks of mortality and allograft failure and year of study, representing potential improvements in patient and graft survivals overtime.

ARTICLE HIGHLIGHTS

Research background

Among renal transplant patients with hepatitis B virus (HBV) (HBsAg positive), there have been reported cases of HBV reactivation, massive liver necrosis due to fulminant hepatitis, and severe cholestatic hepatitis after kidney transplantation. In spite of improvement of HBV care, the outcomes of kidney transplantation including patient and allograft outcomes in recipients with HBV infection remain unclear.

Research motivation

Although hepatitis B is still incurable, there have been significant advancements in antiviral agents resulting in reasonably sustained suppression of HBV replication after kidney transplantation. The results of studies on kidney transplant outcomes in patients with renal transplant patients with HBV (HBsAg positive) were inconsistent. To further investigate outcomes of renal transplant patients with HBsAg positivity, the authors conducted this systematic review and meta-analysis reporting the association between HBsAg positivity in kidney transplant recipients and higher risks of mortality and allograft failure after kidney transplantation.

Research objectives
We conducted this meta-analysis to assess the outcomes of kidney transplantation including patient and allograft outcomes in recipients with HBV infection; and the trends of patient's outcomes overtime.

Research methods
A literature search was conducted using databases (MEDLINE, EMBASE and Cochrane Database) from inception through October 2017. Those studies reported odds ratios (OR) of mortality or renal allograft failure after kidney transplantation in HBV patients (defined as HBsAg positive) were included. HBsAg-negative kidney transplant recipients are the comparison group. The effect estimates from the individual study were extracted and combined.

Research results
The authors demonstrated that HBsAg-positive status in kidney transplant recipients was significantly associated with poor outcomes after transplantation. These associations existed in overall analysis as well as in limited cohort of hepatitis C virus-negative patients.

Research conclusions
The authors found significant associations of HBsAg positive status with poor outcomes after transplantation. Significant negative correlations between the risks of mortality and allograft failure and year of study, representing potential improvements in patient and graft survivals overtime were found.

Research perspectives
This study demonstrated significantly increased risks of mortality and allograft failure in HBsAg-positive kidney transplant recipients. This finding suggests that HBsAg positive status may be an independent potential risk factor for poor outcomes after transplantation. However, there are also potential improvements in patient and graft survivals with HBV infection overtime.

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CASE REPORT

Primary hepatic peripheral T-cell lymphoma associated with Epstein-Barr viral infection

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Author contributions: Ramai D, Ofori E and Nigar S designed the report; Ramai D and Reddy M collected the patient's clinical data; Ramai D wrote the paper; Ofori E, Nigar S and Reddy M edited the manuscript for intellectual content.

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Abstract

Primary hepatic peripheral T-cell lymphoma (H-PTCL) is one of the rarest forms of non-Hodgkin lymphoma. We report a patient who presented with worsening jaundice, abdominal pain, and vomiting. Laboratory values were significant for elevated total bilirubin, alkaline phosphatase, and liver aminotransferases. Following a liver biopsy, histopathology revealed several large dense clusters of atypical T-lymphocytes which were CD2+, CD3+, CD5+, CD7-, CD4+, CD8-, CD56-, CD57-, CD30+ by immunohistochemistry. The proliferation index was approximately 70% by labeling for ki67/mib1. The above histological profile was consistent with peripheral T-cell lymphoma of the liver. Epstein-Barr viral serology indicated a remote infection, a likely risk factor for PTCL. Bone marrow biopsy was negative for malignancy, further supporting hepatic origin.

Key words: Primary lymphoma; Liver cancer; Non-Hodgkin's lymphoma; T-cell lymphoma

Core tip: Primary hepatic peripheral T-cell lymphoma (H-PTCL) is one of the rarest forms of non-Hodgkin lymphoma. We report a patient who presented with worsening jaundice, abdominal pain, and vomiting. Laboratory values were significant for elevated total bilirubin, alkaline phosphatase, and liver aminotransferases. Liver biopsy followed by histopathology confirmed the diagnosis of H-PTCL. Furthermore, bone marrow biopsy was negative for malignancy, further supporting hepatic

origin. Our patient's medical history reported a prior Epstein-Barr viral infection, a risk factor for H-PTCL. In the setting of risk factors, H-PTCL should be born in mind when a patient presents with symptoms of malignancy, and an enlarged and infiltrating liver.

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INTRODUCTION

Peripheral T-cell lymphoma (PTCL) is the rarest of all cases of non-Hodgkin lymphoma (NHL). It constitutes approximately 0.0016% of all extranodal lymphomas[1-3]. PTCL not otherwise specified (NOS) is a heterogeneous subset of nodal T-cell lymphomas which does not satisfy the criteria for the other subtypes of PTCLs, namely, angioimmunoblastic T-cell lymphoma, and follicular T-cell lymphoma[4]. The annual incidence rate for PTCL is 1.56 per 100000 persons in non-Hispanic Whites, 1.32 per 100000 in Blacks, 0.89 per 100000 in Asians/Pacific Islanders, 0.63 per 100000 in American Indians/Alaskan natives, and 0.96 per 100000 in Hispanic Whites[5]. The distribution of PTCL NOS among racial groups is reported to be highest amongst non-Hispanic Whites (2689), followed by Blacks (661), Hispanic Whites (418), Asian/Pacific Islanders (322), and lowest in American Indians/Alaskan natives (20)[5]. When the lesion is localized or arises from the liver, it may also be referred as primary hepatic peripheral T-cell lymphoma (H-PTCL). We present a 37-year-old male with worsening jaundice, abdominal pain, and vomiting who was diagnosed with hepatic peripheral T-cell lymphoma with a Ki-67 of 70%.

CASE REPORT

A 37-year-old male with a past medical history of Epstein-Barr Virus (EBV) infection was admitted for jaundice and right upper quadrant abdominal pain. He reported having worsening symptoms for one month duration. The patient was a non-smoker and non-alcohol drinker. Review of systems was positive for decreased appetite and weight loss of 10 lbs. over the past two months. His family history was unknown. Physical examination was significant for mild scleral icterus and abdominal distension. Heart rate was 92/min, blood pressure was 107/67 mm Hg, respiratory rate was 20/min, oxygen saturation was 94% on room air, and temperature was 98.1 °F. Laboratory results were within normal limits with a white blood cell count (WBC) of 11.4/μL, hemoglobin of 12.2 g/dL, hematocrit of 37%, and platelet count of 291 k/cmm. Total bilirubin was 5.7 mg/dL, alkaline phosphatase (ALP) was 1005 U/L, LDH was 830 U/L, albumin was 3.2 g/dL, aspartate aminotransferase (AST) was 257 U/L and alanine aminotransferase (ALT) was 239 U/L. EBV serology was negative for IgM, and positive for IgG and EBV nuclear antigen, consistent with prior infection.

Abdominal magnetic resonance imaging (MRI) showed mild intrahepatic ductal dilatation, peripheral areas of arterial enhancement in liver felt to be related to vascular shunting, periportal edema, a cut off in the course of the biliary tree at the bifurcation, a simple liver cyst, and enlarged left retroperitoneal nodes. Upper endoscopy showed gastropathy in the gastric fundus and body. Endoscopic ultrasound was unremarkable. Following a liver biopsy, histopathology showed several large dense clusters of atypical T-lymphocytes, which appeared to be centered in the portal areas. The atypical lymphocytes were medium to large in size and were CD2+, CD3+, CD4+, CD5+, CD7-, CD8-, CD56-, CD57-, CD30+, by immunohistochemistry (Figure 1).

The proliferation index was approximately 70% by labeling for ki67/mib1. Labeling for CD68 was seen in Kupffer cells, and in a few scattered histiocytes only. There were rare, scattered, unremarkable small B-lymphocytes (CD20+, CD79a+). Stains for CD138, kappa, lambda, were noncontributory. The above histological profile was consistent with hepatic peripheral T-cell lymphoma. The patient was subsequently transferred to a tertiary care center for further management where he had a bone marrow biopsy which was negative for malignancy, further supporting hepatic origin.

DISCUSSION

H-PTCL is mainly diagnosed by the presence of a hepatic mass in the absence of lymphadenopathy, splenomegaly, bone marrow involvement, and associated with normal tumor markers[6]. After six months following diagnosis, other tissues may become involved including the spleen, lymph nodes, peripheral blood, and/or bone marrow[7]. According to literature, H-PTCL commonly occurs around the fifth decade of life[8].

While the etiology of H-PTCL remains unclear, certain risk factors have been described such as Hepatitis C virus (HCV), Hepatitis B virus (HBV), and Epstein-Barr virus (EBV)[9-12]. In patients diagnosed with H-PTCL, HCV was identified in 20%-60% of cases[9]. This finding hints that viruses such as HCV may play a role in the pathogenesis of H-PTCL. Furthermore, H-PTCL has been diagnosed in immunocompromised patients with Human Immunodeficiency Virus (HIV), Human T-Lymphotropic Virus (HTLV), systemic erythematous lupus (SLE), and immunosuppressive therapy[13]. Our patient was negative for HCV and HBV infections, but his medical history indicated a prior EBV infection. While the tumor was CD30+, we did not pursue in-situ hybridization for Epstein-Barr

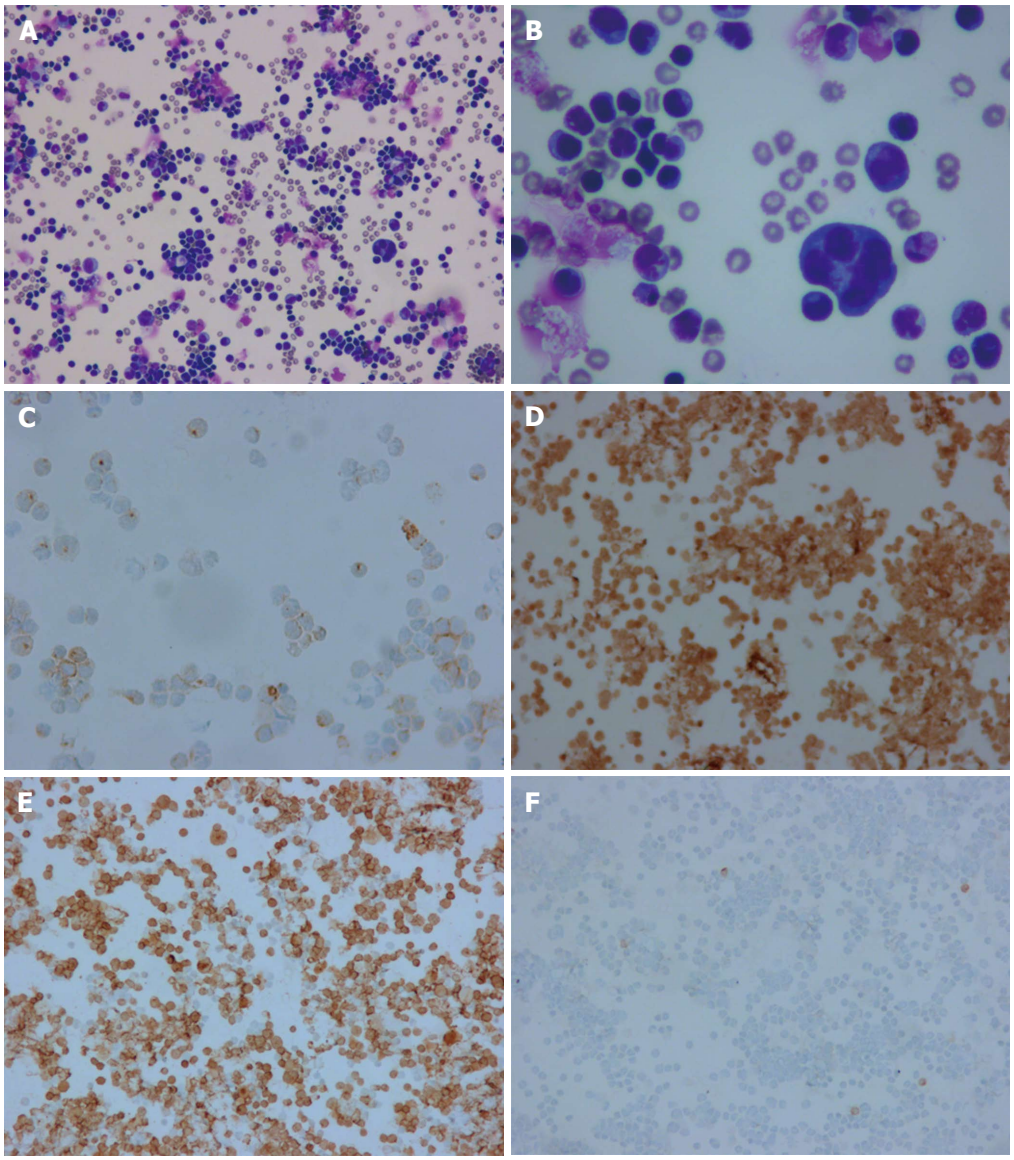

Figure 1 Biopsy results. A: large dense clusters of atypical T-lymphocytes 20 ×; B: atypical T-lymphocytes 40 ×; C: CD2 positive 40 ×; D: CD3 positive 20 ×; E: CD4 positive 20 ×; F: Scantly positive CD79a 20 ×.

virus-encoded RNA in lymphoma cells. Peng *et al*[14] reported the first case of EBV-associated CD30-positive peripheral T-cell lymphoma of cytotoxic phenotype. Our case provides further confirmation of an association of EBV infection and PTCL.

The clinical presentation of H-PTCL is non-specific, with the most reported symptom being abdominal pain in 40%-70% of patients, similar to our patient[3]. About 35% of PTCL patients experience systemic B symptoms including fever, night sweats, and weight loss[15]. Tumor markers alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA) are typically normal in these patients[16]. Abnormal laboratory findings include elevated liver function aminotransferases, bilirubin, γ-glutamyl transferase, ALP and LDH. Approximately 70% of cases present with abnormal liver function enzymes, 30 to 80% with elevated LDH, 90% with elevated β2-microglobulin, and 80% with elevated ALP[17-20]. Mitarnun *et al*[20] reported that out of 100 patients with EBV associated H-PTCL, ALP was found elevated in 80%, while LDH was elevated in 65% of cases. Our patient presented with significantly notable ALP, LDH, total bilirubin, and liver function enzymes.

The proliferation index measured by Ki-67 has traditionally been used in assessing patient prognosis and response to therapy. Went *et al*[21] proposed a prognostic model which incorporated age (> 60 years), high lactate dehydrogenase, poor performance status, and Ki-67 greater or equal to 80%. Their model was significantly associated with patient outcome ($P < 0.0001$). Weisenburger *et al*[15] reported a Ki-67 > 25% was an adverse predictor of survival.

Our case was classified as PTCL-NOS according to guidelines outlined by the World Health Organization[4]. The running differential diagnosis included extranodal NK/T-cell lymphoma, nasal type and adult T-cell leukemia/lymphoma. Extranodal NK/T-cell lymphoma, nasal type, was considered due to a prior EBV

infection, however, it was ruled out after being CD56 negative. CD56 is a diagnostic requisite for extranodal NK/T-cell lymphoma, nasal type[22]. Adult T-cell leukemia/lymphoma was ruled out given that the patient's calcium levels and WBC were within normal limits[23].

Treatment for PTCL requires an aggressive course of chemotherapy, typically cyclophosphamide, hydroxydaunorubicin, oncovin, and prednisone (CHOP). A recent study by Kim *et al*[22] reported that patients with whole blood EBV-DNA were more likely to have aggressive clinical characteristics and inferior survival. Overall, H-PTCL has a poor prognosis due to life threatening complications and tumor progression. Clinical studies report that CHOP therapy can provide up to 60% complete remission, and a 30%-50% five-year survival rate[24-26].

More recently, a prospective study of 499 patients showed that patients who received doxorubicin had a significantly longer survival than those who did not (P = 0.03)[27]. Furthermore, in a study involving 775 patients, better survival outcomes were seen in one third of patients who remained in remission 2 years after diagnosis, especially in younger patients less than 60-years of age[28]. However, Abramson *et al*[29] reported that the most dominant prognostic factor was response to initial therapy, with no overall survival difference based on the choice of upfront regimen. These studies further reemphasizes the need for early detection and treatment.

In conclusion, we report a rare case of H-PTCL in a 37-year old male with a medical history of EBV infection who presented with worsening jaundice, abdominal pain, and vomiting. H-PTCL is an aggressive form of NHL which requires early diagnosis and a robust treatment regimen. However, the diagnosis of H-PTCL remains challenging due to the presence of multiple granulomas, histiocytosis, and focal neoplastic infiltrates. In the setting of worsening symptoms and abnormal liver enzymes of unknown etiology, clinicians should consider performing a differential liver biopsy.

ARTICLE HIGHLIGHTS

Case characteristics

A 37-year-old male with a past medical history of Epstein-Barr Virus infection reported having jaundice, right upper quadrant pain, and decreased appetite and weight loss of 10 lbs over the past two months.

Clinical diagnosis

Abdominal magnetic resonance imaging showed mild intrahepatic ductal dilatation, peripheral areas of arterial enhancement in the liver felt to be related to vascular shunting, periportal edema, a cut off in the course of biliary tree at the bifurcation, simple liver cyst, and enlarged left retroperitoneal nodes.

Differential diagnosis

Cirrhosis, hepatocellular carcinoma, cholangiocarcinoma.

Laboratory diagnosis

Laboratory was significant for total bilirubin of 5.7 mg/dL, alkaline phosphatase of 1005 U/L, albumin of 3.2 g/dL, and AST/ALT of 257/239 U/L.

Imaging diagnosis

Upper endoscopy showed gastropathy in the gastric fundus and body. Endoscopic ultrasound was unremarkable.

Pathological diagnosis

A liver biopsy showed several large dense clusters of atypical T-lymphocytes, which appeared to be centered in portal areas. The atypical lymphocytes were medium to large in size and were CD2+, CD3+, CD5+, CD7-, CD4+, CD8-, CD56-, CD57-, CD30+, by immunohistochemistry. The proliferation index was approximately 70% by labeling for ki67/mib1. Labeling for CD68 was seen in Kupffer cells, and in a few scattered histiocytes only. There were rare, scattered, unremarkable small B-lymphocytes (CD20+, CD79a+). Stains for CD138, kappa, lambda, were noncontributory. The above histological profile was consistent with hepatic peripheral T-cell lymphoma (H-PTCL).

Treatment

The patient was transferred to a tertiary center for chemotherapy (CHOP) treatment.

Related reports

H-PTCL has a poor prognosis due to life threatening complications and tumor progression. Clinical studies reports that CHOP therapy can provide up to 60% complete remission, and a 30%-50% five-year survival rate.

Term explanation

H-PTCL is one of the rarest forms of non-Hodgkin lymphoma. It constitutes approximately 0.0016% of all extranodal lymphomas.

Experiences and lessons

In the setting of worsening symptoms and abnormal liver enzymes of unknown etiology, clinicians should consider performing a differential liver biopsy. Clinicians should also be aware of the risk factors for H-PTCL.

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