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Contents

Monthly Volume 14 Number 10 October 27, 2022

MINIREVIEWS

ORIGINAL ARTICLE

Basic Study

Retrospective Cohort Study

World Journal of Hepatology

Monthly Volume 14 Number 10 October 27, 2022

Contents

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MINIREVIEWS

Natural history and management of liver dysfunction in lysosomal storage disorders

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Abstract

Lysosomal storage disorders (LSD) are a rare group of genetic disorders. The major LSDs that cause liver dysfunction are disorders of sphingolipid lipid storage [Gaucher disease (GD) and Niemann-Pick disease] and lysosomal acid lipase deficiency [cholesteryl ester storage disease and Wolman disease (WD)]. These diseases can cause significant liver problems ranging from asymptomatic hepatomegaly to cirrhosis and portal hypertension. Abnormal storage cells initiate hepatic fibrosis in sphingolipid disorders. Dyslipidemia causes micronodular cirrhosis in lipid storage disorders. These disorders must be keenly differentiated from other chronic liver diseases and non-alcoholic steatohepatitis that affect children and young adults. GD, Niemann-Pick type C, and WD also cause neonatal cholestasis and infantile liver failure. Genotype and liver phenotype correlation is variable in these conditions. Patients with LSD may survive up to 4-5 decades except for those with neonatal onset disease. The diagnosis of all LSD is based on enzymatic activity, tissue histology, and genetic testing. Enzyme replacement is possible in GD and Niemann-Pick types A and B though there are major limitations in the outcome. Those that progress invariably require liver transplantation with variable outcomes. The prognosis of Niemann-Pick type C and WD is universally poor. Enzyme replacement therapy has a promising role in cholesteryl ester storage disease. This review attempts to outline the natural history of these disorders from a hepatologist's perspective to increase awareness and facilitate better management of these rare disorders.

Key Words: Lysosomal; Gaucher; Niemann-Pick; Wolman; Cholesteryl ester; Children

Core Tip: Lysosomal storage disorders have a multisystem involvement. Gaucher disease, Niemann-Pick disease, and lysosomal acid lipase deficiency (Wolman disease and cholesteryl ester storage disorder) may present with predominant liver dysfunction. Those with neonatal-onset, severe extrahepatic and multisystemic presentations often have challenging outcomes. Enzyme replacement therapy and liver transplantation are encouraged in selected patients. Genetic tests and counseling are important aspects of disease management.

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INTRODUCTION

Lysosomes are intracellular organelles that contain multiple enzymes required for the degradation of a range of macromolecules. These enzymes have acidic pH and hydrolyze mucopolysaccharides, glycosphingolipids, and oligosaccharides. Each enzyme is specific for a particular molecule and essential for its catabolism. Lysosomal storage disorders (LSDs) arise from the defect in these enzymes, culminating into the specific substrate accumulation in the lysosomes, which finally causes cellular dysfunction.

Patients with LSDs have variable ages of onset ranging from the perinatal period to adulthood. These are a heterogeneous group of genetic disorders with multisystem involvement. Symptoms vary depending on the most affected organ systems. The most common symptoms are coarse facial features, skeletal dysplasia, hepatosplenomegaly with liver dysfunction, and neuroregression. The combined prevalence of LSDs is 1 per 7000 live births although individual disorders are uncommon[1,2].

There are more than 50 LSDs identified[1]. The classification and cardinal features of some of the important disorders are shown in Table 1. Mucopolysaccharidoses, mucolipidoses, and glycoprotein storage disorders only cause hepatomegaly without causing liver dysfunction. Disorders of sphingolipid and lipid storage disorders such as Gaucher disease (GD), Niemann-Pick disease (NPD), and lysosomal acid lipase deficiency (LAL-D) cause liver diseases ranging from asymptomatic hepatomegaly to cirrhosis and portal hypertension. All these disorders present a different set of challenges as they mimic other liver diseases. Their diagnosis requires enzyme analysis and genetic tests. Therapy is limited and response is variable if treatment exists. Simultaneous involvement of other organ systems often precludes the possibility of liver transplantation (LT). Our review aims to describe the natural history of the liver disease in LSDs. The review is focused on the LSDs that have significant liver dysfunction such as GD, NPD, and LAL-D. The novelty of the review is to comprehensively collate the available literature so that hepatologists will have a better understanding of the disease management.

GD

General aspects

GD is a disorder of sphingolipid storage occurring as a result of the deficiency of acid β-glucosidase enzyme in the nucleated cells. There is defective cleavage of glucosylceramide and glucosylsphingosine, resulting in their accumulation in lysosomes. There is infiltration of macrophages laden with glucosylceramide in visceral tissues such as the bone marrow, spleen, liver, and lymph nodes (Figure 1). These cells, "foamy macrophages" or "Gaucher cells (GC)", have a signet ring with a crumpled-paper appearance as the nucleus is pushed to one side (Figure 2). Neurological involvement is mainly due to damage by the lipids directly rather than the infiltration by foamy macrophages. GD is more common than other LSDs, with a high prevalence of approximately 1 in 855 individuals in the Ashkenazi Jewish population[3].

Depending on neurological involvement, GD is divided into three types: Type 1, no neurological involvement, with a prevalence of 1/40000; Type 2, neurological involvement in infancy, with a prevalence of < 1/100000; Type 3, variable neurological manifestations, with a prevalence of < 1/50000 to < 1/100000[4].

Clinical manifestations and natural history of GD

There are two phenotypes of liver presentation. The first one is milder with hepatomegaly, non-malignant focal liver lesions, and fibrosis. The other is severe, presenting as cirrhosis, portal hypertension, and potential hepatocellular carcinoma (HCC).

Table 1 Lysosomal storage disorders: Classification and clinical features

Diseases	Cardinal features	Degree of hepatosplenomegaly	Liver involvement
Diseases with major liver dysfunction: Sphingolipid and lipid storage disorders			
Gaucher	Bone and lung infiltration, neurological (type II), nonneurological (type I), and intermediate (type III) forms; Paralytic squint (type II), Oculomotor apraxia/horizontal supranuclear gaze palsy (type III); Bone marrow failure, hematolymphoid malignancy (type I); Lymphadenopathy (type I)	Moderate to marked	Neonatal cholestasis, cirrhosis, portal hypertension, adenoma, hepatocellular carcinoma
Niemann-Pick A and B	Lung infiltration, neurological (type A) and nonneurological (type B); Cherry red spot (50%); Lymphadenopathy (type A)	Moderate to marked	Fetal hydrops (type A), cirrhosis, portal hypertension
Niemann-Pick C	Vertical supranuclear gaze palsy; Neurodegeneration	Moderate to marked	Neonatal cholestasis, fetal hydrops, liver failure, cirrhosis, portal hypertension
Lysosomal acid lipase deficiency	Steatorrhea, adrenal calcification (50%); Abnormal lipid profile	Mild to moderate	Asymptomatic hypertransaminasemia, liver failure
Diseases without major liver dysfunction			
Group 1: Sphingolipid and lipid storage disorders			
GM gangliosidosis	Neuroregression, dysostosis, cherry red spot	Absent to mild	Asymptomatic hepatomegaly
Farber	Subcutaneous nodules, joint involvement	Mild to moderate	Asymptomatic hepatomegaly
Group 2: Mucolipidoses			
Mucolipidoses I, II and III	Seizures and cherry red spot (type I), kyphoscoliosis, joint contractures, dysostosis, cardiomyopathy	Mild to moderate	Asymptomatic hepatomegaly
Group 3: Glycoprotein storage disorders			
Galactosialodosis	Mimics GM gangliosidosis, mental retardation	Mild to moderate	Asymptomatic hepatomegaly
Fucosidosis	Angiokeratoma, mental retardation	Mild to moderate	Asymptomatic hepatomegaly
α-Mannosidosis	Deafness, mental retardation	Mild to moderate	Asymptomatic hepatomegaly
Group 4: Mucopolysaccharidoses			
Mucopolysaccharidoses I,II, III, IV, VI, VII	Coarse facies, corneal clouding (types I, IV, VII), mental retardation (types I, III, VII), dysostosis (types I, IV, VI), cardiomyopathy	Mild to moderate. Absent in milder variants	Asymptomatic hepatomegaly

Hepatomegaly occurs as a result of the infiltration of GC in the liver and macrophage lipid accumulation. Liver volumes > 1.25 and 2.5 or > 2.5 multiples of normal are classified as mild, moderate, and severe hepatomegaly, respectively. Moderate or severe hepatomegaly at diagnosis is seen in approximately 80% of patients. Mean liver and spleen volumes are 1.8 and 19.4 times of normal in untreated GD subjects[5]. Hepatomegaly is less massive than splenomegaly in GD[6]. If the liver size outweighs the splenic size, one must carefully evaluate the other causes of liver disease or concurrent comorbidities [7]. Various modalities of assessment report the sizes of the organs variably. Sonographicprevalence of hepatomegaly was noted in 100% of pediatric patients[8]. Magnetic resonance imaging (MRI) has shown that 77%-95% of adults suffering from GD have hepatomegaly of variable degrees[9]. Biochemical liver dysfunction is noted in 19%-55%. Hepatic involvement in GD may lead to portal hypertension and end-stage liver disease. Massive splenomegaly can also produce increased portal flow leading to pre-hepatic portal hypertension and overestimation of the degree of liver disease[10]. The decision for splenectomy as a therapeutic intervention is delicate given the other comorbidities that ensue in splenectomized patients over time[11].

During imaging, multiple hypoechoic and/or hyperechoic lesions are seen in the liver and spleen. Focal hyperechoic liver lesions can be seen in 5% of individuals on sonolography[12]. Small lesions reflect a focal accumulation of GC[13]. These lesions do not merit biopsy as the growth will be slow. Approximately 20% have early focal signal abnormalities on MRI (hypointense on T1, and heterogeneous on T2) and are hypoattenuating on computed tomography in GD[9]. These lesions do not respond to enzyme replacement therapy (ERT) but they must be kept on surveillance. Enlarging lesions on follow-up with rising serum alpha-fetoprotein is a concern. Focal nodular hyperplasia has been reported in GD[14]. HCC is more common in the setting of advanced liver disease with cirrhosis, in the pre-ERT era and splenectomized patients, and also in those with concomitant iron overload[15,16].

Due to bile lipid composition abnormalities, cholesterol gall stones have been reported in GD patients [17]. The reasons for increased stone predilection in splenectomised patients are unclear. Autoimmune hepatitis in the setting of GD has a guarded outcome and requires LT[18]. Viral hepatitis resulting from

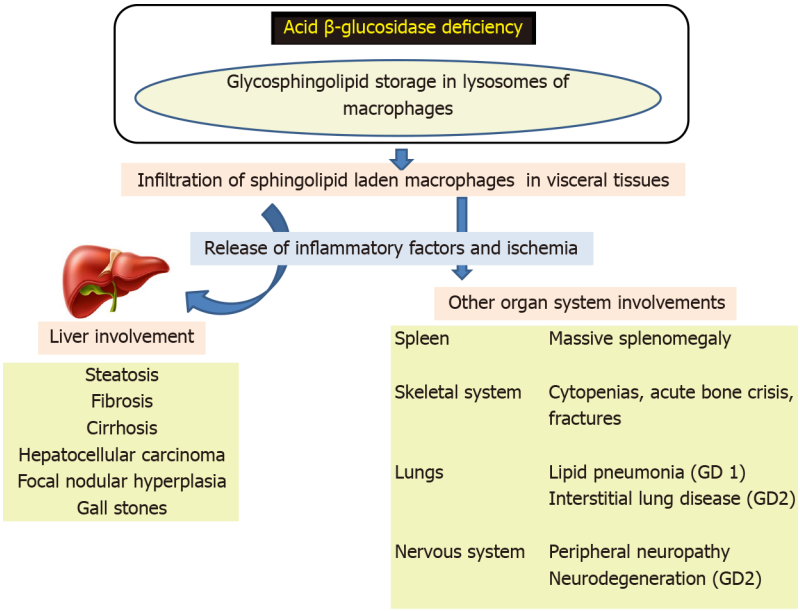

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Figure 1 Pathogenesis of Gaucher disease. GD: Gaucher disease.

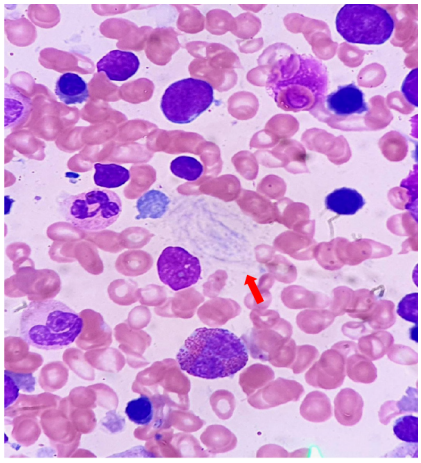

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Figure 2 Histology of Gaucher disease. Red arrow shows Gaucher cells.

transfusion dependency, surgical interventions, and intravenous ERT is a serious concern. Chronic viral hepatitis B and C should be screened at baseline and at follow-up visits in patients with GD[19]. During therapy of concurrent chronic viral hepatitis, GD-related cytopenias may be confounders to anti-viral therapy. Initiating ERT to reduce the bulk of the disease, normalization of counts followed by antiviral therapy is a safe and rational approach[20]. In those with transfusion-related hepatitis C, the present newer antiviral drugs are not associated with cytopenias.

Transient elastography though recommended has no age-specific nomograms for interpretation. It may not distinguish steatosis from infiltrative fibrosis. Liver stiffness is mildly elevated in GD without cirrhosis and those with ERT-related reductions in liver fibrosis[1]. Those with cirrhosis exhibit significant liver stiffness. Since splenectomized patients have a higher degree of disease, their liver stiffness is greater as compared to those with mild disease[2]. Gaucher clinical severity (GD-DS3) scores also correlate well with MRI-measured liver stiffness[21]. Hence, this suggests that elastography may have a significant clinical utility in the management of GD. Abdominal paracentesis is best avoided from the left side due to massive splenomegaly or cautiously performed under sonolographical guidance. The hepatic venous pressure gradient is important to distinguish pre-portal hypertension from cirrhosis. Due to severe hypersplenism, transjugular liver biopsy is often warranted over percutaneous biopsy.

Neonatal cholestasis is an important presentation in GD. Soudek *et al*[22] reported a 4-day-old neonate with GD who presented with cholestasis, low glucocerebrosidase activity (2 nmol/h/mg protein), and deletion of exons 3-12 and c. 1448T>C (p.Leu483Pro) in the *GBA* gene. After failed ERT, the liver dysfunction progressed. LT was performed at 7 mo and liver functions improved. The child died later due to progressive neurological involvement. A review of the literature was performed with nine other previously reported cases. The median age of presentation was 1 (1-51) d of life. The age at diagnosis was 3 (1-6.5) mo. The mean value for total bilirubin was 284 [interquartile range (IQR): 166-304] μmol/L, direct bilirubin was 117 (IQR: 92-265) μmol/L, aspartate transaminase (AST) was 514 (IQR: 420-670) IU/L, alanine transaminase (ALT) was 280 (IQR: 206-441) IU/L, and gamma-glutamyl transpeptidase (GGT) was 208 (IQR: 165-242) IU/L. Six of these patients had neurological presentations by the ages of 2 (0.4-10) mo. Bone marrow and liver biopsies had poor yield. Some of the patients even required a second biopsy for confirmation. The common genetic mutations were c.1342 G>C (p.Asp448His) and c. 1448T>C (p.Leu483Pro). Of the four patients who received ERT, two showed an improvement in bilirubin and platelets. Over a limited follow-up period of 6 mo, the rest seven patients died by the age of 4.75 (IQR: 4-13) mo. Five died due to respiratory causes and two due to gastrointestinal bleeding. Long-term outcomes in this phenotype are not known[23]. There may be an ethical dilemma of LT in children with decompensated liver disease before a fully evolved neurological disease. Parental consent is required in such scenarios.

Liver pathology in GD

GC is characterized by lightly striated eosinophilic cytoplasm and small round vesicular nuclei. There may be some accumulation of intracellular iron. GC has predilection for central zonal distribution (zone 1). Hepatocytes do not accumulate glycosphingolipids as such. However, in close proximity to a cluster of GC, it may undergo degenerative changes. In GD, liver histology shows a wide range of features. In milder disease, there are scattered foci of GC with mild structural parenchymal changes. In advanced cases, there is cirrhosis with dense infiltration of the liver by GC. Most cases have pericellular fibrosis, and 20%-50% have bridging or more severe fibrosis[23]. GC induces inflammatory factors that promote the fibrogenic process. Gaucheromas are large clumps of GC with areas of fibrosis[24-26]. In a single needle biopsy, Gaucheromas pose a diagnostic challenge since they can mimic liver malignancies and have considerable radiological dilemmas[27,28]. Diffuse steatosis in GD occurs as a part of a metabolic syndrome which occurs either *de novo* or as a side effect of long-term ERT. In GD, dyslipidemia and biliary lipid secretion abnormalities can occur[29,30]. Mouse models suggest that suppression of GC levels may be associated with a rise in glycolipids and metabolic derangements[31]. Hepatic fibrosis occurs due to GC infiltration and diffuse low-grade inflammatory processes caused by the activated macrophages or GC[32]. Hepatic microinfarcts result from larger clusters of GC which promote liver fibrosis[33]. Splenectomy may cause liver injury due to the shift of balance of the spleen as the preferred storage organ. Secondary hemochromatosis has been reported due to iron deposits in hepatocytes and Kupffer cells in GD. Elevated ferritin levels reflect iron overload and chronic inflammation[34,35]. Studies in Ashkenazi Jews and animal models have revealed that higher levels of GC may be hepatoprotective in those with liver comorbidities such as hepatitis B and C and non-alcoholic steatohepatitis. Several mechanisms have been postulated to explain the hepatoprotective nature of GC. GC may serve as a glycolipid ligand and is presented to non-killer T cells and dendritic cells *via* CD1 molecules[36]. By changing the cross-talk between these cells and other immune system cells, GC can exert an immunomodulatory effect directly or indirectly on these target cells. They also alter lipid rafts and intracellular signaling machinery, promote regulatory T lymphocytes, and improve immunogenicity. They may function as metabolic intermediates in insulin resistance and promote mucosal immunity [37]. Alpha-glycolipids are hepatotoxic but β-glycolipids are hepatoprotective[38]. Liver biopsy showing GC should be distinguished from liver "pseudo-GC" which is better appreciated in an additional bone marrow examination. The pseudo-GC has been demonstrated in acute lymphoblastic leukemia, myelodysplasia, Hodgkin's disease, thalassemia, and disseminated tuberculosis[39].

Therapy and LT in GD

Therapeutic approaches are ERT or substrate reduction therapy (SRT). Those who are on specific ERT or SRT will most likely experience reductions in liver dysfunction and sizes of organomegaly within 6-12 mo. The newly FDA-approved eliglustat, as a first-line option for GD, can improve liver fibrosis. Conversely, hepatic fibrosis may progress despite high-dose ERT. Advanced liver disease invariably requires LT. Ayto *et al*[18] reviewed outcomes in patients with GD undergoing LT. Good outcomes of LT with concurrent ERT were reported. There was no evidence of GD-related pathology in the liver graft even at 10 years of follow-up. In very rare cases, splenectomy can be considered for portal hypertension if cirrhosis is ruled out.

NPD TYPES A AND B

General aspects

NPD has three types: A, B, and C. The prevalence of NPD-A and NPD-B is 1 in 250 000, which is even higher in Ashkenazi Jews, where it is 1 in 40 000[40]. NPD-A and NPD-B are caused by acid sphingomyelinase deficiency (ASMD). Acid sphingomyelinase cleaves sphingomyelin into ceramide and phosphocholine and its deficiency leads to excessive accumulation of sphingomyelin and its precursor lipids (Figure 3). Foamy histiocytes, which are the characteristic storage cells of NPD, accumulate in visceral organs like the liver, spleen, bone marrow, lungs, and kidneys. Cherry red spots may be seen in the eyes (Figure 4). In NPD-A patients, sphingomyelin is also accumulated in the brain. NPD-A and NPD-B represent different phenotypic spectrums of the same disease. Genetic testing is the gold standard for confirmation of a diagnosis of all NPD. From a hepatologist's point of view, NPD-B and NPD-C are most important for management. NPD-C will be discussed separately due to its unique presentation.

Clinical manifestations and natural history of ASMD

As is well known, NPD-A presents as infantile onset and such patients die early in childhood without much progress in the liver disease. Deaths in NPD-A patients occur predominantly due to extrahepatic manifestations. Terminal liver disease occurs very rarely in NPD-A. NPD-B presents as infantile to adult onset with a significant proportion having progressive liver dysfunction and approximately 40% requiring LT. Those who survive with their native liver die due to pulmonary or neurological disease [40].

McGovern *et al*[41] showed that baseline mean liver volume was 2.1 ± 0.8 times that of normal (MN) (range 0.9-4.6) in ASMD patients. In children, the mean liver volume was 2.2 ± 0.7 MN at baseline, 2.1 ± 0.7 at 1 year, and 1.7 ± 0.4 at the final visit, showing that there was minimal appreciable change. In adults, liver volume is 1.9 ± 0.9 MN at baseline, 1.6 ± 0.5 at 1 year, and 1.5 ± 0.4 at the final visit, showing some reductions over time. However, moderate to severe hepatomegaly (1.25-1.75 MN) was observed in 96% at baseline, 97% at 1 year, and 88% at the final visit. In NPD-B, hepatomegaly (mean liver volume is 1.9 MN) affects up to 70% of patients, less severe than splenomegaly (mean spleen volume is 11.1 MN). Liver volume correlates with splenic volume and severity of extra-hepatic manifestations of NPD-B[42]. In a prospective multicenter longitudinal study, it was shown that ALT and AST were high in 47% and 51% of individuals, respectively. Mean baseline ALT was 86.2 ± 67.8 U/L and 51.5 ± 44.6 U/L in children and adults, respectively. Mean ALT values at the final follow-up were 65.5 U/L in children and 52.7 U/L in adults. Total bilirubin was elevated in 33% at baseline, similar in adults and children (17.5 *vs* 21.8 mg/dL, respectively) and similar at follow-up. Synthetic functions (prothrombin time, platelet count, and albumin level) remained stable or worsened during the study[43-45].Wasserstein *et al*[43] followed 29 patients with NPD-B for 10 years and analyzed liver function at baseline and maximum follow-up visits (at least 9 mo or more apart). In the natural history of these patients (2-64 years of age at study entry), liver dysfunction was common. Approximately 75% of patients had elevated ALT and 65% had elevated AST at the initial visit. At baseline, there were no significant differences in liver function with respect to gender or age. However, total bilirubin was higher in patients > 18 years old. Similarly, there were no statistically significant changes in liver function during the follow-up period. Liver enzymes remained high whereas bilirubin remained normal in most patients throughout the study.

In a single center study of 103 patients with NPD-B over 10 years, six patients had fulminant liver failure, and three showed evidence of cirrhosis on liver biopsy. Two of the patients with liver failure received successful orthotopic LT at 12 and 25 years of age while the rest died from liver failure[42]. In another study by Wasserstein *et al*[43], one patient developed hepatic dysfunction in the first decade and subsequently died of liver failure. Homozygotes for R608, P323A, and P330R had milder disease than other genotypes. In another case report, an adult died at the age of 31 years from refractory encephalopathy related to cirrhosis and hepatic failure[44]. In 13 Chilean children homozygous for the SMPD1 p. (Ala359Asp) (A359D) mutation (associated with moderate to severe NPD B), five patients developed progressive cirrhosis. All five patients had sustained approximately four-fold increases in liver enzymes. Three of these patients died of liver failure and the other two received LT[45]. Cassiman *et al*[46] collected the data from 85 patients who died from NPD type B. They had splenomegaly (96.6%), hepatomegaly (91.4%), liver dysfunction (82.6%), and pulmonary involvement (75.0%). The median age at first symptom onset, age at diagnosis, and age at death or LT were 0.8 (0-60), 2.0 (0.2-78), and 18 (0.58-78) years, respectively. The leading causes of death were respiratory and liver failure (27.7% each) irrespective of age. The authors divided their cohort as chronic visceral *vs* neurovisceral ASMD. In the analysis, chronic visceral ASMD had lower age at first symptom onset (0.5 *vs* 1.25 years), diagnosis (1.7 *vs* 5 years), and death or LT (8 *vs* 23.5 years). Compared to chronic neurovisceral ASMD, 31.8% had progression of neurodegenerative disease along with respiratory disease (both 23.1%) and liver disease (19.2%) leading to death. In the subgroup of 23 patients with terminal liver disease, age of symptoms onset was 0.8 (0.17-5) years and age at diagnosis was 3 (0.2-67) years. Twelve (52.2%; age range 2.5-18 years) and 11 patients (47.8%; age range 21-67 years) died or had LT in childhood. The overall median age at death was 18 (2.5-67) years. Other liver-related deaths were variceal bleeding (n = 4) and hepatocellular carcinoma (n = 2)[46]. McGovern *et al*[41] concluded that individuals with either severe spleno-

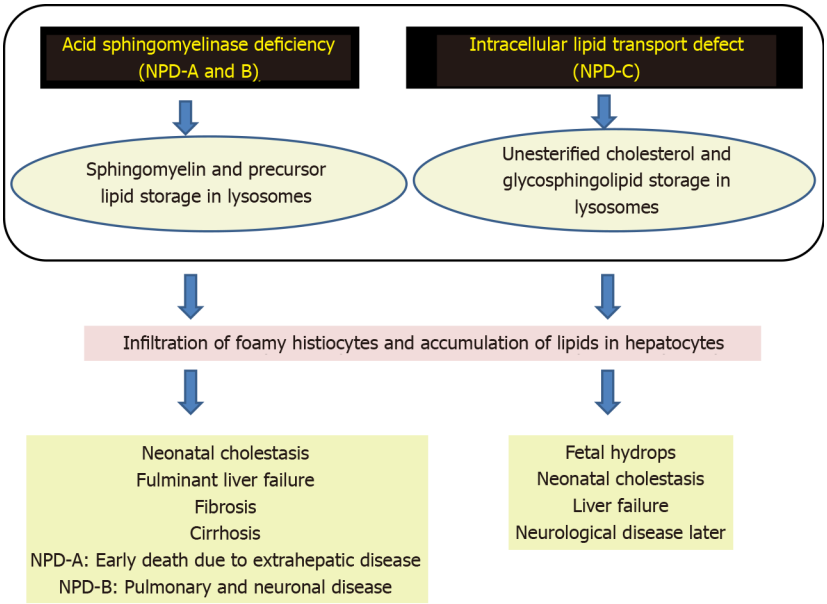

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Figure 3 Pathogenesis of Niemann-Pick disease types A, B, and C. NPD: Niemann-Pick disease.

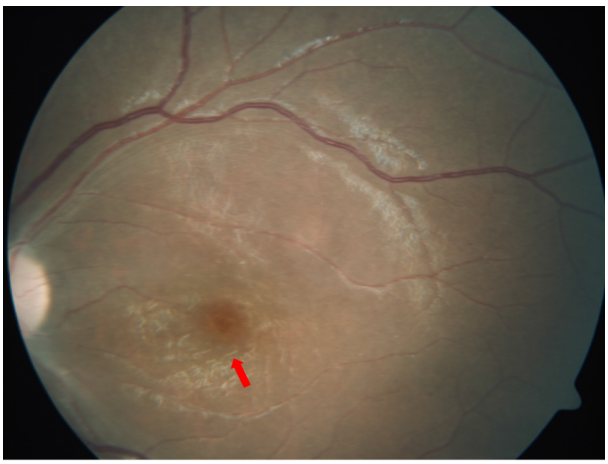

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Figure 4 Fundus examination showing a cherry red spot (red arrow) with a background of white retina.

megaly or prior splenectomy were a significant risk factor of death than those with smaller or intact spleens (odds ratio = 10.29, 95%CI: 1.7, 62.7).

Liver pathology in ASMD

Sphingomyelin accumulation in Kupffer cells and hepatocytes caused hepatomegaly. Hypertransaminasemia does not correlate with the stage of hepatic fibrosis or severity of the liver disease[48]. Evolution of fibrosis is variable. Among 17 NPD-B patients, 88% had fibrosis and 12% progressed to cirrhosis[48]. Sphingomyelin accumulation in sinusoidal Kupffer cells shows an enlarged and foamy appearance (Figure 5). In NPD-B patients with liver fibrosis, the stored sphingomyelin is seen as large collections of foamy Kupffer cells in portal areas as well as within hepatocytes[49].

Therapy and LT in ASMD

Currently, there is no disease-specific treatment for NPD-B. ERT with a recombinant human acid sphingomyelinase (olipudase alfa) is in clinical development. Olipudase alfa reduced liver and spleen volume by 31% and 39%, respectively, in a phase 2 trial evaluating five adults who were followed for 30 mo. It improved respiratory reserve by 35%, lipid profile, and bone health (bone mineral density in spine). Adverse events were headache, nausea, and abdominal pain. Anti-drug antibodies and hematological or cardiac side effects were not present. With olipudase alfa treatment, biomarkers such as chitotriosidase in serum and lysosphingomyelin in dried blood spots decreased remarkably[50].

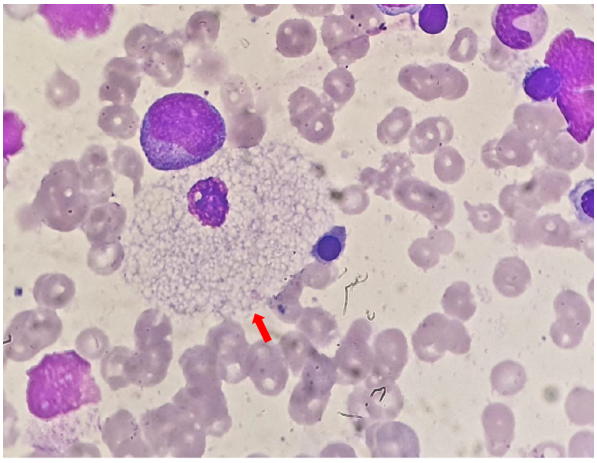

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Figure 5 Histology in Niemann-Pick disease type B. Red arrow shows a foamy vacuolated histiocyte.

Morbidity and disease burden are governed by respiratory disease and organomegaly in chronic ASMD. These are also independent contributors to mortality. The degree of splenomegaly correlates with short stature, atherogenic lipid profile, and hematological abnormalities. Respiratory-related complications are a major cause of mortality in ASMD. Hence Jones *et al*[51] concluded that lung function and spleen volume are meaningful clinical end points for assessing disease burden in ASMD.

Liu *et al*[52]performed LT in seven patients with NPD-B who were symptomatic at 12 (6-14) mo and transplanted at 6.5 (2.2-8.6) years. Among them, four patients received living donor LT, and three received whole-liver orthotopic LT. At a median follow-up of 10 (5-53) mo, all patients were alive with adequate catch-up growth. Liver function normalized within 3 wk after transplantation with improvement in platelet count, leukocyte count, and triglyceride levels. Pulmonary disease ameliorated after transplantation with resolution of interstitial lung disease and improved lung function. However, those with psychomotor improvement and developmental delays had persistent symptoms. The authors concluded that LT was an effective therapy for patients with NPD type B with severe liver and pulmonary dysfunction.

NPD TYPE C

NPD-C is categorized along with other NPDs due to the presence of foamy macrophages but there is no deficiency of acid sphingomyelinase enzyme. In NPD-C, there is an intracellular lipid trafficking defect. There is progressive lipid accumulation (unesterified cholesterol and glycosphingolipids) within the lysosomes. The prevalence of NPD-C is 1 in 120 000 live births. There is variable age of onset along with both visceral and neurological involvement. There are two types of NPD-C, type 1 (NPC1) and type 2 (NPC2). Both types have different genetic mutations, but clinical presentation is similar. NPC1 constitutes 95% while the remaining 5% are NPC2 patients. Elevated plasma chitotriosidase is a useful screening test in young children for NPD-C (and GD) but has a low sensitivity and specificity. Similarly, chemokine (C-C motif) ligand 18 (CCL18) is also a screening test. A positive Filipin stain of bone marrow is seen in 85% of NPD-C cases but it should not be considered a definitive assessment[41].

Age of presentation of NPD-C is variable from the perinatal period to the adult age. Broadly accepted age onset subgroups are perinatal (< 3 mo), early-infantile (3 mo to 24 mo), late-infantile (2 to 6 years), juvenile (6-15 years), and adolescent/adult (> 15 years). As a unique variant, NPD-C that starts early presents as neonatal cholestasis, infantile liver failure, ascites, or hydrops. This variant is very aggressive and the majority of patients have a poor prognosis[53]. In all types, the neurovisceral variant is more aggressive than visceral from the liver point of view. Splenectomy may worsen the liver, just like GD. Other than progressive liver disease, pulmonary complications are common. Most cases die of respiratory insufficiency or chest infections. Reasons for respiratory insufficiency are infiltration of the lungs with foamy cells, worsening organomegaly, and ascites. The respiratory complications are partly related to immune dysregulation in NPD-C[54]. Patients who survive beyond the first month of life without hepatic or respiratory failure, will eventually die of progressive neurological disease[55]. Castaneda *et al*[54] showed that none of the patients had neurological involvement at the time of diagnosis. The deceased patients with delayed developmental milestones have progressive deficits in ambulation, speech, swallowing, and feeding.

Neonatal liver disease has prominent hepatosplenomegaly in NPD-C (Figure 6). In one study, NPD-C accounted for 7.5% of all infants evaluated for cholestasis[56]. Ten patients with NPD-C had an age of

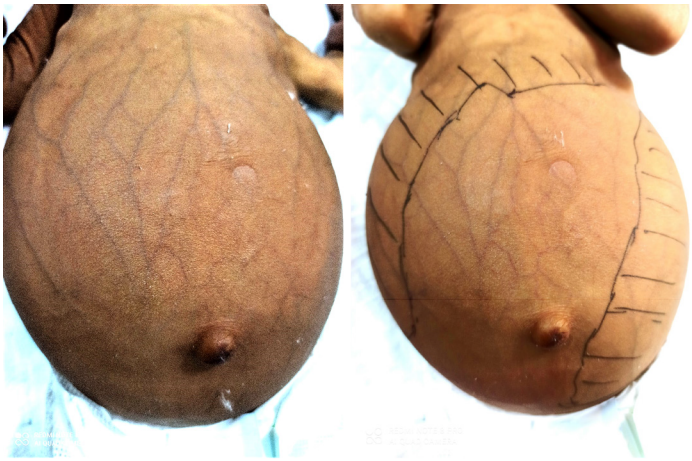

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Figure 6 Infant with Niemann-Pick disease type C presenting as cholestasis, dilated abdominal veins, and massive hepatosplenomegaly.

onset and age at diagnosis of 3.6 (1-10) d and 14.6 (1-30) d, respectively. Total and conjugated bilirubin levels were 13.9 (8-23) mg/dL and 8 (3.4-13.5) mg/dL, respectively. The serum AST level was 300.2 (101-700) U/L, which was nearly three times the upper limit of normal[57]. In most cases, cholestasis is transient for a few months. Hepatosplenomegaly persists for a variable period before the onset of neurological symptoms. These manifestations are important clues toward a possible diagnosis of NPD-C in infancy[58]. Isolated unexplained splenomegaly, with or without hepatomegaly, in a neonate or infant should raise suspicion of NPD-C[59]. Approximately 10% of cases progress to liver failure and usually die before the age of 6 mo in those with early prolonged jaundice and hepatosplenomegaly[54].

Prenatal onset NPD-C is a distinct and severe subgroup of the neonatal-onset NPD-C[60]. Fetal ascites or non-immune fetal hydrops can be seen in the perinatal period. In a study of seven NPD-C patients with prenatal manifestations, it was observed that these patients had a poor postnatal course. Of the two NPC1 patients who presented with fetal ascites at birth, one had prenatal ultrasonography at the 27th week of gestation that showed hydrops fetalis and polyhydramnios. This child later died in the first year of life due to progressive liver failure and pulmonary insufficiency. The second patient with similar clinical findings survived without progressive ascites or liver failure. Siblings with the same molecular defect may have different disease outcomes and variable presentation and severity of perinatal onset NPD-C[61].

There is no curative therapy for NPD-C. Patients with NPD-C are largely managed with supportive treatment and multidisciplinary care. Miglustat can be used as a disease-modifying agent, which is approved by European Union. In a study on 29 patients, after 12 mo of therapy, there was an improvement in horizontal saccadic eye movements, swallowing reflex, auditory acuity, and ambulation[62]. Further extension of the same cohort with 2-year treatment has shown stabilization of neurological symptoms (cognition, ambulation, and swallowing)[63]. Miglustat is more effective in patients with late-onset neurological symptoms as compared to those with early-onset disease.

LAL-D

General aspects

Wolman disease (WD) and cholesteryl ester storage disease (CESD) represent the clinical spectrum of LAL-D. The incidences of WD and CESD are approximately 1:500 000 and 1:40 000, respectively[64]. There is a defect in the *LIPA* gene (chromosome 10q23) which encodes LAL. There is an almost complete deficiency of LAL in WD while in CESD, there is some residual activity. LAL-D leads to impaired metabolism of triglycerides and cholesteryl esters resulting in their accumulation in macrophages and lysosomes of hepatocytes (Figure 7)[65]. There are many clinical differences between CESD and WD. WD has onset by 3 mo age and usually dies within 6 mo, whereas CESD begins in childhood or adulthood and age at death is variable. Compared to mild or absent features in CESD, WD has marked hepatomegaly, splenomegaly, malabsorption, growth failure and dyslipidemia. And 50% of WD have adrenal calcification which is rarely found in CESD[66]. The two entities are described separately.

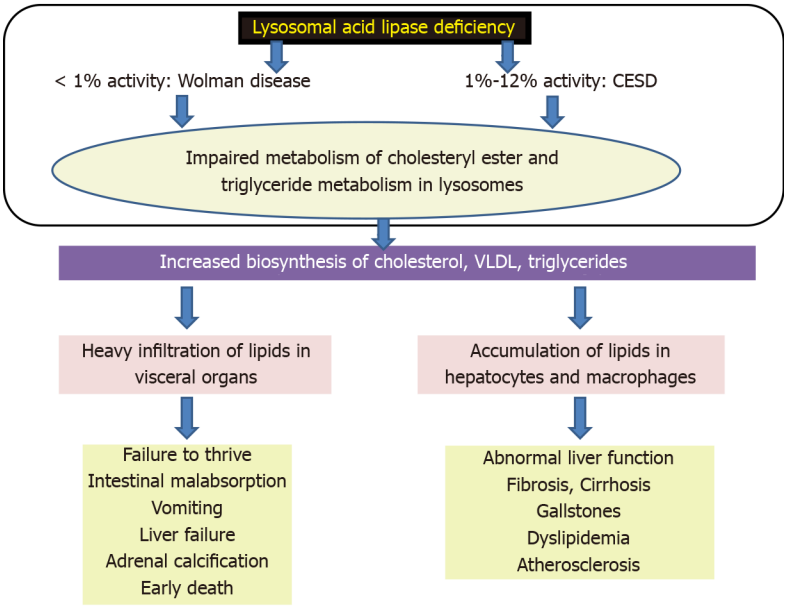

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Figure 7 Pathogenesis of lysosomal acid lipase deficiency. CESD: Cholesteryl ester storage disease; VLDL: Very low-density lipoproteins.

CESD

Clinical features and natural history in CESD

In CESD, residual lysosomal activity is 1%-12%[65]. This is a heterogeneous disorder with variable age of presentation from infancy to adulthood. Disease manifestation is also variable, which includes failure to thrive, vomiting, diarrhea, asymptomatic hepatomegaly, premature atherosclerosis, and cirrhosis. CESD patients often have dyslipidemia in the form of high total cholesterol, low-density lipoprotein (LDL), triglycerides, and low high-density lipoprotein (HDL). Adrenal calcification is usually seen in WD but it can be present rarely in CESD patients. CESD patients usually survive till adulthood.

Clinical settings to suspect CESD

CESD should be suspected in non-obese patients with hepatomegaly and unexplained hypertransaminasemia with abnormal lipid profile (low HDL and high LDL)[65,66].

The clinical situations to suspect CESD are: (1) Lean nonalcoholic fatty liver disease (NAFLD); (2) obese patients with persistent hepatomegaly, elevated transaminases, and abnormal lipid profile not responding to effective body mass index reduction; (3) adolescent and young adults diagnosed with NAFLD but liver biopsy showing microvesicular steatosis; (4) NALFD in a child less than 5 years of age; (5) pediatric cryptogenic cirrhosis; (6) unexplained liver failure in a young child with hepatomegaly or fatty liver; (7) abnormal lipid profile in children without familial dyslipidemias or obesity; and (8) early-onset gall stones or family history of cholecystectomies at a young age.

There is considerable overlap in the features of CESD and NAFLD. Obesity is associated with non-alcoholic steatohepatitis. LAL-D should be ruled out in those obese patients whose lipid profile continue to be deranged despite losing weight on treatment[65]. Microvesicular steatosis on liver biopsy may be mistaken as NAFLD[66]. NAFLD is rare in children < 3 years old, and uncommon at < 10 years of age. Unlike LAL-D, adolescents and adults with NAFLD usually have insulin resistance and hyperglycemia. Hepatomegaly is mild or absent in NAFLD as compared to marked involvement in CESD. Conversely body mass index (BMI) is high (overweight or obesity) in NAFLD as compared to near normal BMI in CESD. In lipid profile, triglycerides are mildly to moderately increased in NAFLD but near normal in CESD. HDL and LDL are mildly deranged in NAFLD as compared to moderate or marked derangement in CESD. Liver biopsy distinguishes macrovesicular steatosis in NAFLD from microvesicular steatosis in CESD[66]. Approximately 5%-15% of pediatric cirrhosis cases are cryptogenic. Screening for LAL-D is recommended in cryptogenic cirrhosis in children and adults. In those with microvesicular or mixed steatosis where Wilson disease is being considered, LAL-D should also be kept in mind. LAL-D should also be considered in unexplained liver failure in early childhood. Another setting for LAL-D is non-familial dyslipidemia resistant to regular treatment. The characteristic lipid profile in these patients is LDL > 130 mg/dL and/ or HDL < 40 mg/dL. These patients are non-obese and had normal fat distribution and normal fasting glucose[67,68]. Biochemical liver abnormalities are present early in the course of LAL-D disease. Low ALT, AST, and albumin with elevated GGT and bilirubin levels are characteristics in the early-onset form which is more aggressive in nature[66].

There is a genotype-hepatic phenotype correlation in CESD. Of the 32 known CESD mutations, 50% are missense, 25% are small deletions/insertions, 16% are non-sense, 6% are consensus splice-site mutations, and 3% have large deletions. The most common mutation, E8SJM$^{-1G>A}$, has been found only in CESD patients. In CESD and WD, nonsense, small deletions/insertions, splicing, and missense mutations can be found. *LIPA* mutations that encode mutant enzymes with residual activity are found in patients with CESD[69]. Individuals of Jewish ancestry (allele frequency of 1 in 32) have the *LIPA* founder mutation G87V (also described as G66V)[68]. E8SJM$^{-1G>A}$ homozygotes have limited genotype-phenotype correlation with diversity in presentation and progression. Almost all E8SJM$^{-1G>A}$ homozygotes in the patients have onset of symptoms in the first years of life, the majority by 6 years of age. Liver diseases among the reported E8SJM$^{-1G>A}$ homozygotes range from microvesicular steatosis to fibrosis and fibrosis with septal bridging, indicative of cirrhosis. Progression of the liver disease occurs later in adult life. Gastrointestinal involvements among E8SJM$^{-1G>A}$ homozygotes are gastrointestinal lipid accumulation, severe, acute, and chronic diarrhea, malabsorption, abdominal pain, and perforated gastric ulcer. Other extrahepatic manifestations are growth failure, anemia, respiratory infections, and coronary artery disease. Disease progression is variable. It is rapid in some patients and slow in others. Ultimate consequence is hepatic fibrosis and complications of atherosclerosis[70]. Several patients are compound heterozygotes for the H129P (histidine to proline) missense mutation (4.6% of normal enzyme activity) and the common E8SJM$^{-1G>A}$ allele (genotype H129P/E8SJM$^{-1G>A}$). This group has adult-onset cirrhosis, portal hypertension, and liver failure by the 4-5th decades[71]. CESD patients with the T288I/T288I (3.6% of normal LAL activity) and G342R/S289C genotypes have a WD-like presentation (infantile-onset, diarrhea, adrenal calcifications, and failure to thrive). However, they have sufficient residual LAL activity to survive into the second or third decades of life and also after LT[72]. Patients homozygous for H295Y (2.9% of normal LAL activity) also have infantile-onset CESD, requiring LT in the second decade[71]. Hence, early onset of disease manifestations may rapidly progress in childhood or adolescence. Patients with slower variant are usually healthy till adulthood when liver failure sets in, resulting in LT or death[73].

Burton *et al*[74] described 32 children with the progression of LAL-D over 13.3 (1.8-38.8) years; 25% of children were aged < 12 and 12-18 years, while 50% were > 18years. The patients had a high frequency of hepatomegaly (84%) and splenomegaly (88%). ALT (89.2 ± 42.1 U/L) and LDL (194.1 ± 63.1 mg/dL) levels were elevated. Age at onset, age at starting antilipidemic therapy, age at first recorded evidence of fibrosis or cirrhosis, and age at LT were 5.8 (0.0-42.0), 9.2 (2.0-43.2), 9.2 (1.9-41.0), and 13.0 (5.8-43.5) years, respectively. The authors concluded that the median time to an event was approximately 3.1 years. Bernstein *et al*[75] reviewed 135 patients with CESD. Age at onset in 35 (27%) severely affected children was between birth and two years, 81 (62%) presented between age 3 and 12 years, and 15 (11%) had an adolescent onset disease. Hepatomegaly and splenomegaly were present in 99.3% and 74%, respectively. Pathologic liver biopsy was reported in 83%, pathognomonic crystals/clefts in 16%, reduced LAL activity in 83%, and mutational diagnosis in 41%. AST and ALT levels were 54 (9-5240) and 52 (15-2340) U/L, respectively. Esophageal varices were reported in 12 patients, including nine from 5 to 20 years of age. Of the 11 reported deaths, 73% were due to liver failure between 7 to 56 years of age. Half of the deaths were under 21 years of age. Two cases of HCC were reported at the ages of 11 and 52 years. Adrenal calcifications were present in nine CESD patients aged < 1 to 10 years.

Pathology in CESD

The liver appears orange-yellow in color on gross examination. Microvesicular steatosis involving hepatocytes, Kupffer cells, and macrophages occurs due to massive lysosomal accumulation of CE and triglycerides. This progresses to fibrosis, and further into micronodular cirrhosis. On light microscopy, there is diffuse, uniform microvesicular steatosis with minimal zonal differences within the hepatic lobule. Foamy macrophages containing lipids and ceroids are present in the sinusoids and portal tracts. In contrast to macrophages, ceroid accumulation does not accompany lysosomal lipid accumulation in hepatocytes. Specific immunostains for the lysosomal lipid accumulation are LAMP1, LAMP2, LIMP2, and cathepsin D[76]. Pathognomonic birefringent CE crystals are observed in hepatocytes and/or Kupffer cells under polarized light. Fifty-eight percent of patients have specifically described birefringent, needle-shaped CE crystals, and 23% additionally cases have CE hepatocyte accumulation. CE deposition may be found in 80%by either frozen biopsy, polarization microscopy, or electron microscopy. Fixed paraffin-embedded sections show remnant clefts where the lipid had been extracted during dehydrating procedures. These crystals and clefts are seen by electron microscopy. They are limited by a single lysosomal membrane or appear free in the cytoplasm. Sixty-four percent of cases have fibrosis and/or cirrhosis. Among this group, sinusoidal, periportal, or septal fibrosis is seen in 50%, cirrhosis in 29%, and hepatocyte necrosis is reported in 7% of patients[76]. There is evidence that the disease is seen in utero. Fetal hepatocytes and syncytiotrophoblasts of the chorionic villi show marked membrane-bound CE accumulation and cholesterol infiltration. Necrosis of enlarged fetal adrenal glands is reported[77]. Gastrointestinal lipid and CE accumulate in the villi of the lamina propria, smooth muscle, vascular pericytes, and lacteal endothelium. Foamy macrophages are seen in the bowel mucosa[75].

Therapy and LT in CESD

ERT in CESD is a viable option. Sebelipase alfa is a recombinant human LAL that is expressed in egg whites from transgenic hen oviduct cells. ERT has considerable success in the late-onset LAL-D[76]. In a trial (LAL-CL01), nine patients were treated with four once-weekly intravenous infusions of sebelipase alfa at a dose of 0.35 mg/kg, 1 mg/kg, or 3 mg/kg[74]. After a median washout period of 15 wk, these patients entered another extension trial (LAL-CL04) with the same dose being continued for another 4 wk before transitioning to infusions (1 mg/kg or 3 mg/kg) every other week for a total of 12 wk. In the seven patients with completed 12 wk therapy, reductions in mean AST and ALT concentrations ($P \leq 0.05$), triglycerides ($P = 0.016$), total cholesterol ($P = 0.047$), and LDL ($P = 0.078$), and increases in HDL ($P = 0.016$) were appreciated from baseline[78]. This cohort was further treated with infusions every other week for 52 wk, demonstrating the long-term efficacy of this treatment. None of the patients developed autoimmunity and maintained favorable liver functions and lipid profiles[79]. In a multicenter randomized phase 3, placebo controlled trial named ARISE, Burton and colleagues showed the effectiveness of sebelipase alfa in 66 adults and children (NCT01757184; 24 patients were aged < 12 years). A dose of 1 mg/kg was infused every other week for 20 wk followed by an open-label period for another 16 wk during which both groups received treatment. Primary outcome of normalization of ALT levels at 20 wk (31% *vs* 7%; $P = 0.03$) was better in the treatment group. Favorable changes from baseline were also seen in the treatment group as compared to placebo regarding LDL cholesterol ($P < 0.001$), non-HDL cholesterol ($P < 0.001$), triglycerides ($P = 0.04$), HDL cholesterol ($P < 0.001$), and AST ($P < 0.001$). The treatment group also had lesser hepatic fat content ($P < 0.001$), steatosis ($P = 0.42$), and reduction in spleen volumes ($P < 0.001$). Liver volume change was not significantly different between the two groups [80]. Subsequently, all the patients entered a 130 wk, open-label extension period, and a 104 wk, open-label expanded treatment period. Age at randomization was 13 (4.7-59) years. Patients who crossed over from placebo to ERT showed improvements in liver enzymes that were similar to the ERT group in the previous double-blind trial. Thirteen patients had infusion-associated reactions and six developed anti-drug antibodies[81].

Bernstein *et al*[82] described 18 childhood-onset LAL-D post-LT. LT was performed for progressive liver dysfunction without ERT pre- or post-LT. Despite LT, extrahepatic progression occurred in 11 patients (61%) and death in six (33%). Liver allograft and post-mortem liver biopsies showed histological recurrence. Hence, it was concluded that LT is required in LAL-D-associated liver failure, but LT cannot prevent disease progression and recurrence. The pathophysiology is predominantly mediated by deficient enzyme activity in bone marrow-derived monocyte-macrophages. Bernstein *et al* [82] also reported a review of cases where LT had been performed in children aged 5 to 14 years. Six post-LT patients were followed from 10 to 36 mo. Except one with an incidental HCC, the rest did not have any complications. One patient who had an LT at five years of age developed rejection and congestive heart failure. In two patients with more than five years of follow-up, one 14-year-old child developed end-stage renal failure due to glomerular sclerosis, tubular atrophy, and interstitial fibrosis. The patient had extensive vascular lipid accumulation resulting in atherosclerosis. The lipid deposition in the renal vascular system signifies systemic lysosomal CE accumulation despite LT[82].

WD

Of the 19 mutations in WD, 37% are small deletions or insertions, 26% non-sense, 21% consensus splice-site mutations, 10% missense lesions, and 5% a large deletion. The two exon 8 splice-junction variants, E8SJM$^{+1G>A}$ and E8SJM$^{+3C>T}$, occur only in WD patients. Most severe *LIPA* gene mutations results in markedly reduced or no LAL activity in patients with WD[69]. Patients present just after birth, most commonly at 2-4 mo of age. Heavy accumulation of cholesteryl esters and triglycerides in visceral organs is a process that starts in utero. The features are adrenal necrosis, polyhydramnios, and microvesicular steatosis[83]. Adrenal infiltration leading to necrosis in the fetal stage leads to adrenal calcification described in about 50% of infants born with the condition (Figure 8). Infants with WD present with profound failure to thrive, hepatosplenomegaly, vomiting, and liver failure. Chronic diarrhea or steatorrhea due to the disease process itself and severe malabsorption are an important feature. Hence, the triad of WD is intestinal malabsorption, liver failure, and adrenal insufficiency[83]. Jones *et al*[84] showed that the median age at death was around 3.7 mo. In the untreated, the survival beyond 12 mo was highly unlikely [estimated probability 0.114 (95%CI: 0.009-0.220)]. Among the patients with evidence of early growth failure, the median age at death was 3.5 mo with even lower estimated probability of survival at 12 mo [0.038 (95%CI: 0.000-0.112)]. Despite hematopoietic stem cell transplant ($n = 9$) or LT ($n = 1$), survival was still poor (median age at death, 8.6 mo). Two open-label studies of ERT with sebelipase alfa were conducted in infants with WD. The VITAL study consisted of infants treated with once-weekly intravenous infusions of sebelipase alfa with a phase 2 dose-escalation study [LAL-CL08 (CL08)]. The analysis population contained 19 patients (9 in VITAL; 10 in CL08). Kaplan–Meier estimates of survival at 12 mo and 5 years of age were 79% and 68%, respectively. Overall, the median age of surviving patients was 5.2 years in VITAL and 3.2 years in CL08. Decreases in hepatosplenomegaly were noted in both studies. Short-term transfusion-free periods were seen in

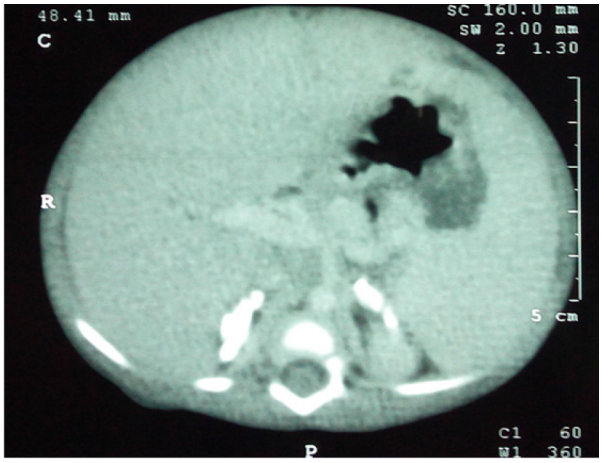

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Figure 8 Computed tomography of the abdomen in an infant with Wolman disease showing bilateral adrenal calcifications.

100% of patients in the VITAL study for a period of 4.6 (0.3-16.6) mo and 70% in the CL08 study for 5.5 (3.7-19.6) mo. None of the patients discontinued therapy. Most infusion-associated reactions (94% in VITAL and 88% in CL08) were mild or moderate in severity[84,85].

OTHER LSDS WITH LIVER DYSFUNCTION

Asymptomatic hepatomegaly is a common component of LSDs but liver dysfunction is not a usual presentation. Few reports suggest that mucopolysaccharidosis type VII (MPS, Sly syndrome) may rarely present with neonatal cholestasis, which may lead to progressive worsening and death. Gillet *et al*[86] diagnosed MPS type VII in a newborn with coarse facies and neonatal cholestasis. The diagnosis was made based on high urinary glycosaminoglycans, Alder-Reilly granules within the granulocytes, and absent β-glucuronidase activity in leukocytes. In another case report, a 55-d-old baby with cholestatic jaundice and coarse facies was diagnosed with MPS type VII on genetic analysis. The patient died at 7 mo of age due to progressive liver disease[87]. Farber's disease type IV has also been reported to present with neonatal cholestasis. Willis *et al*[88] reported two siblings born of nonconsanguineous parents, presenting with jaundice in the early neonatal period with rapid progression of liver disease and death at 32 and 52 d, respectively. The diagnosis was made on liver biopsy in which electron microscopy showed lysosomes containing curvilinear tubular bodies or Farber bodies. Many other LSDs are associated with hepatosplenomegaly in the newborn period, such as sialidosis, galactosialidosis, multiple sulfatase deficiency, I-cell disease, infantile sialic acid storage disease, and prosaposin deficiency[89-92]. Hochman *et al*[93] described a 9-d-old baby with mild jaundice who developed hepatosplenomegaly by 1 mo of age. This was later concluded as bile duct involvement in I-cell disease. One of the mechanisms of infantile hydrops is due to hypoproteinemia caused by liver dysfunction. LSDs associated with congenital ascites have been reported with sialidosis type II, galactosialidosis, isolated sphenoid sinus disease, Salla disease, MPS types IV and VII, GM1 gangliosidosis, I-cell disease, and Farber disease[94,95]. In cases of hydrops, demonstration of highly vacuolated storage cells in proband placental histology can serve as an early diagnostic clue for enzymatic testing in the further pregnancies[80,96].

CONCLUSION

Liver dysfunction in LSD poses a great challenge for pediatric and adult hepatologists. GD, NPD, and LAL-D are the most important LSD that has liver dysfunction. The hepatologist needs to have a high degree of suspicion to differentiate LSDs from other liver diseases. Extrahepatic involvement is the clue to the bedside diagnosis. Unexplained organomegaly, portal hypertension, and fatty liver are important presentations. Neonatal cholestasis and ascites are rare presentations in infants. Those presenting with neonatal or early onset of liver disease have a universally poor prognosis. Diagnosis is mainly dependent on tissue, enzyme activity, and genetics. If available, specific ERT and SRT should be conducted before irreversible organ damage occurs. Vigilance for progression has a key role in management. Those with progressive liver disease require LT. However, extrahepatic progression of the disease is often noted. Future research should preferably focus on long-term data with enzyme replacement, drug chaperone therapy, and gene therapy.

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FOOTNOTES

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MINIREVIEWS

Immunotherapy for hepatocellular carcinoma: A promising therapeutic option for advanced disease

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Abstract

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide, and its incidence continues to increase. Despite improvements in both medical and surgical therapies, HCC remains associated with poor outcomes due to its high rates of recurrence and mortality. Approximately 50% of patients require systemic therapies that traditionally consist of tyrosine kinase inhibitors. Recently, however, immune checkpoint inhibitors have revolutionized HCC management, providing new therapeutic options. Despite these major advances, the different factors involved in poor clinical responses and molecular pathways leading to resistance following use of these therapies remain unclear. Alternative strategies, such as adoptive T cell transfer, vaccination, and virotherapy, are currently under evaluation. Combinations of immunotherapies with other systemic or local treatments are also being investigated and may be the most promising opportunities for HCC treatment. The aim of this review is to provide updated information on currently available immunotherapies for HCC as well as future perspectives.

Key Words: Hepatocellular Carcinoma; Immunotherapy; Hepatocellular Carcinoma management; Hepatocellular Carcinoma therapy; Molecular targeted therapy

Core Tip: Hepatocellular carcinoma (HCC) is associated with high rates of recurrence and mortality. Approximately 50% of the patients require systemic therapies, traditionally consisting of tyrosine kinase inhibitors, with poor outcomes. Recently, immune checkpoint inhibitors have revolutionized the management of HCC, providing new therapeutic options. Despite these major advances, the different factors involved in poor clinical responses and molecular pathways of escape following use of these therapies remain unclear. Other immune strategies, such as adoptive T-cell transfer, vaccination, virotherapy, and combinations of immunotherapy with other systemic or local treatments, are under evaluation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with an estimated global incidence rate of 9.3 per 100000 persons per year and a corresponding mortality rate of 8.5[1]. While the majority of cases are in Eastern Asia, HCC incidence is widely increasing in northwestern Europe as well as in North America. HCC is also the fastest growing cause of cancer-related deaths in US males, tripling in both incidence and mortality rates[2]. The primary risk factors for HCC include underlying cirrhosis (independent of etiology) and chronic infection with hepatitis B virus or hepatitis C virus[3]. Since diabetes, obesity, and metabolic syndrome are also hypothesized to be risk factors associated with the development of metabolic cirrhosis, HCC is expected to become progressively more concerning as a health problem in the near future[4].

HCC is associated with a poor prognosis. A 5-year overall survival (OS) of 50–70% is only attained if the tumor is still resectable, owing to advances in both surgical and medical therapy[5]. Surgical treatments include liver transplantation (LT) and liver resection, with recurrence rates as high as 20% after LT and 70% after liver resection[6]. LT is the most effective curative treatment for cirrhotic patients, but it is reserved for patients who are ineligible for liver resection or radiofrequency ablation (RFA), as well as transplantable patients with recurrent HCC. Due to organ shortages, long waiting time for donors, and the risk of tumor progression, which leads to patient dropout, this is done[7].

Accordingly, liver resection is considered the first-line treatment for HCC in patients with compensated cirrhosis[8]. Thermal ablation is considered to be effective only for lesions smaller than 3 cm and when technically feasible[9]. Unfortunately, less than 30% of patients with HCC are eligible for these procedures because most patients have advanced disease or impaired liver function at the time of diagnosis, thus limiting aggressive treatment[10].

Trans-arterial chemoembolization (TACE) is the treatment of choice for patients with a suitable performance status[11]. Medical therapy is the only viable option for cases with disseminated disease or when other therapies are not feasible. However, HCC is notoriously resistant to chemotherapy and other systemic treatment modalities[12]. To date, systemic therapy is mainly based on the use of sorafenib, a multitargeted kinase inhibitor that improves survival by only 2.3-2.8 mo[13]. Indeed, the global median survival for patients with unresectable HCC is less than 1 year, highlighting the need for novel therapies to treat this disease.

Owing to an improved understanding of the molecular pathways of HCC carcinogenesis, other molecularly targeted approaches are under investigation. However, the intrinsic drug-metabolizing properties of the liver and other factors likely contribute to the limited efficacy of chemotherapeutics in the treatment of HCC[14]. In this review, we summarize novel immunotherapeutic approaches in HCC, reporting the latest evidence, analyzing their main limitations, and summarizing future perspectives that might overcome these drawbacks.

PHYSIO-PATHOLOGICAL BASIS

Liver immune system

Due to its physiological function, the liver has a peculiar and complex anti-inflammatory immune environment that develops tolerance to harmless foreign molecules, such as food antigens[15]. Kupffer cells (KCs), hepatic stellate cells (HSCs), and liver sinusoidal endothelial cells (LSECs) are the main mediators of this tolerogenic process[16]. KCs produce inhibitory cytokines interleukin 10 (IL-10) and prostaglandins and promote the activation of regulatory T cells (Treg)[17]. HSCs produces hepatocyte

growth factor (HGF), that induces the accumulation of Treg cells inside the liver and promotes T-cell apoptosis through programmed death ligand 1 (PDL1) expression[18,19]. LSECs also play a fundamental role in the immune microenvironment of the liver by expressing high levels of PDL1 and actively participating in the induction of Treg cells, mainly through transforming growth factor-β (TGF-β)[20]. KCs, HSCs, and LSECs are antigen-presenting cells (APC)[19,21]. Hepatic dendritic cells (DCs) also contribute to the tolerogenic microenvironment of the liver as they are poor stimulators of effector CD4+ T cells. They express low levels of major histocompatibility complex (MHC) II and co-stimulatory molecules, producing anti-inflammatory prostaglandin E2, which can increase IL-10 secretion and induce Treg cells[22].

The complex physiological immune-tolerating microenvironments of the liver are altered during the formation and progression of HCC. A progressively and persistently downregulated immune gene profile has been identified to occur during HCC progression, which leads to lower tumor immunity in advanced stages of the disease, together with a physical barrier made of collagen and other matrix proteins that protect tumoral cells[23]. Therefore, an interesting strategy could be to use therapeutic compounds to disrupt collagen and promote intratumoral infiltration by CD8+ lymphocytes trapped in the peritumoral zone[24]. Different approaches to modulating this complex immune microsystem are desirable in combination with immunotherapies for HCC.

The tumor microenvironment of hepatocellular carcinoma

The tumor microenvironment (TME) of HCC is the result of complex interactions among hepatic non-parenchymal resident cells, tumor cells, immune cells, and tumor-associated fibroblasts[25]. The TME has important effects on the presence and activity of all signaling molecules, such as cytokines and chemokines, as well as other paracrine factors[26]. This complex cellular interplay has a substantial influence on tumor immune evasion, affecting both innate and adaptive immune responses and often leading to high levels of dysfunctional tumor-infiltrating lymphocytes (TILs) and natural killer (NK) cells[27,28].

The peritumoral environment at the forefront of HCC development is also important. KCs at this stage show higher levels of PDL1, and hyperactivation of HSCs in this TME is associated with a poor prognosis[29,30]. Similarly, PDL1 expression is higher in CD8+ T cells, which is associated with a higher risk of cancer recurrence, metastasis, and death[31]. Other molecules involved in the immune checkpoint have been identified in HCC and have been shown to correlate with poor prognosis, such as T-cell immunoglobulin, mucin-domain-containing molecule-3 (Tim-3), and lymphocyte-activation gene 3 in TILs and tumor-associated macrophages (TAM)[32].

Mechanisms of immune evasion in hepatocellular carcinoma

Several molecular mechanisms of immune evasion have been described in HCC, derived from the alteration of different signaling pathways, although much remains to be explored.

TGF-β is abundant in the HCC TME and can be produced by tumor cells, TAMs, and Treg cells[25,33]. TGF-β can reduce or eliminate antitumor responses by blocking T-cells and NK cells, inhibiting APC cells and TAMs, and activating Tregs[34-36]. High TGF-β expression has been shown to be associated with poor prognosis in HCC patients, and circulating levels are associated with clinical response to sorafenib and pembrolizumab[37,38].

Pro-inflammatory cytokines such as tumor necrosis factor and IL-1 are significantly downregulated in the HCC TME and are associated with increased levels of immunosuppressive cytokines, resulting in immune response dysfunction (IL-4, IL-5, IL-8, and IL-10)[23]. This has been associated with poor prognosis and worse clinical outcomes in several cancer types, including HCC[39]. Another pro-inflammatory cytokine, type I interferon (IFN), can activate the immune response; however, it can also trigger anti-inflammatory signals through the production of IL-10[40]. IL-10 is upregulated in HCC and is produced by TAMs and Treg cells. It can impair the capacity of APCs to recruit T cells and promote the upregulation of PDL1 in monocytes[41]. Furthermore, IL-10 Levels are associated with the number of myeloid-derived suppressor cells (MDSC)[42]. Representing the complexity of the TME, low IFNγ levels are associated with a worse prognosis in HCC[43].

Chemokines also play a fundamental role in recruiting Tregs *via* chemokine receptor 6 (CCR6) and chemokine ligand 20 (CCL20)[44]. The level of both Treg cells and TAMs in the liver is associated with poor prognosis in HCC[45,46]. A rare recently discovered immunosuppressive cell is represented by the T helper 17 cells, that produce high levels of PD1and inhibit NK cell function. Then there are the hepatic neutrophils that can recruit macrophages and Treg cells[47,48].

Vascular endothelial growth factor (VEGF) is a soluble molecule produced by tumor cells and the surrounding stroma[49]. It is known to promote tumor angiogenesis, but it can also act as an immune modulator by inhibiting liver APCs and activating MDSCs and Treg cells[50]. This immunomodulatory action of VEGF inhibitors may play a role into their anti-tumor activity. The presence of many possible immunoregulatory targets in the HCC TME has stimulated the investigation of different immunotherapies in HCC, some of which have been shown to be effective for other malignancies.

IMMUNE CHECKPOINT INHIBITORS

Immune checkpoint inhibitors (ICIs) are monoclonal antibodies directed against extracellular ligands involved in the suppression of antitumor immune responses. These proteins are expressed by both cancer cells and the immune system cells. To date, only two categories of molecular targets have been examined in clinical trials, PD-1 and CTLA-4, and only two checkpoint inhibitors have been approved for use in HCC by the United States Food and Drug Association (FDA)[51,52], while many more promising targets are being investigated.

Nivolumab

Nivolumab is a human anti-PD-1 IgG4 monoclonal antibody targeting PD-1, currently used as a second-line therapy for HCC after approval by the FDA in 2017. Checkmate 040, a phase I/II dose escalation and expansion trial, showed substantial tumor reduction and good tolerability in HCC patients. In the Checkmate040 study, patients showed a median OS of 15 mo (95%CI: 9.6-20.2), with an objective response rate (ORR) of 15% (95%CI: 6-28)[53]. The median duration of response to nivolumab among the 48 patients was 17 mo (95%CI, 6-24 mo), with a 2-year survival rate higher than 80%[53]. As an unexpected result, patients with PD-1- and PD-L1 expression showed better survival outcomes, with a median OS of 28.1 mo (95%CI: 18.2-n.a.) vs. 16.6 mo in the group with low PD-L1 expression (95%CI: 14.2-20.2).

CheckMate 459, a phase III trial, compared the efficacy of nivolumab *vs* sorafenib as a first-line treatment in 743 HCC patients[54]. In the Nivolumab cohort, the OS outcomes were not statistically significant (median survival 16.4 *vs* 14.7 mo, HR 0.85; $P = 0.07$). However, in this study, patients with PD-L1 expression > 1% in the nivolumab arm had a significantly higher ORR (28.2% *vs* 12.2%). Furthermore, the rate of grade 3 or 4 adverse events in nivolumab arm was only 22%, compared to 49% after sorafenib[54]. Currently, other clinical trials are investigating the role of nivolumab for HCC, either as monotherapy or in combination with other modalities (Tables 1 and 2).

Pembrolizumab

Pembrolizumab is an anti-PD-1 IgG4 antibody approved by the FDA in 2018 as a second-line systemic therapy for HCC after sorafenib. A phase II trial, KEYNOTE-224, showed high efficacy and tolerability of pembrolizumab in HCC patients with a significant gain of survival (HR, 0.78; $P = 0.023$), although it did not meet the prespecified statistical threshold[55]. The ORR was 17% (95%CI: 11-26), while1% of the patients showed a complete response to the treatment and 16% experienced a partial response. The median OS was 12.9 mo (95%CI: 9.7-15.5), and the median progression-free survival (PFS) was 4.9 mo (95%CI: 3.4-7.2). Sixty-two percent of patients showed a good disease control (95%CI: 52-71), while the 25% of patients experienced grade 3 or 4 adverse events, with 1 death[55]. More evidences are expected from KEYNOTE-240 and KEYNOTE-394, two phase III trials investigating the role of Pembrolizumab, that are still ongoing.

Similarly, trials evaluating the association between pembrolizumab and other treatments are ongoing. A phase Ib trial showed promising antitumor activity of the combination of pembrolizumab with lenvatinib (a multiple kinase inhibitor against VEGF receptors) as a first-line treatment in patients with unresectable HCC[56]. Furthermore, a phase III trial has investigated the safety and efficacy of pembrolizumab in combination with lenvatinib, while the role of pembrolizumab alone as adjuvant therapy after RFA or radiotherapy is still under evaluation (Tables 1 and 2).

Atezolizumab

Atezolizumab is an engineered IgG1 monoclonal antibody targeting PD-L1 and was recently approved by the FDA and European Medicines Agency in combination with bevacizumab as a first-line treatment for unresectable HCC. In the open-label phase III IMbrave150 clinical trial, 501 patients were randomly assigned at a ratio of 2:1 to receive atezolizumab plus bevacizumab or sorafenib[57]. The combination of atezolizumab and bevacizumab showed significantly higher 12-months OS, 67.2% (95%CI: 61.3-73.1) *vs* 54.6% (95%CI: 45.2-64.0), respectively, with 52% and 40% of patients surviving at 18 months, respectively. Similarly, the median PFS was improved at 6.8 months (95%CI: 5.7-8.3) *vs* 4.3 months (95%CI: 4.0-5.6), respectively ($P < 0.001$).

Further studies investigating the combination of atezolizumab with other treatments are ongoing (Table 2). In particular, the COSMIC-312 phase III study tested atezolizumab plus cabozantinib (an oral tyrosine kinase inhibitor against VEGFR, FLT-3, MET, AXL, KIT, Tie-2, and RET) *vs* sorafenib as first-line therapy. Two phase III studies are enrolling patients to receive atezolizumab plus bevacizumab in combination with TACE or adjuvant therapy after surgery of RFA. Finally, results from several studies investigating other anti-PD-1 antibodies, such as tislelizumab compared to sorafenib, and anti-PD-L1 antibodies, such as durvalumab or avelumab, are expected (Table 2).

Table 1 Clinical trial involving immunotherapic agents as adjuvant therapy for hepatocellular carcinoma

Trial	Included population	Immunotherapy regimen	Target	Control arm	Primary outcome	Sample size
CHECKMATE-9DX (NCT03383458)	Patients at high risk of recurrence after resection or ablation	Nivolumab	PD-1	Placebo	RFS	530
KEYNOTE-937 (NCT03867084)	Patients with complete radiological response after resection or ablation	Pembrolizumab	PD-1	Placebo	RFS, OS	950
EMERALD-2 (NCT03847428)	Patients at high risk of recurrence after resection or ablation	Durvalumab plus bevacizumab anddurvalumab plus placebo	PD-L1	Placebo plus placebo	RFS	888
IMBRAVE-050 (NCT04102098)	Patients at high risk of recurrence after resection or ablation	Atezolizumab plus bevacizumab	PD-L1	Active surveillance	RFS	662

PD-1: Programmed cell death protein 1; RFS: Recurrence-free survival; OS: Overall survival.

Table 2 Ongoing clinical trial involving immunotherapic agents as first line therapy for hepatocellular carcinoma

Trial	Immunotherapy regimen	Target	Control arm	Primary outcome	Sample size
RATIONALE-301 (NCT03412773	Tislelizumab	PD-1	Sorafenib	OS	674
CHECKMATE-9DW (NCT04039607)	Nivolumab plus ipilimumab	PD-1	Sorafenib or Lenvatinib	OS	650
COSMIC-312 (NCT03755791)	Atezolizumab plus cabozantinib	PD-L1/Tyrosine Kinase	Cabozantinib or Sorafenib	PFS, OS	740
ORIENT-32 (NCT03794440)	Sintilimab plus IBI305	PD1/VEGF	Sorafenib	OS, ORR	595
LEAP-002 (NCT03713593)	Lenvatinib plus pembrolizumab	VEGF-R/PD-1	Lenvatinib plus placebo	PFS, OS	750
HIMALAYA (NCT03298451)	Durvalumab plus tremelimumab or durvalumab	PD-L1/CTLA4	Sorafenib	OS	1504
PHOCUS (NCT02562755)	Pexa-Vec plus sorafenib	Thymidine kynase	Sorafenib	OS	459
LEAP-012 (NCT04246177)	TACE plus pembrolizumab plus lenvatinib	PD-1	TACE plus placebo plus placebo	PFS, OS	950
CHECKMATE-74W (NCT04340193)	TACE plus nivolumab plus ipilimumab	PD-1	TACE plus nivolumab plusplacebo or TACE plus placebo plus placebo	TTTP, OS	765
EMERALD-1 (NCT03778957)	TACE plus durvalumab plus; Bevacizumab or TACE plus durvalumab plus placebo	PD-L1/VEGF	TACE plus placebo plus placebo	PFS	710
TACE-3 (NCT04268888)	DEB TACE plus nivolumab	PD-1	DEB TACE	OS	522

PD-1: Programmed cell death protein 1; VEGF: Vascular Endothelial Growth Factor; CTLA-4: Cytotoxic T-Lymphocyte Antigen 4; PFS: Progression-free survival; ORR: Overall response rate; TTTP: Time to TACE progression; RFS: Recurrence-free survival; OS: Overall survival.

VACCINE THERAPY IN HEPATOCELLULAR CARCINOMA

The development of vaccines against different types of cancer aims to enhance existing tumor-specific responses. Due to altered T cell activity in the HCC TME, vaccine therapy is usually investigated in combination with ICI or other therapies[58]. Although the first HCC vaccines were shown to be safe and have immunologic effects, their clinical efficacy is still limited, possibly because of immunological tolerance to self-antigens that causes them to not be completely tumor-specific[59,60]. Thus, new strategies are currently under investigation.

Alpha-fetoprotein peptide

Alpha-fetoprotein (AFP) is a 70-kDa protein expressed during fetal development and in the adult liver. Serum AFP levels are usually not detectable in the adult. However, AFP levels increase in approx-

imately 70% of HCC cases, allowing for its use as a biomarker[61,62]. Butterfield *et al*[63] constructed a human AFP-expressing replication-deficient adenovirus as a new target for T-cell-based immunotherapy. AFP-based therapies have been evaluated in phase I/II trial with two patients with HCC who had an AFP-expressing tumor and who completed a previous treatment for HCC, and their tolerability and safety were confirmed. Additionally, both patients experienced high levels of AFP-specific CD8+ T cells, further confirming their preexisting immunity[64]. Further studies are required to confirm these results.

Multidrug resistance-associated protein 3

Multidrug resistance-associated protein 3 (MRP3) is a carrier-type protein that is highly expressed in several human cancers, including HCC[65]. Interestingly, MRP3 is also associated with resistance to sorafenib toxicity in HCC cells[66]. The safety and the immune response to the vaccine based on an MRP3-derived peptide (MRP3765) were tested in a phase I study involving 12 HLA-A24-positive HCC patients[67]. The vaccine showed a good safety profile, with an immune response in 72.7% of the treated cases and a median OS of 14.0 mo (95%CI: 9.6-18.5). OS was notably better than in patients undergoing hepatic arterial infusion chemotherapy without the MRP3 vaccination (median OS 12.0 12.6 mo)[68].

Glypican-3

HCC cells sometimes overexpress proteins relative to the surrounding healthy tissue, as is the case for glypican-3 (GPC3)[69]. Therefore, the GPC3-derived peptide vaccine was tested and reported as safe in 33 patients with advanced HCC in a phase I clinical study, showing good results in terms of GPC3-specific immune response[70]. However, only 1 patient developed a partial response, while 19 patients had stable disease. Interestingly, another phase II study investigating the GPC3-derived vaccine as adjuvant therapy showed significantly lower recurrence rates than with surgery alone after 1 year (52.4% *vs* 61.9%, P = 0. 387) and 2 years (24% *vs* 48%, P = 0.047)[71].

Oncolytic viruses

Oncolytic viruses are viral units engineered to obtain direct lysis of tumor cells, releasing soluble cancer peptides to induce antitumor neoantigen-specific cytotoxic T lymphocyte responses[72]. A phase II study (NCT00554372) assessed the safety of two doses of JX-594 (Pexa-Vec, by testing both low dose or high dose in 30 patients with HCC. All patients experienced dose-dependent flu-like syndrome with fever, rigor, and vomiting within the first few days[73]. Furthermore, when tested as a second-line treatment, there was no significant survival difference when compared to the standard of care (4.2 *vs* 4.4 mo, 95%CI: 0.78-1.80; P = 0.428)[74]. Other schemes are currently being tested.

Dendritic cell vaccines

DCs can be activated with a specific antigen *in vitro* and then injected into patients to enhance the immune response. Wang *et al*[75] obtained encouraging antitumor effects in murine models treated with DCs activated by tumor cell lysate. A good tolerability profile was reported after a phase I study testing autologous DCs on 10 patients with cholangiocarcinoma or HCC, and after a phase II clinical trial with 35 patients using DCs[76]. Interestingly, DC infusion enhanced a stronger tumor-specific immune responses in combination with TACE, than TACE alone, even if without improved survival outcomes [77]. However, further clinical trials are warranted.

New York esophageal squamous cell carcinoma-1

New York esophageal squamous cell carcinoma-1 (NY-ESO-1) is a promising target antigen owing to its low expression in healthy tissue[78]. A specific CD8+ T cell response to NY-ESO-1b has been reported in 48% of patients with HCC expressing NY-ESO-1 mRNA and HLA-A2[28]. Furthermore, there was a correlation between such response and patient survival. However, no studies have yet been conducted to investigate the clinical response to NY-ESO-1 vaccines in patients with HCC.

ADOPTIVE CELL THERAPY

Adoptive cell therapy (ACT) is a passive therapy in which lymphocytes are activated and/or expanded *ex vivo,* and then re-injected into the patient[79]. The treated cells include lymphokine-activated killer (LAK) cells, cytokine-induced killer (CIK) cells, NK cells, TILs, and redirected peripheral blood T cells. These latter cells are genetically programmed to recognize and attack tumor cells.

Chimeric antigen receptor T cells

An individual's own T cells can be engineered to recognize tumor cell surface proteins and, in turn, cause cancer cell death, as demonstrated by approximately six chimeric antigen receptor T cell (CAR T cell) therapies already approved by the FDA since 2017 (all of them for blood cancers). A similar effect was observed by targeting GPC3-positive HCC cells *in vitro* and in mice[80,81]. Takayama *et al*[82]

published excellent results from a trial of 150 patients who were randomly assigned to receive adjuvant adoptive immunotherapy or no treatment. After a median follow-up of 4.4 years, adoptive immunotherapy decreased recurrence rates by 18% compared with that in controls, with a shorter time to first recurrence [33% (95%CI: 22-4) *vs* 48% (95%CI: 37-59) at 3 years; 22% (95%CI: 11-34) *vs* 38% (95%CI: 22-54) at 5 years; $P = 0.008$]. The immunotherapy group had a significantly longer recurrence-free survival ($P = 0.01$) and disease-specific survival ($P = 0.04$). Several phase I and phase II clinical studies are currently evaluating CAR-GPC3 T-cell therapy alone or in combination with fludarabine, cyclophosphamide, or other treatment options.

Cytokine-induced killer cells

CIK cells are a heterogeneous population of effector $CD3^+CD56^+$ NK cells expanded *in vitro* from peripheral blood mononuclear cells. They are used as pharmacological tools for cancer immunotherapy because they exhibit MHC-unrestricted, safe, and effective antitumor activity. Firsts attempts to develop ACT for HCC were not able to reach the clinical stage owing to the technological complexity and to the low efficacy. A phase II study of 127 patients and a phase III study of 200 patients, in which CIK cells were tested as adjuvant therapy and compared with no adjuvant therapy, showed improved DFS after CIK immunotherapy, although the increase in OS was not statistically significant[83]. Improved OS was observed only in patients diagnosed with tumors > 5 cm in size ($P = 0.0002$). Furthermore, the combination of CIK immunotherapy and minimally invasive therapies in HCC patients with no history of previous surgery was reported to be safe and feasible, as well[84,85]. As a first-line therapy, the CIK cell treatment followed by TACE and RFA group were compared with those treated with TACE + RFA. Although no significant difference in disease control rates was found between the two cohorts, survival analysis showed that patients in the CIK+TACE+RFA group had a significantly longer median OS of 56 mo (95%CI: 38.09-73.91) compared to 31 mo of TACE+RFA alone (95%CI:24.53-37.47)[85].

TILs can also be created from fresh tumor tissue to produce tumor-reactive expanding cells, screened on the basis of the ability to recognize autologous tumor cells, and further expanded to obtain several billion active cells[86]. The safety and feasibility of adjuvant TIL therapy were demonstrated in a phase I trial on HCC patients[87]. Current challenges include the capability of further expand tumor-specific T cells and scaling up the manufacturing process.

OVERCOMING CURRENT LIMITATIONS TO IMMUNOTHERAPY

Enhancing locoregional therapies

Locoregional therapies can be a strong ally in the immunologic war against HCC, owing to several advantages. Their relatively easy accessibility makes HCC an ideal target for local interventions, such as thermal ablations or intra-arterial therapies, and image-guided interventions are a common practice in this setting of patients. Therefore, these approaches can intratumorally deliver immunostimulant agents, allowing not only more potent immunological responses but also potentially decreasing toxicity. Indeed, such agents are often quite toxic when administered systemically, causing sepsis-like cytokine release syndrome and systemic inflammation[88]. Thermal ablation has been shown to activate immune responses and T cell infiltration in HCC[89]. Furthermore, to obtain stronger immune stimulation, different locoregional therapies can be combined sequentially or simultaneously with systemic immunotherapy[90].

Promising results were obtained from the combination of thermal ablation or TACE plus tremelimumab in patients with advanced HCC, with a reported response rate of 26%, a disease control rate of 89%, an OS of 12.3 mo, and 45% of the stabilizations lasting longer than 6 mo[91]. These encouraging data have prompted new clinical trials combining durvalumab/tremelimumab with TACE or RFA, and these trials are ongoing.

Combination immunotherapy strategies

The combination of multiple immunotherapies could be another option to overcome the limitations of single treatments. Although this approach could increase the risk of high-grade adverse events, the initial results are encouraging. A combination of nivolumab (NIVO) and ipilimumab (IPI) was tested as a second-line therapy after sorafenib. Following treatment with this combination, patients showed an ORR twice that of the NIVO mono-treated patients (31% and 14%, respectively). Thirty-seven percent of the patients had grade 3-4 treatment-related adverse events, but only 5% had an event leading to therapy discontinuation[92]. Similar results were reported in another study in which nivolumab was tested with cabozantinib (CABO) and ipilimumab, both as double and triple therapies. The median PFS was 5.5 mo for patients in the NIVO + CABO and 6.8 mo for those in the NIVO + IPI + CABO groups, while the median OS was not reached. However, the triple combination led to grade 3-4 treatment-related adverse events in 71% of patients, with 20% discontinuing therapy[23]. Similarly, a phase II trial investigating the safety and efficacy of a combination of durvalumab and tremelimumab is currently recruiting patients.

Immune checkpoint inhibitors can also be combined with oncolytic viruses; this strategy has been tested in several ongoing randomized trials, but without published results. Another study investigated the combination of activated T cell transfer (ATVAC) with an autologous tumor lysate-pulsed DC vaccine as an adjuvant therapy in HCC patients, showing an improved median PFS of 24.5 mo (95%CI: 7.8-41.2) and OS of 97.7 mo (95%CI: 48.6-146.7), compared to a median PFS of 12.6 mo (95%CI: 6.9-18.3) and OS of 41.0 mo (95%CI: 16.3-65.8) of the other group. No adverse events of grade 3 or more were observed[93]. These encouraging results need to be confirmed in future studies.

Tailoring HCC immunotherapies

As previously discussed, several trials have shown encouraging results in certain subgroups of HCC patients, although the overall outcomes have not improved much. Identification of patient subsets that would benefit from ICI therapy should be a mainstay of current cancer research. Indeed, the identification of the best candidates for immunotherapies and combination therapies can play a fundamental role not only in achieving the best results but also in saving a substantial amount of funding and healthcare resources. At the same time, a better understanding of patient characteristics could help to avoid related toxicities.

Some characteristics of the patients in the KEYNOTE-240 and CheckMate 459 trials have already been reported, identifying Asian patients and those with AFP levels > 200 ng/mL as the patient groups showing the best outcomes[54]. The latter study also showed a better OS among patients with vascular invasion or extrahepatic disease. In the IMbrave150 trial, OS and PFS were worse in patients with a non-viral etiology, high AFP levels, no macro-vascular invasion, and extrahepatic disease[57].

The genetic features of HCC have been identified using next-generation sequencing (NGS), and several biomarkers have been identified as useful for selecting the best candidates for new targeted therapies. NGS analysis detected ten patients with WNT/β-catenin mutations that did not respond to anti–PD-1 or anti–PD-L1drugs, while 50% of CTNNB1 WT cases showed a response[94]. The WNT/β-catenin mutation was also correlated to lower median PFS (2.0 *vs* 7.4 mo; 95%CI: 2.9-28.8; $P < 0.0001$) and OS (9.1 *vs* 15.2 mo; 95%CI: 0.76-8.7; $P = 0.11$) than WNT/β-catenin wild type. Further studies are needed to determine the clinical implications of NGS in HCC therapy.

CONCLUSION

In conclusion, HCC is a widely studied yet challenging disease. Systemic therapies have shown modest results; however, due to tremendous improvements in basic molecular research on anti-tumor immune responses in the TME, a new class of molecular therapies is emerging and changing the HCC therapeutic landscape. Several clinical trials are ongoing, providing hope for an epochal turning point. In our opinion, the development of synergies between immunotherapies and locoregional or radical therapies is likely to be key in the future of HCC therapy. Similarly, another area where a major shift in HCC management may arise is the role of immunotherapy in adjuvant therapy. In fact, immunotherapy used in an adjuvant setting after surgery showed promising results, affecting recurrence rates, which represents a major challenge following surgical therapy. These results suggest the usefulness of immunotherapy, even in early stages, such as in patients undergoing tumor resection or ablation. Importantly, technological advances and recent evidence have also paved the way for the identification of molecular mechanisms involved in sensitivity and resistance to individual agents or combinations, helping advance the era of personalized medicine. We are convinced that these findings may help in the adoption of and adaptation to different types of therapies for individual patients in the near future. Considering the speed and breadth of discoveries in this field, efforts should be made to embed correlative research studies in every new clinical trial.

FOOTNOTES

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MINIREVIEWS

Alcohol use disorder and liver injury related to the COVID-19 pandemic

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Abstract

Alcohol use disorder is a complex and heterogeneous phenomenon that can be studied from several points of view by focusing on its different components. Alcohol is a hepatotoxin whose metabolism creates profound alterations within the hepatocyte. The liver is the central organ in the metabolism of alcohol, a process that also involves other organs and tissues such as the brain, heart and muscles, but the most relevant organ is the liver. The anatomopathological alterations in the liver associated with the prolonged use of alcohol range from the simple accumulation of neutral fats in the hepatocytes, to cirrhosis and hepatocellular carcinoma. Alcohol abuse frequently leads to liver disease such as steatosis, steatohepatitis, fibrosis, cirrhosis, and tumors. Following the spread of coronavirus disease 2019 (COVID-19), there was an increase in alcohol consumption, probably linked to the months of lockdown and smart working. It is known that social isolation leads to a considerable increase in stress, and it is also recognized that high levels of stress can result in an increase in alcohol intake. Cirrhotic patients or subjects with liver cancer are immunocompromised, so they may be more exposed to COVID-19 infection with a worse prognosis. This review focuses on the fact that the COVID-19 pandemic has made the emergence of alcohol-induced liver damage a major medical and social problem.

Key Words: Alcohol use disorder; Alcoholic liver disease; Liver injury; COVID-19; Alcohol abuse; Alcohol dependence

Core Tip: Alcohol use disorder is a complex phenomenon with psychological and physical consequences. Alcohol-associated liver disease is a devastating complication of alcohol use disorder. Following the spread of coronavirus disease 2019 (COVID-19) and consequent lockdown there was an increase in alcohol consumption resulting in a worrying increase in steatosis of the liver, alcohol-related steato-hepatitis and alcohol-related liver disease. In addition, patients with alcohol-associated liver disease may be more susceptible to COVID-19 and a worse prognosis. This review focuses on the emergence of alcohol-induced liver damage as a major medical and social problem during the COVID-19 pandemic.

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INTRODUCTION

Excessive alcohol consumption has a dual harmful effect: It leads to the development of alcohol dependence, withdrawal symptoms and psychosocial problems, but it also elicits a significantly augmented risk of developing acute and chronic dysfunction in multiple organ systems. The liver can be seriously damaged by alcohol as it is mainly metabolized by hepatocytes, but also the brain, gut, pancreas, lungs and the immune system are frequently affected by alcohol abuse. Alcohol may even increase the progression of viral infections, autoimmune diseases and cancer. Augmentation of oxidative stress, aberrant posttranslational modifications of proteins, methylation impairments, alteration in lipid metabolism and signal transduction pathways, represent common mechanisms of alcohol-related organ injury affecting cell survival and function.

The considered tolerable dose of alcohol for women is up to 20 g of pure alcohol per day and for men 30 g of alcohol per day[1]. Alcohol consumption represents a major factor in morbidity and mortality, it ranks fifth as the major cause of death in both men and women and causes up to 139 million disability-adjusted life years[2]. The burden of alcohol-related liver disease (ArLD) has risen in the past two decades, particularly among the young and women. It has been observed that lockdown due to the coronavirus disease 2019 (COVID-19) pandemic has led to a notable increase in alcohol abuse and misuse[3]. In particular, psychological symptoms such as anxiety, fear and stress are correlated with a general increase in alcohol consumption and, in the case of patients with alcohol use disorder (AUD), it has been outlined that social isolation can favor psychological decompensation and increased drinking or relapse[4]. In addition, the inaccessibility of regular clinical monitoring systems and the unavailability of professional help has caused difficulties in the treatment of patients with AUD or chronic liver disease (CLD)[5]. Steatosis of the liver, alcohol-related steato-hepatitis and ArLD are the most common consequences of excessive alcohol consumption[3]. ArLD includes a broad spectrum of disease including fat accumulation, cirrhosis, and hepatocellular carcinoma[6]. Cirrhotic patients or subjects with liver cancer are immunocompromised, so they may be more exposed to COVID-19 with a worse prognosis[7].

There are many factors that contribute to the increased risk of mortality from COVID-19 in patients with ArLD. For example, comorbidity such as malnutrition and metabolic syndrome are frequently observed in patients with ArLD and have been associated with poor clinical outcomes in patients with COVID-19[7]. A large longitudinal population-based study conducted in the Unites States has demonstrated a worrying rise in 60- and 90-d mortality rates in patients with ArLD who attended emergency departments or were inpatients during the pandemic, due to the increase in alcohol use and stress, to the direct impact of COVID-19 but also to its indirect effect on the healthcare system (inadequate medical resources, delays in follow-up visits or presenting for medical attention)[8]. Other studies reported that during the pandemic, the rates of hospitalization, severity at admission and mortality during hospitalization for cirrhosis were not different compared to previous years[9], that in immediate and medium-term lockdown there were no demonstrable adverse outcomes in patients with CLD referred to secondary care[10] or a substantial decline in cirrhosis hospitalizations[11]. These observations could depend on initiatives projected to preserve inpatient resources, and guidance encouraging patients to remain home, and can reflect, in part, the fact that patients avoided hospital presentation until symptoms were severe due to personal concerns regarding COVID-19. An interna-

tional registry study outlined that patients with cirrhosis are at increased risk of death due to COVID-19 and that mortality due to COVID-19 was higher among patients with more advanced cirrhosis and in patients with ArLD[12].

It has been shown that even an increase in alcohol consumption over short-term periods during the pandemic can worsen morbidity and mortality associated with ArLD in the long-term due to several behavioral changes (coping mechanisms to deal with emotional stress and chronic uncertainty)[13]. On the other hand, an epidemiological study conducted on United States mortality data found that ArLD mortality has increased among males and females in almost every age and racial/ethnic demographic, both in rate and absolute count, and before the pandemic (from 2017 to 2020) and this rise has been amplified due to COVID-19[14]. All these data demonstrate that it is pivotal to administer vaccination as a preventive measure in patients with liver disease as soon as possible in order to reduce the risk of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection and severe disease[15]. It should be noted that despite the strong and repeated recommendations, overall vaccination coverage in patients with CLD remains poor and low immunization rates are frequently due to lack of information on vaccine safety, inadequate access to healthcare and poor financial reimbursement for healthcare providers[16].

A simulation model of the long-term drinking patterns of people with lifetime AUD has revealed that if the increase in alcohol consumption registered in the United States in the first year of the pandemic continues with similar characteristics, alcohol-related mortality, morbidity and associated costs will increase considerably over the next 5 years[17]. These observations are a red flag for the necessary improvement in screening for high-risk alcohol use and optimization of early treatment of abuse or misuse and its physical and psychological consequences. Research focusing on the behavioral change after the pandemic in people who already had a problem with excessive alcohol drinking showed how subjects with risky or hazardous consumption increased both quantity and frequency of alcohol assumption in most European countries, underlining the urge to establish regulations to define online and home delivered alcoholic beverages availability and the need to carefully restructure healthcare services[18]. An increase in AUD has been observed in women, racial and ethnic minorities, and in those experiencing poverty in the context of poor access to alcohol treatment, leading to increasing rates of alcohol-associated liver diseases. The diffusion of telemedicine use contributes to provide effective protection to reduce cross-infection between clinicians and patients, but subjects with CLD and ArLD still need regular follow-up examinations to prevent worsening of their clinical condition[15]. It has been demonstrated that ArLD patients with recent hospital admission were more motivated to cut down alcohol consumption, and motivation predicted engagement in alcohol misuse treatment[19].

ALCOHOL AND LIVER INJURY

The most frequent cause of acute liver injury is alcohol (in particular in the form of alcohol binge drinking) followed by hepatitis (A, B, E, autoimmune) and some drugs[20]. Drug-induced liver injury can potentially be caused by several agents, including both prescribed and non-prescribed compounds, herbal and dietary supplements, over-the-counter products and illicit substances[21].

Alcohol has broad effects on hepatic lipid metabolism leading to an increase in hepatic fatty acids pools, which can be esterified and stored in lipids droplets as triglycerides. Chronic alcohol consumption provokes the lipolysis of triglycerides stored in white adipose tissue, which enter the circulation and can be taken up by the liver. Alcohol-induced hepatic lipid metabolism involves altered hepatic lipid uptake, de novo lipid synthesis, fatty acid oxidation, hepatic lipid export, and lipid droplet formation and catabolism[22]. These mechanisms together with other complex effects, some of which are not yet fully understood, contribute to the development of hepatic steatosis[23]. Alcoholic liver injury has a progression from steatosis up to scarring, inflammation and architectural distortion leading to cirrhosis. Hepatocellular carcinoma may occur as a complication of liver cirrhosis[24]. However, only a small percentage of patients with alcoholic steatosis progress to severe liver injury (Table 1).

It is known that the liver plays a homeostatic role in the systemic immune response. Alcoholic steatotic liver is a fragile medium and is more sensitive to drug damage, vascular changes and hypoxia. In fact, alcoholism is considered a proinflammatory condition. Chronic injury and death of hepatocytes lead to the recruitment of myeloid cells, secretion of inflammatory and fibrogenic cytokines, and activation of myofibroblasts. As alcoholic steatotic liver leads to high circulating levels of proinflammatory cytokines, it tends to react to COVID-19 with a massive inflammatory response (the so-called inflammatory "tsunami", induced by both infection and previous alcohol consumption) and to cause excessive expression of apoptotic factors and consequent multi-organ failure[25].

It has been demonstrated that chronic alcohol consumption may augment the risk for severe influenza virus infections through dysregulation of the pulmonary inflammatory environment and CD8 T cell response. In addition, as alcohol reduces oropharyngeal tone, it can lead to an increased risk of aspiration of microbes, may modify alveolar macrophage function and very often causes malnutrition [26].

Table 1 Progression of alcoholic liver injury[24]

Patients with AUD (alcohol use disorder)
90%-100% present with steatosis
10%-35% have alcoholic hepatitis
8%-20% develop cirrhosis
1%-2% develop hepatocellular carcinoma as a complication of cirrhosis

AUD: Alcohol use disorder.

As already mentioned, many studies have confirmed that an increase in alcohol consumption in a short-term period during the COVID-19 pandemic can cause long-term ArLD-related morbidity, hospitalizations and mortality[13-15,17,27], and that abnormal liver biochemical tests are often closely related to the severity and prognosis of patients with COVID-19[28].

SARS-COV-2 EFFECTS ON THE LIVER

Patients with COVID-19 often show liver involvement that may influence disease prognosis and outcome. SARS-CoV-2 is responsible for a direct cytopathic effect on hepatocytes. COVID-19 associated liver injury is defined as liver injury directly due to the virus or its treatment in patients with or without preexisting liver damage[29]. The exact mechanism of liver injury in the presence of SARS-CoV-2 infection is largely unknown[30]. It has been described that this virus enters the cell through angiotensin converting enzyme 2 (ACE2) receptors, which are abundant in many areas of the body, including cholangiocytes and hepatocytes. The consumption of alcohol reduces both innate and acquired immune activity with a probable liver increase in ACE2 receptors. It has been observed that liver dysfunction in COVID-19 is not only due to cholangiocyte dysfunction, but also to the cytokine storm generated by lung damage and to hepatotoxicity related to several drugs used during the treatment of COVID-19[31]. In particular, liver biopsies in COVID-19 patients showed that liver injury is multifactorial: direct cytotoxicity by the virus, hyper-inflammatory reaction to infection, systemic hypoxia and hepatic congestion related to cardiomyopathy and drug-induced liver injury. In fact, the anti-COVID-19 drugs, especially drug-drug or alcohol-drug combinations, cause cellular stress responses and injury to liver cells[32]. In addition, a direct relationship between the grade of liver injury and severity of the disease has been established[33]. Elevated liver enzymes appear to be a risk factor for disease progression, even in the absence of underlying liver disease[30]. Mild aspartate transaminase (AST) elevation is considered an early sign of severe COVID-19, while high alanine transaminase (ALT) levels are considered an independent predictor of prolonged SARS-CoV-2 RNA shedding. AST and ALT levels greater than three times the upper limit of normal have been associated with increased mortality[34].

Acute-on-chronic liver failure (ACLF) has been hypothesized as one of the possible explanations for higher mortality in liver disease patients with COVID-19: It is characterized by two types of liver injury in combination, one acute (liver-specific or systemic) and one chronic (often misunderstood). It has also been observed that the addition of liver and kidney dysfunction in critically ill patients can increase mortality. The MELD (End-Stage Liver Disease) score has been developed to assess risk in patients with liver cirrhosis: it is considered a useful score to deduce both liver and kidney function (based on total bilirubin, creatinine, and International Normalized Ratio-INR) and a possible practical predictor of short- and long-term mortality and morbidity in patients with COVID-19[35]. SARS-CoV-2 infection highlights the pre-existing weaknesses of the individual organ systems; therefore, it is predictable that patients with CLD may be susceptible to more severe respiratory infections or be at increased risk of death. Many studies have shown that hospitalized COVID-19 patients with CLD have an acute rise in liver enzymes, which results in a severe condition requiring mechanical ventilation and even leading to death. There are other plausible pathogeneses in patients with cirrhosis who have a worse disease course and even death following COVID-19, such as excess systemic inflammation, intestinal dysbiosis, cirrhosis-induced immune dysfunction, and coagulopathies[36].

As expected, the presence of AUDs, especially with active alcohol consumption, may worsen the disease course and prognosis[20]. COVID-19 can overlap with pre-existing CLD or induce liver damage directly or indirectly. ACLF patients show a significant increase in inflammatory markers and proinflammatory cytokines, features that are frequently observed in severe SARS-CoV-2 infection. Some studies have claimed that patients with ACLF of alcoholic etiology have significantly prolonged hospital stay, severe COVID-19, admission to the intensive care unit and higher mortality[37,38], while others have shown that ACLF is often triggered not only by ongoing alcohol consumption, but also gastrointestinal bleeding and/or infections, and from a pathophysiological point of view it is charac-

terized by uncontrolled systemic inflammation coupled with paradoxical immunoparesis. ACLF has a clear pathogenesis and epidemiological burden and is different from decompensated cirrhosis; it represents a challenging condition with a rapid clinical course, high short-term mortality and varying clinical phenotypes[39,40].

There is a positive correlation between the stage of cirrhosis and the augmented risk of COVID-19-related liver injury and mortality[41,42]. Cirrhosis results in the liver losing its homeostatic role in controlling bleeding and thrombosis; in parallel one of the features of COVID-19 is hypercoagulability with consequent venous and arterial thrombosis.

Increased alcohol consumption is a consequence that often occurs following a crisis or a traumatic event[43]. An increasingly large number of studies have shown that there has been a substantial increase in the use and abuse of psychoactive substances, alcohol, and tobacco during the COVID-19 pandemic, in particular alcohol intake has risen substantially by 10%-23%[44].

Consumers describe substance use/abuse as a way, albeit problematic and potentially pathogenic, to cope with anxiety regarding COVID-19[44].

Anxiety about COVID-19 is more than just a worry about infection. Scientific research seems to provide evidence that this is a stress syndrome, a disturbing condition with a possible physiognomy. This condition can provoke an anxious and traumatic reaction or a response that appeals to mechanisms of denial and repression, and suggests that the behaviors of addiction have a dissociative nature linked to the management of negative emotions and feelings[45].

Alcohol can be used to alleviate stress related to social isolation, negative emotions, boredom, changes in one's routine, high levels of anxiety and worries, in particular fears of the danger of COVID-19. Furthermore, alcohol can exert an inhibitory effect on the nervous system, generating temporary relief from anxiety, depression, anger, sleep disorders and post-traumatic stress disorder[46].

Those who tend to put in place mechanisms of denial and repression have difficulty in making contact with their emotions and may have an externally oriented cognitive style. These individuals can get used to expressing their sensations, favoring the non-verbal channel, through the development of compensation mechanisms such as compulsive drinking, performing a function of management and avoidance of seemingly uncontrollable emotions.

This reaction to interpersonal trauma, through abuse, can become a dysfunctional coping mechanism that modulates the sensations between the body and emotions, with the risk of dissociative interference in the connections among affects, cognition and voluntary control of behavior.

CONCLUSION

The pressing situation in which the current society finds itself in terms of alcohol consumption, with an exponential increase also in the younger population[47], the multiple opportunities for consumption by anyone who wants to do so and, therefore, the exposure of a considerable number of people to alcohol-related problems of various types[48] requires the adoption of measures to limit the COVID-19 pandemic and the severity of the effects of the disease.

Alcohol consumption is associated with many diseases and is often the cause of injuries and trauma related to road accidents, assaults and episodes of domestic violence. In addition, as a consequence of new consumption patterns of alcohol during lockdown due to the COVID-19 pandemic, many social and psychological issues such as domestic violence, mental diseases, and impairment of family quality have been aggravated[49,50].

A significant problem is acute alcohol intoxication and chronic toxicity, that is, the silent and progressive lesions in vital organs due to prolonged consumption of alcohol even if in moderate doses. The most important point to remember is that alcohol consumption does not protect against COVID-19 in any way, does not destroy the virus and does not prevent becoming infected with it. Conversely, however, those who consume harmful levels of alcohol are at an increased risk of infection. The harmful consumption of alcohol, in fact, affects all components of the immune system; alcohol causes a reduction in the number and functions of B lymphocytes and increased production of immunoglobulins, alters the balance between different T lymphocytes, impairs the number of T lymphocytes and their functioning, and promotes cell apoptosis. Furthermore, alcohol is a potential risk factor for pneumonia *via* other mechanisms: it reduces oropharyngeal tone, increasing the risk of microbial aspiration, and modifies the function of alveolar macrophages, alcohol often causes malnutrition, a condition that increases the risk of infections[26,51,52].

Finally, it should be noted that the elevated risk of infections in addition to the effects of alcohol on the immune system, can also be associated with the presence of ArLD.

Alcohol can perform various "therapeutic effects" from a psychological point of view. Individuals develop the "magical" expectation that psychological difficulties and suffering can be diluted by alcohol, but there is then disillusionment and an even more painful state of helplessness and frustration. People who abuse alcohol try to alleviate intolerable feelings of helplessness and weakness caused by overwhelming emotions. Unconsciously, there is the fantasy that alcohol can substantially change one's psychic state and repair or replace damaged or missing psychological functions[53]. Mc Dougall[54]

believes that alcohol is one of the ways used to escape deep and intolerable anxieties, even of a psychotic nature, caused by the increase in both pleasant and unpleasant affects. The psychic apparatus in particular situations is unable to adequately cope with emotions and affects. Humans are complex and are continuously between different conscious and unconscious states. A balance between the internal world and the external world, and among parts of the internal world itself, is achieved by means of objects that are "transitional" and transformative. In such a perspective, alcohol can become a "transitional object"[55] that seems to offer security and comfort, but conversely tends to be an obstacle to development and integration of self.

While the majority of patients with COVID-19 have no or mild liver function abnormalities during their illness, it is important to closely monitor patients with preexisting liver disease, the elderly, obese subjects or individuals who daily consume high amounts of alcohol[29]. As the COVID-19 pandemic and subsequent lockdown have led to a significant increase in AUD and liver injury worldwide, it seems important to stress that all specialists involved in the field of alcohol addiction and liver disease (specialists in virology, immunology, psychiatry, internal medicine, hepatology, gastroenterology and pharmacology) should interact and strictly collaborate through a multidisciplinary intervention aimed at better management of patients in terms of both prevention and prognosis. Psychological support involving patients and their families/caregivers (locally or *via* telemedicine/telehealth) are of pivotal importance to guarantee the efficacy of treatments[3]. In particular, mental health services should continue to guarantee access to care as usual and alcohol treatment programs should remain available for patients even during a pandemic.

There is a need to accelerate strategies to combat the risks and damage caused by alcohol and therefore it is important to promote measures on the issue of health education.

Reaching general practitioners, stimulating them and training them for short-term interventions in this field, could result in obtaining an important level of care and allow specialists to concentrate on particularly complex situations of discomfort. Furthermore, it is essential to undertake actions aimed at raising awareness in consumers of the risks and harm that the use of alcohol entails, and to provide interventions in relation to personal well-being and quality of life[56-58]. Research has shown that the most effective way to help someone with an alcohol use problem who may be at risk of developing an AUD is to intervene early, before the condition progresses. Seeking help for alcohol abuse is still low, mainly due to stigmatization. It is pivotal to provide policy development, to increase healthcare stakeholders' awareness and skills, and to build relationships with specialist services. Screening on a large scale, including men, women and particularly young people, tailored interventions, appropriate training and support for nursing staff, can guarantee timely and effective care and improve patient satisfaction and health outcomes.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Long-term and non-invasive *in vivo* tracking of DiD dye-labeled human hepatic progenitors in chronic liver disease models

Chaturvedula Tripura, Srinivas Gunda, Sandeep Kumar Vishwakarma, Avinash Raj Thatipalli, Jedy Jose, Mahesh Kumar Jerald, Aleem Ahmed Khan, Gopal Pande

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Abstract

BACKGROUND

Chronic liver diseases (CLD) are the major public health burden due to the continuous increasing rate of global morbidity and mortality. The inherent limitations of organ transplantation have led to the development of stem cell-based therapy as a supportive and promising therapeutic option. However, identifying the fate of transplanted cells *in vivo* represents a crucial obstacle.

AIM

To evaluate the potential applicability of DiD dye as a cell labeling agent for long-term, and non-invasive *in vivo* tracking of transplanted cells in the liver.

METHODS

Magnetically sorted, epithelial cell adhesion molecule positive (1×10^6 cells/mL) fetal hepatic progenitor cells were labeled with DiD dye and transplanted into the livers of CLD-severe combined immunodeficiency (SCID) mice. Near-infrared (NIR) imaging was performed for *in vivo* tracking of the DiD-labeled transplanted cells along with colocalization of hepatic markers for up to 80 d. The existence of human cells within mouse livers was identified using Alu polymerase chain reaction and sequencing.

RESULTS

NIR fluorescence imaging of CLD-SCID mice showed a positive fluorescence signal of DiD at days 7, 15, 30, 45, 60, and 80 post-transplantation. Furthermore, positive staining of cytokeratin, c-Met, and albumin colocalizing with DiD flu-

orescence clearly demonstrated that the fluorescent signal of hepatic markers emerged from the DiD-labeled transplanted cells. Recovery of liver function was also observed with serum levels of glutamic-oxaloacetic transaminase, glutamate-pyruvate transaminase, and bilirubin. The detection of human-specific Alu sequence from the transplanted mouse livers provided evidence for the survival of transplanted cells at day 80.

CONCLUSION
DiD-labeling is promising for long-term and non-invasive *in vivo* cell tracking, and understanding the regenerative mechanisms incurred by the transplanted cells.

Key Words: Chronic liver diseases; Cell transplantation; Cell tracking and imaging; DiD; Hepatic progenitors

Core Tip: Non-invasive tracking of transplanted cells is crucial to understand the homing, distribution, and differentiation into the desired cell types contributing to organ regeneration. Lipophilic fluorescent dye DiD-labeled fetal hepatic progenitor cells (fHPCs) were transplanted into the livers of mice with chronic liver diseases. DiD labeling of cells enabled long-term and non-invasive tracking of transplanted cells *in vivo* up to 80 d. Immunostaining and colocalization using liver-specific markers with DiD confirmed the persistence of transplanted cells in mice liver post-transplantation. Transplanted fHPCs supported liver function recovery, while identification of the Alu gene sequence revealed survival and engraftment of human cells within the mouse liver.

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INTRODUCTION

Chronic liver diseases (CLD) represent one of the leading causes of morbidity and mortality worldwide, specifically in developing countries. They result from the progressive deterioration of liver functions which is caused by the continuous process of inflammation, destruction, and inadequate repair of the liver parenchyma leading to cirrhosis. Liver cirrhosis is characterized by irreversible distortion of the liver architecture in the form of fibrosis, scar formation, the occurrence of several regenerative nodules, vascular reorganization, and immense liver failure. The major aim of the current treatment approaches is to halt the progression of CLD into more severe forms, and further reduce the associated complications representing the need for an appropriate multidisciplinary approach. The clinical signs and symptoms of CLD can be nonspecific; hence the major management perceptions are the elimination of underlying causes, management of portal hypertension, and personalized therapy for each associated condition. Although such strategies provide temporary support to the failing liver; they cannot prevent long-term disease progression. Hence, more effective management approaches are required to overcome such hurdles and bridge the gap of a long-term therapeutic window to improve health-related quality of life.

Cell therapy is an emerging technology and has shown significant promise in addressing the current demand for alternative options to liver transplantation to improve liver functions and act as a supportive bridge therapy in CLD[1,2]. However, it is necessary to ascertain appropriate cell types from widely acceptable sources and resolve several unanswered questions before utilizing cell-based technology in the clinical setting. Since the last two decades, transplantation of different types of cells from various tissue sources such as autologous bone marrow-derived mononuclear cells and mesenchymal stem cells (MSCs), and allogenic fetal hepatic progenitor cells (fHPCs) have shown promising outcomes in clinical studies of CLD[3-7]. In addition, several preclinical studies have also demonstrated the potential applicability of such approaches in immune-compromised/deficient mice to improve the current understanding of the safety, efficacy, and functionality of human stem/progenitor cells post-transplantation[8-11]. While several of these studies have proved the safety and involvement of the transplanted cells in liver recovery, the availability of methods for easy and long-term tracking of infused cells would be extremely beneficial in determining their viability, bio-distribution, homing, and differentiation which represents a major roadblock for cell-based therapies in clinical settings.

The majority of existing strategies employ radioisotopes, magnetic particles, fluorescent tags, or reporter genes as cell labeling agents prior to transplantation in preclinical settings[12]. Furthermore, non-invasive radionuclide imaging methods such as single-photon emission tomography and positron emission tomography using radionuclides [Technetium (^{99m}Tc) and ^{111}In-oxine] are currently employed in clinical settings[4,6,13]. However, the short life of radionuclides limits their wider applicability due to monitoring of the immediate cellular behavior for only a few hours. Magnetic resonance imaging (MRI) is another non-invasive imaging method that has been explored for cell tracing in preclinical CLD models for up to 1-2 wk[14,15]. While MRI offers good spatial resolution and contrast, it is less sensitive and is not effective for follow-up studies due to the gradual loss of signal intensity[12]. Hence, several other tracking methods based on reporter gene expressions, such as fluorescence imaging and bioluminescence imaging are being successfully employed for monitoring the fate of transplanted cells in animal models of liver injury[16,17]. Although this method enables long-term cell tracking, the safety concerns owing to genetic manipulation represent a major hurdle for clinical translation. Hence, direct labeling of the cells without involving genetic manipulation represents a crucial need for a sensitive, relatively safer, and less cumbersome process for tracking transplanted cells in both preclinical and clinical settings.

In the present study, long-term and non-invasive tracking of transplanted fHPCs was evaluated in an experimental severe combined immunodeficiency (SCID) mouse model of CLD using DiD. DiD is a carbocyanine dye having good photochemical properties of strong fluorescence, and stability[18-20]. It is a cationic dye and belongs to the family of lipid intercalating long alkyl side-chain carbocyanine derivatives that have a long-range (540-780 nm) emission. Due to the long-range emission, tissue autofluorescence of DiD is minimum, permitting the use of other fluorochromes such as fluorescein isothiocyanate (FITC) for co-localization studies to evaluate the expression of other essential markers specific to transplanted cells in the recipient tissue. Moreover, the process of labeling the cells using DiD is easy due to its excellent efficiency for integration and diffusion into the cell membranes[18,19,21,22]. Although DiD is insoluble in water, its fluorescence is readily detected when incorporated into the cell membranes. Therefore, it has been classified as one of the most appropriate carbocyanine families of dyes in cell labeling and tracking. After incorporation into cell membranes, it diffuses laterally within the plasma membranes, resulting in staining of the entire cell. Structural similarity with the cell membrane phospholipids and prolonged dye retention within the cells are among the major advantages of DiD for live organisms. Hence, DiD has been used for labeling different types of cells without interfering with cellular differentiation; however, the effects of DiD labeling on human liver cells and its effect on the *in vivo* retention of labeled human liver cells remain to be investigated.

We specifically utilized magnetically sorted fHPCs using epithelial cell adhesion molecule (EpCAM) as a surface marker due to its associated crucial functions such as cell-to-cell adhesion, proliferation, maintenance of pluripotent state, and regulation of differentiation and migration[23]. It has also been demonstrated that EpCAM-positive HPCs are highly proliferative and have diminished class II MHC presentation, and are classified as immature cells suitable for regenerative applications[24]. In our earlier study, EpCAM-positive fHPCs revealed a significant improvement in liver functions and increased disease-free life span in patients with end-stage CLD[6]. Thus, using EpCAM-positive fHPCs could highlight how good DiD is for long-term, non-invasive, and real-time monitoring of cell survival, and structural and functional improvements in preclinical models of CLD post-transplantation. Accordingly, the present study aimed to shed light on the fate of DiD-labeled human liver cells in CLD-SCID mice using live imaging up to 80 d post-transplantation.

MATERIALS AND METHODS

Animals

Experimental animals were obtained from inbred colonies of SCID mice (strain: NOD.CB17-*Prkdc*^{scid}/J) and maintained at the animal facility of the Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India. This study was approved by the Institutional Animal Ethics Committee (Animal trial registration number 20/1999/CPCSEA dated 10/03/1999) of CCMB. All the animal experimental procedures were performed in accordance with the approved ethical guidelines of CCMB for the care and use of animals. All the animals were maintained in standard ventilated cages with a 12 h light-dark cycle and were fed *ad libitum*.

Development of the CLD mouse model

CLD mouse model was generated using 25% carbon tetrachloride (CCl_4, Rankem, India) diluted with mineral oil (Sigma, United States). A sub-lethal dose of diluted CCl_4 was administered according to 125 μL/kg body weight in each animal. A total of 26 mice (either sex) at eight weeks of age were randomly assigned to the vehicle control group (n = 4) and the CCl_4 group (n = 22). The CCl_4 group received intraperitoneal injections of diluted CCl_4 twice a week for 4 wk and the vehicle control mice received only mineral oil. After 4 wk, liver damage was confirmed by changes in liver enzymes and liver tissue histology. For the biochemical evaluation of liver damage, 100-150 μL of blood was collected by orbital

sinus puncture. Serum levels of total bilirubin, glutamate-pyruvate transaminase (SGPT), and glutamic-oxaloacetic transaminase (SGOT) were measured by the Jendrassik-Grof and Reitman-Frankel's methods, respectively, using kits (Coral Clinical Systems, India). The mice after 4 wk of CCl_4 (referred to as CLD-SCID mice) were ready for cell transplantation (TX).

Isolation of human fetal hepatic progenitors

The total fetal liver cells (tFLCs) isolation protocol was approved by the Institutional Ethics Committee of Deccan College of Medical Sciences, Hyderabad. Informed consent was obtained prior to sample collection, and cell processing was performed according to the ethical guidelines for the use of human cells. Briefly, the whole liver was dissected from spontaneously aborted fetuses (n = 3, 10-12 wk gestation), and perfused twice with ice-cold phosphate-buffered saline (PBS) for 5 min to eliminate circulating peripheral blood cells, followed by digestion with 0.025% collagenase in 1× PBS for 5 min at room temperature. Then the liver tissue was minced with a scalpel blade and disintegrated into a single-cell suspension by passing through 40 µm cell strainers (BD Biosciences, United States). Cell viability and counting were performed using the trypan blue dye exclusion test.

Flow cytometry

For flow cytometry analysis, a single cell suspension of 2 × 10^6 tFLCs was fixed in 4% paraformaldehyde for 15 min at room temperature. Following fixation, the cells were washed twice with 1× PBS and stained with anti-human EpCAM (CD326) antibody conjugated with FITC (Miltenyi Biotech, Germany) for 30 min. Cells were washed once with 1× PBS before analyzing on FACS Calibur™ (BD Biosciences, United States) using 488 nm argon laser emission at 530/30 BP filter. The data were analyzed and plotted using Kaluza software 1.5a (Beckman Coulter Inc., United States).

Enrichment of EpCAM-positive fHPCs from tFLCs

To isolate EpCAM-positive cells, a 5 × 10^7 tFLC suspension in 500 µL buffer containing FCR blocking reagent was incubated with anti-human EpCAM antibody (Miltenyi Biotech, Germany) conjugated with magnetic beads at 4°C for 30 min and sorted using the magnetic cell sorter, AutoMACS according to the manufacturer's instructions (Miltenyi Biotech, Germany). Magnetically activated cell sorting (MACS) enriched EpCAM-positive cells were collected and resuspended in RPMI 1640 medium (Sigma, United States) supplemented with 10% fetal bovine serum (FBS, Sigma, United States). Cell isolation and MACS sorting procedures were carried out under sterile conditions in a Class 100 biosafety cabinet.

Immunocytochemistry

MACS-sorted EpCAM-positive cells were fixed in 4% paraformaldehyde, and cytospin preparations were conducted on "Probe-on-Plus" slides (Fisher Scientific, United States). Cells were blocked with 10% goat serum, and stained with mouse monoclonal anti-human EpCAM antibodies directly conjugated with FITC (Miltenyi Biotech, Germany), and co-stained with either anti-cytokeratin (CK) 8+18+19 or anti-c-Met (Abcam Inc., MA, United States) primary antibodies. Alexa 594 (Molecular Probes, United States) was used as the secondary antibody. Images were captured using a confocal laser scanning microscope (Leica, Germany, SP2 AOBS).

DiD-labeling and intra-hepatic cell TX in SCID-CLD mice

MACS sorted EpCAM-positive cells (1 × 10^6 cells/mL) in Hank's buffered salt solution (HBSS) were labeled with DiD dye by adding 5 µL of DiD cell labeling solution (Life Technologies, Eugene, United States) to the cell suspension and incubating for 20 min at 37°C. DiD-labeled cells were washed thrice with HBSS, and resuspended in the same buffer. 1 × 10^5 cells (100 µL) were injected directly into the liver lobes of the CLD mice (n = 14) at a single site using a 26-gauge needle. CLD mice receiving plain HBSS buffer served as non-transplanted (non-TX) controls (n = 5). Post-transplantation, mice were maintained in different cages, imaged, and sacrificed at different time points.

Long-term in vivo and ex vivo imaging

DiD-specific fluorescence from the transplanted animals was detected on the Multispectral FXPRO Fluorescence Imager (Carestream-KODAK, United States) using 630 nm excitation and 670/30 BP emission filters. Highly sensitive fluorescence images were combined with the high resolution X-ray images to precisely locate the DiD-labeled cells. Animals were imaged prior to TX (0 d) and after 7, 15, 30, 45, 60, and 80 d, post-TX. For each imaging experiment, the animals were anaesthetized with Xylazine (5 mg/kg body weight) and Ketamine (25 mg/kg body weight), the abdominal hair was shaved, placed within the chamber, and imaged. After *in vivo* imaging at days 15, 30, and 80 post-TX, mice were sacrificed, liver tissues were excised, and imaged (*ex vivo* imaging) to locate the fluorescing lobe for further processing. The fluorescing area of the liver lobe at day 15 and day 80 was immersed in OCT mounting solution and stored at -80°C. Liver lobes of the non-TX mice were randomly selected and processed similarly. The liver lobes at day 30 post-TX were processed for paraffin embedding and histology.

Histology analysis

The excised liver lobes were fixed in 10% buffered formalin and processed for paraffin embedding. Paraffin-embedded liver tissues were sectioned at 4.0 μm thickness using a rotatory microtome (Leica RM2135, Germany), and stained with Hematoxylin and Eosin, and Sirius Red (Sigma-Aldrich, United States) using standard protocols. Bright-field images were acquired from both control and CLD mice (n = 3) at 10× and 40× using an Olympus inverted microscope (IX3-SSU, Tokyo, Japan). A total of 20-25 random fields captured with the 10× objective was used to calculate the total collagen area using Image J (1.5 2q) software, and expressed as total collagen percent area (% CPA).

Immunostaining

Serial cryosections (7.0 μm thick) of transplanted and non-transplanted mouse liver lobes at 15 and 80 d were stained to determine the expression of hepatic markers using anti-CK, anti-c-Met, and anti-human albumin (MP Biomedicals) primary antibodies, and detected with Alexa 488 (Molecular Probes, United States) secondary antibody. Images were captured using either a confocal laser scanning microscope (Leica, Germany, SP2 AOBS), or the Axioimager Z2 Fluorescence microscope (Zeiss, Germany).

Alu sequence analysis

DNA was isolated from the fHPCs prior to transplantation and from the liver tissues of transplanted and non-transplanted mice at day 80. Isolated DNA was analyzed using human-specific primers for Alu sequence (Forward primer 5′-GGCGCGGTGGCTCACG-3′, Reverse primer 5′-TTTTTTGAGACG-GAGTCTCGCTC-3′). Polymerase chain reaction (PCR) was performed in a 25 μL reaction mixture containing 1 μL DNA, 2.5 μL 10× complete PCR Buffer with $MgCl_2$, 1 μL of 10 mmol/L dNTPs, 0.5 μL forward and 0.5 μL reverse primers, and 0.2 μL Taq DNA Polymerase (5 U/mL) with an initial denaturation step of 94°C for 5 min, followed by 35 cycles of a three-step program of 94°C for 30 s, 54°C for 30 s and 72°C for 45 s, followed by a final extension step at 72°C for 5 min. The PCR products were electrophoresed on a 2% agarose gel and observed under UV with ethidium bromide staining. The images were captured using the Gel Documentation System (BIO-RAD, United States). The PCR product was cleaned using QIAquick Gel Extraction Kit (Qiagen, United States), and sequenced. The amplicon sequences were analyzed using the ClustalW2 online tool (https://www.ebi.ac.uk/Tools/msa/clustalw2/).

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (ver. 5.0). Data are presented as mean ± standard error of the mean. Paired Student t-test at 95% confidence interval for a P value of ≤ 0.05 was considered statistically significant.

RESULTS

The schematic representation in Figure 1 shows the different steps involved in measuring different outcomes throughout the study process. Isolated tFLCs were enriched for EpCAM-positive cells by MACS in step 1 (Figure 1A), labeled with DiD dye in step 2 (Figure 1B), and then transplanted into CLD mouse livers (Figure 1C). Furthermore, live non-invasive near-infrared (NIR) imaging was performed at regular intervals using a small animal imaging system to detect the fluorescence signal (Figure 1D). However, *ex vivo* imaging of the excised liver was performed to confirm the localization of the fluorescence in mice liver post-TX (Figure 1E).

Immunophenotyping of tFLCs to identify the proportion of fHPCs

Human tFLCs stained with FITC-tagged anti-CD326 (EpCAM) antibodies were analyzed using flow cytometry. The gating strategy and the analysis of cell fluorescence *vs* cell size, and the overlaid histogram of the unstained and EpCAM-stained cells were acquired (Figure 2A and B). Of these tFLCs, 49 ± 23% of cells were found to be EpCAM positive and designated as fHPCs.

NIR imaging of DiD-labeled EpCAM-positive cells in vitro

MACS-sorted EpCAM-positive fHPCs were characterized for the expression of specific hepatic cell markers such as CK and c-Met together with EpCAM (Figure 2C). Magnetically sorted EpCAM-positive cells which were labeled with DiD dye showed a separate peak corresponding to the DiD fluorescence (Figure 2D). Furthermore, before transplantation, the DiD-labeled EpCAM-positive cells in the tube were visualized for fluorescence in the multispectral imaging system. The overlay image of the NIR fluorescence and X-ray showed a positive signal only in the tube containing DiD-labeled cells, while the tube with non-labeled cells was devoid of any such fluorescence (Figure 2E).

Long-term tracking of DiD-labeled cells post-TX in CLD-SCID mice

Assessment of the biochemical and histological changes in CLD-SCID mice showed an increase in serum

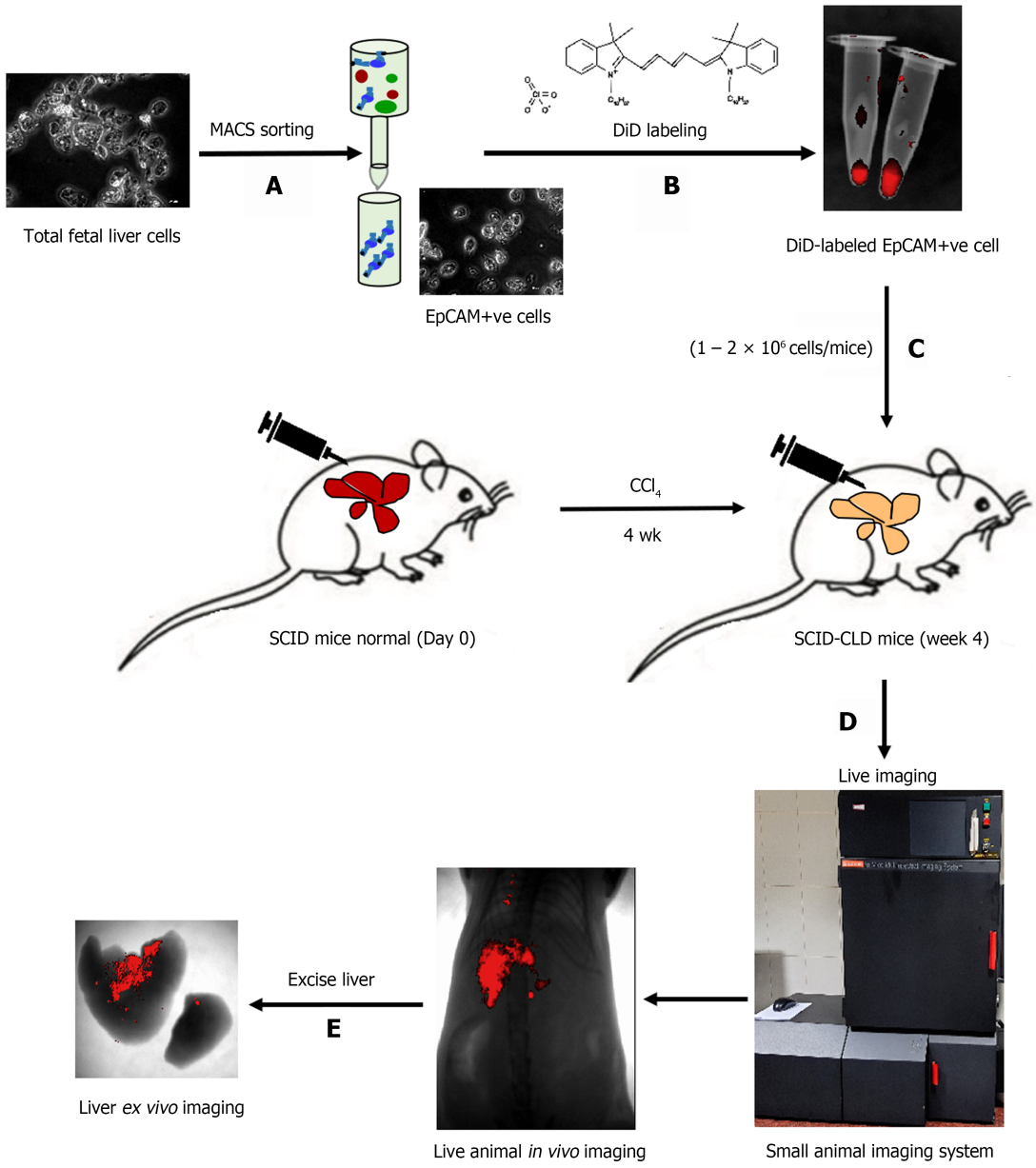

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Figure 1 Schematic presentation of the study. A: Total fetal liver cells were enriched for EpCAM-positive cells by magnetically activated cell sorting; B: Cells were labeled with DiD and visualized in the tube by near-infrared imaging as red fluorescence; C: Cells were intra-hepatically transplanted into injured livers of severe combined immunodeficiency mice; D: *In vivo* imaging of live animals was performed at specified time points; E: *Ex vivo* imaging of liver confirmed the retention of DiD-labeled transplanted cells in the liver. EpCAM: Epithelial cell adhesion molecule; SCID: Severe combined immunodeficiency; MACS: Magnetic activated cell sorting.

parameters (SGOT, SGPT, and bilirubin), and collagen accumulation compared to the control animals, confirming liver injury after 4 wk of CCl_4 injection (Supplementary Figure 1). These CLD mice were utilized for cell transplantation (at day 0), and tracking through *in vivo* imaging on different days from day 0 to day 80 (Figure 3A). NIR fluorescence imaging of CLD-SCID mice before intra-hepatic transplantation of DiD-labeled fHPCs cells at day 0 did not show fluorescence, while mice at days 7, 15, 30, 45, 60, and 80 post-TX showed a positive fluorescence signal of DiD (Figure 3B). The overlay images showed DiD fluorescence in the upper part of the abdominal cavity near the rib cage, suggesting that the cells continue to localize in the liver lobes until day 80 post-TX. The non-TX mouse abdomen lacked such fluorescence signals (Figure 3C). To further confirm the localization, the liver was excised on day 15 and day 80 post-TX, and *ex vivo* imaging was performed which confirmed the localization of DiD-labeled cells within the mice livers (Figure 3D). These results indicated that DiD-labeled cells can be efficiently visualized and tracked for a longer duration post-TX both *in vivo* and *ex vivo*.

Co-expression of hepatic markers with DiD in the transplanted mouse livers

To evaluate the expression of hepatic markers in transplanted cells, frozen mouse liver sections were

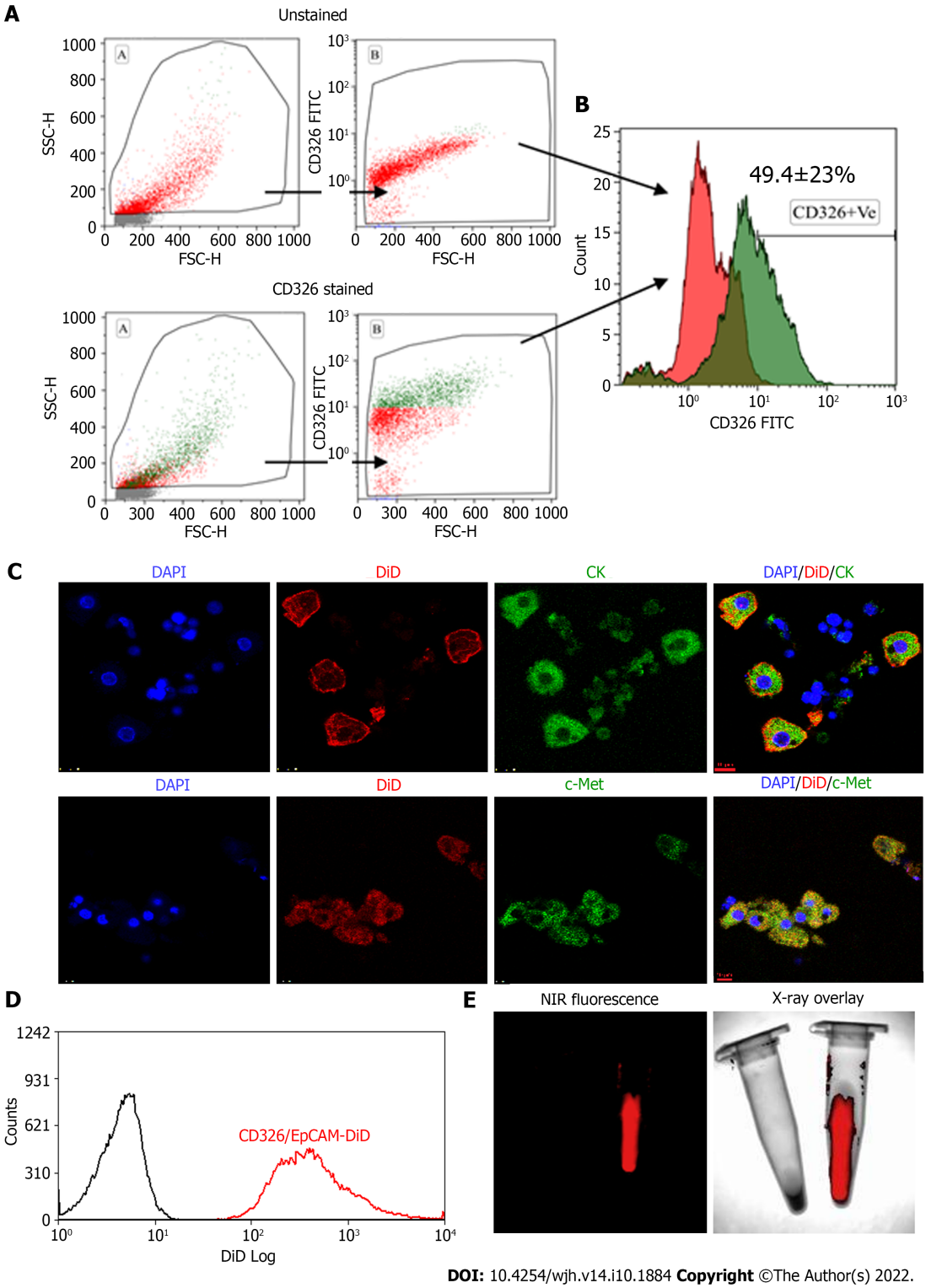

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Figure 2 Flow cytometry characterization of epithelial cell adhesion molecule expressing fetal hepatic progenitors, and DiD labeling of magnetically activated cell sorting enriched epithelial cell adhesion molecule-positive cells. A and B: Total fetal liver progenitors were stained with epithelial cell adhesion molecule (EpCAM)-fluorescein isothiocyanate (FITC) antibody and the representative dot plots of (A) unstained and EpCAM (CD326) stained cells and (B) overlay histogram; C: Colocalization of pan CK and c-Met with EpCAM-FITC expression (Scale bar = 10 μm); D: Overlay histogram of magnetically sorted EpCAM-positive cells labeled with DiD; E: Near-infrared fluorescence imaging and X-ray overlay image of DiD-labeled and unlabeled EpCAM-positive cells in the tube. EpCAM: Epithelial cell adhesion molecule; NIR: Near-infrared; DAPI: 4',6-diamidino-2-phenylindole; MACS: Magneticallyactivated cell sorting.

obtained from the portion of the liver that displayed a DiD-positive fluorescence signal at day 15 and day 80 post-TX. Serial sections of the liver transplanted with DiD-labeled fHPCs and non-transplanted control mice were obtained and stained for CK and c-Met (Figures 4 and 5). The presence of DiD fluorescence only in the transplanted mice confirmed the existence of transplanted cells, while non-transplanted mice did not show DiD fluorescence in liver tissue sections. In addition, positive staining

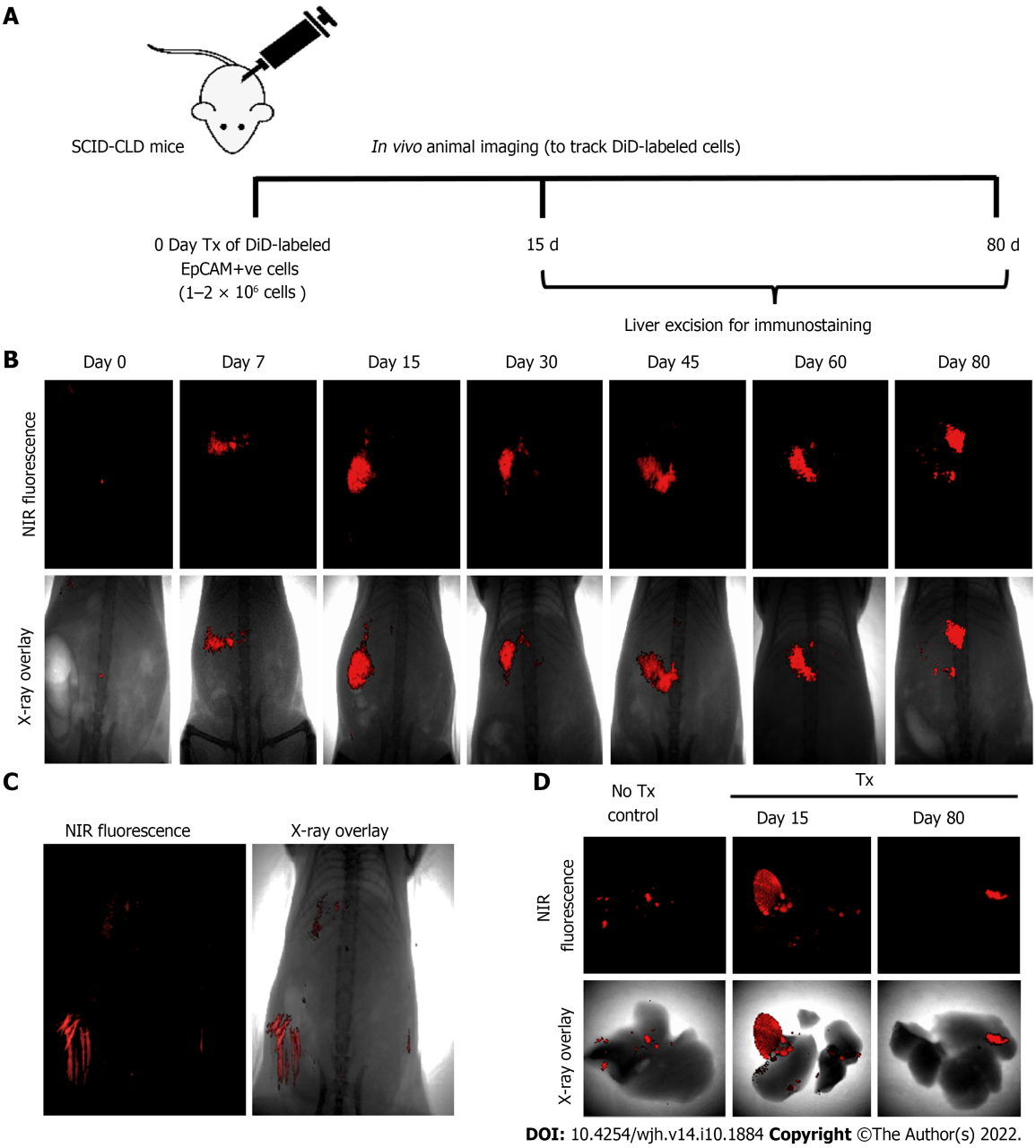

Figure 3 Long-term *in vivo* tracking of transplanted cells. A: Schematic representation showing the timeline for cell transplantation in mice with chronic liver diseases, imaging, and animal sacrifice; B: *In vivo* near-infrared (NIR)-fluorescence images (top panel) overlay with X-ray images (lower panel) of mice post-transplantation at indicated time periods; C: NIR-fluorescence image overlay with X-ray image of non-transplanted control mice (hair autofluorescence can be seen); D: *Ex vivo* imaging of excised livers of non-transplant control mice and transplanted mice at 15 and 80 d post-transplantation.

for both CK (Figures 4A, 5A and 5C) and c-Met (Figures 4B, 5B and 5D) colocalization with DiD fluorescence clearly demonstrated that the fluorescent signal of the hepatic markers, CK and c-Met emerged from the DiD-labeled transplanted cells.

Tracing the effect of transplanted DiD-labeled cells through improved liver function parameters

After 15 d of transplantation, recovery of mouse liver function was analyzed by assaying liver enzymes SGOT, SGPT, and bilirubin. Serum SGOT and SGPT levels were significantly reduced in the transplanted mice compared to the non-transplanted mice (Figure 6A), while serum bilirubin was reduced to normal levels in both groups. Thus, the serum enzyme parameters suggested improved liver function in the transplanted mice compared to the non-transplanted mice. Furthermore, the lower collagen percentage area in transplanted mice compared to the non-transplanted mice suggested that this recovery may be attributed to the transplanted fHPCs (Figure 6B and C).

The above assumption was further confirmed by the positive expression of hu-ALB in CLD-SCID mouse liver cells at day 80 post-TX (Figure 7). ALB-positive cells were also tested for their colocalization with DiD to confirm the effect was only in labeled cells. The non-transplanted mouse livers tested

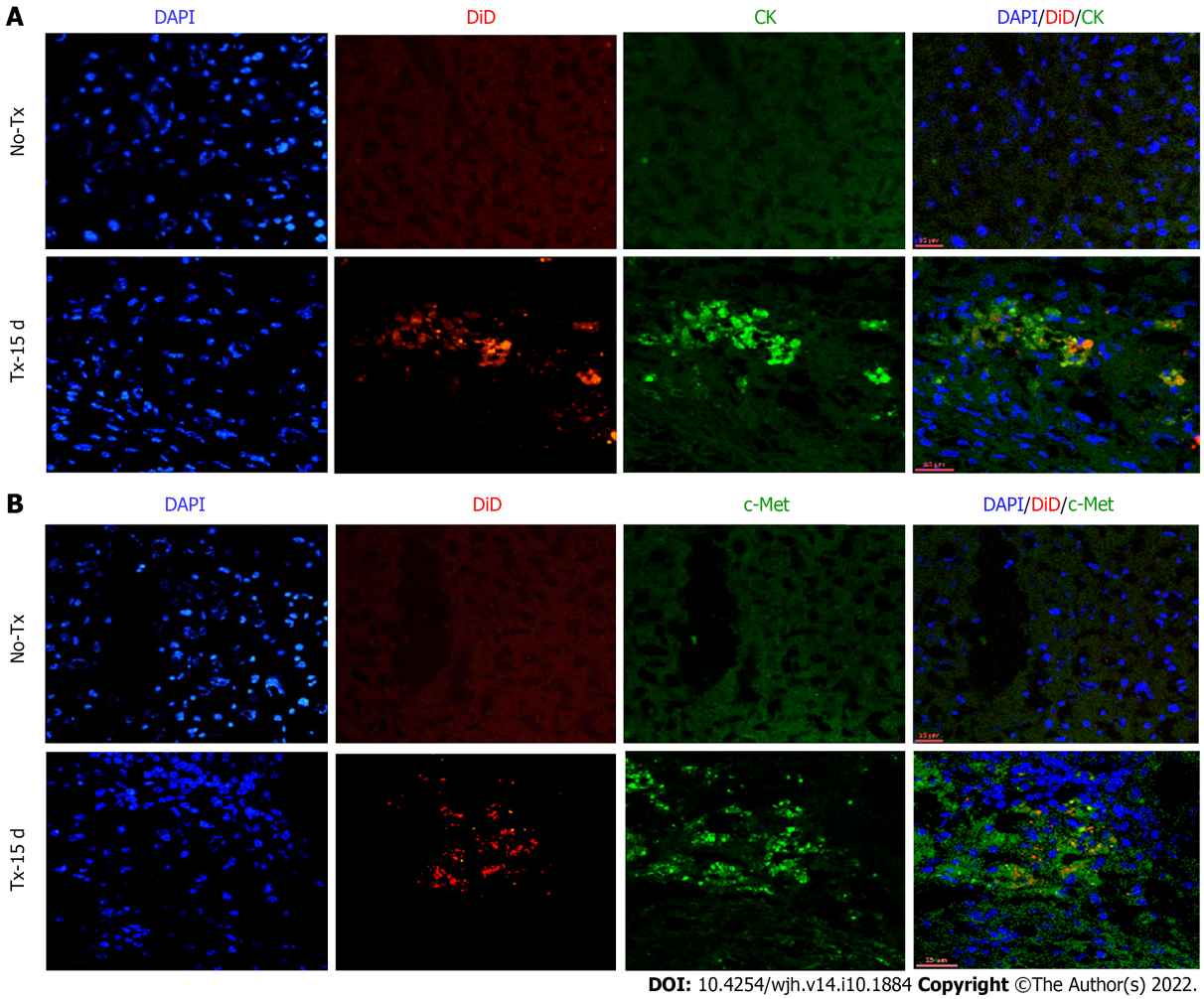

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Figure 4 Co-expression of hepatic markers in the transplanted livers 15 d post-transplantation. Confocal images of liver tissue cryosections of transplanted (TX-15 d) and non-transplanted (No-TX) mice showing expression of CK and c-Met colocalization with DiD-labeling only in the transplanted mice. The non-transplanted mouse livers were negative for CK, c-Met and DiD (Scale bar = 25 μm). A: CK: cytokeratin; B: c-Met; DAPI: 4',6-diamidino-2-phenylindole.

negative for ALB staining. The presence of hu-ALB only in the livers of transplanted mice, but not in the non-transplanted mice further confirmed the differentiation of fHPCs into functional hepatocytes (Figure 7). In addition, the amplification and detection of human-specific Alu-sequence from the transplanted mouse livers provided molecular evidence for the continued presence of human fetal liver cells in mouse liver at day 80. Sequencing analysis of the amplified Alu gene confirmed the presence of human Alu gene sequence in mouse liver tissues (Supplementary Figure 2). These results suggest that the transplanted human EpCAM-positive DiD-labeled cells continue to survive long-term without eliciting serious unfavorable effects in CLD-SCID mice.

DISCUSSION

This study reports a new application of the lipophilic fluorescent dye DiD for non-invasive *in vivo* imaging to monitor transplanted fHPCs in SCID mice with CLD. In our earlier clinical study, transplanted fHPCs in end-stage CLD patients were tracked for only 24 h using radio-labeled ^{99m}Tc-HM-PAO post-TX[6]. In our current study, labeling of fHPCs with DiD enabled long-term *in vivo* monitoring of the transplanted cells, which was further confirmed by *ex vivo* imaging and immunohistological analysis. Using DiD as a cell label, the transplanted cells could be tracked for 80 d non-invasively in CLD-SCID mouse liver. Our findings revealed that DiD labeling is a relatively simple, safe, and effective approach for long-term and non-invasive tracking of fHPCs post-TX in mouse liver. In addition, our results also showed the efficacy of transplanted human fHPCs as a suitable cell type for improving liver functions similar to earlier studies[11,25].

Long-term *in vivo* tracking of transplanted cells is currently required to answer several concerns and to reduce existing controversies in cell-based therapies. Now, it has been identified as an essential component of cell-based therapeutic strategies for optimizing the cell number, route of delivery,

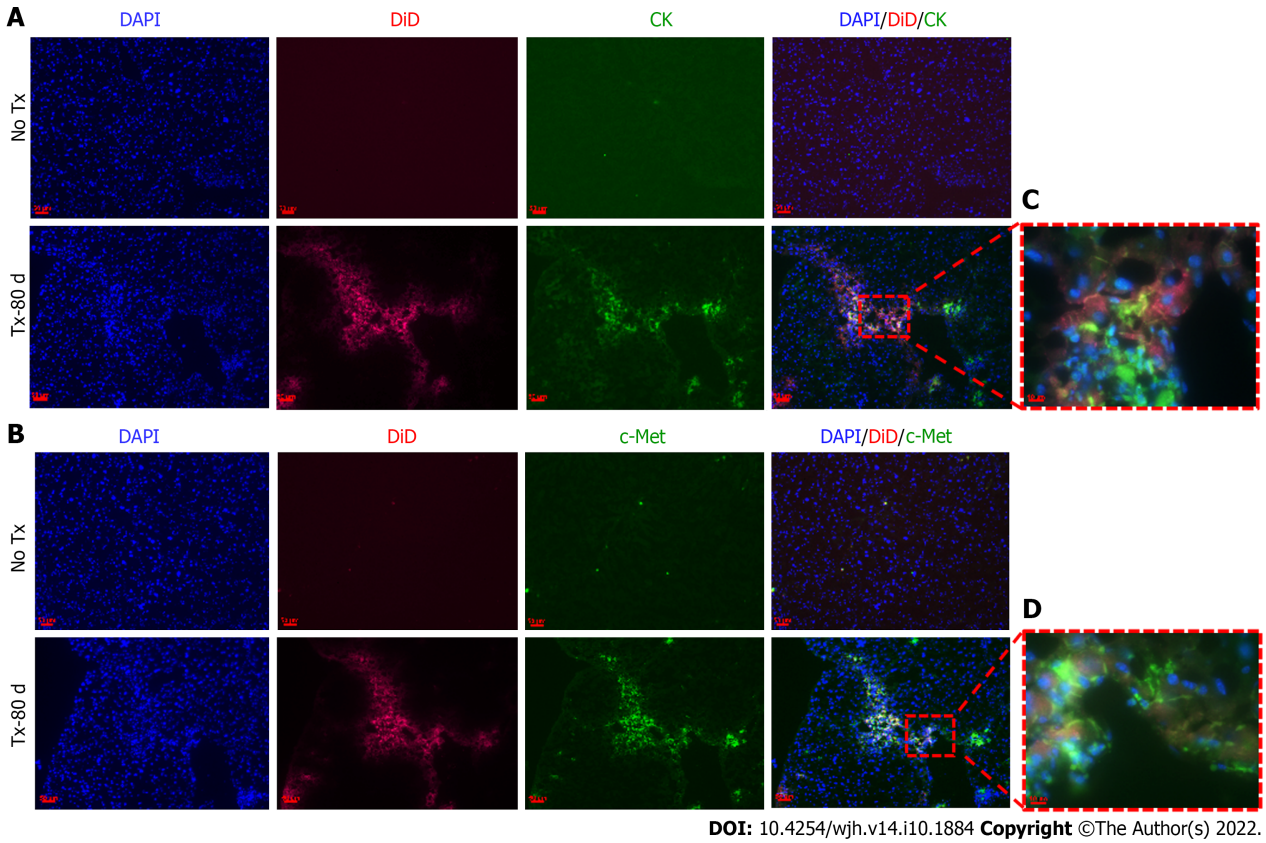

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Figure 5 Detection of hepatic markers in transplanted mouse livers 80 d post-transplantation. A and B: Fluorescent microscopic images of mouse liver tissue cryosections that continue to show DiD signals 80 d post-transplantation and show co-expression of CK and c-Met only in the transplanted (TX-80 d) mouse livers, but not in the non-transplanted (No-TX) livers (scale bar = 50 μm); C and D: 63 × images showing the colocalization of CK and c-Met staining with DiD labeling, respectively (Scale bar = 50 μm and 10 μm). CK: cytokeratin; DAPI: 4',6-diamidino-2-phenylindole.

biodistribution, cell viability post-transplantation, and evaluating the regenerative capabilities, which would aid in accelerating their clinical applications[26]. The depth of penetration, sensitivity, spatial and temporal resolution, ease of availability of the molecular probe, and cost of imaging are some of the important factors to be considered for a suitable imaging approach[26]. Direct labeling of the transplanted cells with fluorescent dyes has been demonstrated in several animal liver disease models to understand their homing and differentiation. For instance, the fluorescent dye PKH26 was used in a CCl_4-induced liver injury model in rats to show stem cell migration, proliferation, and expression of liver-specific markers[27,28]. However, these studies did not involve live cell tracking, and only postmortem analysis of the liver tissue sections was performed to identify the presence of labeled cells. Also, due to the potential cross-transfer of the dye to host cells, PKH26 was not considered ideal as a cell tracer, thus limiting its application in transplantation studies[29].

The carbocyanine dyes, CM-DiI, and DiR have been used to label transplanted cells in animal models of liver injury. Although both CM-DiI and DiR proved to be safe for cell tracking applications, monitoring has only been demonstrated in *ex vivo* settings[30], or if *in vivo*, the signal was reported to have faded within 5 d after infusion[22]. Hence, the above carbocyanine dyes have not provided sufficient evidence of their long-term applicability for cell tracking *in vivo*. In contrast, DiD has been demonstrated to be effective for long-term monitoring of labeled neuronal and cancer cells *in vitro* for up to 4 wk[31,32]. Moreover, DiD at higher concentrations of up to 2 μM also does not affect cell growth, proliferation, migration, and apoptosis, and does not cross-transfer to neighboring cells[33]. In line with this, DiD-labeled MSCs have demonstrated the absence of cytotoxicity and signs of altered functional performance in terms of cytokine production or trilineage differentiation[34], thus assuring the safety of DiD for potential applicability in stem cell tracking. DiD-labeled neural stem cells have also shown promising results following direct injection into the cerebrospinal fluid *in vivo*[35]. Also, among the Vybrant® dye series (DiR, DiI, CM-DiI, DiD), DiD has demonstrated comparatively high intense fluorescence[32]. Thus, these observations support our choice of DiD for cell labeling and long-term *in vivo* imaging.

In the present study, DiD did not show any interference with tissue autofluorescence and FITC due to fluorescence emission close to the NIR region. The quick and easy methodology of staining, non-toxicity, no interference with the functionality of the labeled cells, non-diffusion to adjacent cells, and lack of photo-bleaching were identified as the major advantages of DiD labeling. Furthermore, co-

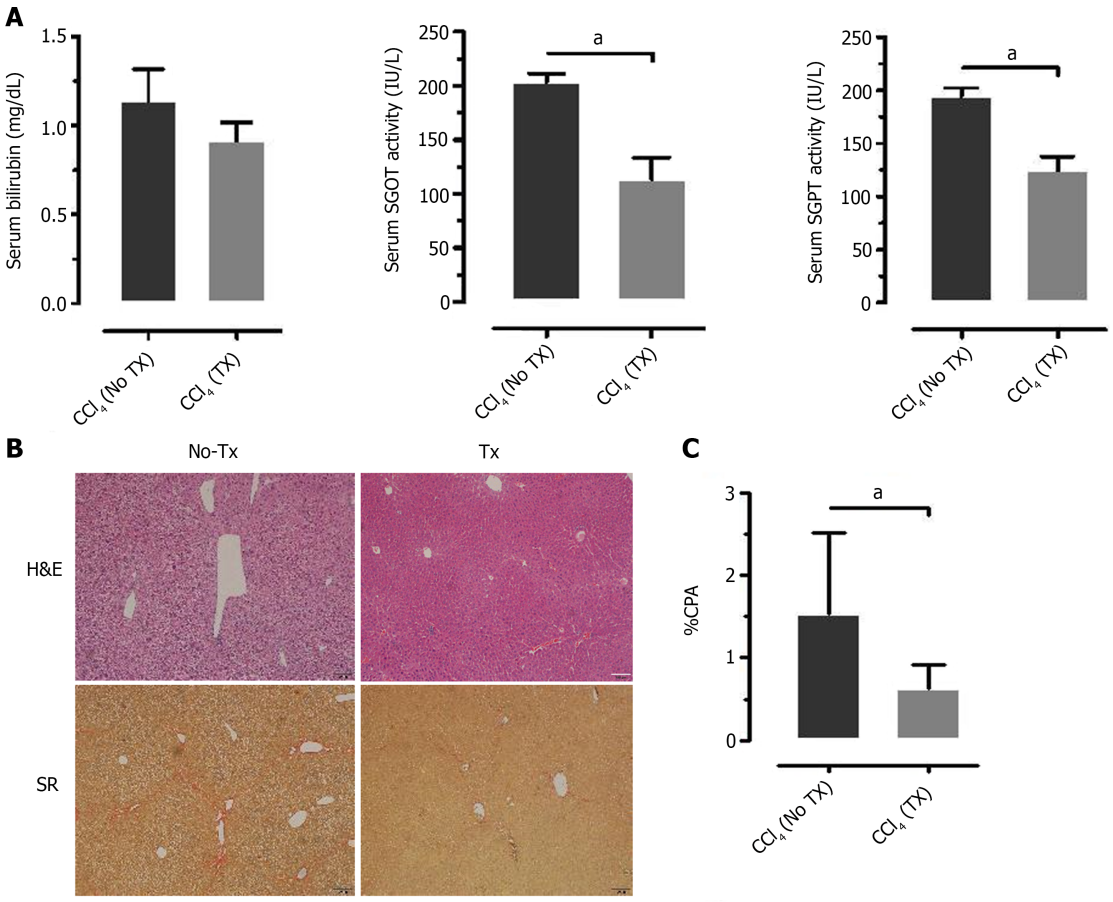

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Figure 6 Liver enzyme and histology assessment of improved liver function parameters. A: Analysis of serum bilirubin, serum glutamic-oxaloacetic transaminase, (SGOT) and serum glutamate-pyruvate transaminase (SGPT) measured 15 d post-transplantation (TX) and compared to the non-transplanted (No-TX) mice (n = 4); B and C: Hematoxylin and Eosin (H&E) and Sirius Red staining of liver tissue sections of non-transplanted and transplanted mouse liver tissue at 30 d post-transplantation showed a significant decrease in collagen percent area in the transplanted mouse livers as compared to the non-transplanted mouse livers (^{a}P < 0.001). H&E: Hematoxylin and Eosin; SR: Sirius Red.

localization of CK and c-Met hepatic markers with the DiD-labeled transplanted cells in the recipient mouse livers showed the long-term persistence of transplanted cells. These observations support the involvement of selected markers in supporting the proliferation, survival, and differentiation of transplanted cells in the recipient's liver regeneration and functional improvements[36-38]. In addition, the detection of hu-ALB-specific expression within the transplanted mouse livers further confirmed that the transplanted fHPCs not only persisted for the longer duration but also differentiated into functional hepatocytes contributing to liver regeneration and functional recovery. In addition, fHPCs successfully attenuated liver fibrosis in mouse liver. However, future studies could aid in deciphering the detailed molecular mechanisms by which fHPCs contribute to liver repair and regeneration.

Overall, the results from our study were supportive of the use of DiD in long-term, non-invasive, *in vivo* tracking of fHPCs in the recipient's liver, but there are certain limitations. Although the results indicate the suitability of DiD for monitoring transplanted fHPCs in the liver, quantification of the signal to correlate the cell number or survival was not reported. Future studies using a larger cohort of animals, varying cell numbers, and quantification of the signal intensity in relation to cell doses would be very useful to understand the efficacy and survival. Moreover, optimizing the route of cell delivery, and assessing the dynamic changes in the expression of EpCAM over time will help in addressing the questions on engraftment and differentiation of fHPCs. Lastly, DiD has been proven to be safe and non-toxic with no effect on the metabolic functioning of cells *in vitro*. However, for regular *in vivo* imaging applications of DiD, it is essential to evaluate the metabolic cycle of the dye for long-term use. Addressing such issues would make DiD labeling more valuable for wider *in vivo* cell tracking applications.

CONCLUSION

Monitoring the fate of transplanted cells through *in vivo* tracking or imaging can help in understanding

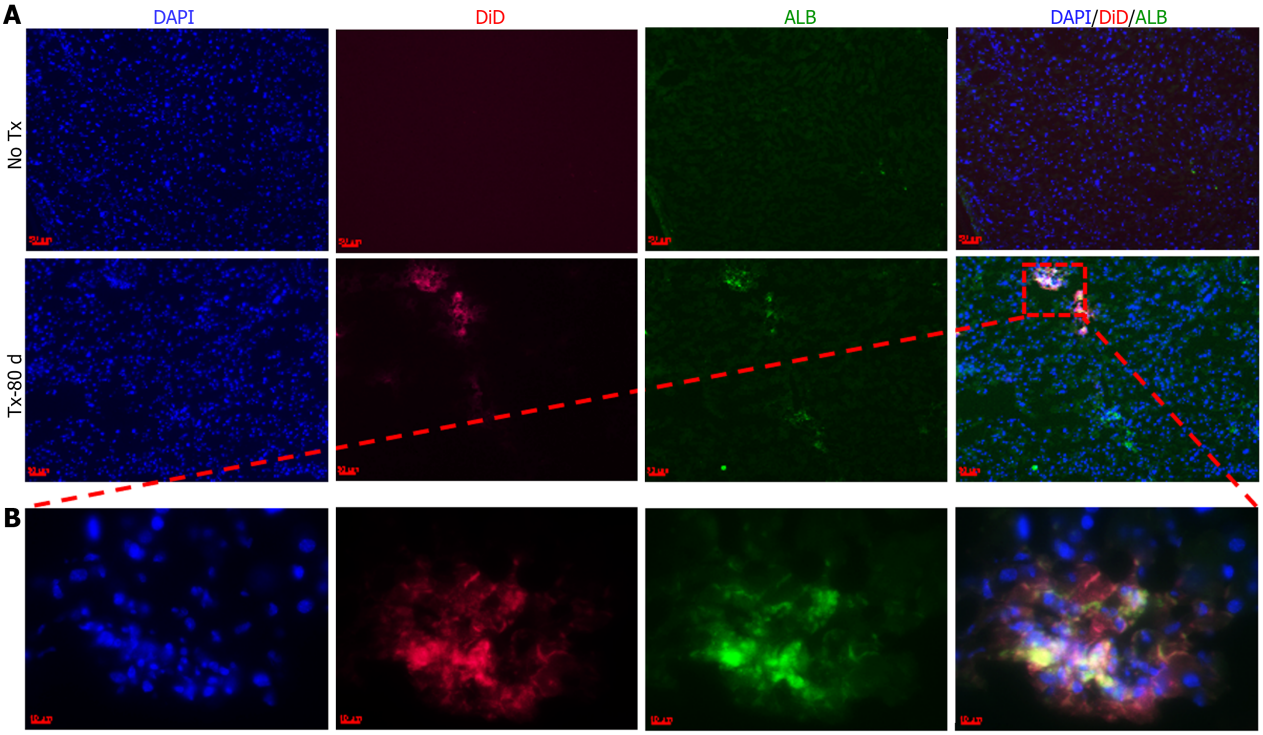

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Figure 7 Expression of hu-ALB in the transplanted mouse livers. Fluorescent microscopic images of mouse liver tissues expressing hu-ALB colocalized with DiD, 80 d post-transplantation. The non-transplanted mouse livers (No-TX) are devoid of any expression (10 ×), while the transplanted livers (TX-80 d) show specific zones of expression (10 × and 63 ×) (Scale bar = 50 μm and 10 μm). A: 10 ×, B: 63 ×. ALB: albumin; DAPI: 4',6-diamidino-2-phenylindole.

the homing, engraftment, long-term survival, and function of the transplanted cells. In this preclinical study, DiD-labeled fHPCs showed efficient long-term cell tracking for up to 80 d. The ease of handling, non-toxicity, and long-term signal retention proved to be major advantages in using DiD as a cell labeling agent for non-invasive, long-term tracking of cells both *in vivo* and *ex vivo*. These findings could pave the way to unravel the underlying regenerative mechanisms and contribution of exogenously transplanted cells in restoring the structural and functional deficits of the liver in CLD.

ARTICLE HIGHLIGHTS

Research background

Determining the fate of transplanted cells *in vivo* through long-term cell tracking remains a crucial field of investigation. Long-term live cell tracking *in vivo* has always been challenging due to the absence of a safe cell-labeling agent.

Research motivation

DiD is a carbocyanine dye having good photochemical properties of strong fluorescence, and stability. Due to the long-range emission of DiD (670 nm), tissue autofluorescence is minimum, permitting the use of other fluorochromes such as fluorescein isothiocyanate, for colocalization studies to evaluate the expression of other essential markers specific to the transplanted cells in the recipient tissue. Moreover, the process for labeling the cells using DiD is easy due to its excellent efficiency for integration and diffusion into the cell membranes. The effects of DiD labeling on *in vivo* retention of labeled human liver cells remain to be investigated.

Research objectives

The present study aimed to shed light on the fate of DiD-labeled human liver cells in chronic liver diseases (CLD)-severe combined immunodeficiency (SCID) mice using live imaging up to 80 d post-transplantation.

Research methods

A chronic liver disease SCID mouse model was developed, which received DiD-labeled EpCAM-positive human hepatic progenitor cells by intra-hepatic infusion. The long-term survival and functional

response of transplanted DiD-labeled cells were investigated up to 80 d.

Research results

This study showed that DiD labeling of human liver cells is easy and efficient for long-term and non-invasive tracking *in vivo* up to 80 d post-transplantation. Using DiD, the fate of transplanted cells was determined. Transplanted human fetal liver cells were able to provide structural and functional improvement in CLD-SCID mice.

Research conclusions

Monitoring the fate of transplanted cells through DiD-based *in vivo* live cell imaging can help in understanding the homing, engraftment, long-term survival, and function of the transplanted cells.

Research perspectives

The findings of the current study may pave the way to unravel the underlying regenerative mechanisms and contribution of exogenously transplanted cells in restoring the structural and functional deficits of the liver in CLD.

FOOTNOTES

Author contributions: Khan AA, Pande G, and Tripura C were responsible for the study concept, design, and supervision; Thatipalli AR, Vishwakarma SK, Jose J, and Jerald MK performed the experiments; Tripura C, and Gunda S were responsible for data acquisition and analysis; Tripura C also performed data organization and manuscript writing; Tripura C, Vishwakarma SK, Khan AA, and Pande G performed editing and revision of the manuscript draft.

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Institutional review board statement: The total fetal liver cells (tFLCs) isolation protocol was approved by the Institutional Ethics Committee (IEC) of Deccan College of Medical Sciences (DCMS), Hyderabad.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Quality of life, depression and anxiety in potential living liver donors for pediatric recipients: A retrospective single center experience

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Abstract

BACKGROUND

Living donor liver transplantation is a safe alternative for patients on a liver transplant list. Donor evaluation goes beyond physical variables to include social, emotional, and ethical aspects. The role of pre-donation sociopsychological evaluation of the donor candidate is as important to the success of the procedure as is the medical assessment. Success implies recovery from the operation and pro -mpt engagement in pre-transplant professional and social activities, without leading to psychological or physical distress. Psychological profiling of potential living liver donors (PLLD) and evaluation of quality of life (QOL) can influence outcomes.

AIM

To evaluate the socio-demographics and psychological aspects (QOL, depression, and anxiety) of PLLD for pediatric liver transplantation in a cohort of 250 patients.

METHODS

This was a retrospective cohort study of 250 PLLD who underwent psychological pre-donation evaluation between 2015 and 2019. All the recipients were children. The Beck anxiety inventory, Beck depression inventory, and 36-item short-form health survey (SF-36) scores were used to evaluate anxiety (Beck anxiety inventory), depression (Beck depression inventory), and QOL, respectively.

RESULTS

A total of 250 PLLD were evaluated. Most of them were women (54.4%), and the mean age was 29.2 ± 7.2 years. A total of 120 (48.8%) PLLD were employed at the

time of evaluation for donation; however, most had low income (57% earned < 2 times the minimum wage). A total of 110 patients (44%) did not finish the donation process, and 247 PLLD answered a questionnaire to evaluate depression, anxiety, and QOL (SF-36). Prevalence of depression was of 5.2% and anxiety 3.6%. Although most of the PLLD were optimistic regarding the donation process and never had doubts about becoming a donor, some traces of ambivalence were observed: 46% of the respondents said they would feel relieved if a deceased donor became available.

CONCLUSION

PLLD had a low prevalence of anxiety and depression. The foundation for effective and satisfactory results can be found in the pre-transplantation process, during which evaluations must follow rigorous criteria to mitigate potential harm in the future. Pre-donation psychological evaluation plays a predictive role in post-donation emotional responses and mental health issues. The impact of such findings on the donation process and outcomes needs to be further investigated.

Key Words: Liver transplantation; Children; Outcomes; Living donation

Core Tip: The role of a pre-donation sociopsychological evaluation of a donor candidate is as important to the success of the procedure as is the medical assessment. This implies recovery from the operation and prompt engagement in pre-transplant professional and social activities without leading to psychological or physical distress. The present study evaluates socio-demographics and psychological aspects of potential living liver donors for pediatric liver transplantation in a cohort of 250 patients. It also investigates specific questions regarding donation, decision-making processes, and feelings of ambivalence.

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INTRODUCTION

The use of living donors has become standard practice in the field of pediatric liver transplantation. The extensive use of this technique has become possible owing to careful patient selection and the proven safety of the procedure. However, donor selection must be explored beyond physical, laboratory, and radiological evaluations. The role of a pre-donation sociopsychological evaluation of a donor candidate is as important to the success of the procedure as is the medical assessment. This implies recovery from the operation and prompt engagement in pre-transplant professional and social activities without leading to psychological or physical distress.

Donor candidates may experience anxiety and other psychological distress during the assessment period[1-3], such as depression, decision ambivalence (coexistence of inconsistent or opposing perceptions)[3], and fear. In a scenario that includes severe disease in a child and the risk of death while on the waiting list, part of the psychological evaluation is to rule out the possibility of coercion, even if the donor candidate is related to the recipient. Furthermore, knowledge of the patient's social status and quality of life (QOL) is crucial for a thorough psychological assessment that will inform the decision-making process, such as acceptance of potential donors.

Little is known about the relationship between eligibility criteria and significant predictors of psychological strain in living donation candidates[4]. Moreover, the predictors of psychological strain may vary in different social scenarios, and may include income, education, and religious orientation.

This study aimed to evaluate the prevalence of depression, anxiety, and QOL in potential living liver donors (PLLD) for pediatric recipients. It also describes the socioeconomic profile of this population.

MATERIALS AND METHODS

A cohort of 250 consecutive PLLD aged > 18 years was selected for the pre-donation evaluation. All the recipients were children. The PLLD underwent psychological assessments during the evaluation process for living donations at Hospital Sírio Libanês, São Paulo, Brazil, between January 2015 and May 2019. This study was conducted in accordance with the principles of the Declaration of Helsinki. The Ethics Committee of Hospital Sírio-Libanês approved this study.

PLLD with a history of drug abuse, dependence, or previous diagnoses of psychiatric disorders were excluded from the analysis. Demographic data were collected through a medical chart review. The patients answered a questionnaire designed to evaluate their perception of the living donation process. QOL was assessed using the 36-item short-form health survey (SF-36) questionnaire[5].

To classify potential donors according to anxiety and depression levels, Beck anxiety inventory (BAI) [6] and Beck depression inventory (BDI)[7] scores were utilized.

The medical outcomes study SF-36 comprises 36 items divided into eight domains: Role-physical (RP), physical functioning (PF), general health (GH), vitality (VIT), social functioning (SF), role-emotional (RE), mental health (MH), and bodily pain (BP). Scaled scores for each domain were the weighted sums of the answers to the domain questions, with each score transformed into a 0-100 scale, with higher scores indicating better functionality or less pain[5].

Demographic variables were also evaluated: Gender, age, relationship to the recipient, education, employment status, religion, marital status, region of origin, and monthly family income.

Specific questions were posed to the PLLD regarding donation and decision-making processes (included as a supplementary material for consultation).

Statistical analysis

The values are expressed as mean ± SD or median, and 25 and 75 percentiles. Categorical data are presented as absolute values and percentages. All analyses were performed using the SPSS 21.0 statistical package (IBM, Inc., Chicago, IL, United States). The statistical review of the study was performed by our institution's biomedical statistics team.

RESULTS

A total of 250 PLLD met the inclusion criteria and underwent psychological evaluation before donation, between January 2015 and May 2019. Most of them were women (54.4%), and the mean age was 29.2 ± 7.2 years. PLLD self-declared as afro-descendent in 49.6% of the cases; 46.3% were married, 51.6% self-declared as Catholic, and 53.7% had a college degree or equivalent. A total of 120 (48.8%) PLLD were employed at the time of evaluation for donation; however, most had low income (57% earned < 2 times the minimum wage) (Tables 1). Most PLLD (51%) lived in the same region as the transplant center, and 214 (85.6%) were family members of the recipient (Table 2). A total of 110 patients (44%) did not finish the donation process. Reasons for not completing the donation processes are described in Table 3.

A total of 247 PLLD answered a questionnaire to evaluate depression (BDI), anxiety (BAI), and QOL (SF-36). Prevalence of depression was of 5.2%. The results of the BDI index were minimal in 237 (94.8%) patients, light in 11 (4.4%), moderate in 2 (0.8%), and severe in none. Prevalence of anxiety was of 3.6%. The results of the BAI index were minimal in 241 (96.4%), light in eight (3.2%), moderate in one (0.4%), and severe in none.

The results of the evaluation of QOL through SF-36 in each domain were: 96.2 ± 8.7 PF, 91.3 ± 22.6 RP, 89.3 ± 15.2 BP, 75.5 ± 10.9 GH, 82.6 ± 16.3 VIT, 90.8 ± 17.9 SF, 88.9 + 24.5 RE, and 86.1 + 13.8 MH.

Although most of the PLLD were optimistic regarding the donation process (motivations and ambivalence questionnaire, Table 4) and never had doubts about becoming a donor, some traces of ambivalence were observed: 46% of the respondents said they would feel relieved if a deceased donor became available.

DISCUSSION

Socioeconomic characteristics have been shown to influence access to LDLT and promote health disparities in both adult and pediatric candidates[5-8]. PLLD profile may vary according to the region or country of origin, as well as the recipient's age group (small children, adolescents, or adults). Most PLLD in this study were female, usually mothers, with a mean age of 29.2 years, and very low annual income. The national minimum wage in December 2021 in Brazil was $196 per month[9]; correspondingly, the annual income for 57% of the PLLD in this cohort was under $4704. This situation reflects the socioeconomic situation of our country, but the majority of liver transplantations were performed under Brazil's publicly-funded healthcare system (Sistema Único de Saúde), which covered all costs for donor and recipient care. A recent study performed in the United States on PLLD for

Table 1 Demographic characteristics of the potential living liver donors

Characteristics	*n* = 250
Sex, female, *n* (%)	136 (54.4)
Age, yr, mean ± SD	29.2 ± 7.2
Race (*n* = 246)	
Afrodescendent	122 (49.6)
White	116 (47.2)
Other	8 (3.2)
Educational level (*n* = 246), *n* (%)	
Post-bac/graduate	61 (24.8)
College/bachelor	132 (53.7)
Basic education or less	53 (21.5)
Employment, *n* (%)	120 (48.8)
Religion, *n* (%)	
Catholic	127 (51.6)
Evangelic	68 (27.6)
Protestant	17 (6.9)
Other	27 (10.9)
None	7 (3)
Marital status, married (*n* = 250), *n* (%)	115 (46.0)
Income (*n* = 246), *n* (%)	
≤ 2 times the minimum wage	140 (57)
2-6 times the minimum wage	91 (37)
≥ 6 times the minimum wage	15 (6)
Family related (*n* = 250), *n* (%)	214 (85.6)
Parents, *n* (%)	183 (73.2)

Table 2 Donor relationship with the recipient

Donor relationship with the recipient	*n* = 247 (%)
Mother/father	158 (64)
Uncle/aunt	30 (12.2)
Brother/sister	10 (4)
Cousin	9 (3.6)
Grandfather/grandmother	4 (1.6)
Nephew/niece	3 (1.2)
Unrelated	33 (13.4)

children found a few different characteristics in these donors, especially a higher mean age (38), and a predominance of white individuals with college degrees[10]. One striking difference was the average age of the PLLD, which was nearly 10 years greater in the United States study than in the Brazilian study. It is conceivable that the PLLD in the present study, most of whom were mothers of the recipients, had children earlier, did not advance as far in their education, and had lower monthly incomes than potential donors in the United States. As for the remaining data in the comparison, although there are some common traits, we must point out that socio-demographic profiles are greatly influenced by cultural differences between countries and even by differences in the transplant centers assessing the patients. Despite the demographic differences encountered, the outcomes for donors and

Table 3 Reasons for not completing the donation process

Reasons for not completing the donation process	*n* = 110 (%)
Medical condition	44 (40)
Abandoned the evaluation	22 (20)
Children died before the transplant	6 (5.4)
Social contraindication	1 (0.9)
Transplant was postponed	1 (0.9)
Unknown/missing	36 (32.8)

Table 4 Motivations and ambivalence in potential living liver donor

Motivations and ambivalence	*n* = 250 (%)
Would prefer that other person donates	25 (10)
Would feel relieved if the recipient was transplanted with a deceased donor	115 (46)
Feels moved about the possibility to donate	59 (23.6)
Wouldn't like to be a donor, would rather care for the recipient after the transplant	11 (4.4)
Voluntary and pro-active decision to donate	195 (78)
Immediate decision to donate	183 (73.2)
Never had doubts about donating	218 (87.2)
Is scared of the surgery	5 (2)
Has a family member who's against the donation	10 (4)
Believes there will be no health-related problems after the donation	241 (96.4)
Would be sad if not compatible to donate	144 (57.6)
Wishes to donate in order to ease the recipient's and the family suffering	152 (60.8)
Believes the donation will be a life changer	210 (84)

PLLD: Potential living liver donor.

recipients reported by our group proved to be safe for donors[11] and with excellent short and long term results for the pediatric recipients[9,12].

Some studies have focused on donors' motivations, concerns, and feelings of ambivalence, as well as the influence that ties and relationships have on their decisions[13-15]. The present study evaluated the quality of the relationship between the potential donor and the recipient and/or the recipient's family, and found that this relationship is satisfactory for a significant part (98.4%) of the sample, regular for 1.2% of the sample, and nonexistent for 0.4%. Regular or nonexistent relationships probably involve PLLD who are not family members. The well-being and expectations of family members are taken into account and influence the decision to donate. In this regard, one study found that 94.1% of donors decide to donate to preserve the well-being of the whole family[15]. Another study[16] approaches the same family setting from a different perspective: family members who somehow show conflict towards the donation present high levels of anxiety, symptoms of depression, and low QOL. Ryu *et al*[15] have found differences in donor motivation levels linked to the donor-recipient relationship. According to the authors, parents donating to their children show higher motivation than children donating to their parents and present lower levels of anxiety and depression during the evaluation period prior to donation.

The World Health Organization (WHO)[17], in their report on prevalence estimates of mental disorders emphasizes some significant data that corroborate the results presented here. The proportion of depression in the world population in 2015 was estimated at 4.4% (*vs* 5.8% in the Brazilian population), being more prevalent among women (5.1%) than men (3.6%), with a total of 322 million people living with depression. There was an 18.4% increase in depression indices between 2005 and 2015, pointing to an increase in the disorder among the global population, including the higher-risk age group (55-74 years). Depression indices in the present study were 5.2% among potential donors. The potential donors are predominantly women, a risk factor for depression.

With regard to anxiety, the WHO[17], in their prevalence estimates of mental disorders, reported that the rate of anxiety disorders among the global population in 2015 was 3.6% (*vs* 9.3% in the Brazilian population) and was also more prevalent among women (4.6%) than men (2.6%), totaling 264 million people living with anxiety. Although the prevalence rates did not show significant differences among age groups, there was a slightly lower prevalence among the older age groups. The global anxiety indices grew by 14.9% between 2005 and 2015, suggesting an increase in disorders among the total population. Anxiety indices in the present study were 3.6% among potential donors. Similarly, the age group in our study presented a risk for anxiety, according to the WHO data. In regards to the SF-36 questionnaire, potential donors showed a lower impairment of QOL in GH, VIT and MH domains, and a greater impairment in the PF domain.

LDLT requires the donor to submit to surgery that has no medical benefit[15]. However, the process of preparing for the donation and the post-operative period may provide significant psychological benefits, such as finding new meaning in life and a feeling of plenitude for helping a loved one (in the case of a family member) or a person in need (in case of non-familial donors). This study evaluated the meaning that PLLD derived from the process and found the following benefits: improving the recipient's QOL (25.2%), saving a life (22.7%), a gesture of love (21.1%), a second chance for the recipient (14.9%), and doing good for someone else (13%). A separate question about their expectations regarding the donation obtained responses such as "a feeling of well-being with myself", "a new meaning for life through the donation", "better life conditions", and "family and social recognition" in 37.5% of cases. These data encourage important ethical questions, since a careful assessment of the donor, including a favorable physical and psychological evaluation, will minimize potential risks for the donor and are aligned with the bioethical principle of non-maleficence. Furthermore, the psychological benefits highlighted by this study and the spiritual benefits of a new meaning in life are in agreement with the bioethical principle of beneficence. Even though the reasons for not completing the donation process were not directly related to psychological aspects, it is difficult to know if in some instances it had a causative effect, such as in those who abandoned the evaluation during the donation process.

CONCLUSION

The scientific community places marked attention on the post-transplantation period, including the progress made by recipients and donors. However, the foundation for effective and satisfactory results can be found in the pre-transplantation process, during which evaluations must follow rigorous criteria to mitigate potential harm in the future. Pre-donation psychological evaluation is effective and plays a predictive role in post-donation emotional responses and mental health issues. Also, the socio-demographic findings of this particular population indicate the complexity of the donation process in a setting of low resources. The results of this study indicate the need for the transplantation community to take heed of the benefits of a thorough psychological evaluation of the potential donor, which will play a central role in predicting their emotional progress and the donation experience itself.

ARTICLE HIGHLIGHTS

Research background

The role of pre-donation sociopsychological evaluation of the living donor liver candidate is as important to the success of the procedure as is the medical assessment. Psychological profiling of potential living liver donors (PLLD) and evaluation of quality of life (QOL) can influence outcomes.

Research motivation

Adequately profiling potential donors may help the transplant team to better guide them through the donation processes. Detection of depression and anxiety among potential donors may influence the pre donation evaluation.

Research objectives

Evaluate QOL, depression and anxiety among PLLD.

Research methods

This was a retrospective cohort study of 250 consecutive PLLD who underwent psychological pre-donation evaluation between 2015 and 2019. All the recipients were children. The Beck anxiety inventory (BAI), Beck depression inventory (BDI), and 36-item short-form health survey (SF-36) scores were used to evaluate anxiety, depression, and QOL, respectively.

Research results

A total of 250 PLLD were evaluated. Most of them were women (54.4%), and the mean age was 29.2 ± 7.2 years. A total of 120 (48.8%) PLLD were employed at the time of evaluation for donation; however, most had low income (57% earned < 2 times the minimum wage). Family members were the majority of the. A total of 110 patients (44%) did not finish the donation process. A total of 247 PLLD answered a questionnaire to evaluate depression (BDI), anxiety (BAI), and QOL (SF-36). Prevalence of depression was of 5.2% and anxiety 3.6%.

Research conclusions

The socio-demographic findings of this particular population indicated the complexity of the donation process in a setting of low resources. PLLD had a low prevalence of anxiety and depression. Pre-donation psychological evaluation plays a predictive role in post-donation emotional responses and mental health issues. The impact of such findings on the donation process and outcomes needs to be further investigated.

Research perspectives

Almost half of the evaluated potential donors did not complete the donation processes. Reasons for not completing the donation process should be further evaluated in other centers as well. To further study the impacts of donation among living liver donors, our group aims to evaluate the post-donation psychological outcome in these donors.

FOOTNOTES

Author contributions: Reine PK contributed to study design, data collection, manuscript writing; Feier F contributed to data analysis, manuscript writing; Hernandes RG contributed to data collection, manuscript final version critical analysis; da Fonseca EA contributed to manuscript writing, manuscript final version critical analysis; Seda-Neto J contributed to study design, manuscript writing, data analysis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Hepatic involvement in children with acute bronchiolitis

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Abstract

BACKGROUND

Respiratory syncytial virus (RSV) is a prevalent cause of lower respiratory tract infections. It may be associated with hepatocellular involvement, as indicated by increased liver enzymes aspartate aminotransferase and alanine transaminase (ALT).

AIM

To evaluate the rate of increased liver enzyme levels in children with acute bronchiolitis and correlate them with clinical, laboratory, and radiological variables.

METHODS

The study was a retrospective review of the medical records of children who presented with acute bronchiolitis when admitted to the Pediatric Department, Salmaniya Medical Complex, the Kingdom of Bahrain, between 2019 and 2020. We collected the demographic data, the clinical presentation, the laboratory and radiological findings, and the clinical outcomes. We compared the patients with elevated liver enzymes to those with normal levels at the time of presentation and at follow-up.

RESULTS

We included 166 (57.8%) of 287 patients with acute bronchiolitis who fulfilled the inclusion criteria. Ninety-three (56%) patients were males. The median age at presentation was 3.4 (interquartile range 1.1 to 12.4) mo. Fifty-four (28%) patients tested positive for RSV, which was confirmed in 15 of them (28%) by PCR. Laboratory findings of 161 patients tested at presentation showed high ALT levels in 14 (8.7%) patients and normal ALT in 147 (91.3%). Coagulation profiles were measured in 46 (27.7%) of 166 patients. High prothrombin time was present in 15 (32.6%), a high international normalized ratio was present in 13 (28.3%), and high activated partial thromboplastin time was present in three (6.5%). Thrombin time was elevated in nine (27.3%) of 33 patients. Five (21.7%) of 23 patients with available radiological data had hepatomegaly; one of them had findings suggestive of fatty infiltration. High ALT had a significant association with lengthy hospital stays ($P < 0.05$) and positive urine culture ($P < 0.05$). Seventy (42.2%) patients had documented follow-up with liver function tests over a median follow-up period of 10.2 (IQR, 2.4-23.3) mo. Total serum protein and serum globulin levels were normalized at the follow-up time, with a significant P value of < 0.05.

CONCLUSION

This study showed a low prevalence of liver function involvement in patients with acute bronchiolitis with a benign course. However, there was a rising trend in ALT during follow-up. Prolonged hospital stay and positive urine cultures were associated with elevated liver enzymes.

Key Words: Children; Acute bronchiolitis; Liver function tests; Respiratory syncytial virus

Core Tip: How frequent is hepatic involvement in children with acute bronchiolitis? Furthermore, what are the predicted factors? To answer these questions, we conducted this retrospective study. Despite the low prevalence of impaired liver function in patients with acute bronchiolitis and the benign course, there was a rising trend in alanine aminotransferase levels during follow-up. Elevated liver enzymes were linked to an extended hospital stay and positive urine cultures. Therefore, children with acute bronchiolitis should be monitored and followed up with liver function tests.

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INTRODUCTION

Respiratory syncytial virus (RSV) is one of the most common causes of lower respiratory tract infections in children[1]. By the age of two years, most children have had at least one episode of RSV bronchiolitis [2]. It is a single-stranded RNA virus belonging to the Paramyxovirus family that affects 4-5 million children annually, leading to more than 125000 pediatric admissions annually in the United States[3,4]. Premature infants and infants with chronic lung disease or congenital heart diseases have much higher morbidity and mortality due to acute bronchiolitis[2].

RSV has been found to affect the gastrointestinal system in addition to respiratory tract infections. It has been associated with hepatocellular involvement, causing an increase in liver enzymes, including aspartate aminotransferase (AST) and alanine transaminase (ALT). Several types of research have proven that the elevation of transaminases is associated with more severe cases of RSV in children[1,5]. The hepatocellular involvement is reported to be due to different mechanisms, for example, high viral load causing dispersion into the systemic circulation, which can lead to acute hepatitis, hepatic congestion, or ischemia[5].

Data on hepatic involvement in children with acute bronchiolitis are scarce. There have only been a few reports describing this issue[1,3]. Unfortunately, there are no previously published data regarding hepatic involvement in children with acute bronchiolitis in the Kingdom of Bahrain. In this study, we aimed to assess the rate of impaired liver functions in children with acute bronchiolitis and to detect the predicted clinical, radiological, and laboratory variables.

MATERIALS AND METHODS

Study design and setting

In this study, we retrospectively reviewed the electronic medical records of all children with a clinical diagnosis of acute bronchiolitis admitted to the pediatric department at Salmaniya Medical Complex (SMC), the Kingdom of Bahrain, between September 1, 2019, and February 29, 2020.

Study participants

The study included children aged five years or less admitted to the hospital with the clinical diagnosis of acute bronchiolitis who had a reported nasopharyngeal swab for RSV direct antigen detection, PCR test, and liver function tests (LFTs). Children were diagnosed with acute bronchiolitis according to the diagnostic criteria of the American Academy of Pediatrics[6]. Signs and symptoms suggestive of bronchiolitis include running nose, tachypnea, cough, wheezing, rales, and indications of increased respiratory effort such as nasal flaring, grunting, and intercostal and/or subcostal retractions. These are the necessary criteria for clinical diagnosis. We did not routinely rely on radiographic or laboratory results to confirm acute bronchiolitis diagnoses[6].

Data collection

We collected patients' demographic information, including age at the time of the study, age at presentation, sex, nationality, gestational age, clinical presentation, and length of hospital stay. Fever was diagnosed with a rectal temperature of 38 °C (100.4 °F) or an axillary temperature of 37.5 °C (99.5 °F)[7]. In our hospital, the patient's temperature was measured rectally if the patient's age was below one year or axillary in those older than one year after adding 0.5 °C to the measured temperature.

The results of laboratory investigations included complete blood count, LFTs, blood, urine, and cerebrospinal fluid (CSF) culture, coagulation profile, and direct detection of RSV antigen in the nasopharyngeal swabs. We also gathered the results of the respiratory microbial serology panel (immunoglobulin G and M) for *Mycoplasma pneumonia*, *Legionella pneumophilia*, *Coxiella burnetii*, *Chlamydia pneumonia*, RSV, adenovirus, influenza A and B, parainfluenza, Cytomegalovirus, and Epstein-Barr virus and hepatitis profile. Senior radiologists reported and documented radiological findings from abdominal ultrasound, and computed tomography (CT) scans. Medical treatments included antibiotics and steroids. Patient outcomes and complications were also documented.

Statistical analysis

The data were entered into an Excel 2010 worksheet and then transferred to Statistical Package for the Social Sciences (SPSS) version 21 for statistical analysis. Demographic data are presented as frequencies and percentages. Continuous variables are presented as mean and standard deviation (SD) if found to be normally distributed or as a median and interquartile range (IQR) if not normally distributed. We divided the patients according to LFT results into two groups: group 1 included patients with hepatic involvement and group 2 with normal LFTs. High and average values were defined based on the ALT level being the best enzyme that reflects liver injury. An elevated ALT level of more than 41 U/L was considered high, while an ALT of less than or equal to 41 U/L was considered average. We compared the two groups regarding the demographic data, the clinical presentation (presence of fever, cough, and other symptoms and signs), laboratory results (blood, urine, and CSF cultures, complete blood count, and RSV swab for direct antigen detection and PCR test), radiological results (ultrasound and CT when performed), antibiotic and steroid use, and the clinical outcomes. Children with a single viral infection were compared with those with multiple infections in terms of their mean ALT level. The Chi-Square and Fisher's tests were used to compare categorical variables. Student's *t*-tests or Mann-Whitney *U*-tests were used to compare continuous variables. P value < 0.05 was considered statistically significant. The confidence interval was set at 95%.

Ethical approval

We performed the current study in accordance with the Helsinki declaration. The study was ethically approved by The Research and Research Ethics Committee of the Salmaniya Medical Complex, Government Hospitals, Kingdom of Bahrain (with IRB number: 27130220). As the study is retrospective without exposure of patient personal data, we did not require patient consent.

RESULTS

Figure 1 shows the flow chart of the study. We studied 287 infants admitted with the clinical diagnosis of acute bronchiolitis. One hundred and sixty-seven patients (58.2%) had LFT results available in their medical records. One patient was excluded due to his underlying mitochondrial myopathy and elevated gamma-glutamyl transferase. Of the remaining 166 (57.8%) patients, 161 (56.1%) were tested for liver function at presentation, 66 (22.9%) were tested both at presentation and follow-up LFTs, and four (1.4%) patients only at follow-up.

Table 1 shows the demographic data of the included patients. Ninety-three (56%) were males; 117 (70%) infants were Bahraini, and 49 (29.5%) were not Bahraini (seven from Yemen, five from Pakistan, four from India, one from Indonesia, Jordan, Saudi Arabia, Libya, and the Philippines, and 28 without nationality data). Most patients presented with cough (n = 124, 74.7%), followed by fever (n = 118, 71.1%), and other signs of severe respiratory distress such as shortness of breath (n = 31, 18.7%), cyanosis (n = 8, 4.8%), and tachypnea (n = 2, 1.2%) as shown in Figure 2. Thirty-three (19.9%) patients had vomiting, 31 (18.7%) had reduced feeding, 13 (7.8%) had jaundice, 6 (3.6%) were found to have clinical hepatomegaly, and 2 (1.2%) had abdominal pain.

The results of the laboratory investigations are shown in Table 2. One hundred and sixteen (69.9%) patients were anemic; 83 (50%) had leukocytosis; six (3.6%) had leukopenia; 74 (44.6%) had thrombocytosis; and eight (4.8%) patients had thrombocytopenia. ALT levels were high in 14 (8.7%) patients and normal in 147 (91.3%) patients. 46 of 166 patients (27.7%) had documented data regarding their coagulation profile. High prothrombin time (PT) was found in 15 (32.6%), high international normalized ratio (INR) was found in 13 (28.3%), and high activated partial thromboplastin time (APTT) was found in three patients (6.5%). Fibrinogen levels were tested in 34 (20.5%) patients; 16 of them (47.1%) had low levels. Thrombin time was tested in 33 (19.9%); nine of them (27.3%) had prolonged thrombin time. Random glucose levels were available for 54 (32.5%) patients; five (3%) were found to have hyperglycemia, and two (1.2%) had hypoglycemia. Lactate dehydrogenase was determined in six (3.6%) patients; three of them (50%) had high levels. The iron level was available for nine (5.4%) patients; seven (77.7%) of them had low levels. C-reactive protein (CRP) levels were high in 116 (69.9%) patients, while 50 (30.1%) patients had normal levels.

Of the 137 (82.5%), patients who were checked for bacterial co-infections, 20 (14.6%) patients were confirmed to have bacterial infections. Twelve (8.7%) patients had positive blood cultures; 7 (5.2%) had positive urine cultures, and one (0.7%) had positive CSF culture by lumbar puncture.

The hepatitis profile was conducted for two patients and was found to be negative. EBV was tested in seven patients; five were positive for IgG, indicating past infection, and two showed negative results. Six patients were tested for CMV, three had normal results, and three had reactive IgG and non-reactive IgM, indicating previous infection.

Of 163 patients who were tested for RSV *via* nasopharyngeal swabs, 124 (74.7%) were tested by rapid antigen test only, one (0.6%) by PCR alone, and 38 (23%) patients were tested by both. Positive RSV results were found in 54 (28%) patients; 15 of them (28%) were confirmed by PCR. A respiratory microbial serology panel was accomplished in 42 (25.3%) infants; 14 of them (33.3%) had positive findings. Four (29%) of the 14 infants were positive for more than one virus. As for IgG, it was positive in six (14.3%) patients tested for adenovirus, six (14.3%) with RSV, three (7%) with influenza B, and one (2%) with influenza A and parainfluenza. IgM was positive in one (2%) patient with adenovirus, RSV, influenza B, and *Mycoplasma pneumonia*, respectively.

Radiological findings were available for 23 patients; 21 (12.7%) underwent abdominal ultrasound, and two (1.2%) had abdominal CT. Abdominal ultrasound results were normal in 12 (57%) patients, and abnormal in nine (43%) patients. Five patients (23.8%) had hepatomegaly: One of them had mild diffuse increased parenchymal echogenicity suggestive of fatty infiltration; one patient had an unvisualized gall bladder; one patient had subhepatic free fluid and dilated bowel loops, and one had a slightly edematous gallbladder wall. Abdominal CT in one patient showed a cyst between the liver and kidney, and the other patient had a normal CT. Both patients who had CT scans had an associated underlying disease. The first patient was a seven-year-old female with retroperitoneal neuroblastoma, and a CT scan was performed as a follow-up for her previous condition. She had chemotherapy that was stopped years before she had acute bronchiolitis. Her original medical condition did not affect her liver function as her ALT levels were normal at the time of admission and on follow-up. The second patient was a four-year-old male diagnosed with polycystic kidney disease, and the CT scan was performed as he was scheduled for a nephrectomy. His CT report did not show any cystic lesions in the liver, and he was also admitted with normal ALT (22 U/L). All the infants were managed with normal saline and bronchodilator inhalation. Antibiotics were given to 128 (77.1%) patients. The antibiotics were cefotaxime (n = 61, 46.9%), ampicillin (n = 47, 36.1%), gentamycin (n = 20, 15.3%), ceftriaxone and clindamycin (n = 9, 6.9% each), amoxicillin/clavulanic acid (n = 8, 6.1%), vancomycin (n = 7, 5.3%), clarithromycin (n = 5, 3.8%), metronidazole, meropenem and ceftazidime (n = 4, 3.1% each), piperacillin/tazobactam (n = 3, 2.3%), and ciprofloxacin, rifampicin, and sulfamethoxazole/trimethoprim (n = 1, 0.7% each). Some patients received a combination of antibiotics. In these patients, paracetamol was the antipyretic given as syrup, suppositories, or intravenously, and they were rarely given ibuprofen.

Table 1 Demographic data of children with acute bronchiolitis (*n* = 166)

Demographic data		No. of patients (%)
Sex	Male	93 (56)
	Female	73 (44)
Nationality	Bahraini	117 (70.5)
	Other	49 (29.5)
Gestational age (*n* = 140)	Term	111 (79.3)
	Preterm	29 (20.7)
Age at presentation (mo), median (IQR)		3.4 (1.1-12.4)
Current age (mo), median (IQR)		30.3 (28.31-39.8)
Length of stay (d), median (IQR)		6.0 (3.0-11.0)

Values are presented as *n* (%) for categorical variables and median (interquartile range) for continuous variables. IQR: Interquartile range.

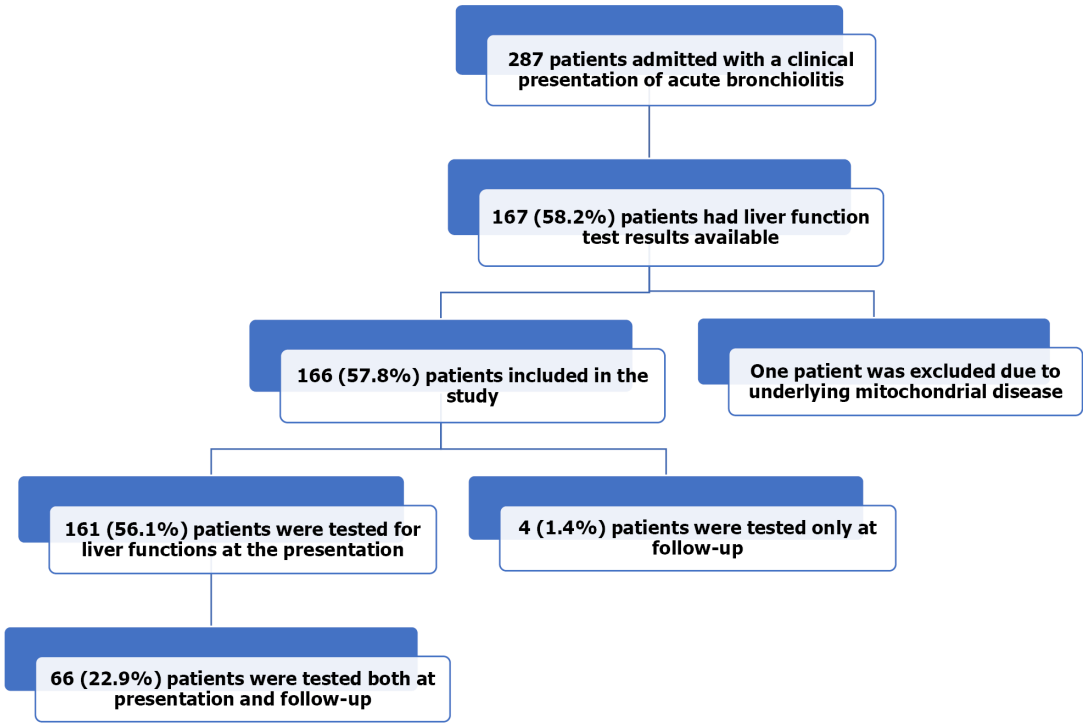

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Figure 1 The study flow chart describes hospitalized infants with acute bronchiolitis.

The outcome was documented for all hospitalized patients. Eleven patients (6.70%) required admission to the pediatric intensive care unit (PICU), eight (4.80%) required total parenteral nutrition, five (3.01%) had a surgical procedure during their admission (tracheostomy was performed in two infants, while insertion of a central line, intercostal drain tube, and nasojejunal tube were performed in one infant each). Three infants (1.8%) needed endotracheal intubation with ventilatory support. Unfortunately, nine infants (5.4%) died. No patients developed fulminant hepatic failure or required liver biopsy or liver transplantation.

A comparison between group 1 (high ALT level) and group 2 (average ALT level) is shown in Table 3. Patients with high ALT levels had extended hospital stays (P = 0.025) and were found to have more frequent positive urine cultures (P = 0.030) than those with normal ALT levels. Other parameters such as nationality, sex, gestational age, age at presentation, current age, white blood cell count, platelet count, other LFT variables, coagulation profile, positive RSV swabs, positive blood and CSF cultures, antibiotic use, complications, PICU admission, mortality, and outcomes were not significantly different between the two groups.

Table 2 Results of laboratory investigations in the 166 children who presented with acute bronchiolitis

Variable	Normal range	Mean	SD	Median	Minimum	Maximum	Tested patients (%)
White blood cell count (× 10^9/L)	3.6-9.6	10.8	5.4	9.7	0.80	27.8	166 (100)
Hemoglobin level (g/dL)	12.0-14.5	11.5	2.3	11.0	5.7	20.0	166 (100)
Platelet count (× 10^9/L)	150-400	410	179.7	383.5	14.5	971.0	166 (100)
Total serum protein (g/L)	64-82	57.8	8.7	58.0	25.0	96.0	161 (97)
Serum albumin (g/L)	38-54	41.5	10.7	41.0	17.0	143.0	161 (97)
Serum globulin (g/L)	15-30	18.0	10.7	17.0	6.0	140.0	161 (97)
Total bilirubin (μmol/L)	5-21	37.4	61.2	10.0	2.0	482.0	161 (97)
Direct bilirubin (μmol/L)	0-5	12.0	14.4	11.0	1.0	122.0	71 (42.8)
Indirect bilirubin (μmol/L)	< 18	56.0	46.0	27.0	1.0	454.0	71 (42.8)
Alkaline phosphatase (U/L)	150-420	242.6	141.7	198.0	58.0	841.0	161 (97)
Alanine aminotransferase (U/L)	< 41	25.6	30.2	19.0	6.0	247.0	161 (97)
Gamma glutamyl transferase (U/L)	< 18	63.2	80.0	32.0	6.0	526.0	145 (87.3)
Prothrombin time	10-14	13.7	2.8	12.9	9.5	25.2	46 (27.7)
INR	0.6-1.2	1.1	0.3	1.1	0.79	2.2	46 (27.7)
PTT (sec)	28-43	28.2	9.7	26.8	13.2	63.3	46 (27.7)
Fibrinogen (mg/dl)	217-496	285.9	213.8	233.8	0.0	1115.2	34 (20.48)
Thrombin time (sec)	15.6 -18.4	17.9	4.1	17.3	4.1	13.0	33 (19.87)
Random blood sugar (mmol/L)	3.6-8.9	5.7	1.4	5.2	4.0	10.0	53 (32)
Ammonia (μmol/L)	11-35	80.6	61.2	62.0	7.0	230.0	11 (6.6)
Lactic acid (mmol/L)	0.5-2.2	3.1	3.3	1.5	1.0	10.0	7 (4.2)
Lactate dehydrogenase (units/L)	250-650	1030.1	1281.9	531.5	3.0	3509.0	6 (3.6)
Iron profile (μg/dL)	11.6-31.3	9.4	5.7	8.0	4.0	19.0	9 (5.4)
CRP (mg/L)	0.3	26.8	39.2	9.5	10.0	297.0	166 (100)

SD: Standard deviation; INR: International normalized ratio; PTT: Partial thromboplastin time; CRP: C-reactive protein.

In this study, 13 (7.8%) patients tested positive for more than one viral infection. There was no significant difference between children with single viral infection and those with multiple infections in mean ALT level (25 U/L ± 30 U/L *vs* 23 U/L ± 17 U/L, respectively) (P = 0.872).

Of the 166 patients, 70 (42.2%) were followed up for LFTs with a median follow-up period of 10.2 (IQR, 2.4-23.3) mo. A comparison between LFTs at the time of presentation and at the time of follow-up is shown in Figure 2 and Table 4. It was found that only total serum protein and serum globulin levels were normalized at the follow-up time with a significant P value of 0.008. However, no significant changes were found in other LFTs. Of 72 patients with available data on ALT levels at the follow-up time, 11 (15.3%) still had high levels (Figure 3).

These patients were further investigated to exclude other causes of ALT elevation. Eight were diagnosed at the follow-up time with other underlying diseases, which may have been related to their elevated ALT. The first patient had Dandy-Walker syndrome, skeletal dystrophy, and ventricular septal defect; the second patient had tracheomalacia, severe combined immune deficiency (SCID), and hypothyroidism; while the third patient had polycystic kidney disease on hemodialysis; the fourth patient had isolated SCID; the fifth patient had cystic fibrosis; the sixth patient had progressive familial intrahepatic cholestasis; the seventh patient had biliary atresia with cirrhosis and ascites, and the eighth patient had intestinal volvulus with gangrene. One patient died from an unknown cause, and two patients had no apparent medical reason for elevated ALT.

DISCUSSION

This study showed that symptoms and signs of hepatic involvement, such as jaundice and hepato-

Table 3 Comparison of patients with and without liver function test abnormalities

Variable		Alanine aminotransferase level (*n* = 161)		*P* value
		High (> 41 U/L), 14 (8.7)	Normal (≤ 41 U/L), 147 (91.3)	
Sex	Female	9 (64.3)	61(41.5)	0.157[a]
	Male	5 (35.7)	86 (58.5)	
Nationality	Bahraini	9 (64.3)	104 (70.7)	0.760[a]
	Non-Bahraini	5 (35.7)	43 (29.3)	
Gestational age (*n* = 135)	Term	8 (57.1)	98 (66.7)	0.683
	Preterm	1 (7.1)	28 (19.0)	
Age at presentation (mo), mean ± SD		10.1 ± 12.8	10.6 ± 17.3	0.535[b]
Age at the time of study (mo), mean ± SD		37.5 ± 13.2	37.8 ± 17.5	0.394[b]
Length of hospital stay (d), mean ± SD		18.0 ± 19.3	13.4 ± 41.1	0.025[b]
History of fever		9 (7.9)	105 (92.1)	0.552[a]
White blood cell count (× 10^9/L), mean ± SD		12.2 ± 7.0	10.6 ± 5.3	0.764[b]
Hemoglobin (g/dL), mean ± SD		11.6 ± 2.1	11.5 ± 2.4	0.796[b]
Platelet count (× 10^9/L), mean ± SD		335.4 ± 194.8	417.6 ± 178.5	0.109[b]
Total serum protein (g/L), mean ± SD		56.6 ± 7.3	57.9 ± 8.9	0.895[b]
Serum globulin (g/L), mean ± SD		16.71 ± 3.69	18.1 ± 11.2	0.801[b]
Serum albumin (g/L), mean ± SD		39.86 ± 5.89	41.63 ± 11.1	0.971[b]
Total bilirubin (μmol/L), mean ± SD		29.4 ± 41.2	38.11 ± 62.81	0.794[b]
Direct bilirubin (μmol/L), (*n* = 71), mean ± SD		24.1 ± 39.9	10.5 ± 5.7	0.722[b]
Indirect bilirubin (μmol/L), (*n* = 71), mean ± SD		22.5 ± 17.9	62.0 ± 75.1	0.184[b]
Alkaline phosphatase (U/L), mean ± SD		271.9 ± 217.2	239.9 ± 133.1	0.935[b]
Gamma-glutamyl transferase (U/L), (*n* = 145), mean ± SD		92.4 ± 88.2	60.6 ± 79.0	0.080[b]
Prothrombin time, (*n* = 46), mean ± SD		13.2 ± 1.6	13.8 ± 3.0	0.809[b]
INR, (*n* = 46), mean ± SD		1.1 ± 0.1	1.2 ± 0.3	0.831[b]
PTT, (*n* = 46), mean ± SD		25.5 ± 4.9	28.8 ± 10.4	0.579[b]
Fibrinogen, (*n* = 34), mean ± SD		326.4 ± 387.2	277.3 ± 166.4	0.581[b]
Thrombin time, (*n* = 33), mean ± SD		21.4 ± 7.3	16.9 ± 2.0	0.131[b]
Random blood glucose, (*n* = 52), mean ± SD		6.0 ± 1.0	6.0 ± 1.0	0.905[b]
Positive blood culture (*n* = 127)		2 (14.3)	10 (6.8)	0.278[a]
Positive urine culture (*n* = 88)		3 (21.4)	4 (2.7)	0.030[a]
Positive cerebrospinal fluid culture (*n* = 23)		0 (0.0)	1.0 (0.7)	1.000[a]
Positive RSV test (*n* = 159)		6 (42.9)	46 (31.3)	0.356[a]
Antibiotic use (*n* = 157)		11 (78.6)	115 (78.2)	1.000[a]
Complications		2 (14.3)	28 (19)	1.000[a]
Admission to the intensive care unit		1 (7.1)	10 (6.8)	1.000[a]
Mortality		1 (7.1)	8 (5.4)	0.569[a]

[a]Fisher's exact test was used for categorical variables.
[b]Mann-Whitney *U* test was used for continuous variables.
Values are presented as *n* (%) or mean ± SD. Bold indicates a statistically significant difference with $P < 0.05$. SD: Standard deviation; INR: International normalized ratio; PTT: Partial thromboplastin time; RSV: Respiratory syncytial virus.

Table 4 Comparison of patients with acute bronchiolitis who were tested for liver enzymes at the time of presentation and at the time of follow up

Variable	Normal range	*n*	At the time of the presentation	At the time of follow-up	*P* value[a]
Total serum protein (g/L)	64-82	65	59.0 ± 11.0	62.0 ± 11.0	0.011
Serum albumin (g/L)	38-54	65	42.0 ± 9.0	42.5 ± 7.0	0.467
Serum globulin (g/L)	15-30	65	18.0 ± 5.0	20.0 ± 6.0	0.008
Total bilirubin (μmol/L)	5-21	64	37.0 ± 49.0	28.0 ± 91.0	0.376
Direct bilirubin (μmol/L)	0-5	16	18.0 ± 28.0	51.0 ± 157.0	0.322
Indirect bilirubin (μmol/L)	< 18	15	56.0 ± 46.0	35.0 ± 50.0	0.103
Alkaline phosphatase (U/L)	150-420	64	240.0 ± 143.0	242.0 ± 121.0	0.897
Alanine aminotransferase (U/L)	< 41	61	31.0 ± 43.0	42.0 ± 88.0	0.371
Gamma-glutamyl transferase (U/L)	< 18	50	61.0 ± 75.0	41.0 ± 91.0	0.234

[a]*P* value was calculated by the paired *t*-test.

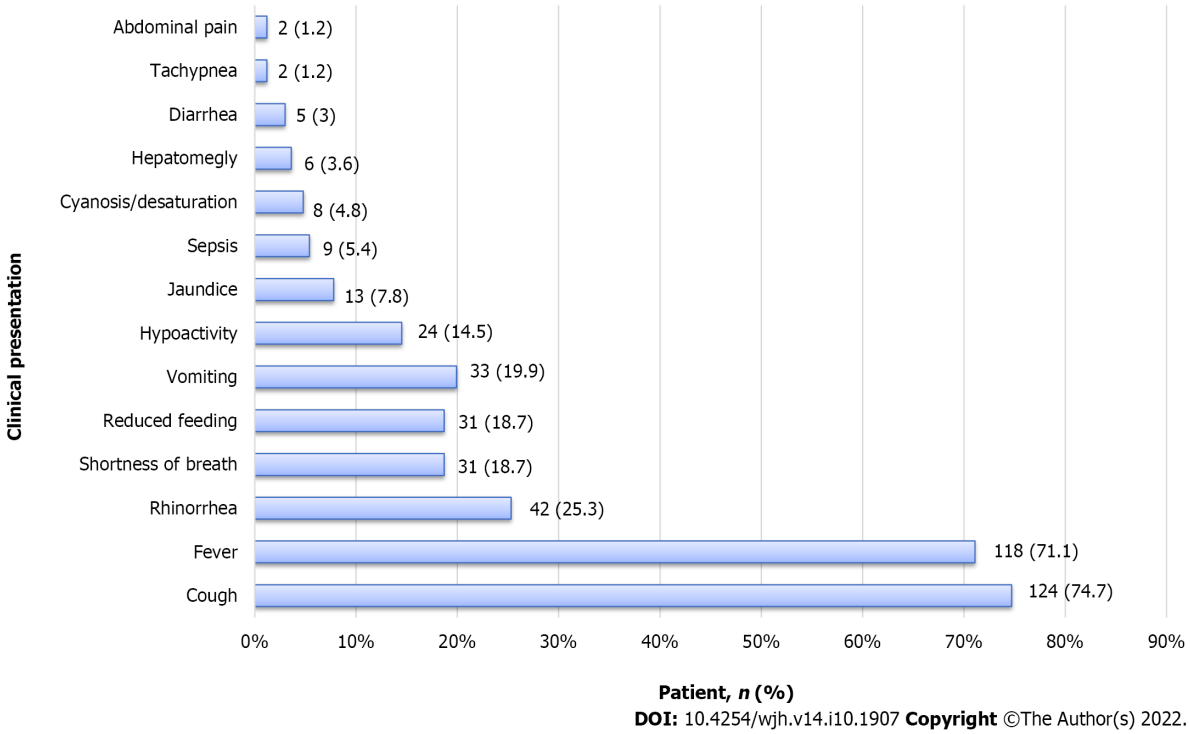

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Figure 2 Clinical characteristics of 166 infants admitted with the diagnosis of acute bronchiolitis.

megaly, are infrequent among patients hospitalized for acute bronchiolitis. Jaundice was found in 7.8% (*n* = 16 out of 166) of our patients, while hepatomegaly was observed in only 3.6% (*n* = 6/166). Jaundice was also reported by Nadal *et al*[8] in a seven-month-old infant who presented with acute bronchiolitis and cholestatic jaundice. RSV was detected in his liver biopsy. Al-Maskari *et al*[3] and Kirin *et al*[9] reported two infants with acute bronchiolitis and hepatomegaly; one was 11 mo, and one was 13 mo. However, these patients were pooled from case reports with weak evidence. With a rigorous literature review, we found that the current study was the first to investigate the prevalence of hepatomegaly and jaundice in a large cohort of children with acute bronchiolitis.

In the present study, ALT levels were high in 8.7% of our patients. This percentage is considered low compared to several previous studies. The Oh *et al*[10] study showed that 16% of the RSV-positive children had high ALT levels. Thorburn *et al*[5] showed that 19% of ventilated children with acute bronchiolitis had high transaminase levels. In 2002, Eisenhut *et al*[11] studied 55 ventilated patients with RSV bronchiolitis. They found that 46% had high transaminase levels (ALT and/or AST). Two years later, in 2004, Eisenhut *et al*[12] published a follow-up study and reported a higher percentage of

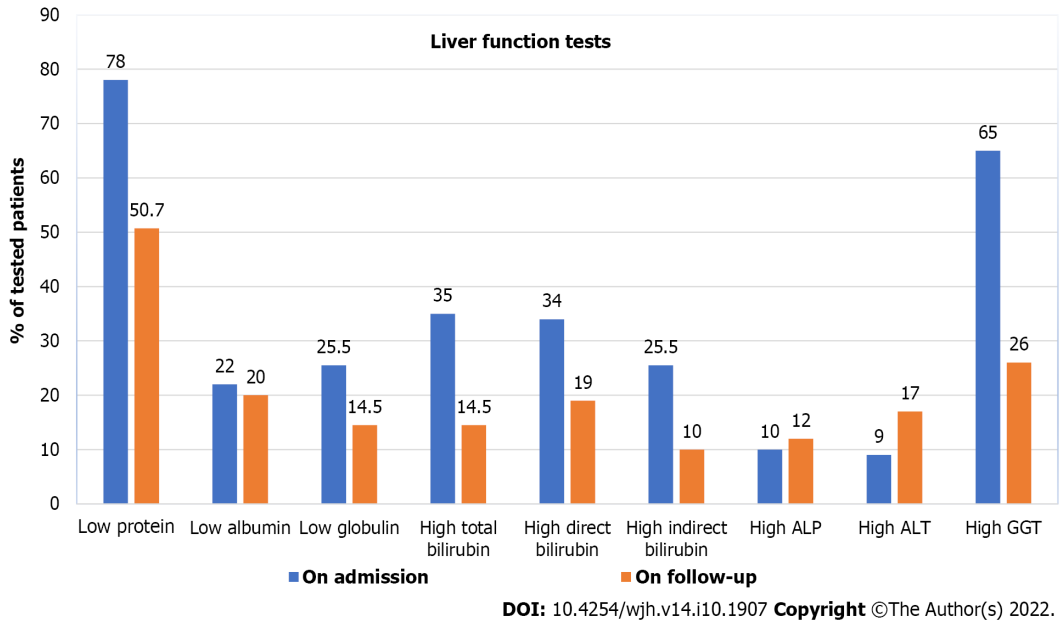

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Figure 3 Comparison of liver function tests in infants admitted with acute bronchiolitis at admission and during follow-up. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase.

elevated transaminase levels, reaching up to 49%. Giordano *et al*[1] also reported three male children hospitalized for mild to moderate RSV bronchiolitis. All their patients' laboratory tests showed elevated levels of transaminases (ALT/AST). The differences in the percentage of patients with elevated ALT levels between the current study and other studies could be attributed to differences in the patients included, as most other studies focused on sick, ventilated patients admitted to the PICU. Consequently, high transaminase levels could reflect the disease severity[5].

Virus-induced, ischemic, or hypoxic hepatitis are the possible causes of increased transaminase levels in patients with RSV bronchiolitis[5]. Similar to the other Paramyxoviridae, RSV can attach to specific receptors on the non-epithelial cell surfaces to infect them. RSV can be detected and isolated from the liver, human myocardial tissue, and cerebrospinal fluid[11]. Direct liver invasion by RSV in an immunocompetent infant has been documented by the successful isolation and culture of RSV from liver tissue obtained by biopsy[8]. The higher the transaminase levels are, the higher the viral load, causing spillover of the virus, which spreads from the lungs into the systemic circulation with subsequent extrapulmonary manifestations[12]. RSV infection was the causative agent in 28% of our patients with acute bronchiolitis. However, we did not find significant differences in the ALT levels between RSV-positive patients and RSV-negative. On the other hand, Do *et al*[13] found a high prevalence of elevated AST in isolated RSV infections. Moreover, Oh *et al* reported that children with RSV infection had the highest prevalence of elevated ALT levels (16%) compared to other viruses[10].

In the current study, patients with high ALT levels had extended hospital stays ($P = 0.025$) compared to those with average ALT levels. Similarly, Oh *et al*[10] 's study showed that children with the highest elevated ALT had more extended hospital stays than those with normal ALT levels. However, Thorburn *et al*[5] reported that ventilation duration and the length of PICU admission were significantly longer ($P < 0.05$ for both) in patients with elevated AST but not with elevated ALT. Eisenhut *et al*[11] also found a significantly longer duration of ventilation in patients with elevated transaminases ($P = 0.02$).

This study observed an abnormal coagulation profile, such as high PT, high INR, high APTT, low fibrinogen, and prolonged thrombin time in 32.6%, 28.3%, 6.5%, 47.1%, and 27.3%, respectively. Eisenhut *et al*[11], Bakalli[14], and Lee *et al*[15] noted a deranged coagulation profile affecting PT, INR, APTT, and fibrinogen. In addition, Al-Maskari *et al*[3] also noted similar findings, yet this coagulopathy recovered within one week with supportive management.

The current study found that disturbed glucose homeostasis was infrequent, as only 3.0% of our patients developed hyperglycemia, and 1.2% had hypoglycemia. Fares *et al*[16] studied 49 patients admitted with acute bronchiolitis and found that hyperglycemia was not frequent. They related this finding to the non-standardized timing of blood glucose analysis. On the other hand, Branco *et al*[17] studied 50 children. They showed that hyperglycemia was frequent in children with bronchiolitis who required mechanical ventilation, as 98% of the patients had peak glucose levels higher than or equal to 6.1 mmol/L and 72% had peak glucose higher than or equal to 8.3 mmol/L.

In this study, all the patients were managed with steam and bronchodilator inhalation. Unfortunately, we found that bronchodilators are still used in cases of acute bronchiolitis in our hospital despite most of the studies and guidelines recommendations, including that of the American Academy of

Pediatrics (AAP), showing that their use was ineffective in reducing hospitalization rate, oxygen requirement, mechanical ventilation, the duration of illness, or hospital stay[16]. This behavior can be explained by the limited therapeutic options that can be delivered to children with acute bronchiolitis, which puts the physician under pressure to give inhaled bronchodilators even if they are aware of the recommendations[18].

In the present study, confirmed bacterial co-infection was found in 14.6% of the patients. Bacterial co-infections, especially urinary tract infections, were associated with elevated ALT levels in the current study (*P* 0.030 =). Thorburn *et al*[5] also showed that 32% of their patients had bacterial co-infection with elevated transaminase levels (AST and/or ALT). However, Fares *et al*[16] found no difference between patients with isolated RSV infection and those with bacterial co-infection with elevated liver enzymes. Despite the low rate of confirmed bacterial co-infections (14.6%) in the current study, there was a high rate of antibiotic use in children with acute bronchiolitis (77%). However, this rate was comparable to the previously reported rates in infants with severe bronchiolitis (79.9%)[19]. Unnecessary antibiotic use in infants with acute bronchiolitis is common due to challenging differentiation between infants with isolated viral infections and those with invasive bacterial co-infections[19]. The antibiotic prescription was common in infants with high CRP levels, even though these high levels could not predict the presence of alveolar condensation on chest X-rays[20]. Some radiological findings of acute bronchiolitis and elevated CRP levels might deceive physicians. These findings might push physicians to use antibiotics unnecessarily in a viral-induced disease. However, all the patients in this study were tested for liver function at presentation, even before any medications were given to them. Some antibiotics and antipyretics can cause impaired liver function. Accordingly, the decision to use antibiotics in infants with acute bronchiolitis should be based on more meticulous evidence, such as positive tracheal aspirate, blood, urine, or cerebrospinal fluid cultures[21]. In this study, abdominal ultrasound was not performed as a routine investigation for patients with acute bronchiolitis. It was only performed in 12.7% of patients. However, 43% of them showed positive findings. Nonetheless, Giordano *et al*[1] described three cases of acute bronchiolitis. All had normal abdominal ultrasound findings.

In the current study, 6.7% of patients required PICU admission compared to 5.1% in the study by Papoff *et al*[7]. Thorburn *et al*[5] focused on patients admitted to the PICU with acute bronchiolitis to establish a relationship between transaminase levels and disease severity. They discovered that patients with elevated transaminase levels require more ventilation and spend more time in the PICU. Eisenhut *et al*[11] also confirmed the same observation.

Although no patient in this study deteriorated into fulminant hepatic failure, Al-Maskari *et al*[3] reported that an 11-month-old female infant with RSV-induced acute bronchiolitis developed acute fulminant hepatic failure and hepatic encephalopathy. Bakalli also described a one-month-old boy with RSV-induced acute bronchiolitis who was admitted to the PICU with liver failure. He related the presence of liver dysfunction to the effects of the initial tissue hypoperfusion and the consequences of shock[14].

The limitations of the study

As this was a retrospective study, some demographic and laboratory data of some patients were missing. In this research, we looked at the presence or absence of fever as a clinical presentation, but we did not specify the degree of fever as this was not the aim of the study. In addition, the hepatitis profile was available for some but not all the patients included in the study, which could impact the study's results, as hepatic involvement by other viruses could not be entirely excluded. Antibiotic use or the presence of bacterial co-infections could also be a factor in increased liver enzymes. Nevertheless, most of the patients in this study received antibiotics after LFTs. Another confounder in patients with high ALT on follow-up is the 10-mo duration, which is long enough for complete recovery from a viral illness such as acute bronchiolitis. Looking for alternative causes for this ALT elevation is essential as a new illness with another viral infection, which is common in children and unrelated to the original infection, might be the cause.

Moreover, the number of published studies that have attempted to find an association between acute bronchiolitis and hepatic function involvement is minimal, making comparing this study's findings with those of other studies challenging. Another significant limitation is that this is a single center-based study, so we cannot generalize our data. Despite these limitations, we think this study's findings are significant, being the first to investigate this issue in the Kingdom of Bahrain.

CONCLUSION

This study showed a low prevalence of liver function impairment in patients with acute bronchiolitis. Even those with hepatic impairment had a benign course, as most of them improved liver function with time. However, there was a rising trend in ALT during follow-up. Extended hospital stays and the presence of urinary tract infections were linked to elevated hepatic enzyme levels. Therefore, further studies are needed to assess the relationship between disease severity and liver function involvement.

ARTICLE HIGHLIGHTS

Research background

Acute bronchiolitis caused by Respiratory syncytial virus (RSV) is the most common type of lower respiratory tract infection of viral etiology. It is occasionally associated with hepatocellular involvement, as indicated by the increase in liver enzymes, including aspartate aminotransferase and alanine transaminase.

Research motivation

Due to the limited data on liver involvement in acute bronchiolitis, we were motivated to study the prevalence of liver involvement in RSV-induced acute bronchiolitis and the associated factors.

Research objectives

To assess the frequency of impaired liver functions in infants with acute bronchiolitis and to detect the predicted clinical, radiological, and laboratory variables.

Research methods

We retrospectively reviewed demographic data, clinical presentation, laboratory results, radiological findings, and outcomes of infants with acute bronchiolitis admitted to the pediatric department, Salmaniya Medical Complex, Kingdom of Bahrain, collected from medical records in 2019-2020. Infants with high liver enzymes were compared to those with normal levels at the time of presentation and at follow-up.

Research results

One hundred sixty-six (57.8%) out of 287 patients with acute bronchiolitis fulfilled the inclusion criteria. Ninety-three (56%) patients were males. The median age at presentation was 3.4 (interquartile range 1.1 to 12.4) mo. Fifty-four (28%) patients tested positive for RSV, which was confirmed by PCR in 15 of them (28%). High ALT levels were found in 14 (8.7%) patients and ALT was normal in 147 (91.3%). Coagulation profiles were measured in 46 (27.7%) of 166 patients. High PT was present in 15 (32.6%), high INR was present in 13 (28.3%), and high APTT was present in three (6.5%). Thrombin time was elevated in nine (27.3%) of 33 patients. Five (21.7%) of 23 patients with available radiological data had hepatomegaly; one of them had findings suggestive of fatty infiltration. High ALT was significantly associated with lengthy hospital stay ($P < 0.05$) and a positive urine culture ($P < 0.05$). Seventy (42.2%) patients had documented follow-up with liver function tests over a median follow-up period of 10.2 (IQR, 2.4 -23.3) mo. Total serum protein and serum globulin levels were normalized at the follow-up time, with a significant P value of < 0.05.

Research conclusions

This study showed a low prevalence of liver function involvement in patients with acute bronchiolitis with a benign course. However, there was a rising trend in ALT during follow-up. Prolonged hospital stay and positive urine cultures were associated with elevated liver enzymes.

Research perspectives

A proper evaluation of liver function during acute bronchiolitis is needed. Both diagnostic and therapeutic approaches are required to alleviate hepatic involvement in children with acute bronchiolitis.

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FOOTNOTES

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