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REVIEW

Neoadjuvant treatment strategies for intrahepatic cholangiocarcinoma

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Abstract

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver malignancy and is increasing in incidence. Long-term outcomes are optimized when patients undergo margin-negative resection followed by adjuvant chemotherapy. Unfortunately, a significant proportion of patients present with locally advanced, unresectable disease. Furthermore, recurrence rates are high even among patients who undergo surgical resection. The delivery of systemic and/or liver-directed therapies prior to surgery may increase the proportion of patients who are eligible for surgery and reduce recurrence rates by prioritizing early systemic therapy for this aggressive cancer. Nevertheless, the available evidence for neoadjuvant therapy in ICC is currently limited yet recent advances in liver directed therapies, chemotherapy regimens, and targeted therapies have generated increasing interest its role. In this article, we review the rationale for, current evidence for, and ongoing research efforts in the use of neoadjuvant therapy for ICC.

Key Words: Biliary tract cancer; Preoperative therapy; Conversion therapy; Down-staging; Hepatectomy; Liver resection

Core Tip: Liver resection is the primary component of curative-intent treatment for patients with localized intrahepatic cholangiocarcinoma (ICC). However, a majority of patients present with locally advanced disease and even those who undergo resection are at high risk of recurrence. Neoadjuvant therapy may successfully downstage a

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subset of patients to resectable disease and improve the long-term outcomes of patients treated with multimodality therapy. As such, the benefits of neoadjuvant treatment strategies aimed at down-staging the tumor and increasing resection rates are of great interest. While high-level evidence regarding the efficacy of neoadjuvant therapy in ICC is lacking, emerging evidence from case control series, as well as recent advances in systemic therapies, liver-directed treatments, and targeted therapies based on an improved understanding of cholangiocarcinogenesis have led to increasing interest in its use.

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INTRODUCTION

Cholangiocarcinomas (CCA) are a heterogeneous type of biliary tract cancer (BTC) arising from the epithelial cells of the intrahepatic and extrahepatic biliary tracts[1]. CCAs are classified as distal bile duct, perihilar, or intrahepatic based on their anatomic location[2]. Intrahepatic cholangiocarcinoma (ICC) arises distal to the secondary biliary radicals and comprise approximately 20% of all CCAs. ICCs have distinct molecular, anatomic, clinical, and prognostic characteristics compared with other BTCs. Although relatively rare, the incidence of ICC has been increasing over the past decade and ICC is currently the second most common type of primary liver cancer. The optimal management of ICC includes surgical resection; unfortunately, a majority of patients will present with metastatic or locally advanced disease and are therefore not candidates for surgery. Even those patients with localized disease who undergo margin-negative resection are at high risk for recurrence, highlighting the need for effective systemic therapies. While the optimal systemic therapy given following resection (*i.e.*, adjuvant therapy) continues to evolve, there is growing interest in the use of neoadjuvant therapy (NT) for ICC. Such strategies may effectively downstage patients with locally advanced disease in order to achieve surgical resection while prioritizing the early and guaranteed delivery of systemic therapy in order to improve long-term oncologic outcomes for this aggressive cancer. Recent advances in the molecular understanding of ICC, as well as the development of effective systemic and liver-directed therapies, have also increased interest in the use of NT. In this study, we review the rationale for, evidence of, and ongoing research efforts in the use of NT for ICC.

MANAGEMENT OF ICC

Surgical resection

Surgical resection remains the only treatment option with curative intent in the management of ICC[3,4]. Unfortunately, only about 20%-30% of patients present with resectable disease. Given that most patients present with advanced disease, patient selection is critical to ensure that patients will benefit from surgery. All patients require comprehensive evaluation along three domains: Anatomic, biologic, and physical condition. Patients must have appropriate performance status to undergo major liver surgery without prohibitive medical comorbidities. Complete staging with cross-sectional imaging and tumor markers should be performed to ensure the absence of metastatic disease. In general, distant, contralateral hepatic, peritoneal, and lymph node metastases beyond the porta hepatis are contraindications to resection[5]. As such, some guidelines recommend a diagnostic laparoscopy prior to resection[5,6]. Dedicated, liver-protocol, contrast-enhanced imaging is needed to assess resectability. In general, a technically safe hepatic resection requires a future liver remnant (FLR) defined as at least two contiguous liver segments with intact arterial and portal venous inflow, intact hepatic vein outflow and intact biliary drainage. In general, an FLR of at least 20% is necessary for patients with normal liver function. For patients undergoing

neoadjuvant chemotherapy or those with significant steatosis, an FLR of at least 30% is required. And for patients with underlying liver cirrhosis, an FLR of at least 40% is required[7]. FLR is traditionally calculated by volumetric analysis using CT, MRI, or Scintigraphy. Several strategies can be employed to improve the FLR, including ligation of the feeding portal vessels or embolization[8,9].

Like most solid organ tumors, obtaining a margin negative (R0) resection is an important oncologic goal and one of the few metrics potentially under control of the surgeon. In a small series of 50 patients with locally advanced tumors by Lang *et al*[10], the median survival after R0 resection was 46 mo *vs* 5 mo for R1 resection[10]. In a larger series of 224 patients, Yeh *et al*[11] reported a median survival of 26.1 mo and 1-year, 3-year, and 5-year OS of 78.5%, 43.3%, and 28.6%, respectively in patients who underwent R0 resection *vs* a median survival of 11.4, and 1-year, 3-year, and 5-year OS of 47.5%, 6.8%, and 4.5% for patients who had an R1 resection. The results are even worse for R2 resection in which the median survival was 5.8 mo and 1-year, 3-year, and 5-year OS was 24.0 %, 6.0%, and 0%, respectively[11]. These results have been confirmed in larger studies and meta-analyses[12,13]. While an R0 resection margin should clearly be the goal of surgery, the optimal margin width remains controversial with some data suggesting a wider margin (≥ 10 mm) is associated with improved outcomes[14-18]. Portal lymphadenectomy is also recommended as part of the surgical approach to ICC. Lymphadenectomy is essential for accurate staging, determining prognosis, and guiding the use of adjuvant therapies[19,20]. Removal of at least six lymph nodes is recommended by the National Comprehensive Cancer Network[21], although this is commonly not achieved[20]. Limited data support the use of minimally invasive approaches at experienced centers and the use of vascular resection, when indicated, in order to achieve negative margins.

Adjuvant therapy

Even among patients who undergo a complete macro- and microscopic margin negative resection, patients can still experience a high incidence of recurrence. Indeed, ICC is an aggressive malignancy, and overall survival remains poor[22]. For this reason, there has been a longstanding interest in the development of effective adjuvant therapies to reduce cancer recurrence. Fortunately, several recent prospective trials have provided new data on this controversial issue. The French PRODIGE 12–ACCORD 18 Trial was a phase III randomized trial that randomized patients to adjuvant chemotherapy (Gemcitabine/Oxaliplatin, GEMOX) *vs* observation following R0 or R1 resection of BTCs (43% ICC). The investigators reported no difference in overall survival or relapse-free survival[23]. The BILCAP trial randomized patients with resected ICC to capecitabine or observation following an R0/R1 resection. In this trial, 19% of the patients had ICC, 38% of whom had R1 resection, and 47% had nodal metastases. Patients who received adjuvant capecitabine experienced improved median overall survival (51.1 mo *vs* 36.4 mo)[24]. The ACTICCA-1 trial[25] and JCOG1202[26] are currently ongoing. Adjuvant radiotherapy and transarterial chemoembolization (TACE) have also been investigated, but the data are still lacking[27-31]. Based on these data, current guidelines recommend the use of adjuvant capecitabine following resection of any BTC, including ICC.

Metastatic disease

Unfortunately, most patients with ICC present with metastatic disease as many will not develop symptoms until an advanced stage. As surgical resection is not appropriate in the setting of metastatic disease, treatment goals focus on improving local control, treating symptoms, and extending survival. To that end, various systematic chemotherapy regimens and liver-directed therapies have been tried with varying success[32]. The ABC-02 trial was a prospective randomized trial of 410 patients with locally advanced or metastatic BTCs (intrahepatic or extrahepatic cholangiocarcinoma, gallbladder cancer, or ampullary carcinoma) who were randomized to cisplatin plus gemcitabine (Gem-Cis) or gemcitabine alone. Patients treated with Gem-Cis had significantly better overall survival and progression-free survival, establishing this doublet regimen as the standard chemotherapy for advanced BTC[33]. Recent trials have explored triplet gemcitabine-cisplatin-nab-paclitaxel, although further data are needed[34]. Several mutations in IL-6, ErbB2, K-ras, BRAF, and COX-2, p53, P16, cyclin D1, and DNA repair enzymes have all been linked to ICC and provide a basis for targeted therapies. Most of the morbidity and mortality among patients with metastatic ICC is due to liver disease and liver failure. Therefore, the selective use of liver-directed therapies, even in the presence of metastatic disease, may improve local disease control, health related quality of life, and potentially overall survival[35].

RATIONALE FOR NEOADJUVANT THERAPY

While the use of NT has been increasing in other common cancers, its use in ICC remains relatively rare[36-39]. Thus, a sound rationale for the use of NT in BTCs is necessary as empirical data accrue (Table 1). As previously detailed, only a minority of patients presenting with ICC are eligible for resection; fewer patients successfully undergo an R0 resection. Yet the ability to achieve margin-negative resection is a primary determinant of long term survival outcomes[11,12,40,41]. Therefore, a major impetus for pursuing NT is the potential to downstage locally advanced cancers and convert to resectable disease (Figure 1). Thus, NT given for this purpose is commonly referred to as downstaging or conversion therapy[42].

A second major motivation for the use of NT is to ensure its early and near-universal use. While current guidelines recommend the use of adjuvant therapy for essentially all patients with resected ICC, a significant proportion of patients are unable to initiate and/or complete adjuvant therapy due to postoperative complications or poor performance status following major liver surgery. For example, in a retrospective series of 72 patients who underwent resection for ICC, only 35% of the patients received chemotherapy[43].

Not only does the delivery of systemic therapies prior to surgery ensure receipt is not prevented by postoperative complications, it also prioritizes the use of systemic therapies for an aggressive cancer with a strong tendency for systemic recurrence. For example, a recent multi-institutional review of patients with resected ICC reported that approximately 22% of patients experienced very early recurrence (defined as within six months of surgery), which ultimately led to poor survival outcomes[43]. It is likely that micrometastatic disease was present in these patients at the time of surgery and early systemic therapy may have been beneficial. Similarly, the use of NT also facilitates the appropriate selection of patients for a major surgery by ensuring that the rapid progression of metastatic disease does not occur while on systemic therapy. Recent prediction models may be useful in determining which patients with ICC may be best suited for NT[44,45]. Finally, given the genomic, phenotypic, and clinical heterogeneity of patients with ICC, monitoring the response to NT radiographically, biochemically, and histopathologically provides important prognostic and therapeutic information. This *in vivo* test will only become more important with the development of more effective targeted therapies in the era of personalized medicine.

EVIDENCE FOR NEOADJUVANT THERAPY IN ICC

Systemic chemotherapy

There have been no prospective randomized trials evaluating the benefit of neoadjuvant chemotherapy among patients with BTC, including ICC. However, there are some case reports, single-institution studies, and some retrospective data to suggest its benefit (Table 2). Chemotherapy regimens often use a combination of Gem-Cis, based on data extracted from the ABC-02 trial[33]. In some early studies of patients with locally advanced and unresectable tumors receiving Gem-Cis, 36.4% (8/22) in one study[46] and 25.6% (10/39) in another study[47] were able to be appropriately downsized and undergo resection. In a large single-center study of 186 patients with locally advanced ICC, 39/74 (53%) of patients who received NT were able to undergo resection[48]. In a large meta-analysis of 18 studies and 1,880 patients, including eight studies with chemotherapy. Patients who underwent resection following downstaging had significantly longer median survival compared with patients who did not (29 mo *vs* 12 mo, $P < 0.001$)[49]. In another systematic review of 132 patients, 27 patients (20.5%) were downstaged to surgical resection candidates[42]. In sum, these data support the use of systemic therapy among patients with locally advanced ICC as an attempt to downstage tumors to become resectable since achieving resection following conversion therapy is associated with improved long-term survival.

While the use of NT in locally advanced disease is indicated, the routine use of systemic therapy prior to surgery for patients with resectable disease is not well established. Yadav *et al*[50] evaluated the National Cancer Database and performed a propensity-matched comparison of patients who received NT prior to surgical resection to individuals receiving surgery and adjuvant therapy. Acknowledging the limitations in this type of retrospective review, patients who received NT before surgery experienced improved OS (median OS: 40.3 mo *vs* 32.8 mo; $P = 0.01$)[51]. However, in a multi-institutional study of 62 patients who received NT (44% chemotherapy, 29% transarterial therapy) prior to curative-intent resection for ICC, a

Table 1 Rationale for the use of neoadjuvant therapy in intrahepatic cholangiocarcinoma

	Rationale for the use of neoadjuvant therapy in ICC
1	Downstaging of locally advanced tumors
2	Improve margin-negative resection rate
3	Increase receipt of systemic therapy given challenges in delivering postoperative chemotherapy
4	Prioritize the early systemic treatment of potential micrometastatic disease
5	Enhance patient selection for major surgery
6	Facilitate an *in vivo* test of chemotherapy's effectiveness

ICC: Intrahepatic cholangiocarcinoma.

Table 2 Select studies on neoadjuvant systemic chemotherapy for intrahepatic cholangiocarcinoma

Ref.	Study type	Intervention	Sample size	Conversion to resection	Tumor response
Kato *et al*[46], 2013	Retrospective	Gemcitabine	22	8 (37%)	3 PR, 11 SD, 8 PD
Kato *et al*[47], 2015	Retrospective	Gemcitabine plus cisplatin	39	10 (26%)	9PR, 21 SD, 9 PD
Rayar *et al*[71], 2017	Retrospective	Gemcitabine and/or platinums; Y-90 TARE	45	10 (22%)	NR
Konstantinidis *et al*[79], 2017	Retrospective	Bevacizumab + FUDR HAI	104	8 (8%)	NR
Omichi *et al*[110], 2017	Retrospective	Gemcitabine based therapy	43	43 (100%)	NR
Le Roy *et al*[48], 2018	Retrospective	Gemcitabine plus oxaliplatin	74	39 (53%)	18 PR, 33 SD, 23 PD
Sumiyoshi *et al*[112], 2018	Retrospective	S-1 + IMRT	7	5 (71%)	4 PR, 1 SD, 2 PD

NR: Not reported; PR: Partial response; SD: Stable disease; PD: Disease progression; ICC: Intrahepatic cholangiocarcinoma; TARE: Transarterial radioembolization; IMRT: Intensity-modulated radiation therapy.

comparison of propensity-score matched patients who underwent upfront surgery did not find any significant difference in survival[51]. Clearly, more data are needed, preferably through well-designed prospective clinical trials, in order to define the indications for NT among patients with resectable ICC.

TACE

While TACE is routinely used for patients with HCC[52] and neuroendocrine liver metastases[53], its appropriate use in ICC remains undefined. ICC is a hypovascular tumor and thus less responsive to TACE[54,55]. However, since most of the morbidity and mortality associated with ICC results from overwhelming liver disease and liver failure, locoregional therapies such as TACE can be useful in controlling locally advanced disease. As such, in patients with ICC, TACE is traditionally used for those that are not eligible for surgery, as a palliative option, though it has been explored with downstaging intent (Table 3)[56,57]. One of the early successes in the use of TACE as a conversion therapy was reported by Burger *et al*[58] Seventeen patients with unresectable ICC under conventional TACE using cisplatin, doxorubicin, and mitomycin-C. Six of the patients had previously faced systemic therapy. TACE resulted in 75% tumor necrosis in 8 patients and tumor downstaging in 3 patients. Two of these patients were able to undergo surgical resection[58]. Herber *et al*[59] investigated the role of TACE using mitomycin C in 15 patients with unresectable disease and noted stable disease in 9 patients, partial response in 1, and tumor progression in 4 patients; no conversions to resection were reported[59]. Gusani *et al*[60] compared patients who received combination systemic gemcitabine and TACE to gemcitabine alone, and reported significant improvement in survival among patients who received TACE (13.8 mo *vs* 6.3 mo, respectively; $P = 0.0005$)[60]. A large retrospective review of 198 patients with advanced ICC undergoing transarterial therapies reported a complete or partial response in 25.5% of patients and stable disease in another 61.5%,

Table 3 Select studies on neoadjuvant transarterial chemoembolization for intrahepatic cholangiocarcinoma

Ref.	Study type	Intervention	Sample size	Conversion to resection	Tumor response
Burger *et al*[58], 2005	Retrospective	Cisplatin, doxorubicin, and mitomycin-C	17	2 (12%)	NR
Herber *et al*[59], 2007	Retrospective	Mitomycin-C	15	BR	1 PR, 9 SD, 4PD
Gusani *et al*[60], 2008	Retrospective	Gemcitabine-based	42	NR	20 SD, 15 PD
Hyder *et al*[61], 2013	Retrospective – multi-institutional	cTACE (64.7%), DEB-TACE (5.6%), bland embolization (6.6%), or Y-90 (23.2%)	198	NR	56 PR, 77 SD, 29 PD
Vogl *et al*[62], 2012	Retrospective	Mit-C (20.9%), Gem. (7%), Mit-C +Gem (47%), Gem+ Mit-C and Cisplatin (25.1%)	115	NR	10 PR, 66 SD, 39 PD
Alibertti *et al*[64], 2017	Retrospective	DEB-TACE and PEG-TACE	127	4 (4%)	19 PR, 101 SD, 7 PD
Schiffman *et al*[65], 2011	Retrospective	EBIRI or DEB-DOX therapy	24	3 (13%)	1CR, 1PR, 13 SD, 3 PD
Kuhlmann *et al*[66], 2012	Prospective	Irinotecan (iDEB-TACE), mitomycin-C (cTACE)	41	1 (4%)	2 PR, 12 SD, 19 PD
Poggi *et al*[67], 2009	Retrospective	DEB-TACE	9	3 (33%)	4 PR, 5 SD

NR: Not reported; PR: Partial response; SD: Stable disease; PD: Disease progression; ICC: Intrahepatic cholangiocarcinoma; DEB: Drug-eluting bead

suggesting a role for the use of TACE as neoadjuvant treatment (of note, 23.2% of the patients received yttrium-90 radioembolization)[61]. In another large series by Vogl *et al*[62], there was no difference in survival between different TACE regimens[62].

Drug-eluting bead chemoembolization (DEB-TACE) allows for the delivery of highly concentrated doses of chemotherapy in addition to conventional chemoembolization. The beads limit the systemic availability and systemic toxicities of chemotherapy. In a retrospective study from Italy, 127 patients with advanced ICC underwent DEB-TACE or polyethylene glycol drug-eluting microspheres (PEG-TACE). Of the 109 patients treated with DEB-TACE, 7% had a partial response, 88% had stable disease, and 5% had progressive disease. Four patients (3.8%) in the DEB-TACE group were downsized and successfully underwent resection[63,64]. Multiple studies have evaluated DEB-TACE for unresectable ICC[65-67]; in general, few conversions to resectability have been reported.

Taken together, these findings suggest a potential role for TACE in the neoadjuvant treatment of patients with locally advanced ICC. However, while radiographic responses are observed, the majority of patients demonstrated stable disease, and conversions to resectable disease are the exception. Future studies may consider combination regimens that aim to enhance the response rate while treating/preventing the risk of systemic disease.

Transarterial radioembolization/selective internal radiation therapy

Transarterial radioembolization with yttrium-90 (Y-90) is an alternative transarterial therapy that is used in the management of locally advanced ICC and may downstage patients to resectability (Table 4). In an early open-label trial of Y-90 for ICC, 24 patients with advanced and unresectable ICC were treated with Y-90. Of the 22 patients with follow-up imaging, 6 patients demonstrated a partial response, 15 had stable disease, and 1 patient had progressive disease; 1 (4%) patient was downstaged and underwent resection[68]. In 2013, Mouli *et al*[69] reported on a series of 60 patients with ICC treated with Y-90 transarterial radioembolization (TARE). By EASL criteria, 33 patients had partial or complete response disease, and 12 patients showed stable disease. In this cohort, 5 patients successfully underwent an R0 resection[69]. In 2015, Al-Adra *et al*[70] performed a pooled analysis of several studies reporting on the use of Y-90 for patients with unresectable ICC. In this pooled data of 73 patients, the partial response rate following Y-90 treatment was 28%, and 54% had stable disease at 3 mo; 7 (10%) patients underwent surgical resection post TARE[70].

Rayar *et al*[71] combined Y-90 with systemic therapy as an option to downstage

Table 4 Select studies on neoadjuvant transarterial radioembolization/selective internal radiation therapy for intrahepatic cholangiocarcinoma

Ref.	Study type	Intervention	Sample size	Conversion to resection	Tumor response
Ibrahim *et al*[68], 2008	Prospective	Y-90	24	1 (4%)	6PR, 15 SD, 1PD
Mouli *et al*[69], 2013	Retrospective	Y-90	46	5 (11%)	11 PR, 33 SD, 1 PD
Rayar *et al*[71], 2015	Retrospective	Gemcitabine followed by Y-90	10	8 (80%)	NR
Saxena *et al*[72], 2010	Retrospective	Y-90	25	1 (4%)	6 PR, 11 SD, 5 PD
Rafi *et al*[73], 2012	Prospective	Y-90	19	NR	2 PR, 13 SD, 4 PD
Hoffman *et al*[74], 2012	Prospective	Y-90	33	NR	12 PR, 17 SD, 5 PD
Riby *et al*[75], 2020	Retrospective	Y-90	19	19 (100%)	NR
Edeline *et al*[76], 2019	Phase II Trial	GemCis + Y-90	26	9 (22%)	NR

NR: Not reported; PR: Partial response; SD: Stable disease; PD: Disease progression; ICC: Intrahepatic cholangiocarcinoma.

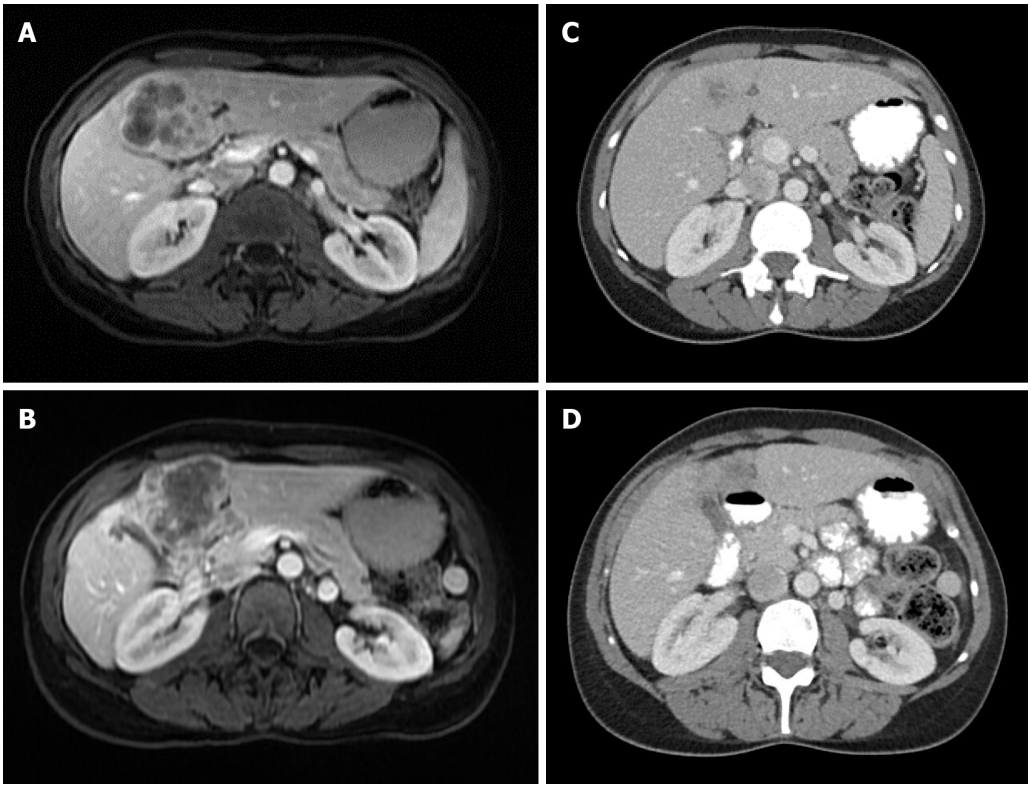

Figure 1 Locally advanced intrahepatic cholangiocarcinoma. A and B: 48F with large, multifocal intrahepatic cholangiocarcinoma who received 5 cycles of neoadjuvant gemcitabine/cisplatin; C and D: She experienced an excellent response and underwent extended left hepatectomy with pathology showing T2N0 moderately differentiated cholangiocarcinoma with negative margins.

unresectable ICC. Of the 45 patients treated with the combination regimen, ten were downstaged to potentially resectable, and 8 (17.8%) patients underwent resection[71]. Other studies have reported similarly low conversion rates with neoadjuvant Y-90 treatment[72-74]. In a large retrospective single-institution study of 169 patients, Riby *et al*[75] compared patients who underwent upfront resection to those who received downstaging chemotherapy with or without selective internal radiation therapy (SIRT) prior to surgical resection. Interestingly, patients with unresectable disease at presentation who became resectable after downstaging had similar median overall survival as patients with resectable disease who underwent upfront surgery (32.3 NT *vs* 45.9 primary surgery, $P = 0.54$). In addition, patients who received SIRT as part of their downstaging NT were more likely to undergo an R0 resection[75]. This approach has recently been validated *via* the MISPHEC trial, which was a prospective, multi-

institutional trial of 41 previously untreated patients with locally advanced ICC. The patients received concomitant cisplatin and gemcitabine chemotherapy, followed by TARE with Y-90 microspheres. The response rate was 39% by RECIST criteria and 93% by Choi criteria. 9 patients (22%) were downstaged to resectable candidates, and 8 (20%) of them subsequently underwent an R0 resection. Additionally, subgroup analysis showed that 30% of patients in the trial with disease involving only 1 hemi-liver disease could be downstaged[76].

In summary, these findings suggest that while TARE provides good locoregional control, however the low response rates, when used alone, limits its application as a downstaging treatment for neoadjuvant intent. Recent studies combining TARE with systemic therapy hold promise and should be the subject of future trials.

Hepatic artery infusion

Given the toxicities associated with systemic chemotherapy, hepatic artery infusion (HAI) pumps were initially developed for the management of colorectal liver metastases but, more recently, trialed in patients with ICC (Table 5). One of the earliest experiences with HAI therapy in the management of ICC was performed by the Memorial Sloan Kettering Cancer Center in a phase II trial. Twenty-six patients with unresectable ICC were treated with HAI FUDR therapy. Fourteen patients (53.8%) experienced a partial response, 11 (42.3%) had stable disease, and 1 patient (3.8%) had disease progression[77]. In a follow-up study, the authors added systemic bevacizumab to HAI therapy which resulted in worsened toxicity without improved outcomes[78]. In 2015, Konstantinidis *et al*[79] reported on large series of 167 patients with advanced/unresectable, 104 of whom had disease confined to the liver, and 63 had regional nodal disease. Patients had either received HAI or HAI plus systemic therapy. Although there was no significant difference in tumor response by RECIST criteria, patients who received HAI plus systemic chemotherapy had better overall survival (30.8 mo) compared with patients who received systemic therapy alone (18.4 mo)[79]. Eight patients from the cohort (4 from each group) underwent resection with curative intent. Despite the low rate of conversion, the results remain promising. In another smaller series reported by Massani *et al*[80], 11 patients with unresectable disease underwent HAI therapy with fluorouracil and oxaliplatin, 5 patients had a partial response of whom 3 underwent resection. 2 of these patients had more than 70% tumor necrosis on pathology[80].

In summary, HAI therapy may induce higher response rates for ICC, especially when combined with systemic chemotherapy. However, conversion rates remain low, and, unlike other transarterial therapies, HAI therapy requires surgical placement of an implanted pump, which carries morbidity and delays the use of systemic therapy. In addition, data supporting its use are largely retrospective and have not been studied among patients undergoing true neoadjuvant intent. Future multi-institutional trials are needed to validate this approach and compare it to other approaches.

FUTURE DIRECTIONS

Targeted therapies

The prognosis of patients with locally advanced, recurrent and/or metastatic ICC remains poor even with contemporary systemic therapy. As such, there is great interest in the development of novel targeted therapies[81]. Next-generation sequencing of patients with advanced and refractory tumors have led to an improved understanding of the genetic changes driving cholangiocarcinogenesis. For example, mutations in KRAS have been identified and are an independent predictor of worse survival after hepatectomy[82]. Mutations in BRAF[83], EGFR[84], PI3K[85], and TP53[86] have also been reported with varying percentages. In addition, there are novel antitumor therapies directed at the fibroblast growth factor receptor 2 fusion protein (FGFR)[87,88], isocitrate dehydrogenase-1 (IDH1), and IDH2[89], BAP1[90], BRAF V600[91], and Her2/neu mutations[82]. Immunotherapies, including anti-PDL1 and anti-CTLA-4 inhibitors have been trialed inBTCs as well including ICC. About 10% of ICC lack mismatch repair mechanisms and as such are good targets in immunotherapy[92,93]. In the Phase II KEYNOTE-158 Study, 41% of patients with cholangiocarcinoma had an objective response[94]. While most of these targeted therapies are currently being investigated in patients with advanced disease and early phase trials, these therapies hold great potential for use in the neoadjuvant and perioperative setting.

Table 5 Select studies on neoadjuvant hepatic artery infusion for intrahepatic cholangiocarcinoma

Ref.	Study type	Intervention	Sample size	Conversion to resection	Tumor response
Jarnagin *et al*[77], 2009	Phase II trial	HAI	26	1 (4%)	14 PR, 11 SD, 1PD
Kemeny *et al*[78], 2011	Phase II trail	HAI + bevacizumab	18	3 (17%)	7 PR, 11 SD
Konstantinidis *et al*[79], 2015	Retrospective	HAI + chemotherapy	93	8 (4%)	NR
Massani *et al*[80], 2015	Retrospective	HAI	11	3 (27%)	5 PR, 2 SD,
Tanaka *et al*[113], 2002	Retrospective	HAI	11	1 (9%)	7 PR, 2 SD, 2 PD
Shitara *et al*[114], 2008	Retrospective	HAI	20	NR	1CR, 9PR, 8 SD, 2PD
Ghiringhelli *et al*[115], 2013	Retrospective	HAI	12	2 (17%)	8 PR, 3 SD, 1 PD

NR: Not reported; PR: Partial response; SD: Stable disease; PD: Disease progression; ICC: Intrahepatic cholangiocarcinoma; TACE: Transarterial chemoembolization.

Liver transplantation

While liver transplantation (LT) is indicated in the treatment of HCC meeting Milan criteria[95], the role of LT in the management of ICC remains controversial and, in general, remains limited to specialized centers and for patients on clinical trials. A challenge in interpreting the current literature is that most of the early studies combined hilar cholangiocarcinoma and ICC, making the results difficult to interpret. But even in those studies, transplant outcomes for ICC were generally very poor[96,97]. Interestingly, the standardization of transplantation protocols that include strict inclusion criteria and NT regimens has led to increased transplant rates for hilar cholangiocarcinoma[98-100]. Indeed, NT regimens for hilar cholangiocarcinoma are incorporated into the preoperative protocol at most LT centers[101,102]. It is therefore assumed that the development of effective programs for LT for ICC will similarly require effective neoadjuvant therapies.

One of the earliest successful experiences was reported by Goss *et al*[103] In their review of 127 patients who underwent LT for primary sclerosing cholangitis (PSC), ten patients (8%) had incidental CCA on explant pathology. More importantly, they had equivalent survival to those without ICC (100%, 83%, and 83% at 1, 2, and 5 years, respectively)[103] A recent large multicenter trial by Sapisochin *et al*[104] found that patients with tumors less than 2cm did not have any added risk of recurrence. 13 patients (8 TACE, 3 RFA and 2 PEI) received some neoadjuvant therapy. Preoperative treatment had no association with outcomes[104]. A more recent larger experience with pre-transplant regimens for ICC was reported by Lunsford *et al*[105] Patients with non-metastatic locally advanced ICC were treated with a gemcitabine-based chemotherapy regimen. After six months of radiographic response or stability, patients were listed for transplantation. The median duration to transplantation was 26 mo. Overall survival was 100% (95%CI: 100-100) at 1 year, 83.3% (27.3-97.5) at 3 years, and 83.3% (27.3-97.5) at 5 years. Three patients developed recurrent disease at a median of 7.6 mo (IQR 5.8-8.6) after transplantation, with 50% (95%CI: 11.1-80.4) recurrence-free survival at 1, 3, and 5 years[105]. Rayar *et al*[71] reported on another case of an unresectable lesion that was downstaged with multimodal therapy, including Y-90 TARE, systemic chemotherapy, and external beam radiation. The patient was transplanted after several months of disease stability. Although several barriers exist, these recent studies highlight the potential for improved outcomes for patients with advanced ICC using LT. Future research will require the design of effective multidisciplinary programs for patients that incorporate NT protocols, not only to bridge patients while on the waitlist, but also to ensure appropriate oncologic selection for transplantation.

Biomarkers

The design and validation of effective NT protocols also require the identification of appropriate biomarkers to guide its use and gauge response to therapy. The discovery of somatic mutations in ICC has led to renewed interest in the use of these as potential prognostic and predictive biomarkers[106,107]. For example, KRAS mutations, one of the most frequently seen mutations in ICC, is also associated with worse survival after resection in some studies[83,108]. MUC-44 has been linked to poor outcomes in mass forming ICC subtype[109]. The neutrophil-to-lymphocyte (N:L) ratio has equally been associated with worse survival in ICC[110]. CEA and CA-19 have very wide-ranging

sensitivities and specificities in ICC[111]. Recent studies have highlighted the ability of machine learning to identify which patients with ICC may be best suited for NT[44,45]. Despite these recent advances, research on biomarkers for ICC is still lacking, and future trials are needed.

Ongoing trials

While interest in neoadjuvant approaches in other gastrointestinal cancers continues to drive new clinical trials, prospective trials of NT for ICC remain limited. An ongoing trial (NCT03579771) evaluating the benefit of neoadjuvant gemcitabine, cisplatin, and nab-paclitaxel is currently accruing. Another trial (NCT03867370) aims to investigate the benefit of neoadjuvant Toripalimab (a PD-1/PD-L1 immune checkpoint inhibitor) in patients with resectable HCC and ICC, although this study has not started accrual yet.

CONCLUSION

In conclusion, a sound rationale for the use of NT exists in ICC, particularly among patients with locally advanced disease. Given the importance of a margin-negative resection on overall prognosis, NT with a downstaging intent should be given in these patients. While numerous systemic and transarterial therapies have been reported in limited series, strong evidence for the superiority of one approach over another is lacking. Recent evidence has offered support for the use of combination strategies (*e.g.*, systemic therapy with TARE) in order to augment the response and increase the proportion of patients downstaged to resectability. Future comparative effectiveness studies are needed to evaluate the optimal neoadjuvant approach, and ongoing research into targeted therapies for ICC may offer new opportunities for personalized neoadjuvant treatment. Finally, while margin-positive resection rates and disease recurrence is common even among patients with resectable disease, the evidence of NT in this patient population is extremely limited. Thus, the role of routine NT in patients with resectable ICC should be limited to patients with high-risk disease and preferably as part of a clinical trial. In the meantime, oncologic surgery that includes a margin-negative resection with formal lymphadenectomy followed by adjuvant chemotherapy remains the recommended approach.

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REVIEW

Metabolic syndrome and liver disease in the era of bariatric surgery: What you need to know!

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Abstract

Metabolic syndrome (MS) is defined as the constellation of obesity, insulin resistance, high serum triglycerides, low high-density lipoprotein cholesterol, and high blood pressure. It increasingly affects more and more people and progressively evolves into a serious issue with widespread healthcare, cost, and quality of life associated consequences. MS is associated with increased morbidity and mortality due to cardiovascular or chronic liver disease. Conservative treatment, which includes diet, exercise, and antidiabetic agents, is the mainstay of treatment, but depends on patient compliance to medical treatment and adherence to lifestyle modification recommendations. Bariatric surgery has recently emerged as an appropriate alternative treatment with promising long-term results. Sleeve gastrectomy and Roux-en-Y gastric bypass constitute the most commonly performed procedures and have been proven both cost-effective and safe with low complication rates. Liver transplantation is the only definitive treatment for end-stage liver disease and its utilization in patients with non-alcoholic steatohepatitis has increased more than fivefold over the past 15 years. In this review, we summarize current state of evidence on the surgical treatment of MS.

Key Words: Metabolic syndrome; Bariatric surgery; Sleeve gastrectomy; Gastric bypass; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Liver transplantation

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Core Tip: Metabolic syndrome (MS) is increasingly common in developed countries, and is associated with cardiovascular disease, hyperlipidemia, and non-alcoholic steatohepatitis. Diet, exercise, and weight loss are the milestones of conservative management. Bariatric surgery has emerged as a promising treatment in severely obese patients or in patients with MS resistant to conservative measures. Sleeve gastrectomy and Roux-en-Y gastric bypass are the most commonly performed bariatric procedures. The only definitive treatment in patients with MS and end-stage liver disease secondary to non-alcoholic steatohepatitis is liver transplantation (LT). The optimal timing for bariatric surgery, when required along with LT, has yet to be determined.

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INTRODUCTION

Metabolic syndrome (MS), also known as syndrome X, is a complex entity consisting of insulin resistance, obesity, hypertriglyceridemia, increased waist circumference and hypertension[1,2]. According to National Heart, Lung and Blood Institute, at least three of the following metabolic risk factors should be met to establish the diagnosis of MS: (1) Obesity (waist circumference ≥ 102 cm for men and ≥ 88 cm for women); (2) Triglycerides ≥ 150 mg/dL; (3) High-density lipoprotein (HDL) cholesterol < 40 mg/dL for men and < 50 mg/dL for women; (4) Systolic blood pressure ≥ 130 mmHg and/or diastolic ≥ 85 mmHg; and (5) Fasting serum glucose ≥ 100 mg/dL[2-4]. The incidence of MS, following the patterns of obesity and type 2 diabetes mellitus (T2DM), is approximately 30% in the adult population in the United States[1,5,6]. Data suggest that even populations with relatively lower body mass index (BMI), such as Asian Americans, can be affected by MS[7]. The prevalence of MS has significantly increased over the last decades, with less physically active and older individuals being increasingly affected[6]. It is quite evident that MS evolves into a global epidemic health problem that mandates timely and effective action[1,8]. Therefore, we sought to review the complications associated with MS with a particular focus on diseases of the liver, as well as the available treatment options focusing mostly on bariatric and liver surgery.

COMPLICATIONS ASSOCIATED WITH MS

MS has been associated with an increased risk of cardiovascular morbidity and mortality, and has been identified as an independent predictor of nonfatal stroke, ischemic heart disease, and cardiovascular death[9]. Wilson *et al*[10], in a prospective study of 3323 adults followed over 8-years, reported an increased incidence of cardiovascular disease in patients who developed MS, while 30% of all myocardial infarctions and coronary heart disease deaths in men and 16% in women could be attributed to MS.

MS can also lead to insulin resistance, and consequently T2DM. The mean weight, BMI, and prevalence of obesity in the United States population have increased significantly from 1960 to 2000[11]. The prevalence of T2DM has also increased from 1.8% to 5.8% over the same period, due to the increased prevalence of obesity, as well as due to the increased detection and awareness in previously undiagnosed patients[11]. In addition to obesity, other factors play a key role in the rising T2DM trend, such as the lack of physical activity, dietary changes, and other environmental factors[8,11].

Moreover, MS may affect the liver resulting in a wide spectrum of clinical conditions ranging from non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) to cirrhosis and, eventually, hepatocellular carcinoma (HCC). NAFLD is the most common cause of chronic liver disease in western countries[12,13],

and is defined as ≥ 5% fatty permeation of the liver parenchyma in the absence of an alcohol abuse history[12]. It occurs in up to one-third of the general population[14], and in up to 80% of patients with MS[15]. Risk factors predisposing to NAFLD include older age (> 50 years), hypertriglyceridemia, insulin resistance, and central obesity[13,16]. Liver steatosis is considered to be one of the earliest signs of MS[17], and early diagnosis and management are warranted to prevent the occurrence of irreversible histopathologic changes of the liver parenchyma[15], which can lead to NASH, cirrhosis, and HCC[12,14]. Notably, Ekstedt *et al*[18] reported three out of 129 (2.3%) recruited NAFLD patients developed HCC. Data suggest that the excessive fat stored in the hepatocytes promotes the release of pro-inflammatory cytokines, such as tumor necrosis factor, which stimulate pro-oncogenic pathways, including the nuclear factor kappa-light-chain-enhancer of activated B cells and the c-Jun N-terminal kinase pathways[19]. Additionally, it has been shown that the loss of function of several tumor suppressor genes is involved in this process[12]. The definitive pathophysiologic mechanisms predisposing to the development of HCC in patients with NAFLD have yet to be elucidated.

CONSERVATIVE MANAGEMENT

According to the current state of evidence, the cornerstone of MS management consists of lifestyle changes, such as restricted consumption of calories combined, regular exercise, and weight loss[20]. Previous studies have shown promising results with pioglitazone, metformin and vitamin E for the management of NASH[20,21]. Recently, obeticholic acid was found to be effective in the FLINT and REGENERATE trials and is expected to become the first FDA approved drug for the treatment of NASH[22-26]. Amphetamine derivatives, such as phentermine and desoxyephedrine, as well as statins have been occasionally utilized and showed promising results against NASH[27,28]. The addition of liraglutide to lifestyle changes has demonstrated better results than lifestyle changes alone[29]. Finally, vitamin D and zinc sulfate seem to be beneficial in children with MS[30,31].

Nevertheless, the overall impact of lifestyle changes in MS and NASH highly depends on patient compliance, while conservative treatment is mostly effective in limiting the progression of obesity[32]. Despite the progress in pharmacologic treatment, non-surgical treatment is not always adequate to yield fruitful outcomes in obese patients (BMI > 30).

SURGICAL MANAGEMENT

Bariatric surgery can result in significant weight loss, and potentially complete resolution of MS. In addition, operative MS management results in reduced rates of hypertension, cardiovascular risk, and plasma lipids, while it may also lead to improvements in glucose tolerance[27]. In addition, surgery has a significant advantage over conservative methods in lowering the level of hemoglobin A1c (HbA1c) in T2DM patients[33]. Regarding its latter effect, bariatric surgery may even result in the complete remission of T2DM[34-36]. In fact, the duration of T2DM and preoperative serum C-peptide levels have been identified as predictive factors of postoperative benefit in glucose tolerance[37,38]. Recent recommendations suggest lifelong supplementation after all bariatric surgeries[39]. The loss of weight after bariatric surgery is also beneficial for patients with NAFLD and NASH, considering that a loss of ≥ 10% of body weight might facilitate a significant decrease in liver fibrosis[40].

Bariatric surgical management was historically classified into restrictive and malabsorptive procedures. Restrictive procedures aim to decrease the amount of ingested food through a modification of the stomach capacity, while malabsorptive procedures aim to remove or bypass part of the small intestine thus leading to a decrease in gastrointestinal absorptive surface. In general, malabsorptive procedures are more beneficial in terms of lipid parameters than restrictive procedures[41]. Usually, both types of procedures are utilized in the management of MS. However, recent data suggest that factors other than restriction or malabsorption mediate the benefits of bariatric surgeries. For instance, gut hormones and enteroplasticity have been proven to play also an important role in terms of weight loss[42], while alteration of the intestinal microbiome, gut hormone production, neural signaling, hepatic and pancreatic function, and gastrointestinal nutrient-sensing affect the glucose homeostasis and insulin sensitivity[43]. Bariatric procedures are most commonly

performed laparoscopically (96%) and include the following: Roux-en-Y gastric bypass (RYGB), sleeve gastrectomy (SG), biliopancreatic diversion (BPD) with duodenal switch, and placement of laparoscopic adjustable gastric band (LAGB)[44].

RYGB

RYGB involves the formation of a 50-mL gastric pouch, as well as an antecolic Roux-en-Y gastrojejunostomy. Immediate effects of RYGB include the restricted intake of calories, the rapid entry of nutrients into the small intestine, and the increased nutrient and bile delivery to the distal small intestine, while it concurrently excludes the proximal intestine from nutrients[43]. RYGB can lead to significant mean weight loss (from 136.9 kg to 100.6 kg) and decrease in BMI (from 45.5 kg/m^2 down to 33.3 kg/m^2) at 4 years postoperatively[38]. MS resolution during the first postoperative year can occur in up to 75.8% of the patients[45], while a beneficial effect on blood pressure can be seen in up to 65% of MS patients[45]. Similar effects have been observed in other parameters associated with MS, including fasting lipids and glucose metabolism. In T2DM patients with a mean HbA1c level of 8.6%, the 1-year postoperative remission of T2DM after RYGB has been reported to be as high as 73.5%[40]. However, data suggest that during the 5-year postoperative period, the observed T2DM benefits may abate, mostly due to the insufficient amount of pancreatic beta cells reservoir in some patients[33]. Similarly, the beneficial effects on hyperlipidemia and hypertension are greater during the first 2 years after surgery, while these conditions may reemerge at 10 years post-procedure[46]. This finding signifies the potential importance of lifelong treatment with antihypertensive and lipid-lowering medications.

RYGB is considered a safe and effective therapeutic modality with low rates of postoperative complications, such as anastomotic leakage (0.63%), hemorrhage (0.52%), and bowel obstruction (0.4%)[38,47]. A common type of hernia observed after RYGB is Petersen's hernia, which is characterized by the herniation of a small bowel helix through the mesenteric gap created during the operation[48]. A recent meta-analysis revealed that this complication can be prevented with routine mesenteric gap closure after laparoscopic RYGB, with similar results in terms of other complication rates or weight loss[49]. Other postoperative complications include vitamin B_1, vitamin B_{12}, iron and calcium deficiency, as well as peptic ulcer disease[50]. Moreover, the need for reoperation or endoscopic intervention (anastomotic leak, infection, internal hernia, small bowel obstruction, insufficient weight loss) in patients undergoing RYGB is up to 22.1%[51]. Additional complications that may affect the quality of life include postprandial dumping syndrome, hypoglycemia, calcium oxalate nephrolithiasis and chronic kidney disease[52-54]. The 5-year postoperative mortality rate is around 3%[55].

SG

SG is mainly a procedure that results in caloric restriction, rapid entry of nutrients in the small intestine, and enhanced nutrient and bile delivery to the distal jejunum and ileum[43]. SG involves resection of approximately 80% of the stomach. SG is the most widely applied surgical procedure in the management of MS worldwide and results in improvement of all MS constituents, except for hypertriglyceridemia[47].

Although both RYGB and SG can lead to weight loss and decrease in BMI, these effects are less pronounced with SG. In a meta-analysis, comparison of RYGB and SG demonstrated significantly higher percentage excess weight loss in RYGB patients (65.7% *vs* 57.3%, $P < 0.0001$)[52]. Despite being a simple procedure, SG offers significant benefits, including improved glycemic control, weight loss, improved insulin sensitivity, and decreased need for hypoglycemic agents, in patients with MS, diabetes, and obesity. Postoperatively, most insulin-dependent patients tend to reduce or even stop taking their insulin dose, and their management can be changed to oral hypoglycemic agents only[56-58]. At 6 mo after surgery, up to 84% of diabetic patients present with resolution or remission of T2DM[59]. However, 30% to 50% of patients exhibit recurrence of diabetes in the long-term[60]. The effectiveness of SG in lowering glucose levels might be associated to the fact that fasting glucagon-like peptide 1, an incretin that promotes glucose homeostasis through insulin secretion, increases significantly after SG[61]. Significant improvements after SG have also been reported in terms of HDL cholesterol levels and hypertension[57]. In addition, when compared to RYGB, SG does not increase the risk of nephrolithiasis and chronic kidney disease[53,62].

SG is considered to be safer than RYGB, but both share the same spectrum of postprocedural complications except for the nutritional deficiencies, which are typically seen in lower rates after SG compared to RYGB. However, SG patients may present with postoperative iron, vitamin B12, and vitamin D deficiencies and ongoing monitoring with supplementation is necessary[63,64]. Reflux esophagitis is a relatively common complication after SG, while it is also deemed to be a contraindication for

SG[52]. In the long-term, a small percentage of patients may require a supplementary endoscopic or surgical intervention[65], while some patients complain of nausea and vomiting after excess food intake[50]. In extremely obese patients (BMI > 60 kg/m²), the rate of postoperative complications appears to be high and comparable to the rate of other bariatric procedures[52].

BPD with duodenal switch

BPD with duodenal switch resembles RYGB, both in terms of procedure and mechanisms that mediate the effect on glucose homeostasis and weight loss. BPD is the most effective procedure in terms of weight loss, but requires higher levels of expertise and surgical skill and is considered as the least safe bariatric procedure. BPD can be effective in extremely obese patients (BMI > 60 kg/m²) or in patients with MS resistant to other modalities, since it provides very strong metabolic effects and durable 35%-45% weight loss[66]. BPD with duodenal switch constitutes only 1.5% of all bariatric procedures performed worldwide[67].

Postoperatively almost 90% of BPD patients achieve normal HDL cholesterol levels[68], while fasting serum glucose levels may remain normal for up to ten years[69]. Serum total cholesterol and triglycerides levels commonly normalize too, while complete resolution of hypertension has also been documented with three-fourths of the patients presenting with normal blood pressure values at ten years postoperatively[69]. An up to 70% weight loss may also be achieved and it may be preserved for more than ten years[70].

Immediate postoperative complications include wound infection, anastomotic leak, and bowel obstruction[70]. Extensive small bowel resection can result in severe malabsorptive complications, such as anemia, nutritional deficiencies, hypoproteinemia, and bone demineralization[47,70]. BPD patients will require strict lifelong nutritional supplementation, including supplementation of lipid soluble vitamins, since they commonly exhibit vitamin A, D, E, and K deficiency[52,71]. Similarly to RYGB, patients undergoing BPD are at increased risk of nephrolithiasis[53,62].

LAGB placement

LAGB procedure involves the placement of an inflatable silicone device over the cardia of the stomach, which results in the formation of a small gastric pouch. This device includes a subcutaneous port to adjust the gastric band and the width of the gastric pouch[72]. LAGB can result in sufficient weight loss, while the reduction in BMI can be as high as 6.56 kg/m² in only 1 mo after the operation[48]. One significant advantage of LAGB placement over the other bariatric procedures is that the LAGB placement does not induce renal damage nor promotes renal stone formation[72,73]. In fact, urinary oxalate excretion was reported to be lower after LAGB placement than after RYGB, and similar to that of normal controls[74].

Nevertheless, this technique is infrequently used mostly due to complications, including erosion, infection, band slippage, esophagitis, esophageal dilation, and port dysfunction[50]. LAGB placement may be technically easier than the other bariatric operations, but it has been associated with a higher reoperation rate[15] with approximately 20% of the patients requiring a reoperation at 4.5 years postoperatively[72,75]. In addition, although LAGB placement can achieve a significant loss of weight, the results are inferior to those seen with either SG[35] or RYGB[50]. Other aspects of MS are less improved, and these findings are to a certain extent attributed to the unchanged postoperative plasma ghrelin levels[40]. Last but not least, LAGB placement has not been deemed effective in the management of NASH[15].

Comparison of bariatric procedures

Despite the large number of patients in need of bariatric surgery, no official guidance on patient allocation to the various treatment modalities has been published to date. There is a growing body of evidence that the several bariatric procedures could be ranked in ascending order based on their effectiveness (weight loss percentage and duration of weight loss maintenance) as follows: (1) LAGB placement; (2) SG; and (3) RYGB, and (4) BPD with duodenal switch[67]. Despite its increased effectiveness, BPD with duodenal switch has been associated with high rates of postoperative complications, while all bariatric procedures require varying lifelong supplementation due to nutritional deficiencies. In general, higher rates of morbidity and mortality have been observed in bariatric patients with comorbidities associated with MS, especially in the first 30 d after surgery[76]. These data render BPD suitable only for extremely obese patients, when RYGB and SG are thought of as inadequate or for patients suffering from less severe MS-related conditions. The use of LAGB placement has been

decreasing in western countries, since SG and RYGB can achieve superior rates of MS resolution with much lower morbidity rates, and a decreased need for postoperative monitoring. As previously mentioned, SG is currently the most frequently performed bariatric procedure worldwide.

The role of bariatric surgery in NAFLD/NASH

Although recommendations from the American Association for the Study of Liver Diseases[77] advocate for the use of vitamin E (in non-diabetics) and pioglitazone for NASH, caution is warranted with these agents due to their long-term risk of prostate and bladder cancer development, respectively[78,79]. Although nonsurgical weight loss can effectively improve all histological features of NASH and NAFLD (including fibrosis), most patients had early-stage fibrosis[80]. Therefore, other options including bariatric operations have been explored for the management of NASH and NAFLD. In fact, NAFLD at all stages is more common in those who meet criteria for bariatric surgery, which can indeed lead to sustained weight loss[77]. The most commonly used system for the assessment of necro-inflammatory lesions in NAFLD is the NAFLD Activity Score (NAS) from the NASH Clinical Research Network, which is comprised of 4 semi-quantitatively assessed histology features [steatosis (0-3), lobular inflammation (0-2), hepatocellular ballooning (0-2), and fibrosis (0-4)] and 9 histologic features recorded as either present or absent[81].

A recent prospective study demonstrated the bariatric surgery, namely one-anastomosis gastric bypass, led to a significant decrease in the grades of fatty infiltration, cell ballooning, lobular inflammatory changes and total NAS at 15 mo postoperatively[82]. More specifically, the histological features of NASH disappeared in 41.7% of NASH cases and in 50.0% of borderline NASH cases[82]. Another recently published prospective study supports these findings by demonstrating histological resolution of NASH with no worsening of fibrosis in 84.4% of the patients[83]. There is a growing body of evidence suggesting that the vast majority of patients with NAFLD and NASH will experience improvements in histology after any type of bariatric surgery (Table 1). On the other hand, compared to those without cirrhosis (0.3%), caution is warranted when recommending bariatric surgery for patients with compensated or decompensated cirrhosis due to the higher mortality rates (0.9% and 16.3%, respectively[84]. In a systematic review summarizing the outcomes of bariatric surgery in 122 cirrhotics (96.5% Child-Pugh A, and 3.4% Child-Pugh B), early and late mortality were found to be 1.6% and 2.45%, respectively[85].

The American Association for the Study of Liver Diseases recommends considering bariatric surgery in otherwise eligible obese NAFLD or NASH patients[77]. However, the current state of evidence does not allow us to deduce meaningful conclusions whether bariatric surgery can be used for the management of NASH specifically, but experienced bariatric surgeons can offer this option in eligible patients with compensated NASH on a case-by-case basis[77].

Liver transplantation and bariatric surgery

A significant percentage of patients may eventually require both bariatric surgery and liver transplantation (LT) for MS-related liver conditions; however, the sequence and appropriate interval between bariatric surgery and LT are still under investigation. The typical approach includes bariatric surgery one year prior to LT[86]. The main advantage of this approach is that the bariatric procedure can act as a "bridge" for patients to reach the predetermined BMI requirement for LT. Besides, data suggest that LT may result in a 5 kg weight gain at one year and a 10 kg weight gain at three years post-LT[72]. Theoretically, this approach would improve the LT outcomes and would result in fewer postoperative complications, less final weight, and lower graft rejection rates. On the other hand, serious adverse events associated with the bariatric operation, such as portal hypertension[86], anastomotic leakage, wound infection, bleeding, and kidney injury[87] could possibly complicate the subsequent LT. It has also been shown that patients with non-compensated cirrhosis have an increased mortality rate after bariatric surgery (16.3%), in contrast to patients with compensated cirrhosis or patients without liver disease (< 1%)[84]. Severe hepatic dysfunction has also been noted as a complication after RYGB[88]. Therefore, this likelihood for an increased mortality in cirrhotic patients, renders this "bridging" strategy questionable[84,88-90].

Recently, the "simultaneous" approach for LT and bariatric surgery has emerged as an alternative to the "bridging" approach[86,91]. The bariatric procedure most commonly performed along with LT is SG, because it does not involve manipulations around the biliary tract, while malabsorption is also not typically seen after SG[92]. This approach may lead to decreased length of hospital stay, postoperative pain, cost, and stress[44]. Postoperative complications of SG during LT include the leak from gastric staple line,

Table 1 Bariatric surgery studies with histological assessment of liver biopsy

PMID	First author	Year	Country	Study design	Patients	Type of surgery	Steatosis[1]	Hepatocyte ballooning[1]	Inflammation[1]	Fibrosis[1]	NAS[1]	Deterioration[2]	Follow-up (mo)
32553765	Lassailly	2020	France	P	180	RYGB, LAGB, BPD, SG	Yes	Yes	Yes	Yes	Yes	Yes	60
32556752	Salman	2020	Egypt	P	67	OAGB	Yes	Yes	Yes	Yes	Yes	No	15
32153044	Salman	2020	Egypt	P	81	SG	Yes	Yes	Yes	Yes	Yes	Yes	18
32152677	Salman	2020	Egypt	P	71	SG	Yes	NA	NA	Yes	Yes	Yes	30
32124215	Nikai	2020	Japan	R	28	SG	Yes	Yes	Yes	Yes	Yes	No	24
32360804	Bazerbachi	2020	United States	P	20	IGB	Yes	Yes	Yes	No	Yes	Yes	6
29126863	Garg	2018	India	P	32	RYGB, LAGB, SG	Yes	Yes	Yes	Yes	NA	Yes	12
27697327	Manco	2017	Italy	P	20	SG	Yes	Yes	Yes	Yes	Yes	No	12
27405478	Aldoheyan	2017	Saudi Arabia	P	27	SG	Yes	Yes	Yes	Yes	Yes	No	3
26077701	Froylich	2016	United States	R	25	RYGB, SG	Yes	Yes	Yes	Yes	Yes	Yes	18
27594839	Schneck	2016	France	P	9	RYGB	Yes	Yes	Yes	Yes	Yes	Yes	55
25537957	Taitano	2015	United States	R	160	RYGB, LAGB	Yes	NA	Yes	Yes	NA	Yes	31
26003897	Praveen Raj	2015	India	P	30	RYGB, SG	Yes	Yes	Yes	Yes	Yes	No	6
25917783	Lassailly	2015	France	P	30	RYGB, LAGB, BPD, SG	Yes	Yes	Yes	Yes	Yes	No	12
25379859	Caiazzo	2014	France	P	413	RYGB, LAGB	Yes	NA	Yes	Yes	Yes	NA	60
22161114	Tai	2012	Taiwan	P	21	RYGB	Yes	Yes	Yes	Yes	Yes	Yes	12
23355916	Vargas	2012	Spain	P	26	RYGB	Yes	Yes	Yes	Yes	Yes	No	16
22108808	Moretto	2012	Brazil	R	78	OAGB	Yes	Yes	Yes	Yes	NA	Yes	NA
20460923	Weiner	2010	Germany	R	116	RYGB, LAGB, BPD	Yes	NA	Yes	Yes	NA	No	19.4
19409898	Mathurin	2009	France	P	211	RYGB, LAGB, BPD	Yes	Yes	No	No	Yes	Yes	60
17376042	Furuya	2007	Brazil	P	18	RYGB	Yes	Yes	Yes	Yes	Yes	No	24
16076987	Clark	2005	United States	R	16	RYGB	Yes	Yes	Yes	Yes	Yes	No	10.2

[1]Did the parameter improve after the bariatric operation?

[2]Did any patient experience worsening in any of the parameters after the bariatric operation?

BPD: Biliopancreatic diversion with duodenal switch; IGB: Intragastric balloon placement; LAGB: Laparoscopic adjustable gastric banding; NA: Not available; OAGB: One-anastomosis gastric bypass; P: Prospective; R: Retrospective; RYGB: Roux-en-Y gastric bypass; SG: Sleeve gastrectomy.

and rarely excessive weight loss[86].

Performing a bariatric procedure after LT is not considered to be an optimal option. The most important complication, wound dehiscence, is attributed to the use of corticosteroids and immunosuppressive medications in LT recipients[86]. It has been proven that immunosuppressive regimens are a strong predictive factor for 30-day mortality in patients undergoing bariatric surgery[86,93]. Post-LT adhesions might also turn a routine bariatric procedure into a particularly challenging operation[44,72,86].

Currently, LT is the only treatment option that can definitively lead to complete resolution of NASH in bariatric patients. It is worth mentioning that in the early 2000s, only 3% of the LTs were performed for end-stage liver disease secondary to NASH, while in 2011 this percentage increased to 19%[12]. By 2020, NASH is expected to come first as a cause for LTs, at least in western countries[92]. The 5-year survival rate after LT for end-stage liver disease attributed to NASH is 60%-85%[94].

Despite of these promising results, LT in NASH patients has been associated with increased risk of postoperative complications compared to patients undergoing LT for other indications, such as renal dysfunction, sepsis, cardiovascular complications, wound infection, and prolonged mechanical ventilation. In the long-term, hypertension, obesity and hyperlipidemia may also deteriorate post-LT, mostly due to the state of immunosuppression, while recurrence of MS has been observed in around 50% of LT patients with preoperative MS[89]. Interestingly, up to 12% of transplant patients may require re-transplantation: (1) Due to NASH recurrence, which can be attributed to genetic causes, immunosuppressive agents, and the presence of excess adipose tissue[44,70,95]; or (2) Due to acute graft rejection, which is also higher compared to that seen after LT for other conditions[12,92].

CONCLUSION

MS is a common disease entity, particularly in western countries. It is usually accompanied by cardiovascular disease, dyslipidemia, and NASH, and is associated with increased morbidity and mortality. Although diet, exercise and weight loss are the cornerstone of initial management, bariatric surgery has emerged as an alternative approach, particularly in severely obese patients or in those with MS resistant to conservative treatment. SG and RYGB are the most commonly utilized bariatric procedures. The only definitively therapeutic modality in MS patients with end-stage liver disease secondary to NASH is LT, while the optimal time frame for bariatric surgery, when required in combination with LT, has yet to be determined.

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REVIEW

Combined liver-kidney transplantation for rare diseases

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Abstract

Combined liver and kidney transplantation (CLKT) is indicated in patients with failure of both organs, or for the treatment of end-stage chronic kidney disease (ESKD) caused by a genetic defect in the liver. The aim of the present review is to provide the most up-to-date overview of the rare conditions as indications for CLKT. They are major indications for CLKT in children. However, in some of them (*e.g.*, atypical hemolytic uremic syndrome or primary hyperoxaluria), CLKT may be required in adults as well. Primary hyperoxaluria is divided into three types, of which type 1 and 2 lead to ESKD. CLKT has been proven effective in renal function replacement, at the same time preventing recurrence of the disease. Nephronophthisis is associated with liver fibrosis in 5% of cases and these patients are candidates for CLKT. In alpha 1-antitrypsin deficiency, hereditary C3 deficiency, lecithin cholesterol acyltransferase deficiency and glycogen storage diseases, glomerular or tubulointerstitial disease can lead to chronic kidney disease. Liver transplantation as a part of CLKT corrects underlying genetic and consequent metabolic abnormality. In atypical hemolytic uremic syndrome caused by mutations in the genes for factor H, successful CLKT has been reported in a small number of patients. However, for this indication, CLKT has been largely replaced by eculizumab, an anti-C5 antibody. CLKT has been well established to provide immune protection of the transplanted kidney against donor-specific antibodies against class I HLA, facilitating transplantation in a highly sensitized recipient.

Key Words: Combined liver-kidney transplantation; Methylmalonic aciduria; Hereditary complement C3 deficiency; Glycogen storage diseases; Homozygous protein C deficiency; Primary hyperoxaluria; Atypical hemolytic uremic syndrome; Sensitization; Donor-

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specific antibodies

Core Tip: Combined liver and kidney transplantation (CLKT) provides replacement of both liver and kidney function in both organ end-stage diseases, or in end-stage kidney disease with origin in the genetic defect in the liver. It has been proven as an invaluable treatment option in the range of rare diseases such as primary hyperoxalurias, atypical hemolytic uremic syndrome, lecithin cholesterol acyltransferase deficiency, alpha 1-antitrypsin deficiency, hereditary complement C3 deficiency, nephronophthisis glycogen storage diseases. In this review, we provide an overview of rare indications for CLKT.

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INTRODUCTION

Combined liver and kidney transplantation (CLKT) is indicated for patients with failure of both organs, or for the replacement of genetic defect in the liver in the presence of advanced or end-stage chronic kidney disease. In the latter case, liver transplantation represents a form of gene therapy. CLKT is well established, although still presents a rare type of transplantation. In the United States, CLKT represented 8.6% of all adult and 2.9% of all pediatric liver transplants in 2018[1]. Only 3.1% of all adult kidney transplants and 2.2% of all pediatric kidney transplants consist of CLKT. The most frequent indications for liver transplantation in CLKT are cirrhosis caused by hepatitis C, cryptogenic cirrhosis, alcoholic cirrhosis, and polycystic liver disease, while most frequent renal indications are chronic glomerulonephritis, diabetic nephropathy, polycystic kidney disease, and hypertensive kidney disease[2]. In children, epidemiology is different, with rare diseases such as primary hyperoxaluria and congenital liver fibrosis/polycystic kidneys being the major indications for CLKT[3]. In this review, our goal is to provide the most up-to-date overview of the rare conditions as indications for CLKT (Table 1).

PRIMARY HYPEROXALURIA AND CLKT

Primary hyperoxalurias (PHs) (reviewed in Cochat *et al*[4]) are autosomal recessive disorders that result in increased oxalate generation leading to hyperoxaluria, nephrocalcinosis, renal stone formation, urinary infections, chronic kidney disease (CKD) and finally to systemic oxalosis. Type 1 hyperoxaluria results from a deficiency of alanine-glyoxylate aminotransferase (AGT), which facilitates transamination of glyoxylate to glycine in the liver. Thus, AGT deficiency results in the accumulation of glyoxylate and overproduction of oxalate and glycolate. Type 2 PH is a consequence of deficiency of a primarily hepatic enzyme, glyoxylate reductase-hydroxypyruvate reductase (GRHPR). GRHPR deficiency results in the accumulation of oxalate and L-glycerate. PH type 3 is caused by a deficit of 4-hydroxy-2-oxoglutarate aldolase, resulting in the accumulation of oxalate. The most frequent among the PHs is type 1. The clinical course is also the most aggressive in PH1, while PH3 is the mildest form of the disease, without systemic oxalosis and with the uncommon occurrence of nephrocalcinosis, kidney stones, and renal failure. PHs are very rare diseases. It has been estimated that their combined prevalence by genetic analysis is approximately 17 per million population[5].

Patients with PH1 and PH2 present with kidney stones or nephrocalcinosis early in life; median age at symptoms appearance was in recent studies 5.6 years in PH1 and 3.2 years in PH2. Mean age of patients at first liver/kidney transplant was 16.5 for PH1 and about 40 years in PH2[6,7]. Isolated kidney transplantation in patients with PH1 is

Table 1 Rare indications for combined liver-kidney transplantation

Disease	Indication(s) for CLKT
Monogenic diseases with primary hepatic expression without significant parenchymal damage	
Atypical hemolytic-uremic syndrome	Renal failure and alternative complement pathway activity
AIP	Renal failure and recurrent medically non-responsive AIP attacks
Primary hyperoxaluria	Renal failure and metabolic control of the disease
Homozygous protein C deficiency	Renal failure and coagulation control
Hereditary complement C3 deficiency	Renal failure and risk reduction of recurrent infections (?)
Monogenic diseases with primary hepatic expression with parenchymal damage	
Alpha-1-antitrypsin deficiency	Renal failure and liver failure (cirrhosis)
Glycogen storage disease	Renal failure with hepatocellular adenomatosis/carcinoma and metabolic control of the disease
Monogenic diseases with hepatic and extrahepatic manifestation	
Nephronophthisis associated with liver fibrosis	Renal failure and liver failure (cirrhosis)
Lecithin cholesterol acyl transferase deficiency	Renal failure and metabolic control of disease
Methylmalonic acidemia	Renal failure and metabolic decompensation
Other	
Antibody mediated rejection of the kidney	Renal failure and in the presence of positive CDC cross-match (?)

CLKT: Combined liver and kidney transplantation; AIP: Acute intermittent porphyria; CDC: Complement-dependent cytotoxicity.

associated with greatly decreased renal graft survival, because of the rapid recurrence of the disease[8,9]. Liver transplantation corrects genetic defects both in PH1 and PH2[10-12]. Although there are reports on preemptive liver transplantation in patients, who do not have advanced CKD, majority of patients receive CLKT[6,7,13,14]. CLKT (or sequential liver and kidney transplantation from a living donor[15]) has been consistently shown to provide better renal graft and patient survival, as compared to the isolated kidney transplantation. However, since a large amount of oxalate accumulates in the body over the years of disease, it is important to prevent rapid oxalate deposition in the renal graft. Namely, it can further lead to acute oxalate nephropathy and irreversible renal failure. This is achieved by high-intensity renal replacement therapy in the perioperative and early post-transplant period[16], even in patients with good immediate renal function. Long-term in the post-transplant period, high urine output with urine alkalization (potassium citrate) and avoidance of dehydration should be maintained. Another option for PH1 patients may represent sequential liver and kidney transplantation, where liver is transplanted first and in the second procedure at least several months apart kidney is transplanted from the same living, or different deceased donor[17,18]. The rationale for sequential LKT is to decrease oxalate production prior to kidney transplantation.

A separate question is whether entire liver tissue replacement is required for patients with primary hyperoxalurias. Since genetic defect that results in oxalate hyperproduction resides in hepatocytes and there is no significant transport of oxalate into hepatocytes, common opinion has been that hepatectomy and orthotopic liver transplantation (whole, or a segment) are required[19]. However, we and others have reported that auxiliary partial orthotopic liver transplantation (as part of the CLKT) was sufficient to prevent recurrent kidney injury in patients with PH1[20-22]. This is important because auxiliary liver transplantation would be safer than native hepatectomy and liver transplantation, in the case of post-transplant liver graft failure. The effect of auxiliary CLKT *vs* total CLKT on systemic oxalosis is currently unknown.

ATYPICAL HEMOLYTIC-UREMIC SYNDROME AND CLKT

Atypical hemolytic-uremic syndrome (aHUS) is a rare disease caused by enhanced activity of the alternative complement pathway. It is characterized by microangiopathic hemolytic anemia and thrombocytopenia, accompanied by acute kidney injury. aHUS results in death or end-stage kidney disease in up to 80% of patients within 3-10 years from the onset of the disease[23]. A recent systematic review reported prevalence in populations younger than 20 years old of 2.2-9.4 per million population (pmp), with an incidence in this population of 0.26-0.75 pmp. In all age groups, based on limited information, the prevalence was 4.9 and the incidence was 0.23-1.9 per million population[24]. Being constitutively active, the alternative complement pathway is controlled by several regulatory proteins, among which, some are synthesized in the liver[25]. A great majority of aHUS cases are caused by genetic abnormalities in complement proteins or their regulators, which results in uncontrolled activation of the alternative complement pathway. The most frequent cause of aHUS is factor H deficiency. In a great majority of patients, the deficiency is caused by mutations in factor H gene, with autoantibodies to factor H being responsible for up to 10% of cases[23]. Other causes may be mutations of factor I, B, and membrane cofactor protein (CD46), as well as mutations in C3. Factor H, together with factor I participates in the regulation of constitutive alternative pathway activity. They are both produced mainly by the liver. Mutations in factor H are responsible for about 30% of aHUS[26].

Historically, recurrence of the disease following kidney transplantation was very frequent, which almost universally led to graft loss[27-31]. Liver transplantation can correct the genetic abnormality in patients with aHUS due to factor H deficiency. The first report of CLKT in aHUS in a 2-year-old child was published in 2002[32]. Subsequent results of CLKT, following a protocol of peritransplant plasma-exchange, were favorable[33-35]. Although CLKT appeared promising in patients with end-stage kidney disease due to aHUS, it was largely replaced by eculizumab, an anti-C5 antibody[36,37]. Eculizumab is currently the standard treatment of aHUS before and after kidney transplantation according to national and international guidelines[38-42]. However, high cost of eculizumab and uncertainty of the needed duration of eculizumab treatment, as well as relapse in rare patients following renal transplantation under eculizumab, leave doors for CLKT in select aHUS patients still open[43,44].

HEREDITARY COMPLEMENT C3 DEFICIENCY AND CLKT

Hereditary complement 3 deficiency is an extremely rare autosomal recessive disease, which is present in less than 1 per million people[45]. It is associated with recurrent bacterial infections and complement-mediated glomerulonephritis (C3 glomerulopathy) although end-stage renal disease (ESRD) is uncommon[46,47]. In the complement system, complement C3 is central to classical and alternative complement pathways, and it is predominantly synthesized in the liver[48], but extra-hepatic synthesis such as monocyte- and kidney-derived is present as well[49,50]. Therefore, in case of kidney transplantation and inevitable immunosuppression post-transplant, the patient may be additionally compromised with the recurrence of bacterial infections.

Thus, the rationale behind the simultaneous liver-kidney transplantation lies in the long-term restoration of plasma C3 levels. So far only one case has been published of an adult with complete complement 3 deficiency due to homozygous mutation in C3, with a complete restoration of circulating C3 levels and good functioning both grafts 24 mo after simultaneous liver-kidney transplantation[51].

ALPHA-1-ANTITRYPSIN DEFICIENCY AND CLKT

Alpha-1-antitrypsin deficiency (A1AD) is a genetic condition caused by mutations in *SERPINA1* gene, resulting in synthesis and aggregation of misfolded alpha 1 antitrypsin (AAT) in the liver and its low serum level. Accumulation of abnormal protein leads to liver injury, while deficiency of protease inhibitor function disturbs antiprotease activity, primarily in the lungs. Some homozygous and compound heterozygous mutation patients typically develop liver disease in childhood and/or lung disease in adulthood, the later especially in smokers. Nowadays, A1AD with the prevalence of 24/100000 people[52], is the most common genetic cause of pediatric LT,

and the third metabolic liver disease in adults which may also lead to cirrhosis and LT[53-55].

In some A1AD patients, membranoproliferative glomerulonephritis (MPGN) develops as a rarely associated co-morbidity. The explanation for this pathology is not fully understood. It has been hypothesized that altered protein released from damaged hepatocytes becomes antigen for immunological reaction forming deposits in glomeruli. MPGN may progress to end-stage renal disease (ESRD) with a necessity for kidney transplantation. There have been literature reports of successful CLKT in A1AD patients with concomitant end-stage liver and advanced kidney disease[56,57]. Although, in A1AD cases with liver failure and MPGN, but without ESRD, an isolated LT may lead to the recovery of MPGN making renal transplantation unnecessary[58]. Therefore, for A1AT patients with end-stage liver disease and MPGN, careful evaluation of the kidney function and severity of damage should be done before deciding what would be the better option, isolated LT or CLKT[59].

LECITHIN CHOLESTEROL ACYLTRANSFERASE DEFICIENCY AND CLKT

Lecithin cholesterol acyltransferase (LCAT) deficiency is a rare autosomal recessive disorder, occurring in less than 1/million people[60] and it is caused by mutations in the *LCAT* gene (16q22.1), which encodes LCAT enzyme. It catalyzes the formation of cholesterol esters in lipoproteins. A deficiency of LCAT leads to increased plasmatic free cholesterol and lecithin with severe reduction of plasma HDL cholesterol, which in turn affects the metabolism of other lipoproteins and results in lipid-containing deposits in various tissues. Familial LCAT with the total loss of enzyme activity usually manifests in early adulthood with corneal opacifications, hemolytic anemia, and renal injury with proteinuria[61,62]. Enlargement of the liver, spleen, and lymph nodes may be found in addition to atherosclerosis[63]. Renal failure typically appears in the second or third decade and progresses to end-stage kidney disease, necessitating renal replacement therapies including kidney transplantation[62]. As kidney transplantation alone does not affect the levels of plasma LCAT nor the abnormal lipid profile, recurrence of the disease in the graft is to be expected within days after transplantation. However, long-term graft function is well-maintained despite the presence of deposits[61,64-66].

Since LCAT is produced within the liver, CLKT provides a plausible treatment option. Though, so far only one report addressed the LCAT deficiency in a 29-year old man treated with kidney transplant combined with a year apart sequential auxiliary partial orthotopic liver transplant from the same living donor. The improvement in HDL and triglycerides was only present up to 1 year after transplantation, but the long-term follow-up showed no histological signs of LCAT nephropathy[67].

GLYCOGEN STORAGE DISEASE AND CLKT

Glycogen storage diseases (GSDs) are a group of inherited metabolic disorders of glycogen metabolism. GSDs affect liver and/or muscles. Subsequently, they are commonly divided into GSDs with the mainly hepatic presentation, which main features include hepatomegaly and hypoglycemia (0, I, III, IV, VI, IX, XI) and GSDs with the neuromuscular presentation, typically with muscle weakness and hypotonia (II, III, IV, V, VII, IXd)[68].

Glycogen storage disease I, also known as Von Gierke's disease, is one of the most common types with the incidence of 1/million people[69]. GSD I is an autosomal recessive disease resulting in glucose-6-phosphatase (G6P) deficiency. Normally, G6P catalyzes the last step of glycogenolysis and it is expressed in the liver, kidney, and scarcely in intestines, resulting in glycogen deposits in the aforementioned organs[70]. Approximately 80% of patients have type Ia deficiency, with the G6P catalytic unit defect on the endoplasmic reticulum, while the rest have type Ib due to a G6P translocase defect[71,72]. Patients with GSDs usually present in infancy with poor tolerance to fasting, hepatomegaly, and growth retardation. Their characteristic laboratory findings include hypoglycemia, hyperlactacidemia, hyperlipidemia, and hyperuricemia[73]. GSD Ib patients additionally have frequent neutropenia, which creates common difficulties with recurrent infections[74]. Neutropenia in GSD Ib is possibly caused by abnormal neutrophil function since neutrophils energetically considerably depend on glycogenolysis[74]. The patients are usually diagnosed by molecular diagnostics[73] and prescribed with a strict diet regime (frequent meals, slow-

absorption carbohydrates) yielding in majority normoglycemia and good metabolic control[70]. The long-term course of GSD I is frequently complicated with hepatocellular adenomas (16%-75%), that appear in the second or third decade of life and progress in size and number[71]. The occurrence of hepatocellular carcinoma is also possible, and its incidence increases along with the increasing survival of the well-metabolically controlled patients[75]. Accordingly, hepatocellular adenomatosis and carcinoma are the main indications for LT in GSD I patients, which also provides good glucose homeostasis[71,76].

However, several reports suggest that immunosuppression after LT may severely worsen renal function in these patients. Namely, pre-existent renal complications as focal segmental glomerulosclerosis, proximal and distal tubular dysfunction are also common GSDs trait[71,77]. Several authors thus report CLKT as a successful treatment for GSD I patients with hepatic adenomatosis and kidney failure[78-81]. As a treatment modality, CLKT provides GSD I patients correction of the metabolic defect, and consequently effective metabolic control. Hence, some investigators have even broadened the hepatic indications for CLKT, including poor metabolic control and severe growth retardation[82]. In the literature, there are also several reports of GSD I patients who underwent CLKT for terminal kidney failure, without hepatic adenomatosis being present, but with poor metabolic control[83-86]. As isolated kidney transplantation does not improve liver glucose metabolism and hence potentially presents a ground for reoccurring kidney failure, CLKT was recommended as a better option in those cases[83,85] Patients who underwent CLKT so far, were all adults aged 19-42 years and they all have recovered well after transplantation[84,85]. CLKT meant great advance for two women with GSD I, who managed to conceive and successfully bear healthy children, despite the low fecundity and pre-transplantation metabolic disturbances[85,86].

LT might provide a successful metabolic balance in poorly regulated GSD I patients, although CLKT might maintain that balance, particularly in severe cases with renal failure.

NEPHRONOPHTHISIS ASSOCIATED WITH LIVER FIBROSIS AND CLKT

Nephronophthisis (NPHP) is a renal ciliopathy with the autosomal recessive inheritance of cystic kidney disease and with a prevalence of 1/100000 people[87]. It may progress to ESRD and is the most common genetic cause of renal failure in children and young adults[88]. In 10%-20% of NPHP there is an association with extra-renal manifestations; neurological, ocular, skeletal, hepatic, cardiac, and pulmonary, sometimes manifesting in specific clinical syndromes. Boichis syndrome is characterized by the simultaneous development of hepatic fibrosis, in up to 5% of patients with NPHP[89]. These patients are candidates for CLKT as injury of both organs may end in irreversible organ failures. One case report and case series of CLKT in patients with Boichis syndrome have been published so far. In all of the four reported patients, both liver and kidney functions recovered initially, but two died (one 2 wk after CLKT due to pneumonia and intracranial hemorrhage, and the second 10 years later due to cardiovascular disease)[18,90]. Sequential liver-kidney transplantation was also described in the literature. The liver transplantation was performed first, followed by kidney transplantation 4 mo later. Both grafts came from the same donor and retained good function after transplantation[91].

HOMOZYGOUS PROTEIN C DEFICIENCY AND CLKT

Homozygous protein C deficiency (HPCD) is a rare autosomal recessive disorder, which results in a hypercoagulable state due to very low levels of active protein C caused by a mutation in the *PROC* gene. Clinically significant HPCD occurs in 1/20000 people[92]. HPCD typically presents as neonatal *purpura fulminans* with thrombosis of major blood vessels that lead to ophthalmologic, neurological, and renal complications with high rates of mortality[93]. The condition is managed by substitution of protein C and anticoagulation therapy, although long-term survival without major co-morbidity is rarely reported[93]. Several reports demonstrate that liver transplantation, as it is the organ of protein C synthesis, provides a good therapeutic option. Namely, LT leads to the reconstitution of protein C activity, which in the long-term is both clinically effective and cost-effective[93-97]. To date, only one report has addressed CLKT in an 8-year-old patient with HPCD and bilateral renal vein thrombosis resulting in renal

failure. The patient underwent auxiliary liver with combined renal transplantation, which improved her quality of life and removed the need for protein C infusions and hemodialysis[95].

ACUTE INTERMITTENT PORPHYRIA AND CLKT

Acute intermittent porphyria (AIP) is an autosomal dominant disorder resulting in the deficiency of porphobilinogen deaminase (PBGD), an enzyme involved in heme synthesis[98]. AIP has a prevalence of 1/20000 people[99]. Only a minority of symptomatic patients with AIP (< 10%) develop potentially life-threatening recurrent acute attacks, which can be triggered by various metabolic and environmental factors as a consequence of accumulated phototoxic and neurotoxic heme precursors (PBG)[98,100,101]. Acute attacks are characterized by severe abdominal pain, nausea, vomiting, hypertension, and sometimes neurological manifestations (neuropathies, encephalopathy, convulsions, anxiety), while major chronic complications include chronic kidney disease and development of hepatocellular carcinoma[100,102].

Current treatment for acute attacks consists of intravenous hemin and carbohydrate loading[101], though repeated hemin therapy may complicate patients' conditions, causing vascular thromboses and restricting venous access[103]. Thus, liver transplantation (LT), as a major source of PBG production, should be considered for patients with AIP, who suffer from recurrent, medically non-responsive attacks that substantially impair the life quality[104].

The outcome of the few LTs performed in AIP patients has been excellent so far, while the transplanted patients have not experienced further acute AIP attacks. Their biochemical PBG results markedly improved and the 5-year survival rate was nearly 80%[105]. However, a high incidence (40%) of hepatic artery thrombosis has been observed after LT and the patients with long-term neuropathies did not have significant neurological improvement[105]. Despite extrahepatic PBGD deficiency, there were no observed induced AIP attacks due to immunosuppressants[106]. A few successful renal transplantations have also been performed in patients with uncomplicated AIP and renal failure[107,108]. Even though, most recommendations for patients with frequent acute attacks, progressive neuropathies and deteriorating renal function suggest performing CLKT[101] yet only two published cases (aged 24 and 55 years) have undergone CLKT[109]. Wahlin *et al*[109] showed that their clinical and biochemical AIP markers significantly improved after CLKT, while one of them exhibited bile leakage as a post-transplant complication. Prior to CLKT, they had both presented with a diverse degree of neuropathies that subsequently completely resolved in the post-transplant period.

Although there were descriptions of unpredictable course of AIP and possible amelioration during the time[110], current recommendations advise early LT as a cure, since recurrent attacks may prompt severe neurological deficits and end kidney failure. In advanced cases, CLKT may be a considerable treatment option if the patients are adequately clinically stable.

METHYLMALONIC ACIDURIA AND CLKT

Methylmalonic aciduria (MMA) refers to a rare group of inherited disorders of the catabolic pathway of branched-chain amino acids and odd-chain fatty acids resulting in methylmalonic acid (MA) accumulation. The incidence of MMA varies from 1/50000 to 1/100000 of people[111]. MMA is inherited in autosomal recessive pattern, affecting MUT gene encoding methylmalonyl-CoA mutase or genes encoding key enzymes for the metabolism of the cofactor cobalamin[112]. MUT enzyme is normally expressed primarily in mitochondria of the liver, but also in kidneys, endocrine tissue, brain, and muscles[113]. Furthermore, the accumulation of MA provokes episodes of metabolic instability[114].

The intensity of the clinical picture depends on the particular genetic mutation. In the most severe cases, shortly after birth patients experience recurrent episodes of metabolic acidosis and hyperammonemia, leading to neurologic, hematologic, renal, sometimes gastrointestinal, heart and vision impairment, followed with growth failure and developmental delay. In others, symptoms may not be so intense and its appearance may be postponed to even adulthood. Some triggers like fasting, infection, surgery, or any other stress may provoke sudden worsening and metabolic crisis[114-117]. Such episodes are also exacerbated by unrestricted protein intake, therefore high-

energy low-protein diet (low in propiogenic amino acid precursors) is crucial throughout life. Administration of L carnitine, vitamin B12, and symptoms-based treatment are also part of conventional therapy[118]. However, despite all measures, MMA may not be under sufficient control, therefore the organ injuries may also progress. In such patients, with frequent metabolic decompensation episodes, in spite of proper conservative management, better metabolic stability may only be accomplished with liver, kidney or CLKT. It is advisable to perform it before the appearance of irreversible neurologic damage[58,119].

In the context of MMA, organ transplantation is not a curative option, but it can be a complementary therapeutic solution. Namely, liver transplantation reduces the systemic accumulation of MA, while kidney transplantation facilitates the clearance of MA[114]. Even more so, the amount of normal enzyme is higher in CLKT and in addition kidney rejection may be better controlled[59,120].

After CLKT levels of MA decrease by 80%-97%[121,122] and are lower than after isolated LT with better metabolic outcomes and reduced number of hospitalizations[123-125]. The majority of reports show improvements in neuro-development (enhanced motor skills, learning abilities, social engagement) and improved quality of life after CLKT[18,114,118,121,123-128]. Yet, in most cases, MA levels after CLKT are still 1000x higher than normal[124,125]. Besides, neurologic and/or muscle impairments may continue despite normal graft function[59,117]. It is also of great importance to maintain close metabolic monitoring and dietary measures after the transplantation, as extrarenal and extrahepatic production of MMA continues to derive from skeletal muscles[117]. In some cases in the post-transplant period, a relaxed dietary protein diet may safely occur[128].

To date, only several dozen CLKT cases performed in MMA have been described in the literature[129] with promising results. The age of MMA patients considered for CLKT ranged between 2 and 28 years[116,124,125,127]. Combined transplants were almost entirely performed in patients with mut0 type of MMA and their survival rates were excellent[114,116,118,121-128,130,131]. Two deaths after CLKD have been reported; one caused by a metabolic crisis after the transplant[132] and the other by early post-transplant complications[125].

Preoperative treatment should be precisely planned to prevent catabolism afterward. Surgical complications are more frequent after an extensive procedure such as LKT and the procedure carries more risks[123-125]. Most of the immunosuppression regimens for CLKT in MMA patients consisted of calcineurin inhibitors (CNI) (tacrolimus and cyclosporine) steroids and mycophenolate-mofetil[116,124,125]. In addition, neurological complications such as seizures, tremor, ataxia, worsening vision, and altered mental status were common and occurred in 15%-40% of patients after CLKD. Early after the procedure, the patients with their pre-existent mitochondrial dysfunction are particularly prone to the development of CNI-neurotoxicity while later neurological symptoms are more probably metabolically induced[115,116,121,122].

Though not curative, CLKT is generally a highly effective additional therapeutic option for MMA patients who cannot be stabilized only with regular dietary and pharmacological therapy, in spite of the higher risk for post-transplantation complications in this population. Decisions on whether or not CLKT will be performed should be individualized on a case by case basis balancing advantages and post-transplantation risks[115].

ANTIBODY-MEDIATED REJECTION OF THE KIDNEY

The long-term practice of many transplant centers was to proceed to CLKT even in the presence of positive complement-dependent cytotoxicity (CDC) crossmatch, as hyperacute kidney rejection in CLKT is extremely rare. Now, it has been well established that CLKT may confer partial kidney allograft protection against donor-specific antibody (DSA)-mediated rejection[133]. For example, in a recent retrospective study, CLKT patients with preformed DSA had lower rates of acute and chronic antibody-mediated rejection (AMR) as compared to isolated kidney transplant recipients[133]. In addition, recipients of CLKT had reduced the incidence of T-cell mediated rejection (TCMR) of kidney grafts as compared to kidney-transplant alone recipients[133]. This protection is predominantly related to anti-HLA class I DSA, as anti-HLA class II DSA has been associated with increased risk of graft loss and patient death in CLKT[134]. *De novo* DSAs following LT, that are in majority of cases directed against HLA class II, are associated with increased risk of antibody-mediated rejection, long-term graft failure and patient death, similar to their association with antibody-

mediated rejection and decreased long-term graft survival following kidney transplantation. However, some studies failed to demonstrate an increased risk for graft loss in liver transplant recipients with *de novo* DSA[135]. Currently, it is not well known whether patients with CLKT have a different incidence of *de novo* DSA and lower risk of AMR, as compared to kidney only transplant recipients.

Mechanisms providing immune protection of kidney grafts in CLKT are incompletely elucidated but may include HLA class I antigen shedding by the liver grafts, DSA absorption by the liver, and increased activation of tissue integrity/metabolism pathways in the kidney[133].

To date, CLKT in highly sensitized recipients was performed only in patients with conventional indications for CLKT. Though, since acute AMR is associated with a high risk of renal graft loss and of progression into chronic AMR[136], one could hypothesize that simultaneous auxiliary liver and kidney cross-match positive transplantation for an extremely sensitized patient in need of kidney only transplantation might improve short- and long-term kidney transplant outcomes. It is unknown whether the same degree of protection would be seen in the recipient of simultaneous auxiliary LKT, because of a smaller transplant liver tissue mass. That would be in line with the hypothesis, that the capacity of the liver graft providing immune protection for the kidney would be limited in the presence of too high levels of DSA[137]. Such auxiliary, partial, CLKT performed to facilitate HLA-incompatible kidney transplantation in a difficult to desensitize recipient remains to be reported.

CONTRAINDICATIONS FOR CLKT

As in other forms of solid organ transplantation, absolute contraindications for CLKT include active infection, recent or active malignancy, severe irreversible heart or respiratory failure, severe non-adherence, and psychiatric disorder impairing consent or adherence[138]. In patients with liver cirrhosis, an additional contraindication is moderate or severe portopulmonary hypertension[139]. Timely referral for CLKT is essential, as long-standing chronic kidney disease may lead to the progression of cardiovascular disease. In addition, both long-standing chronic kidney disease and liver disease are associated with increased frailty, and consequently increased risk for poor transplant outcome[140].

CONCLUSION

Simultaneous liver and kidney transplantation has proven to be a life-saving procedure in the simultaneous failure of both organs. Though, besides the well-established indications for end-stage liver and kidney disease, it also represents a therapeutic option for numerous rare diseases. Namely, by replacing mutated genes, liver transplantation provides a cure for genetic diseases with origin in the liver. Although nowadays there are enzyme replacement therapies, in general, they are still too expensive and thus less cost-effective than LT. Furthermore, the indications for CLKT in rare metabolic disorders have even broadened, in order to achieve better metabolic control and improve the quality of life.

Advances in surgical technique, as well as improvement in immunosuppression, led to better long-term CLKT transplant outcomes. The risk of the procedure may be even lower in partial orthotopic auxiliary liver transplantation, which merits further evaluation in candidates for CLKT when a genetic disease with origin in the liver is present. Yet, meticulous estimation of risk is necessitated, including consideration of possible short- and long-term complications after transplant. Accordingly, it is also complex to decide on the time-point of CLKT. Despite the complexity of the procedure, CLKT has better outcomes in patients with metabolic diseases and renal failure than isolated LT, or isolated kidney transplantation, and in addition reduction of extrahepatic synthesis of metabolites may in some cases also be reduced. After the CLKT, careful monitoring for extrarenal and extrahepatic metabolic manifestations is necessary. As CLKT itself is not a frequent procedure, we believe that it should be performed only in high volume transplant centers. That is even more important in case of rare indications, where an experienced multidisciplinary team is a prerequisite.

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MINIREVIEWS

Hepatocellular carcinoma Liver Imaging Reporting and Data Systems treatment response assessment: Lessons learned and future directions

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Abstract

Hepatocellular carcinoma (HCC) is a leading cause of morbidity and mortality worldwide, with rising clinical and economic burden as incidence increases. There

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are a multitude of evolving treatment options, including locoregional therapies which can be used alone, in combination with each other, or in combination with systemic therapy. These treatment options have shown to be effective in achieving remission, controlling tumor progression, improving disease free and overall survival in patients who cannot undergo resection and providing a bridge to transplant by debulking tumor burden to downstage patients. Following locoregional therapy (LRT), it is crucial to provide treatment response assessment to guide management and liver transplant candidacy. Therefore, Liver Imaging Reporting and Data Systems (LI-RADS) Treatment Response Algorithm (TRA) was created to provide a standardized assessment of HCC following LRT. LI-RADS TRA provides a step by step approach to evaluate each lesion independently for accurate tumor assessment. In this review, we provide an overview of different locoregional therapies for HCC, describe the expected post treatment imaging appearance following treatment, and review the LI-RADS TRA with guidance for its application in clinical practice. Unique to other publications, we will also review emerging literature supporting the use of LI-RADS for assessment of HCC treatment response after LRT.

Key Words: Hepatocellular carcinoma; Liver Imaging Reporting and Data Systems Treatment Response Algorithm; Locoregional therapy; Liver Imaging Reporting and Data Systems Treatment Response equivocal; Arterial phase hyper enhancement; Stereotactic body radiotherapy

Core Tip: Liver Imaging Reporting and Data Systems (LI-RADS) Treatment Response Algorithm (TRA) provides a new framework to describe treatment response for each individually treated hepatocellular carcinoma (HCC). Emerging evidence for its use in clinical practice is promising for ablation and non-radiation arterial-based therapies (*i.e.*, transarterial chemoembolization). However, LI-RADS TRA should be applied cautiously when assessing HCC treated with radiation-based therapies (*i.e.*, transarterial radioembolization, stereotactic body radiotherapy), in which early post-treatment persistent arterial phase hyperenhancement is common, and expected, and can confound treatment response.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and third leading cause of cancer related mortality worldwide[1]. The incidence of HCC continues to rise in the United States[2], largely due to the increasing rate of cirrhosis from obesity, alcohol use and chronic viral hepatitis[3]. Historically, curative treatment options for HCC include liver transplantation, surgical resection or thermal ablation for tumors less than 3 cm in size[4]. However, approximately 80% of patients are not surgical candidates; for them, locoregional treatment (LRT) options include: Thermal ablation [*e.g.*, microwave ablation (MWA), radiofrequency (RFA), cryoablation], percutaneous ethanol injection (PEI), transarterial chemoembolization (TACE), transarterial bland embolization (TAE), transarterial radioembolization (TARE), and stereotactic body radiotherapy (SBRT)[5-7]. LRT can be used alone, in combination with each other, or in combination with systemic therapy, and has been shown to improve disease-free and overall survival (OS) in patients who cannot undergo surgery[8-10].

Furthermore, LRT can help prolong time to progression, extend survival, palliate symptoms, keep lesions from progressing outside of Milan criteria to maintain liver transplant candidacy (bridge to transplant), and convert non-transplant candidates to transplant candidates based on Milan criteria (downstage to transplant)[11-13]. Treatment decisions are usually made by a multidisciplinary liver tumor board, and depend on various patient factors, including tumor location, size and multiplicity, disease stage, liver function, performance status, technical feasibility and potential for future transplant candidacy[14,15].

Following LRT, it is imperative to provide accurate treatment response assessment to help guide clinical management. While numerous validated imaging based treatment response classification systems exist, [*i.e.*, European Association for the Study of Liver Disease (EASL)[16], modified Response Evaluation Criteria in Solid Tumors (mRECIST)][17], they are based on tumor response assessment at the patient-level. However, HCC is unique in that tumors are often isolated to the liver, and LRT can be used to target the tumor(s) directly. Different LRTs may also be performed to different lesions within the same liver. Furthermore, liver transplant candidacy is based on assessment of each lesion. Consequently, the Liver Reporting and Data System (LI-RADS) Treatment Response Algorithm (TRA)[2], was created to provide a standardized assessment of each HCC treated by LRT, a feature which makes this treatment response classification unique to the existing ones. Furthermore, this allows LI-RADS TRA to be more applicable from a clinical perspective in patient management.

In this manuscript, we provide a brief overview of the various LRTs for HCC, describe the expected post treatment imaging appearances after LRT, review the definitions within the LI-RADS TRA and provide guidance for their use in clinical practice. We will also review the emerging literature supporting LI-RADS for assessment of HCC treatment response after LRT. This review is unique to other publications because it provides a comprehensive overview of the LI-RADS TRA and guidance for its application in clinical practice based on expected post treatment imaging findings, as well as critically reviews current literature supporting this algorithm.

LOCOREGIONAL THERAPIES FOR HCC

There are many treatment options for HCC, depending on stage of disease, as well as other factors mentioned above. LRTs for liver limited disease have proliferated in recent years, and are generally categorized as follows[18-20]: (1) Loco-ablative therapy: Chemical ablation (PEI), physical ablation utilizing energy sources [Heat: RFA, MWA; Cold: Cryoablation; Electrical: Irreversible electroporation (IRE)]; (2) Arterial based therapy (non-radiation): TAE, conventional trans-arterial chemoembolization (cTACE), drug eluting beads TACE (DEB-TACE); and (3) Radiation-based therapy: TARE and SBRT.

Loco-ablative therapy

Historically, the first ablative therapy used for HCC was PEI, which consists of injecting ethanol directly into the tumor under image guidance to achieve tumor death *via* coagulative necrosis and ischemia[21]. Studies show that PEI has a high safety profile, good overall efficacy, and low complication rates with complete necrosis of small HCC tumors; however, limitations of PEI include the need for multiple treatments[22]. In 1999, the first thermal ablation was performed with RFA, showing a high safety profile, good overall efficacy, and 5 year survival rates similar to surgical resection for tumors less than 3 cm in size[23]. Thermal ablation modalities, including RFA and MWA, use energy at different frequencies to create high temperatures with rapidly oscillating field strength *via* percutaneous insertion of electrodes (RFA)/antennas (MWA) into the tumor through image guidance. This results in cell death *via* coagulation necrosis[24]. Multiple randomized controlled trials have been performed showing superiority of RFA to PEI in terms of OS, complete response (CR) and local recurrence (LR)[25]. A recent meta-analysis demonstrated that RFA significantly increases survival in patients with HCC by 3-years as compared with PEI[26]. Although there is still a role for PEI in some cases where ablation is technically challenging or cannot be performed safely, thermal ablation is much more commonly performed worldwide.

Arterial based therapy (non-radiation)

The goal of non-radiation arterial-based therapy is to prevent local progression in intermediate and advanced stage HCC, as a form of palliative therapy or as a method for downstaging/bridging to resection or transplant. By providing arterial delivery of embolic material, either bland (TAE) or chemotherapy coated (cTACE/DEB-TACE), to HCC *via* the hepatic artery, arterial inflow to the tumor is eliminated, resulting in cell death[18,27,28]. When TACE is performed, tumor cell death (necrosis) occurs by two mechanisms: Ischemic injury from arterial embolization and chemotoxic injury from the administered chemotherapeutic agent[29]. Historically, TACE is indicated in non-surgical patients with large multifocal HCC and Child-Pugh Score A without extrahepatic spread[30]; although recently, the scope for using TACE has expanded, and now includes treating small and/or solitary tumors. The presence of portal vein obstruction by bland thrombus or intravascular tumor is a relative contraindication to non-radiation arterial based therapy[31,32]; in this instance, both the arterial and portal venous flow to the liver would be compromised, resulting in ischemia of the hepatic parenchyma within the embolization zone[33]. Non-radiation based trans-catheter intra-arterial therapies include: (1) Conventional TACE: Utilizes an emulsion of iodized oil mixed with a chemotherapeutic agent followed by administration of gelatin sponge or embolic microparticles to near complete stasis[34]; (2) DEB-TACE: Utilizes microspheres coated with chemotherapeutic agent, which will elute into local tissues over time[35]; and (3) Bland TAE: Utilizes polyvinyl alcohol particles or microspheres to occlude the arterial supply without a chemotherapeutic agent[36]. Multiple randomized control studies have shown that neither TACE nor DEB-TACE improved tumor objective response or provided survival benefit when compared to TAE[9,37,38]. However, conventional TACE remains the current standard of care for unresectable intermediate or advanced stage HCC in patients with preserved liver function based on BCLC guidelines[39].

Radiation based therapy

TARE and SBRT are the most common radiation-based treatment modalities used today[40]. As in any form of LRT, patient selection, including assessment of patient's disease burden, biochemical parameters and performance status, are critical to determining which form of therapy is preferred. TARE is ideal if the disease is limited to less than half of the liver[40]. Other lab parameters are also important for patient selection, including bilirubin < 2 mg/dL and albumin > 3 g/dL[40,41]. TARE involves injection of Y90 microspheres (20-60 microns in diameter) into the hepatic arteries, which delivers targeted radiation[42]. Portal vein thrombus (bland or tumor) is not a relative contraindication for TARE[41], since this form of intra-arterial therapy does not result in arterial embolization[42].

SBRT consists of applying multiple tightly focused high energy beams of radiation to treat HCC, allowing for the delivery of higher doses of radiation with relative sparing of adjacent parenchyma when compared to other options, albeit with the limitation of multiple treatment sessions over days.

HCC TREATMENT ASSESSMENT BASED ON LI-RADS TRA

Following LRT for HCC, cross-sectional imaging with multiphasic MRI and/or CT (including pre contrast and dynamic arterial, portal venous and delayed phase imaging) is routinely performed to assess treatment response and to identify new or developing sites of disease in the untreated liver. Routine time intervals for follow-up varies depending on institutional protocol, type of LRT performed, and transplant status for patients being down staged or bridged. Most major transplant centers and large institutions perform imaging 1-mo post-treatment, followed by imaging at 2-3 mo intervals. Imaging after radiation-based therapy (TARE/SBRT) begins 3 mo after treatment and about every 3 mo thereafter. While the choice of imaging modality (CT or MRI) can vary depending on patient factors and institutional preference, it is important to try and maintain consistency in the modality and technique for imaging performed before and after LRT. Accurate interpretation of post-treatment imaging is essential for guiding further management decisions and requires comparison of post-treatment with pre-treatment imaging to appreciate the original tumor dimensions and enhancement characteristics.

EXPECTED POST TREATMENT IMAGING APPEARANCE

Imaging appearances of HCC after LRT will vary depending on the treatment modality, with different expected findings for the various forms of therapy. Thus, it is imperative to become aware of the expected treatment specific enhancement patterns in order to prevent inaccurate interpretation of residual or recurrent neoplasm. While the expected imaging appearances of the treated tumor are similar for ablation and non-radiation arterial-based therapy, as tumoral necrosis is expected immediately after LRT, imaging findings are distinct after radiation-based therapy (TARE and SBRT), as tumoral necrosis develops over time.

The creation of an ablation margin of greater than 5-10 mm around the tumor is considered essential for adequate ablation; thus, an ablation zone larger than the original tumor dimension is an expected finding. Furthermore, the ablation zone should not demonstrate residual enhancement because of the anticipated coagulation necrosis and cell death within the treatment cavity; this frequently results in the development of a central zone of hyper-intense signal on pre-contrast T1 weighted MRI and a hyperdense appearance on unenhanced CT, both are expected post-treatment findings[43]. Subtraction (MRI) and non-contrast (CT) imaging are essential to avoid interpreting these imaging characteristics as areas of arterial phase hyperenhancement (APHE, Figure 1). Since the ablation zone represents devitalized liver parenchyma and tumor, reporting measurements of the ablated zone is not mandatory, rather, the residual nodular areas of enhancement suspicious for viable tumor should be described.

A uniform thin peripheral rim of enhancement is an expected post-ablation finding. Additionally, there can be geographic APHE within the parenchyma surrounding the treatment zone, which usually resolves, but can persist on portal venous and delayed phase imaging (Figure 1). APHE which resolves on portal venous and delayed phase of imaging is referred to as transient hepatic intensity/attenuation difference (THID/THAD), postulated to be secondary to arterioportal shunts created during needle puncture or coagulated portal vein branches resulting in compensatory increased arterial flow[44]. Over time, the ablation cavity is expected to involute and stabilize in size. Imaging features suggestive of residual viable tumor post-treatment include: Thick peripheral irregular nodular APHE with or without washout appearance, "washout" alone, enhancement characteristics similar to pre-treatment tumor, or discontinuity in the smooth thin peripheral rim of enhancement[45] (Figure 2).

As with ablation, non-radiation arterial-based therapies have a similar evolution of post-treatment appearances. TAE and TACE create ischemic and/or cytotoxic effects that result in cell necrosis; the tumor usually does not change in size early post-treatment, although rarely can slightly increase in size as a result of edema and hemorrhage. As with ablation, the treated tumor should become immediately non-enhancing after transarterial therapy. Often, there is a pronounced surrounding geographic enhancement pattern that persists on portal venous and delayed imaging, which represents perfusional changes secondary to inflammation and arterial embolization[17].

One unique transarterial post-treatment feature is seen when iodized oil is used for embolization. In these instances, the treatment zone appears extremely hyperdense on unenhanced CT, secondary to iodized oil deposition within and around the tumor, limiting assessment for tumor viability on post contrast CT images[46]. Evidence does suggest that the degree of iodized oil deposition within the tumor is an indicator of tumor necrosis, thus could possibly be used as an indirect feature for tumor response assessment; nonetheless, evaluation for residual tumoral enhancement is limited by the hyperdense appearance of the iodized oil. The iodized oil is not apparent on MRI, and thus MRI is preferred to evaluate for APHE in and around the treatment zone to assess for recurrent/viable disease. Just as with ablation, locally recurrent or residual viable HCC presents as irregular, nodular areas of APHE, APHE plus "washout", "washout" alone, or enhancement similar to the pretreatment tumor, within or along the margin of the treated tumor (Figure 3). Some studies have reported that recurrence after TACE and RFA could result in dedifferentiation into more aggressive infiltrative tumor[47-49], which tends to have an atypical appearance on post-therapy imaging (Figure 4); thus one must pay close attention for any changes in the treated tumor, particularly in size.

Radiation-based therapies result in post-treatment imaging appearances distinct from other therapies, particularly on the arterial phase of imaging. For example, early after SBRT and TARE transient increases in tumor size can be seen. Furthermore, tumor shrinkage is often delayed and slow, secondary to the cytostatic effects of TARE[50]. Therefore, size measurements within 3-mo of treatment are not reliable for

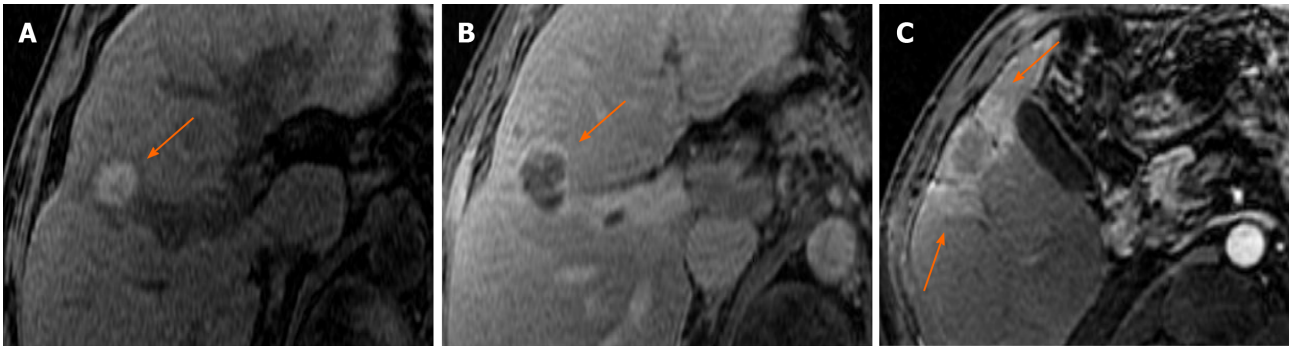

Figure 1 Spectrum of expected post treatment imaging appearances after successful LR TR Nonviable microwave ablation of LR 5 hepatocellular carcinoma in different patients. A: Hyperintense signal on pre-contrast T1 weighted image, which does not enhance (subtraction not shown); B: Thin continuous smooth rim of enhancement surrounding the ablation zone on portal venous phase of imaging; C: Mild peri-tumoral ill-defined geographic areas of arterial phase hyperenhancement within the parenchyma adjacent to the treatment cavity on arterial phase image, which becomes isoenhancing to the parenchyma on delayed phase of imaging (not shown).

prediction of tumor response[18]. Enhancement patterns after TARE are also highly variable, as successfully treated tumors can demonstrate a range of imaging findings, including: (1) Persistent intra-tumoral APHE; (2) Geographic or nodular peri-tumoral APHE; (3) Thin rim of peri-tumoral APHE, and (4) Complete lack of enhancement (Figure 5). Of note, persistent central arterial hyperenhancement can be seen at 3 mo in tumors that have been treated by TARE, specifically in cases that show progressive decrease and eventual lack of enhancement over time from the delayed effects of radiation therapy. Thus, evaluation of these tumors poses a diagnostic and interpretation challenge. Key features suggestive of residual tumor after TARE includes new or enlarging nodular or mass-like APHE within or around the treated tumor and growth over time, particularly when identified more than 6 mo after treatment. Care should be taken not to mistake TARE-related peri-tumoral perfusional change with viable tumor. Close evaluation and comparison of the pre-treatment imaging to identify the tumor margins is essential; additionally, peritumoral parenchymal perfusion tends to be geographic in shape, and either persists on portal venous and delayed phases of imaging or becomes isoenhancing to the remainder of the hepatic parenchyma.

After SBRT, APHE with or without "washout" can persist for up to a year, and even longer, but these imaging features gradually decrease over time[51] (Figure 6). Early post treatment, geographic APHE surrounding the treated tumor is a common finding and likely represents hyperemia; this eventually converts to progressive delayed phase geographic enhancement, likely secondary to radiation fibrosis, usually with associated findings of capsular retraction and peripheral intrahepatic biliary dilatation[52]. The treated tumor should gradually decrease or stay stable in size during the time period where treatment response is evolving (Figure 6). Imaging features suggesting recurrent disease after SBRT include: Increase in size of the treated tumor or new or increasing intensity of APHE after treatment[53]. Although the treated HCC often demonstrates persistent APHE after SBRT, the degree, or intensity, of APHE often decreases as it resolves. Thus, in treated HCC which originally demonstrates persistent but decreasing degree of enhancement or resolution of enhancement, the development of increasing intensity of enhancement or new APHE, is a feature suggesting LR.

LI-RADS TRA

LI-RADS TRA was created to improve the consistency and standardization in reporting treatment response after liver-directed therapy and, unlike other response assessment systems, it has a distinct advantage of providing assessment on a lesion-by-lesion basis, an approach which can potentially improve communications for individualized management considerations. LI-RADS TRA is modelled after mRECIST, as it primarily relies on post treatment APHE to identify viable tumor. However, LI-RADS TRA is unique, because in addition to APHE, the definition of viable tumor includes washout appearance or enhancement similar to that seen before treatment. This may render this advantageous when interpreting treatment response

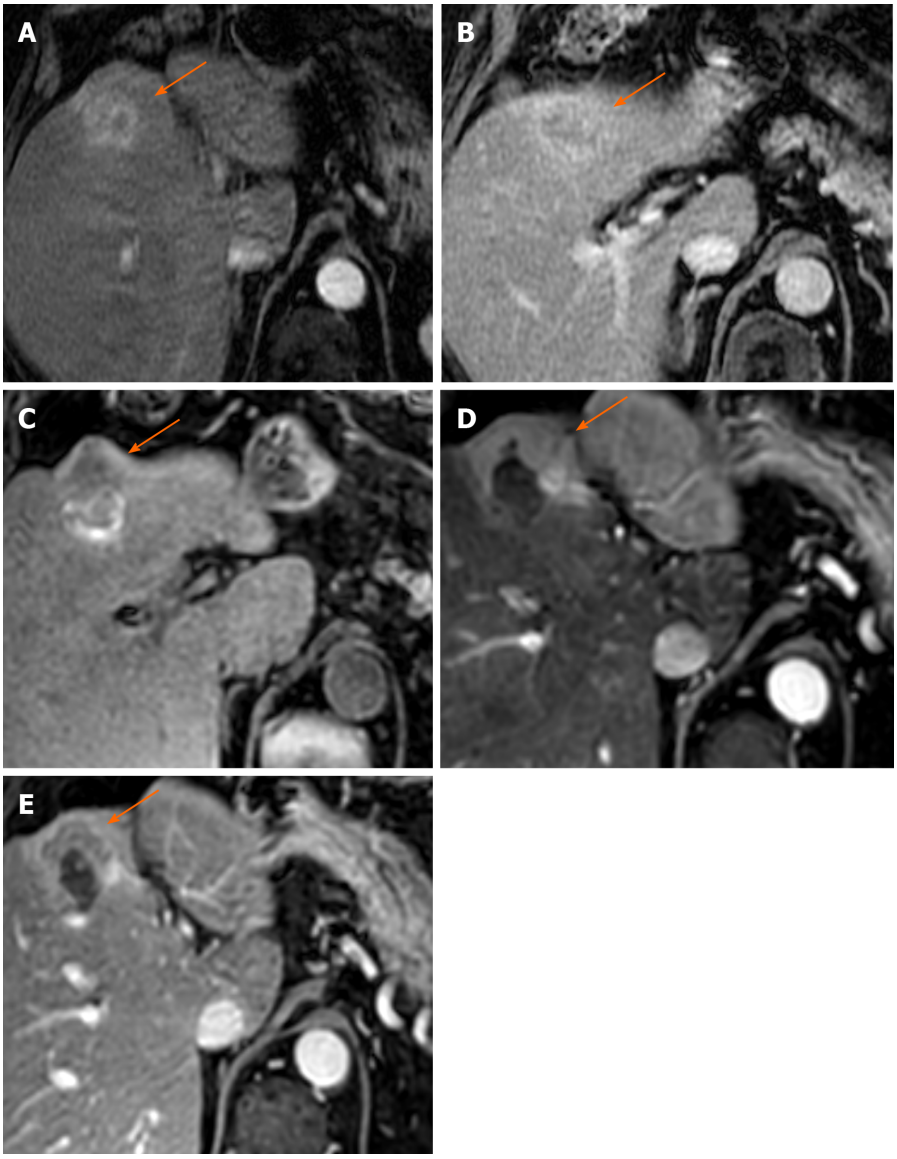

Figure 2 Eighty-three-year old male with nonalcoholic steatohepatitis related cirrhosis presented with a 3.5 cm mass demonstrating arterial phase hyperenhancement (APHE) (A) and "washout" (B), compatible with an LR-5 hepatocellular carcinoma; C: Pre-contrast T1 images 6 mo post microwave ablation demonstrate a hypointense nodular area along the anterior margin of the ablation cavity, with thick irregular nodular APHE on arterial phase (D) and "washout" and "capsule" on portal venous phase (E), LR-TR Viable.

after radiation-based therapies in particular.

During image interpretation of treated HCC, each treated liver observation should be reported separately according to the LR-TR categories[54]. Treatment response categories include: "LR-TR Nonviable", "LR-TR Equivocal" and "LR-TR Viable". In instances where technical limitation precludes characterization of the tumor, an "LR-TR Nonevaluable" category can be assigned[2,55].

LR TR Nonviable

If a treated lesion exhibits no tumoral enhancement or only shows a treatment specific enhancement pattern (which is unique for each LRT, such as thin rim enhancement after ablation), an LR-TR Nonviable category can be assigned[54]. After ablation (MWA/RFA/cryoablation/PEI/IRE) and non-radiation arterial-based therapies (TAE/cTACE/DEB-TACE), a nonviable tumor category is assigned when there is complete tumor devascularization, *i.e.*, complete loss of APHE. One must carefully evaluate the margins of the treated lesion for the presence of nodular or irregular APHE and/or a discontinuous appearance of the thin rim of expected post-treatment enhancement, which suggests viable disease.

In contradistinction, after SBRT and TARE, there is often, but not always, persistent APHE and "washout" within the treated tumor, which can be seen for up to a year,

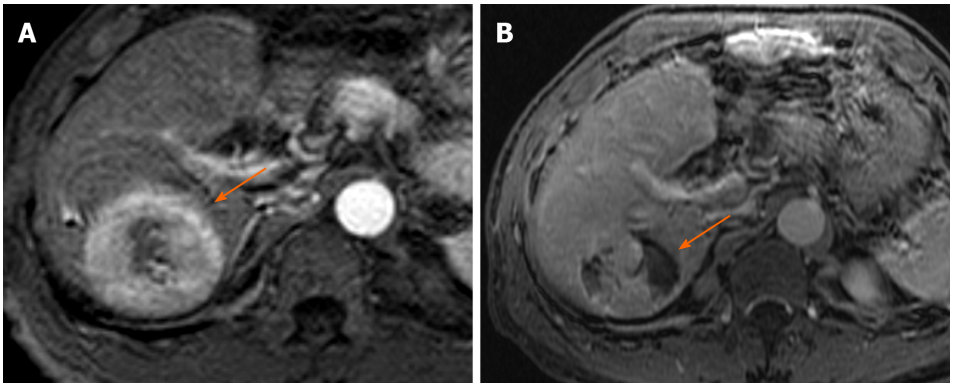

Figure 3 Fifty-four-year old male with hepatitis C virus presents with 5.4 cm hepatocellular carcinoma in the right lobe of the liver demonstrating arterial phase hyperenhancement on arterial phase of imaging (A), LR 5; 1 mo post-transarterial chemoembolization there is significant residual viable enhancing tumor with areas of necrosis on arterial phase imaging (B), LR-TR Viable.

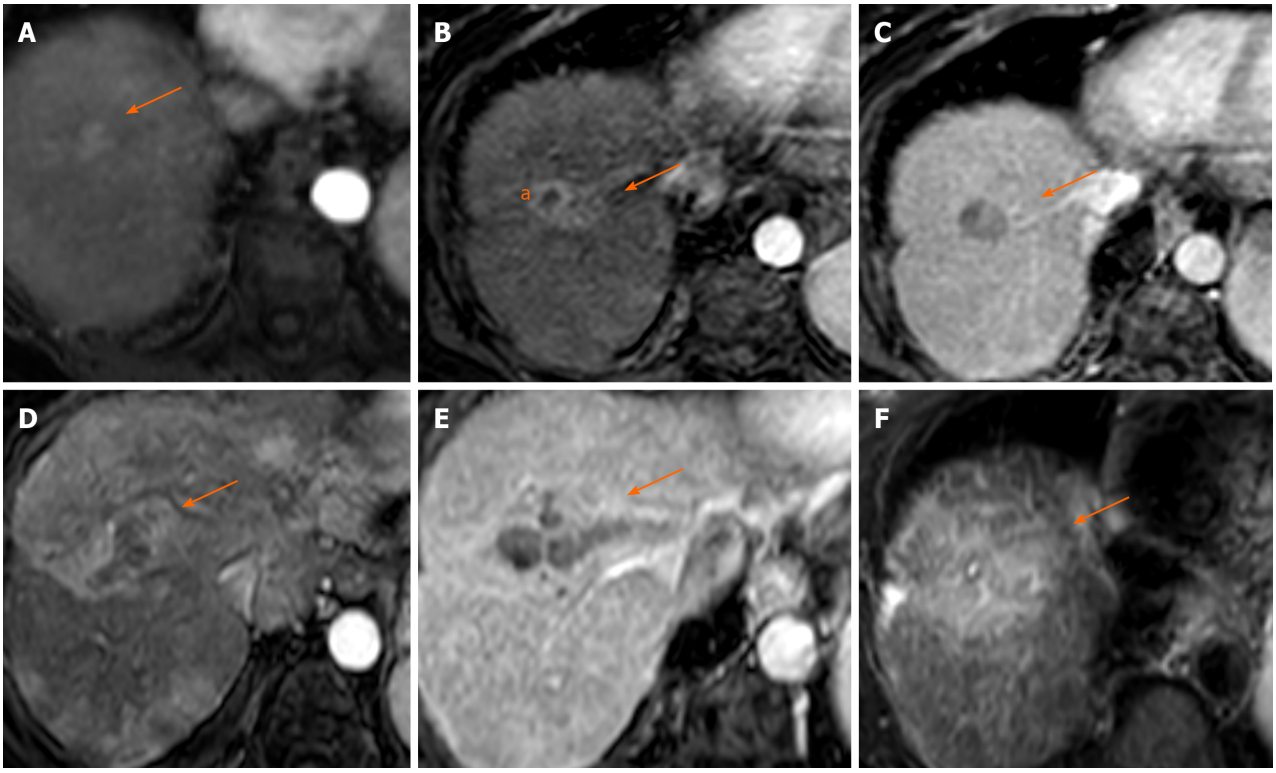

Figure 4 Seventy-three-year old woman with history of nonalcoholic steatohepatitis related cirrhosis presented with 1. 5 cm mass in segment 8 with arterial phase hyperenhancement (APHE) (A) and washout (not shown), LR 5; 1 mo post transarterial chemoembolization (TACE) there is central necrosis with a smooth rim of enhancement ([a]) (B); however, along the medial edge of the treatment cavity is new ill-defined APHE (arrow) (B) with washout (C), LR TR Viable; 3 mo post re-TACE, there was interval development of infiltrative tumor with APHE on arterial phase (D), "washout" on portal venous phase (E) and T2W hyperintensity (F). In addition, new tumor thrombus was seen in the right portal vein (E), LR TR Viable with tumor in vein.

and sometimes longer. This creates a diagnostic dilemma during image interpretation since persistent APHE, albeit a treatment-specific expected appearance, denotes viable disease for other LRTs. Lack of radiology-pathology correlation studies in patients treated with SBRT for HCC limits interpretation of imaging features that translate to true viability or nonviability. Despite early post-treatment APHE after radiation therapy, this frequently resolves over time without additional therapy, and with subsequent decrease in size of the treated lesion (Figure 6). Thus, early post-treatment, a category of LR TR Viable may be misleading and result in unnecessary retreatment if the referring clinician does not understand the time course for tumoral necrosis resulting from radiation. Alternatively, even though APHE is an "expected" imaging feature, categorization as LR TR Nonviable may also be inaccurate if there is ongoing necrosis but residual viable tissue on pathology. Therefore, there is a current gap in

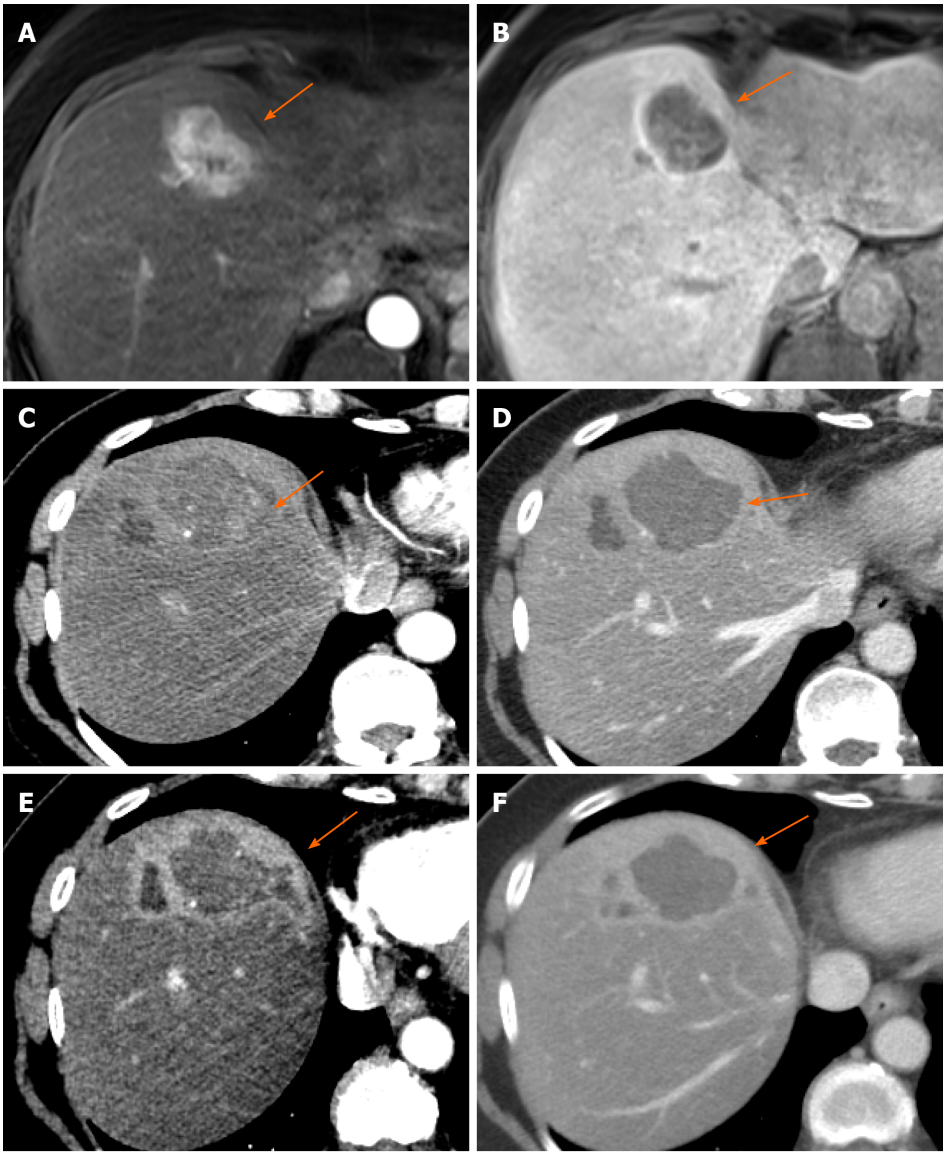

Figure 5 Sixty-two-year old female with LR 5 hepatocellular carcinoma within segment 4a of the liver, demonstrating arterial phase hyperenhancement (APHE) (A) and "washout" (B); 3 mo post transarterial radioembolization (TARE) there is persistent arterial phase nodular enhancement (arrow) (C) with associated "washout" on portal venous phase (D) within the largely necrotic treatment cavity, LR TR Equivocal; 6 mo post-TARE, the treatment cavity decreases in size and the nodular area of APHE is no longer identified on arterial phase (E) and portal venous phase (F), LR TR Nonviable; geographic APHE in the surrounding parenchyma is compatible with post-radiation changes (E and F).

knowledge in the application of LI-RADS TRA after radiation therapy, given the absence of radiology-pathology correlation. Notably, this feature of APHE renders TRA after radiation therapy a challenge with all existing treatment response classification systems.

LR TR Viable

The LR-TR Viable category is considered if there is presence of nodular, mass-like or thick irregular tissue within or along the treated tumor with any of the following features: APHE, washout appearance or enhancement similar to pre-treatment imaging[54]. After ablation or non-radiation arterial-based LRT, this is fairly straightforward; however, for the reasons mentioned above, assigning LR-TR Viable to lesions early after SBRT and TARE therapy remains a diagnostic challenge as APHE is an expected post-treatment imaging finding that can evolve into nonviable disease on subsequent exam[56]. The expected temporal evolution of HCC treated with radiation-based LRT results in decreasing degree/intensity of enhancement with a gradual decrease in size. If there is new or increasing enhancement of the treated tumor or an increase in size of the enhancing tumor post SBRT or TARE, then the LR TR Viable

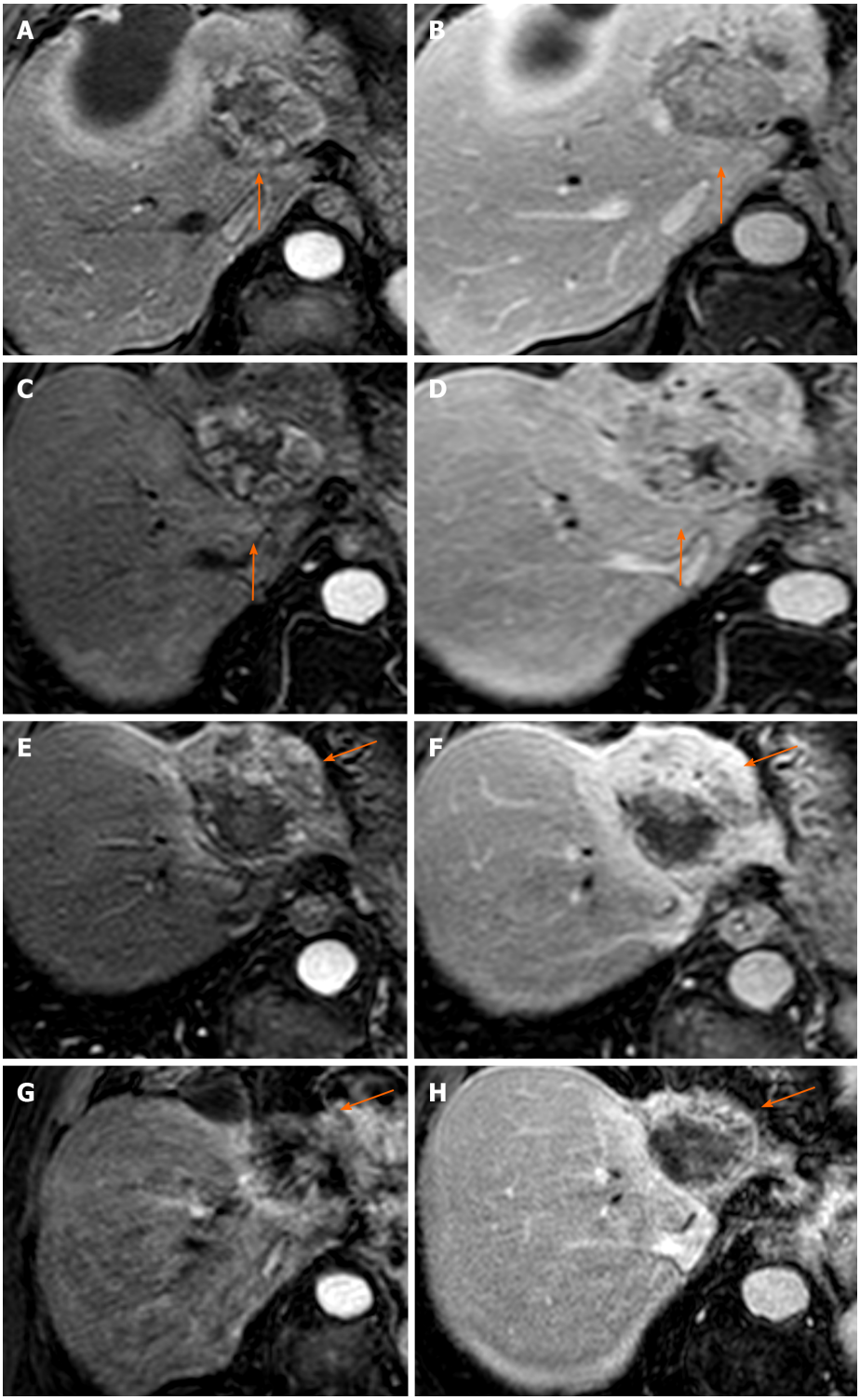

Figure 6 Fifty-eight-year old male presenting 3 mo post transarterial chemoembolization for follow up of a 7.2 cm LR 5 hepatocellular carcinoma. Persistent thick nodular peripheral arterial phase hyperenhancement (APHE) of the treated tumor (A) with "washout" on portal venous phase (B), LR-TR Viable. This lesion was subsequently treated with stereotactic body radiotherapy (SBRT). Three months post SBRT there is persistent APHE on arterial phase (C) and "washout" on portal venous phase (D), with no change in size, LR-TR Viable or Equivocal. Six months post SBRT, there is decreasing size of the tumor to 6.3 cm with decreasing APHE and increasing necrosis, albeit persistent APHE (E) and "washout" (F), LR-TR Equivocal. Fifteen months post SBRT, further decrease in size to 5.1 cm, now with minimal residual APHE (G) and "washout" (H), LR-TR Nonviable.

category should be assigned.

LR-TR Equivocal

A unique aspect of the LI-RADS TRA is the addition of a novel category, LR TR Equivocal. This category allows reporting of lesions when there is uncertainty in viability or nonviability, allowing short-term follow-up for re-evaluation. This is

particularly useful given the increasing complexity in post-treatment imaging appearances, particularly after radiation-based therapies.

The LR-TR Equivocal category is defined as enhancement atypical for treatment specific enhancement pattern and not meeting criteria for probably or definitely viable tumor[54]. There are instances when imaging findings are equivocal following LRT, particularly after arterial-based and radiation-based therapies, which also affect the hepatic parenchyma adjacent to the targeted tumor. The result is the presence of abnormal areas of APHE as a result of altered perfusion around the treated tumor, which can mimic viable disease. In these cases, it may be prudent to assign an LR-TR Equivocal category unless the perfusional alteration is clearly geographic in appearance. While this category may result in increased frequency of follow-up imaging, as well as the risk that viable tumor is left untreated, HCC is generally a slow growing tumor with a double timing of 85.7-117 d[57,58]. Thus, a "wait and watch" approach with 2-3 mo interval imaging can help distinguish true residual disease from benign parenchymal perfusional alterations.

EMERGING EVIDENCE

While the LI-RADS TRA was designed to complement other existing treatment response systems, its utility is limited, as it needs to be validated in clinical practice. There have been a number of recent publications evaluating the performance of LI-RADS TRA. These studies compare imaging to pathologic data, as well as measure the reliability of the TRA categories with inter-reader studies.

Evidence from recent literature suggests moderate inter-reader agreement in assigning LR-TR categories following LRT (ablation and non-radiation arterial-based therapies) for HCC. Two recent studies, Cools *et al*[59] and Chaudhry *et al*[60], have shown high inter-reader agreement in determining LR TR category after thermal ablation (RFA/MWA), with an inter-reader reliability of 90% and 95% and kappa of 0.75 (Standard Error ± 0.09) and 0.71 (95%CI: 0.59-0.84), respectively. Inter-reader agreements are slightly lower when comparing LR TR categorization after non-radiation arterial-based therapy for HCC. Seo *et al*[61], in which 78.6% of tumors were treated with TACE and imaged with either CT or MRI and Shropshire *et al*[62] in which all tumors were treated with TAE and imaging with MRI, reported kappas of 0.69 (CT) and 0.56 (MRI), and 0.55, respectively. These differences in inter-reader agreement between thermal ablation and arterial therapy is not surprising, since the expected imaging appearance post-ablation is simpler compared to the often complex imaging features seen after transcatheter arterial based therapies.

In addition to inter-reader reliability studies, validation studies evaluating the sensitivity and specificity of LR TR algorithm to predict tumor necrosis with radiology-pathology correlation are necessary. Chaudhry *et al*[60] reported that 81% of HCC post-TACE which were categorized as LR-TR Nonviable demonstrate 100% necrosis on pathology. Similarly, Shropshire *et al*[62], reported that 67%-71% of tumors categorized as LR-TR Nonviable after TAE were 100% necrotic at pathology. The reported incidence is not surprising, since the gold standard histopathology would call anything less than 100% necrotic as viable disease. Thus, while microscopic viable tumor is present in a moderate percentage of treated HCC that are deemed nonviable based on imaging features, the clinical significance based on local tumor progression, disease free survival and impact on OS are yet to be determined. Microscopic viable tumor may be of little clinical significance, particularly since national and international guidelines accept the presence of viable tumor, at a specific size threshold, in patients undergoing transplantation. Although, post-liver transplant, achieving a complete pathologic response has been shown to strongly predict tumor-free survival[63].

These studies also reported high sensitivity and specificities when evaluating the radiology-pathology concordance with LR TR Viable categorization. Chaudhry *et al*[60] report 73% of treated lesions characterized as viable disease had < 99% necrosis and Shropshire *et al*[62] report 60%-65% of disease reported as LR TR viable had < 99% necrosis.

The LR-TR Equivocal category has a relatively low sensitivity for predicting tumor necrosis. Radiology-pathology correlation after thermal ablation report that 83% of treated tumors categorized as LR TR Equivocal demonstrate viable neoplasm at pathology; similarly, after TAE, 71% of treated lesions categorized as LR TR Equivocal demonstrate viable neoplasm at pathology[60,62]. Seo *et al*[61] report that 93%-100% of the HCC's treated with TACE, RFA or in combination, which were categorized as LR-TR Equivocal, demonstrated viable disease. All three studies thus report similar findings

with a high percentage showing viable tumor at histopathology when treated tumor is assigned LR-TR Equivocal category. When LR-TR Equivocal categorization was treated as equivalent to viable disease in one study, sensitivity and specificity of detecting viable disease increased from 40%-77% to 81%-85%, across readers[60]. As previously mentioned, these findings are likely related to the ability of pathology to determine microscopic viable tumor which is not evident on imaging. Of note, the ACR LI-RADS manual states that the LR-TR categories were designed to help provide a probability of the presence of viable tumor and do not correspond to histologic viability; hence the presence of microscopic tumor cannot be excluded based on imaging alone[54]. As mentioned above, the impact of microscopic viable HCC in the setting of cirrhosis is yet to be determined in relation to a patient's OS, disease free survival and time to local progression. Although the results show that there is a high rate of viable disease in the LR-TR Equivocal category, further data is needed before its elimination as a concept.

The above studies include HCC treated only with thermal ablation or non-radiation arterial-based therapies. As mentioned earlier, HCC treated with radiation-based therapy has unique post-treatment imaging features of persistent APHE which confounds image interpretation and can result in a high false positive rate of LR TR Viable categorization. The current challenge is the limited number of studies evaluating radiology-pathology correlation after radiation-based LRT. A study by Mendiratta-Lala *et al*[64], in which 10 SBRT treated HCC's had corresponding explant or AFP values as a surrogate biomarker, reported imaging findings of persistent APHE (4/10) and washout (9/10) at 12 mo, in which all of the treated HCC showed complete tumor necrosis on explant pathology or normalization of AFP values. Moore *et al*[65] evaluated the role of SBRT as bridge to liver transplantation for early stage inoperable disease in a small cohort, and reported no SBRT-related mortality or recurrence. Amongst the explants, there were 3 (27%) that showed CR, 6 (54.5%) pathological partial response and 2 (18%) pathological stable disease. Currently, no inter-reader agreement studies have been performed assessing LI-RADS treatment response categories in patient treated with radiation-based therapies and explant pathology to determine actual tumor necrosis. Similar gaps in knowledge are present for the use of mRECIST in patients treated with SBRT.

Riaz *et al*[66] evaluated the degree of tumor necrosis on explant pathology in 37 lesions post TARE and found complete pathologic necrosis in 61% of the lesions, even in the setting of residual nodular enhancement on imaging pre-transplant. Radiologic findings of these treated lesions were compared to the pathologic findings to determine the predictability of actual tumor necrosis by imaging. WHO and EASL treatment response categories were assigned as CR in 78% and 100% of lesions at a median time of 34 d (95%CI: 29-43) and 126 d (95%CI: 80.2-313.2), respectively. It was also noted that the longer the time to liver transplant, the greater the degree of tumor necrosis identified within the lesion, with the least percentage of tumor necrosis seen in explants at 3 mo post TARE[66]. Thus, it is possible that residual APHE in the early post TARE treatment period does correspond to some viable disease, but it is viable disease that will decrease over time as the radiation effects progress. In this context, LR-TR Equivocal may be the best option for TARE treated lesions in the first three months of treatment. As mentioned previously, currently all of the treatment response classification systems are limited in their ability to accurately assess treatment response after radiation-based therapy given the persistence of APHE early post-treatment.

MANAGEMENT BASED ON LR-TR CATEGORIES

With the advent of different types of LRT for treatment of HCC, it is extremely challenging to develop a dedicated management pathway that can be applicable to all patients. Thus, while no specific management recommendations exist, LI-RADS TRA provides lesion by lesion assessment which, when discussed in a multidisciplinary setting, may allow improved communication and management in this cohort of patients. However, unlike mRECIST, EASL, WHO and RECIST which have a multitude of validating literature, the LR-TR algorithm is relatively new. Future anticipated studies validating the LR-TR algorithm will therefore improve our ability to create standardized guidelines for post treatment management and better predict outcomes.

CURRENT LIMITATIONS OF LI-RADS TRA

The main limitation of LI-RADS TRA is the small number validation studies, given its recent introduction in 2017, although the published sensitivity/specificity for a subset of LRTs is promising. Further studies investigating its use in tumors treated with radiation-based therapy are sorely needed. Second, the LR TR algorithm is not yet applicable to tumor treated with systemic and/or biologic therapy. Given that LRT is increasingly used in combination with systemic therapy, this will remain a challenge to address. Third, there is no dedicated post-treatment specific imaging follow-up interval recommendation, partly due to the variable evolution of post treatment necrosis after different forms of LRT, and institution-specific imaging protocol. Fourth, the long-term utility of the LR-TR Equivocal category remains to be seen, given the evidence that most LR-TR Equivocal lesions are viable in the studies published to date.

CONCLUSION

With the increasing incidence of HCC and the increasing number of LRTs available, the complexity in assessing treatment response will also rise. Nevertheless, post treatment imaging will always play a critical role in providing the clinician a road map to direct further management. It is thus essential for diagnostic radiologists to understand interpretation of post-treatment imaging findings specific to each form of LRT. Current existing treatment response classification systems such as RECIST, mRECIST, EASL and WHO are fraught with their own unique limitations when assessing LRT for HCC, including lack of change in size post-treatment (thus rendering RECIST and WHO limited), and persistent post-treatment enhancement after radiation-based therapies (thus rendering mRECIST and EASL limited). LI-RADS TRA provides a new framework to describe treatment response for each individual lesion and the emerging evidence is promising for ablation and non-radiation based arterial therapies. LI-RADS TRA should be used cautiously for radiation-based therapies (TARE, SBRT) in which early post-treatment persistent APHE is common and expected. Its current limitations will be addressed as future studies investigate its performance and inform refinements of future versions.

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MINIREVIEWS

Tumor necrosis family receptor superfamily member 9/tumor necrosis factor receptor-associated factor 1 pathway on hepatitis C viral persistence and natural history

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Abstract

Hepatitis C virus (HCV) infection is an excellent immunological model for understanding the mechanisms developed by non-cytopathic viruses and tumors to evade the adaptative immune response. The antigen-specific cytotoxic T cell response is essential for keeping HCV under control, but during persistent infection, these cells become exhausted or even deleted. The exhaustion process is progressive and depends on the infection duration and level of antigenemia. During high antigenic load and long duration of infection, T cells become extremely exhausted and ultimately disappear due to apoptosis. The development of exhaustion involves the impairment of positive co-stimulation induced by regulatory cytokines, such as transforming growth factor beta 1. This cytokine downregulates tumor necrosis factor receptor (TNFR)-associated factor 1 (TRAF1), the signal transducer of the T cell co-stimulatory molecule TNFR superfamily member 9 (known as 4-1BB). This impairment correlates with the low reactivity of T cells and an exhaustion phenotype. Treatment with interleukin-7 *in vitro* restores TRAF1 expression and rescues T cell effector function. The process of TRAF1 loss and its *in vitro* recovery is hierarchical, and more affected by severe disease progression. In conclusion, TRAF1 dynamics on T cells define a new pathogenic model that describes some aspects of the natural history of HCV, and sheds light on novel immunotherapy strategies for chronic viral infections and cancer.

Key Words: Hepatitis C virus; Tumor necrosis factor receptor-associated factor 1; CD8; Exhaustion; Tumor necrosis family receptor superfamily member 9; Chronic hepatitis

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Core Tip: Tumor necrosis factor receptor-associated factor 1 (TRAF1) is the signal transducer of the positive checkpoint tumor necrosis family receptor superfamily member 9 (4-1BB), essential in the activation of adaptive immune response. During persistent hepatitis C virus (HCV) infection, this transducer is down-regulated *via* transforming growth factor beta 1, linked to T cell exhaustion. Interleukin-7 can restore TRAF1 expression and improve T cell reactivity but only in patients with mild evolution, while cases with a more aggressive progression also need the modulation of other negative co-stimulatory molecules. Therefore, TRAF1 dynamics defines a new pathogenic model that explains the different level of T cell exhaustion and progression during HCV infection and supports the rationale for immunotherapeutic strategies in chronic viral infections.

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INTRODUCTION

Hepatitis C virus (HCV) evolution is heterogenous as a result of the particular interplay between the virus and the immune system[1]. The outcome of the fight between host and pathogen depends on the balance of the host-microbe interaction, which causes varying degrees of progressive liver damage[2-5]. The fine-tuning of this equilibrium can induce either rapid or slow disease progression, which depends on the degree of impairment of the adaptive immune system[6]. During persistent non-cytopathic viral infection, the antigen (Ag)-specific T cell response is exhausted and unable to clear infection despite achieving partial viral control[7,8]. The correct activation of this response relies on the interaction with Ag-presenting cells (commonly known as APCs) in the proper cytokine environment with the right co-stimulation[1,9,10]. Non-cytopathic viruses manipulate T cell co-stimulation for their own benefit, favoring the induction of negative co-stimulatory receptors and inhibiting positive co-stimulatory pathways[11-13]. Tumor necrosis factor receptor (TNFR) superfamily member 9 (4-1BB) is a TNFR-associated factor 1 (TRAF1)-binding checkpoint molecule that is normally absent from resting cells but is induced by T cell receptor (TCR) signaling[14]. It is a positive activator of the T cell response, which is key during viral infection and cancer. TRAF1 is the major signal transducer after 4-1BB triggering[15], and its downregulation on T cells is used by pathogens as a mechanism to evade specific adaptive immune responses[2,16].

In this review, we present an update on the current knowledge of the role of the 4-1BB/TRAF1 pathway in the outcome of HCV infection, and how it can be manipulated to overcome T cell exhaustion. Although this immunotherapeutic strategy is no longer needed in the era of direct acting anti-viral (commonly referred to as DAA) medications[17,18], lessons obtained from this persistent infection model can be extrapolated to other viral infections, such as hepatitis B virus (known as HBV) and human immunodeficiency virus (HIV), or cancer.

ROLE OF T CELLS IN THE NATURAL HISTORY OF HCV

HCV is a highly variable, positive-sense, single-stranded hepatotropic non-cytopathic RNA virus of the family *Flaviviridae*[19,20], with parenteral, vertical, and sexual transmission capacities[21]. HCV induces progressive liver damage that can lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma[3,22]. About one-third of patients spontaneously clear the virus but in the remaining two-thirds, the infection persists unless an anti-viral treatment is administered[5]. Currently, the infection is easily controlled by using DAA drugs[17]. Nevertheless, it is still possible to learn from

HCV about the host-pathogen interaction in chronic viral diseases, which can be applied to other chronic viral infections and cancer.

During the natural history of untreated, persistent HCV infection, there are three different progression groups: Slow, mild, and rapid fibrosers. Slow fibrosers do not develop significant fibrosis during their life, whereas rapid fibrosers can progress to cirrhosis, portal hypertension, or hepatocellular carcinoma in as quickly as 10-20 years after primoinfection[23]. Host factors such as sex, age of infection, alcohol consumption, co-infection with HIV or HBV, steatosis, and insulin resistance[23,24], as well as the quality of the adaptive immune response[1], are involved in the different evolution patterns of HCV. HCV-specific cytotoxic T cells play a central role in controlling HCV infection[25,26]. During persistent HCV infection, however, the cytotoxic T cell response becomes dysfunctional, with cells presenting markers of exhaustion and apoptosis[27-30]. Nevertheless, these HCV-specific CD8 T cells can still partially control viral replication[31].

Interestingly, it is not HCV-specific CD8 T cells but other inflammatory cells recruited to the infected liver that are ultimately responsible for persistent liver damage[32,33] (Figure 1). Therefore, long-lasting infection linked to a weak CD8-specific T cell response can induce permanent non-specific inflammatory infiltrates that can promote the rapid progression of liver fibrosis[33,34]. In fact, a high level of prolonged antigenemia induces a hierarchical loss of effector functions and ultimate apoptosis of T cells[35]. During persistent HCV infection, the level of specific T cell impairment positively correlates with the speed of liver fibrosis progression. These data suggest that stronger T cell exhaustion may facilitate rapid fibrosis progression. In support, rapid fibrosers with long-lasting infection lack detectable peripheral HCV-specific cytotoxic T cells, which although exhausted, are present in slow fibrosers and short-term disease[2]. Consequently, it may be possible to restore specific T cell responses to improve viral control, and in addition, to prevent liver damage by reducing pro-inflammatory chemokines and cytokines secreted in the infected liver.

During chronic hepatitis C, some pro-fibrogenic and immunoregulatory cytokines, such as transforming growth factor beta 1 (TGF-β1) are increased. *In vitro* analysis has shown that after Ag encounter, HCV-specific CD8 T cells secrete TFG-b1, which is linked to effector dysfunction and can be rescued by anti-TGF-β1 blocking antibodies[36]. Moreover, HCV itself is able to induce liver cells to express TGF-β1, and the number of TGF-β1-secreting regulatory T cells is also enhanced during chronic hepatitis C infection[37,38]. Among its immunoregulatory properties, TGF-β1 has been linked with the negative modulation of the positive co-stimulatory checkpoint 4-1BB/TRAF1 in some chronic viral infections, such as those by HIV, HCV, and lymphocoriomeningitis virus[2,16].

In the next sections of this review, this specific pathogenic axis will be discussed in detail.

4-1BB/TRAF1 PATHWAY

4-1BB, also called CD137, is a co-stimulatory checkpoint that is predominantly expressed on activated CD8 T cells and natural killer cells[39], and in lower levels on CD4 T cells, dendritic cells, granulocytes, and mast cells[40]. It binds to 4-1BB-ligand (4-1BBL, CD137L, or L/TNFR9), which is present on such APCs as activated B cells, dendritic cells, and macrophages[41]; the 4-1BB/TRAF1 pathway is shown in Figure 2. 4-1BBL trimer has a three-bladed propeller structure and binds to three 4-1BB receptor monomers[42]. 4-1BB translocates to the membrane after Ag encounter on $CD8^+$ T cells[43], recruiting the TRAF family members TRAF1, 2, and 3[44]. Signaling through the 4-1BB receptor depends on the association with TRAF1 and 2 molecules, as evidence shows that the lack of any of them blocks 4-1BB/4-1BBL downstream transduction[16,45].

TRAF 1, 2, and 3 can form heterodimers and interact with adaptor proteins (*i.e.*, ubiquitin ligases, proteases, kinases), creating a three-dimensional structure complex where enzymatic processes can be carried out[46]. TRAF1 differs from the other members of its family, as it lacks the N-terminal RING finger domain, which prevents it from acting as an E3 ubiquitin ligase. However, TRAF1 acts as a bridge between a wide range of adaptor proteins, regulating their activity[47] and interacting with several TNFR members, prompting their stimulation or inhibition. TRAF1 has a role in T cell activation through the canonical nuclear factor-kappa B (NF-κB) pathway and an alternate pathway. These two different mechanisms of action regulate the physiology of T cells. In the canonical pathway, TRAF1 is inducible after cell activation through NF-κB[48], and is present in a restricted group of cells in which activated lymphocytes

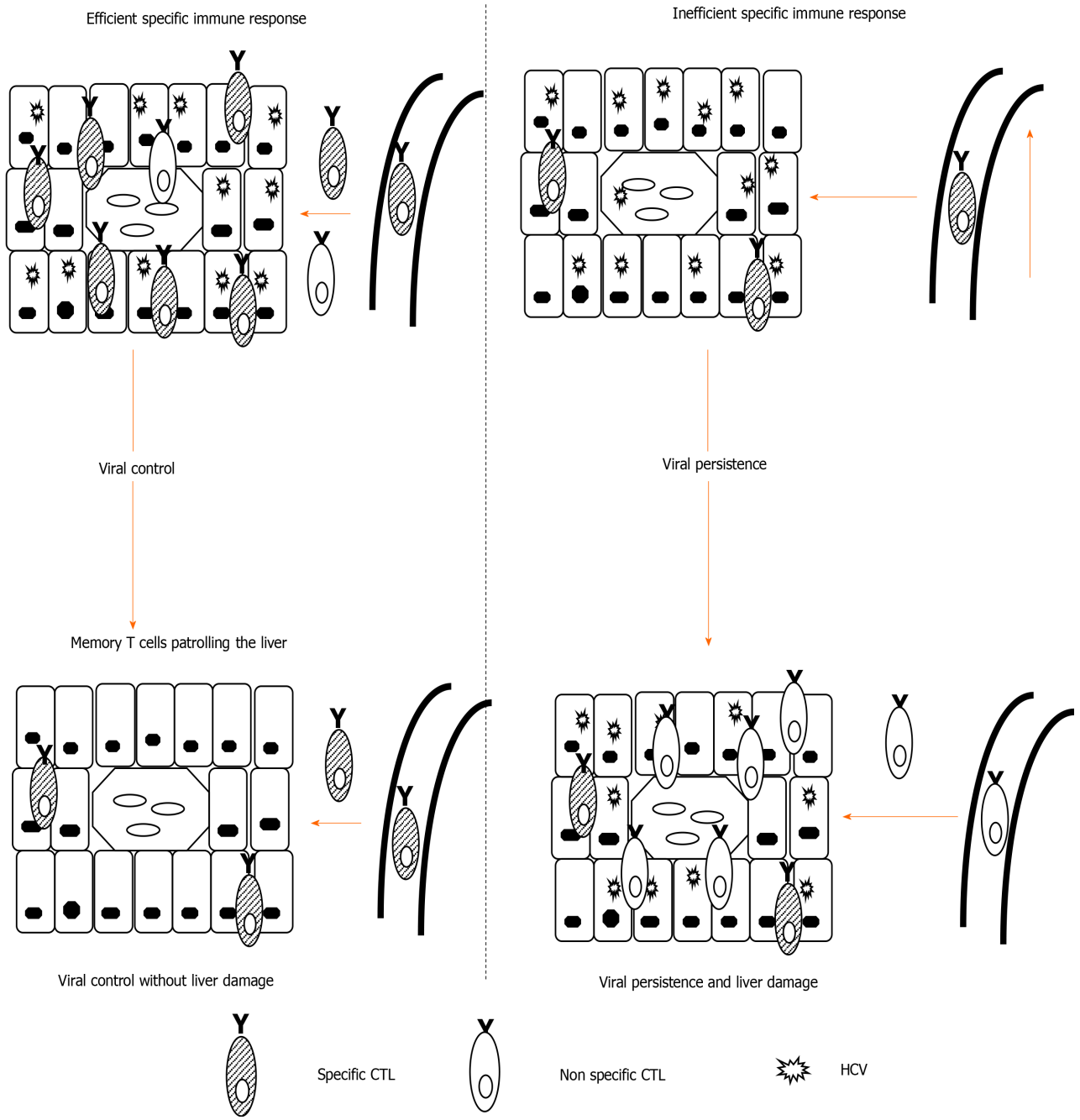

Figure 1 Theoretical model of liver damage during chronic viral hepatitis due to non-specific inflammatory infiltrate. Left-side: Depiction of an efficient hepatitis C virus (HCV)-specific cytotoxic T cell (CTL) controlling HCV in the liver; Right-side: Depiction of HCV-specific exhausted CTLs unable to control HCV replication. Hepatocytes steadily secrete chemokines that attract specific and non-specific infiltrate, the latter of which is responsible for liver damage. CTL: Cytotoxic T cell; HCV: Hepatitis C virus.

are included[49]. TRAF1 regulates survival signals mediated by TRAF2, modulating their ability to mediate sustained activation of NF-κB and c-Jun N-terminal kinase[50]. Specifically, TRAF1 is implicated in extracellular signal-regulated kinase (ERK) activation mediated by leukocyte-specific protein 1[51].

ERK phosphorylates Bim, eliciting its elimination by the proteasome and abrogating its anti-apoptotic effects[52]. The formation of two heterotrimers TRAF1:TRAF2 results in the recruitment of cellular inhibitor of apoptosis protein (cIAP) as well as the interaction with other adaptor proteins and protein kinases, which leads to activation of the NF-κB pathway[53]. TRAF2 can also dimerize to activate E3 ubiquitin ligases through their RING finger domains. Evidence indicates that the interactions among different TRAFs heterodimers allow them to adopt an octagonal superstructure where many 4-1BB/4-1BBL act simultaneously. This structure has been called the 4-1BB signalosome and could provide a model to design novel 4-1BB analogues as immunotherapeutic strategy[46]. Downstream signaling leads to the phosphorylation of

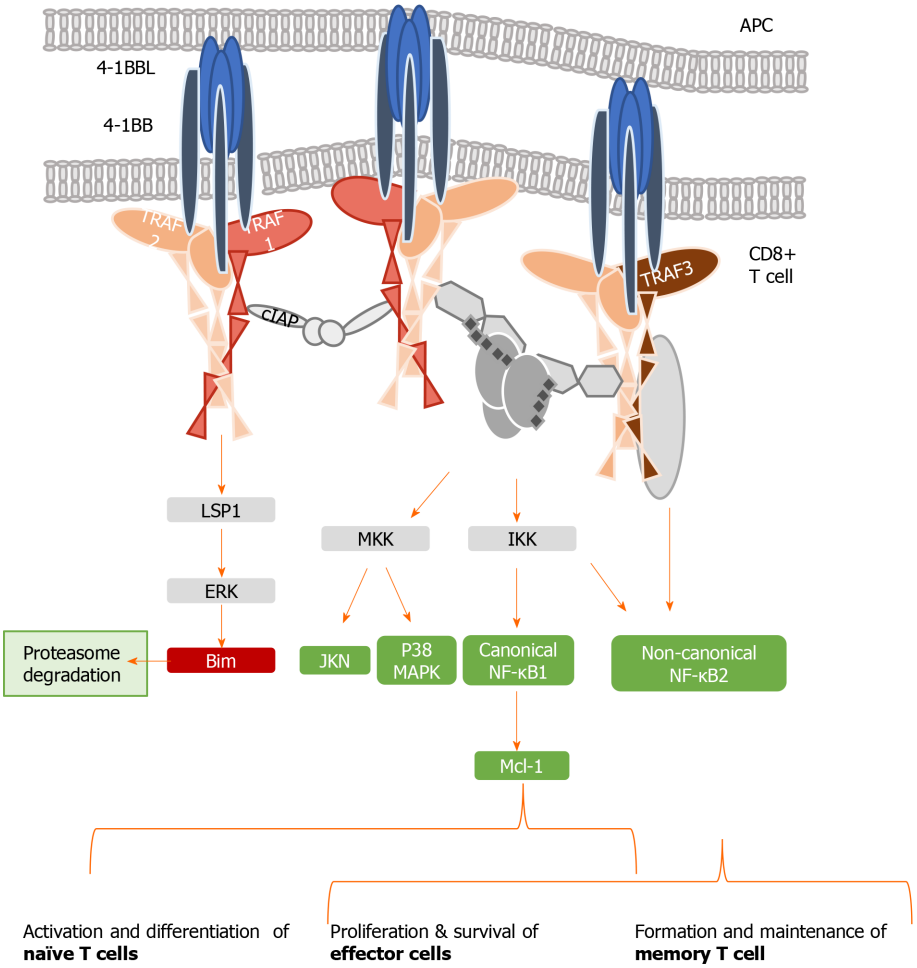

Figure 2 Tumor necrosis family receptor superfamily member 9/tumor necrosis factor receptor-associated factor 1 signaling complex. Schematic representation of tumor necrosis family receptor (TNFR) superfamily member 9 (4-1BB) signaling pathways, indicating the interaction between the trimeric 4-1BB ligand presented by the antigen presenting cell and the three molecules of the receptor 4-1BB. The signal transduction occurs through tumor necrosis factor receptor-associated factor (TRAF) 1. Representative combinations of TRAF1, 2, and 3 and their interactions with adaptor proteins are presented. Canonical activation of nuclear factor kappa B (NF-κB) leads to the activation of naïve T cells, which differentiate into effector cells and proliferate after antigen encounter. Non-canonical NF-κB bestows proliferation and survival of effector cells and also drives the generation and maintenance of memory T cells in a delayed manner. APC: Antigen-presenting cell; 4-1BB: Tumor necrosis family receptor superfamily member 9; 4-1BBL: 4-1BB-ligand; TRAF: Tumor necrosis factor receptor-associated factor; cIAP: Cellular inhibitor of apoptosis protein; ERK: Extracellular signal-regulated kinase; MKK: Mitogen-activated protein kinase kinase; IKK: Inhibitory kappa B kinase; MAPK: Mitogen-activated protein kinases; NF-κB: Nuclear factor kappa B; Mcl-1: Myeloid leukemia cell differentiation protein.

inhibitor of kappa B kinase subunit β and subsequent activation of canonical NF-κB[54], ERK1/2[55], and p38 mitogen-activated protein kinase[56]. Collectively, this 4-1BB-dependent modulation results in CD8 T cell proliferation and survival.

When TNFR signaling is active, TRAF1 also engages the non-canonical NF-κB pathway by degrading TRAF3[54,57,58]. Initiation of the non-canonical NF-κB pathway is delayed with respect to the canonical one, which may play a role in T cell activation and memory differentiation[56]. Thus, in contrast to the rapid and transient activation of the canonical NF-κB pathway, activation of the non-canonical NF-κB pathway is characteristically slow and persistent. On the other hand, TRAF1 also regulates the canonical pathway by preventing TRAF2 degradation or enhancing cIAP recruitment, degrading NF-κB-inducing kinase, which is necessary for activation of the alternate NF-κB pathway[14,58]. Therefore, TRAF1 is a key transducer involved in initial T cell activation and proliferation by the canonical NF-κB pathway, but also in the generation of the memory and effector pool in a delayed manner through the non-canonical NF-κB pathway[54,56].

Figure 2 summarizes the different pathways involved in 4-1BB signaling.

4-1BB/TRAF1 AND SPECIFIC CYTOTOXIC T CELL RESPONSE

Cytotoxic T cells carry out an essential task in non-cytopathic virus control[59,60]. This population is able to recognize infected cells and clear the virus by cytopathic and non-cytopathic mechanisms. Follow-up of healthcare workers after accidental needlestick HCV exposure showed that in those who naturally controlled the virus, HCV-specific CD8 T cells initially destroyed some hepatocytes but later removed the virus by releasing interferon-g[60]. These immune cells become activated by the combination of three different signals. First of all, the interaction between the APC and the TCR is necessary[61]. Thereafter, the interleukin (IL)-2 receptor is upregulated and its subsequent activation promotes T cell proliferation[62]. These two signals must be combined with the activation of early and late positive co-stimulatory checkpoints. Early positive co-stimulatory CD27 and CD28 counteract the inhibitory effects of negative checkpoints such as programmed cell death protein-1 (PD-1)[63-65]. Late positive co-stimulatory molecules such as 4-1BB play an important role in boosting the T cell response and inducing memory generation[14,66].

The 4-1BB/TRAF1 pathway promotes T cell memory formation[67] and survival[55,68] but also regulates effector T cell trafficking into the infected organ[69]. The triggering of this pathway can also improve T cell effector function by mitochondrial morphological and functional reprogramming[12,70,71]. Noteworthy, 4-1BB co-stimulation activates glucose and fatty acid metabolism to enhance CD8 T cell reactivity[72]. As noted above, the role of 4-1BB in T cell survival is mainly mediated *via* ERK by the downregulation of the pro-apoptotic protein Bim[55,73,74]. Thus, pharmacological intervention of this pathway can improve the T cell response by increasing survival and reactivity.

Tumors and persistent viral infections counter positive co-stimulation by early induction of negative checkpoints and inhibition of the positive checkpoints[7]. During non-cytopathic persistent viral infections, specific CD8 T cells are characterized by the expression of negative co-stimulatory molecules such as PD-1, T cell immunoglobulin and mucin-domain containing-3, and cytotoxic T-lymphocyte protein 4[11,27,75]. In addition, these viruses can impair downstream signaling of 4-1BB by causing the loss of its signal transducer TRAF1[16], which explains why positive immunotherapeutic modulation of 4-1BB has failed to boost the virus-specific CD8 T cell response[76]. During chronic lymphocytic choriomeningitis virus infection in mice, TRAF1 Loss on specific CD8 T cells is caused by TGF-β1-induced TRAF1 degradation, and this effect can be counter-regulated by common-g chain receptor cytokines, such as IL-7[16].

Interestingly, similar data have been reported for some human infections. Particularly, in chronic progressors during HIV infection, TRAF1 expression is lower than in elite controllers[16]. T cells from those elite controllers are more active in controlling HIV-infected cells and the process is correlated with TRAF1-mediated Bim downregulation. Indeed, the T cell response during HCV infection shares many features with HIV, and consequently, TRAF1 signaling could also be involved in HCV-specific T cell exhaustion, as will be discussed in the next section.

TRAF1 INVOLVEMENT IN HCV T CELL EXHAUSTION

Exhausted HCV-specific cytotoxic T cells are characterized by the high expression of negative checkpoint proteins, such as PD-1, and low expression of the IL-7 receptor CD127[27] (Figure 3). Lack of CD127 makes these cells less sensitive to the pro-survival cytokine IL-7, which stabilizes the anti-apoptotic protein myeloid leukemia cell differentiation protein (Mcl-1)[28] (Figure 3). IL-7/IL-7R signaling positively regulates Mcl-1 *via* signal transducer and activator of transcription 5[77] but also increases TRAF1 level[16] (Figure 4). As previously stated, 4-1BB/TRAF1 also counters Bim *via* ERK signaling[55] (Figure 4). Moreover, during persistent HCV infection, TGF-β1 Level is increased, and this cytokine downregulates TRAF1 expression on T cells. Hence, during HCV infection, the combination of low IL-7 sensitivity linked to the higher TGF-β1 Level could be the "perfect storm" to desensitize 4-1BB signaling *via* TRAF1 Loss. This suggests that, as in HIV infection[16], the loss of TRAF1 in HCV-specific CD8 T cells during chronic hepatitis C is central to the aforementioned imbalance between Bim and Mcl-1[28] (Figures 2 and 3). Therefore, HCV-specific T cells could be poorly reactive and prone to apoptosis due to the lack of signaling by IL-7 and 4-1BB.

TGF-β1 Levels are increased during persistent HCV infection[2,36,37] and there is low IL-7 receptor expression on T cells. TRAF1 is positively and negatively regulated by IL-7 and TGF-β1, respectively[16]. With this in mind, we hypothesize that high TGF-β1 Level during HCV infection could downregulate TRAF1, impairing 4-1BB signaling

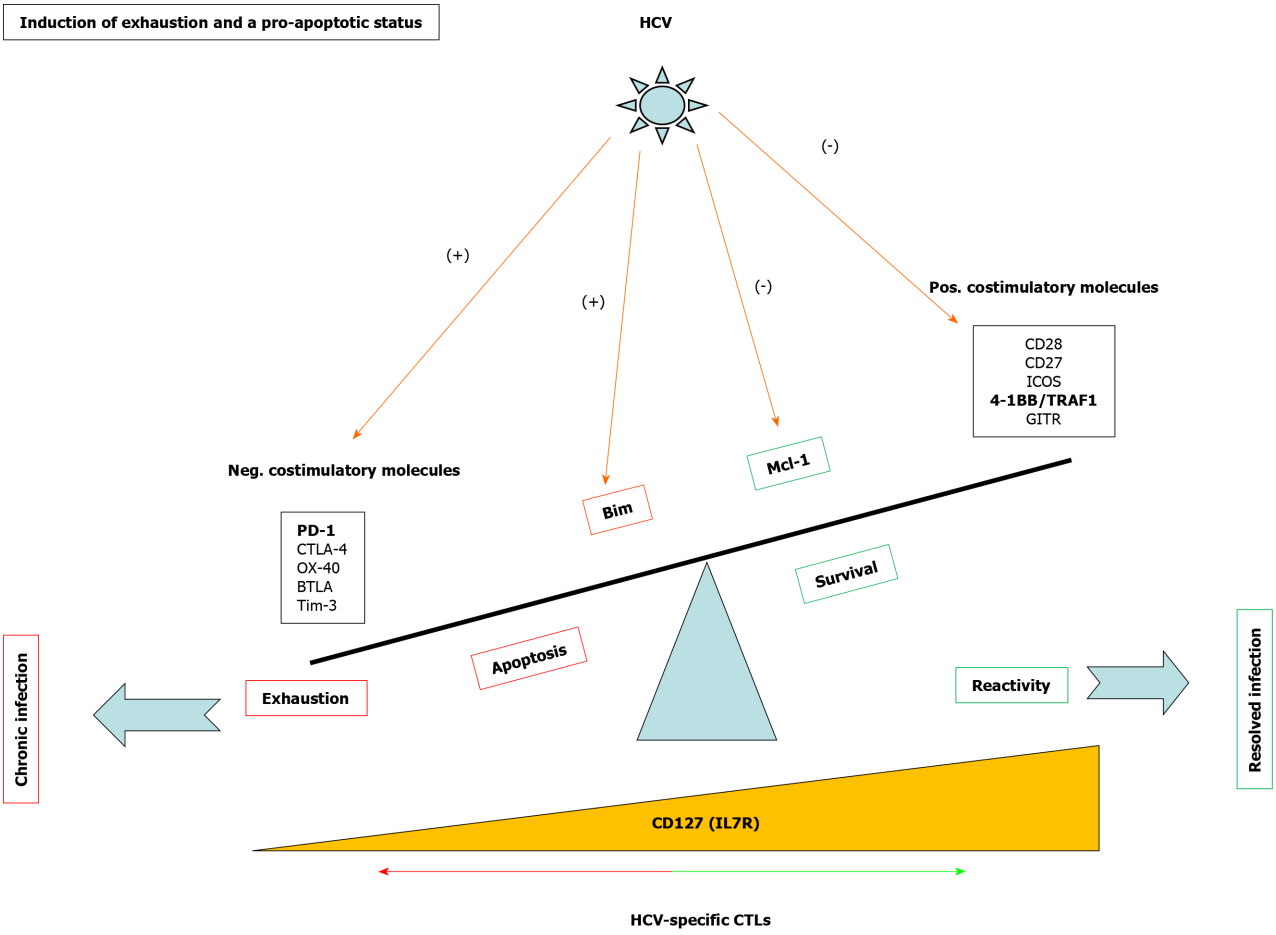

Figure 3 Mechanisms involved in T cell exhaustion and apoptosis during persistent hepatitis C virus infection. Scheme showing positive and negative checkpoints and proteins involved in CD8 T cell reactivity and apoptosis during hepatitis C virus infection. In TextTitle are highlighted the pathways discussed in the current review. HCV: Hepatitis C virus; 4-1BB: Tumor necrosis family receptor superfamily member 9; TRAF: Tumor necrosis factor receptor-associated factor; GITR: Glucocorticoid-induced tumor necrosis factor receptor-related protein; CTL: Cytotoxic T lymphocyte; Neg: Negative; Pos: Positive; PD-1: Programmed cell death protein-1; Mcl-1: Myeloid leukemia cell differentiation protein; IL: Interleukin.

and upregulating Bim. Furthermore, low CD127 expression on HCV-specific CD8 T cells would also reduce Mcl-1 Levels. The combination of low Mcl-1 and high Bim levels would synergize to negatively affect T cell proliferation, cytotoxicity, and survival (Figure 4).

To test this hypothesis, our group detected TRAF1 expression directly *ex vivo* on HCV-specific CD8 T cells from chronically-infected and treated patients. As was expected, those individuals with persistent viral replication had lower TRAF1 expression than HCV controllers[2]. Moreover, TRAF1 expression was inversely correlated with the exhausted and pro-apoptotic phenotypes and directly correlated with T cell reactivity. Low TRAF1 expressing T cells were PD-1^{high}, Mcl-1^{low}, and CD127^{low}, and did not expand after Ag encounter. Analysis of the supernatants of Ag-specific T cell cultures showed that those cases with less proliferative potential had higher levels of TGF-β1. Moreover, a negative correlation was also observed between serum TGF-β1 Level and TRAF1 expression on Ag-specific CD8 T cells. Furthermore, TGF-β1 *in vitro* treatment of HCV-specific CD8 T cells from resolvers induced TRAF1 downregulation, and this effect was counteracted by IL-7 treatment. Although the CD127 expression level is low in the effector progeny subset, the low frequency progenitor pool still maintains this receptor, and it is this population that is suitable for immunotherapy[78,79]. Moreover, IL-7 at a therapeutic dose can antagonize multiple cellular and molecular networks[80]. These data suggest that during persistent HCV infection, TGF-β1 downregulates TRAF1 in T cells, which can be reversed by *ex vivo* IL-7 treatment.

Consequently, we developed an IL-7 and 4-1BBL combination treatment to improve T cell reactivity; IL-7-dependent upregulation of TRAF1 restored 4-1BB signaling to fully enable the agonist actions of 4-1BBL over 4-1BB. We observed a hierarchical response that was dependent on the stage of HCV infection; only cases with less severe

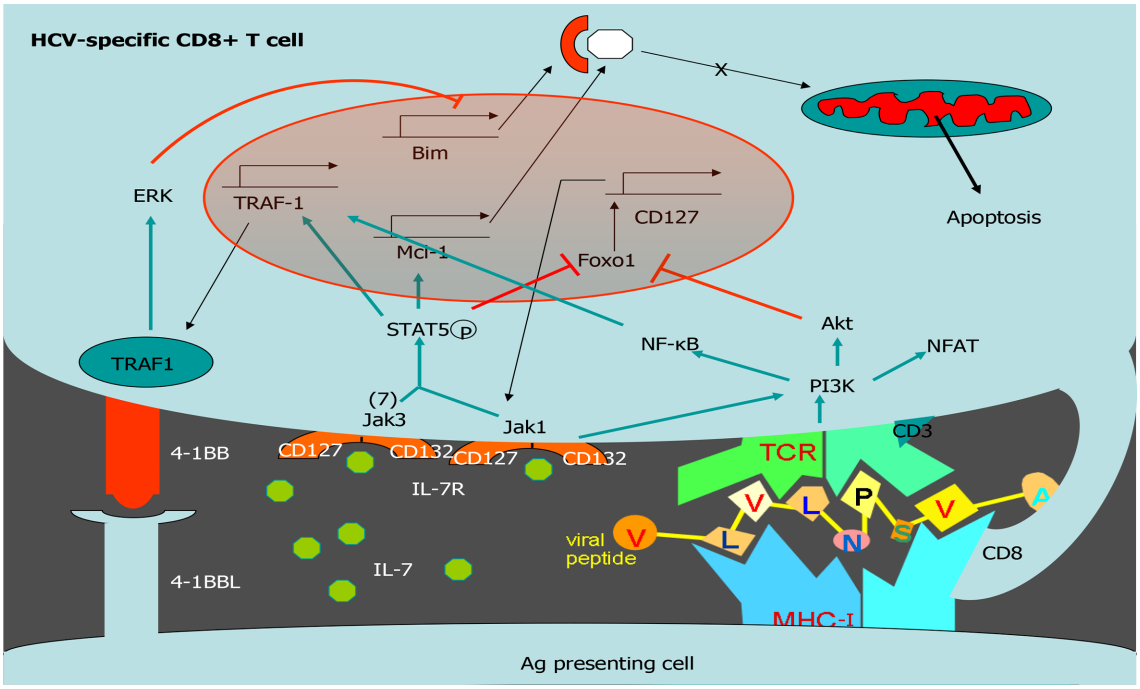

Figure 4 Tumor necrosis factor receptor-associated factor 1 pathways involved in T cell survival. Scheme of T cell survival pathways. Interleukin (IL)-7/IL-7 receptor (CD127) increases the level of the anti-apoptotic molecule myeloid leukemia cell differentiation protein (Mcl-1) *via* signal transducer and activator of transcription 5. After T cell receptor activation, tumor necrosis factor receptor (TNFR)-associated factor 1 (TRAF1) level is upregulated *via* nuclear factor-kappa B. TRAF1 is the signal transducer of the positive checkpoint TNFR superfamily member 9 (4-1BB). 4-1BB stimulation downregulates Bim *via* extracellular signal-related kinase. IL-7 induces TRAF1 expression, increasing its anti-apoptotic effect by improving 4-1BB signaling. Together, 4-1BB and CD127 balance Bim and Mcl-1. HCV: Hepatitis C virus; 4-1BB: Tumor necrosis family receptor superfamily member 9; ERK: Extracellular signal-regulated kinase; TRAF1: Tumor necrosis factor receptor-associated factor 1; Mcl-1: Myeloid leukemia cell differentiation protein; IL: Interleukin; NF-Kb: Nuclear factor kappa B; MHC: Major histocompatibility complex; TCR: T cell receptor.

fibrosis and lower evolution responded favorably to the 4-1BBL/IL-7 combination[2]. We speculated that cases with worse progression probably had higher burden of exhausted T cells with increased PD-1 expression, leading us to add anti-PD-L1 treatment to the IL-7/4-1BBL combination[81]. After the combined treatment, we were able to restore two other groups of cases: Those with low fibrosis progression but long-term infection, and those with rapid-progression and short-lasting disease. Unfortunately, those cases with less favorable factors, specifically rapid fibrosis progressors with long-term infection, were not responsive to the treatment[2]. This may have been due to the loss of these T cell populations from apoptosis (Figure 5).

CONCLUSION

The HCV-specific T cell response impacts infection outcomes. Mid-slow fibrosis progressors have less exhausted T cells, but the length of infection also influences the impairment of the T cell response. Worse T cell reactivity is observed the longer the infection lasts, and the faster liver fibrosis takes place. T cell response impairment is mediated by an exhausted and pro-apoptotic status that is characterized by the upregulated expression of negative checkpoints and the inhibition of positive co-stimulatory molecules. Among the latter is 4-1BB signaling *via* its effector TRAF1. This pathway regulates downstream Bim *via* ERK and is involved in T cell activation and survival. TRAF1 is induced by IL-7 and downregulated by TGF-β1. During persistent HCV infection, TGF-β1 Level is increased and can contribute to T cell exhaustion by TRAF1 loss. Depending on the stage of the infection, IL-7 *ex vivo* treatment can restore TRAF1 expression and T cell reactivity (Figure 5).

4-1BB/TRAF1 has a pathogenic role in chronic HCV infection that describes a new mechanism of T cell exhaustion and explains different infection outcomes. Modulation of 4-1BB/TRAF1 can be useful as an immunotherapeutic strategy in chronic viral infections and cancer.

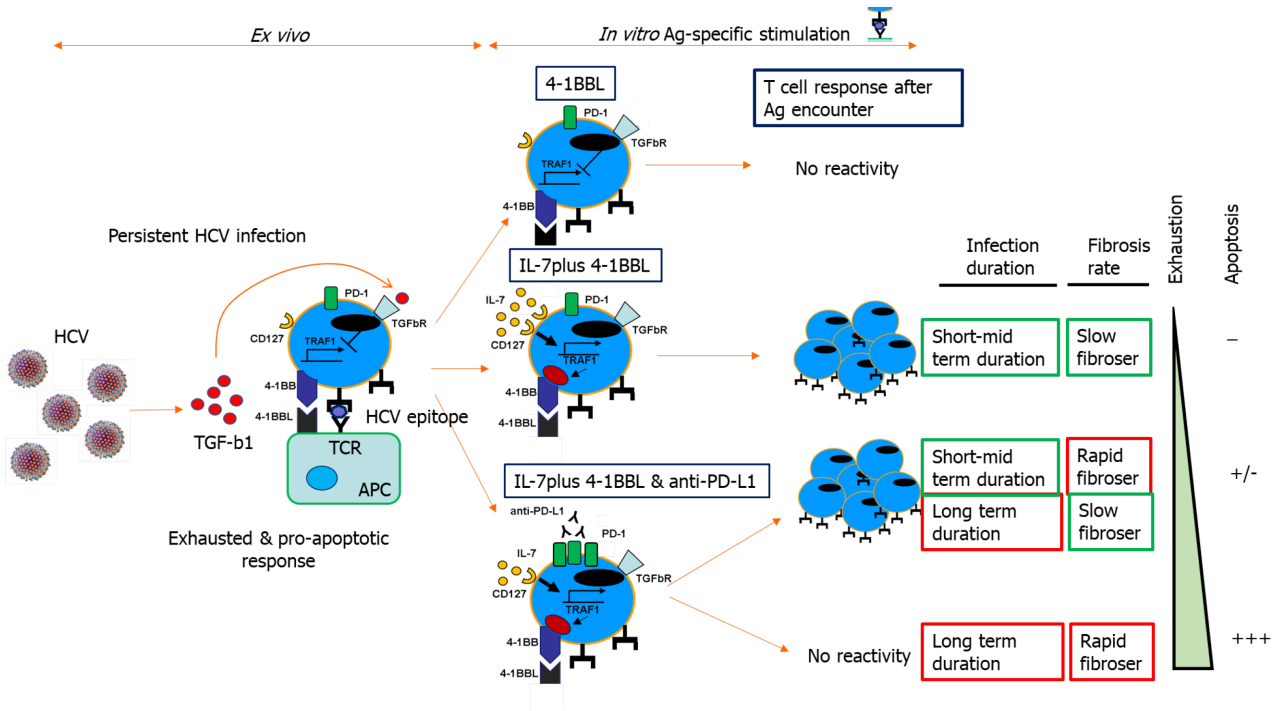

Figure 5 Tumor necrosis factor receptor-associated factor 1-related pathogenic mechanism involved in T cell exhaustion and liver fibrosis progression during persistent hepatitis C virus infection. Scheme showing transforming growth factor beta 1-mediated CD8 T cell impairment during chronic hepatitis C virus infection due to tumor necrosis factor receptor-associated factor 1 (TRAF1). In patients with mild clinical progression, T cell reactivity can be restored by TRAF1 upregulation with interleukin (IL)-7 treatment. Those with rapid fibrosis or with long-term infection need IL-7 treatment combined with programmed cell death protein 1 blockade. Cases with rapid fibrosis and long infection duration cannot be restored, probably due to T cell deletion. Ag: Antigen; 4-1BB: Tumor necrosis family receptor superfamily member 9; 4-1BBL: 4-1BB-ligand; PD-1: Programmed cell death protein-1; HCV: Hepatitis C virus; TRAF1: Tumor necrosis factor receptor-associated factor 1; TGF: Transforming growth factor; IL: Interleukin; APC: Antigen-presenting cell; TCR: T cell receptor.

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MINIREVIEWS

Apatinib as an alternative therapy for advanced hepatocellular carcinoma

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Abstract

Angiogenesis plays an important role in the occurrence and development of tumors. Registered tyrosine kinase inhibitors targeting vascular endothelial growth factor reduce angiogenesis. Apatinib, a tyrosine kinase inhibitor, can specifically inhibit vascular endothelial growth factor receptor 2, showing encouraging anti-tumor effects in a variety of tumors including advanced hepatocellular carcinoma (HCC). This article intends to review the clinical research and application prospects of apatinib in the field of HCC.

Key Words: Apatinib; Hepatocellular carcinoma; Angiogenesis; Vascular endothelial growth factor receptor 2

Core Tip: Apatinib, as a tyrosine kinase inhibitor, has a good inhibitory effect on

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advanced hepatocellular carcinoma (HCC). In this article, we will introduce the role of apatinib in advanced HCC from the aspects of structure and mechanism, pharmacokinetics, preclinical studies, clinical trials, side effects, and combined drug use.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common malignant tumor in China. Its 5-year survival rate is only 14.1%, which seriously threatens people's health and life[1]. Asymptomatic or insignificant symptoms are common in the early course of the disease. About 70%-85% of patients are in advanced stage at the time of diagnosis[2], and the natural survival time is only 4.2 mo in the Asia-Pacific region and 7.9 mo in Europe[3,4]. For patients who have no opportunity for surgery or metastasis after treatment, effective systemic treatment is necessary.

In the "Guidelines Insights: Hepatobiliary Cancers, Version 2.2019", first-line targeted drugs for palliative systemic therapy include sorafenib and lenvatinib[5]. As a multi-target kinase inhibitor, sorafenib can inhibit the proliferation of HCC cells through the RAF/MEK/ERK signaling pathway and block the angiogenesis by inhibiting vascular endothelial growth factor receptor (VEGFR) and platelet-derived growth factor receptors (PDGFRs)[6]. Two phase III clinical trials confirmed that sorafenib prolonged the overall survival (OS) by 2.3-3.2 mo, while the objective response rate (ORR) was 2% to 3.3%[3,4]. The effect of lenvatinib is not inferior to sorafenib, while OS and progression free survival (PFS) are improved compared with the latter. However, the therapeutic effect is still not very satisfying[7].

Apatinib mesylate (YN968D1) is a highly specific small molecule VEGFR-2 tyrosine kinase inhibitor, preventing its downstream signaling pathways, blocking the migration and proliferation of vascular endothelial cells, reducing tumor microvessel density, and inhibiting tumor angiogenesis[8-11]. With the announcement of the results of phase I and phase II clinical trials, the China Food and Drug Administration (CFDA) approved apatinib as the third-line treatment for advanced gastric cancer or adenocarcinoma of the gastroesophageal junction in October 2014.

In this review, we summarize the structure, mechanism and pharmacokinetic characteristics of apatinib, overview the current data of apatinib in clinical studies, and propose future development directions of HCC.

STRUCTURE AND MECHANISM

Angiogenesis plays an important role in the occurrence and development of tumors[12]. Vascular endothelial growth factor (VEGF) and its receptor VEGFR have been thought to play a central role in angiogenesis and tumor growth[13]. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PLGF). Similarly, there are three subtypes of receptor family, including VEGFR-1, VEGFR-2, and VEGFR-3[14]. The combination of VEGF-A and VEGFR-2 is considered to be mainly involved in the generation of blood vessels in solid tumors[15-18]. VEGF-A binds to the Ig-like domains 2 and 3 of VEGFR-2 to dimerize the receptor, which in turn causes the tyrosine kinase of receptor to undergo autophosphorylation[15] (Figure 1). Subsequently, several different molecular pathways are activated simultaneously: The RAF/MEK/ERK pathway promotes endothelial cell proliferation and survival; the p38-MAPK pathway increases the migration and invasion of endothelial cells, and enhances chemotactic and homing of bone marrow-derived vascular precursor cells; and the PI3K/AKT/mTOR pathway improves endothelial cell survival and vascular permeability[14,15,18-21].

Apatinib mesylate is a derivative of valatinib. Its predecessor is YN968D11 (N-[4-(1-cyano-cyclopentyl) phenyl]-2-(4-pyridylmethyl) amino-3-pyridine carboxamide

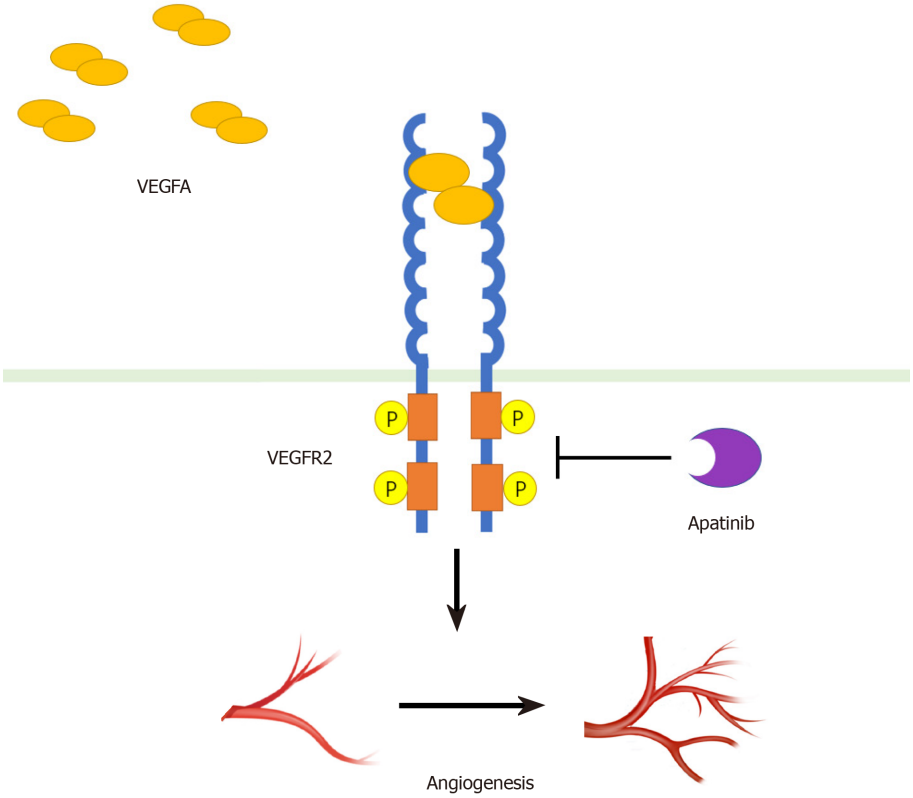

Figure 1 Schematic illustration of the mechanism of apatinib as an inhibitor of vascular endothelial growth factor receptor 2. VEGFR2: Vascular endothelial growth factor receptor 2.

mesylate). It highly specifically binds to the intracellular ATP binding site of VEGFR-2, preventing receptor phosphorylation. Apatinib has a strong affinity for VRGFR2 (IC_{50} = 2), which is ten times that of other anti-angiogenic drugs such as sorafenib (IC_{50} = 90)[8,9,22,23].

PHARMACOKINETICS

The pharmacokinetic analysis showed that the time to maximum plasma concentration level after administration was about 3-4 h with an average half-life of 9 h[9]. There are many main pathways of apatinib biotransformation, in which M1-1 is the main metabolite and shows the strongest inhibitory effect on VEGFR-2, and it is most closely related to the anti-angiogenic effect of apatinib. In contrast, M9-2 has no obvious inhibitory effect on the above enzymes. The oxidative metabolites of apatinib are mainly formed in the liver in a NADPH-dependent manner. The process is mainly mediated by the CYP3A4/5 enzyme, followed by CYP2D6, CYP2C9, and CYP2E1. After 96 h of oral apatinib, drug excretion rate was 76.8%, including 69.8% in stool and 7.0% in urine[24].

PRECLINICAL STUDIES

In vitro experiments

Apatinib can effectively inhibit the activity of VEGFR-2 kinase and block its downstream signaling by specifically competing for the ATP binding site in the cell[8]. Apatinib also inhibits the proliferation, migration, and tube formation of human umbilical vein endothelial cells (HUVEC), blocking the germination of rat aortic rings[8,25].

In HCC, apatinib can induce cell cycle arrest at the G2/M phase, promoting apoptosis of HCC cells *in vitro*, and its inhibitory effect is related to the expression level of VEGFR[26]. Li *et al*[27] have found that apatinib promotes tumor cell apoptosis

and inhibits metastasis, which may be related to the down-regulation of PDGFR-α, IGF-IR, and AKT phosphorylation levels. Similar results have been observed in SMMC-7721 cells, in which apatinib promoted apoptosis by inhibiting the phosphorylation level of PI3K/AKT[28]. In pancreatic cancer, apatinib promotes apoptosis of pancreatic cells by down-regulating hypoxia inducible factor-1α (HIF-1α) and increasing reactive oxygen levels[29]. In thyroid cancer, apatinib inhibits the expression of angiopoietin through tumor cell AKT/GSK3β/ANG pathway, thereby inhibiting tumor angiogenesis[25]. Apatinib inhibits cell invasion and migration by inhibiting the RET/SRC signaling pathway, suggesting a potential role in treating KIF5B-RET-driven tumors[30]. Apatinib can also promote the apoptosis of tumor cells of extrahepatic bile duct cancer[31], esophageal cancer[32], colon cancer[33], osteosarcoma and glioma[34], and B and T cell acute lymphoblastic leukemia[33].

In vivo experiments

In an immunodeficiency mouse xenograft model of HCC, apatinib was administered orally three times a week, and the inhibition rate of tumor growth was 71% after 30 d, and no significant weight loss or treatment-related death was observed[27]. Liang *et al*[35] evaluated the therapeutic effect of apatinib and sorafenib in HCC by multimodal molecular imaging. The results showed that apatinib inhibits the growth and angiogenesis of HCC, which is equivalent to sorafenib but has fewer side effects[35]. Apatinib can also cause metabolomics changes. After apatinib treatment, 3-hydroxybutyric acid (3-HB) is significantly increased in serum, tumor, and the liver, which aids antitumor effect of apatinib[36].

Apatinib alone or in combination with chemotherapeutics can effectively inhibit a variety of established human tumor xenograft models with less toxicity. The combination of apatinib with docetaxel and adriamycin significantly inhibits the growth of transplanted lung cancer, which is significantly different from the apatinib group and the chemotherapy drug group. In addition, the combination of apatinib with oxaliplatin and fluorouracil also showed a significant inhibitory effect in colon cancer[8]. Tong *et al*[37] selected a subset of K562 leukemia cells with higher doxorubicin resistance as the object of observation. The experimental results showed that apatinib could significantly reduce the IC_{50} value of doxorubicin in this subgroup of cells and significantly increase the sensitivity to chemotherapy drugs. It was also confirmed in a tumor xenograft model that apatinib could reverse ABCB1 and ABCG2-mediated multidrug resistance (MDR) by directly inhibiting ABCB1 and ABCG2 function, leading to the rise of intracellular concentrations of chemotherapeutic drugs. The reversal of MDR further supports the potential role of combining apatinib with other conventional anticancer drugs in overcoming clinical resistance[38].

CLINICAL RESEARCH OF ADVANCED HCC

Qin *et al*[39] reported a prospective, randomized, open label, nationwide, multicenter, phase II clinical trial of apatinib as second-line therapy for advanced HCC. The primary endpoint of the study was time to disease progression (TTP). The secondary endpoints included OS, ORR, disease control rate (DCR), quality of life, and serum alpha-fetoprotein (AFP) levels. A total of 121 patients with advanced HCC were enrolled and randomly assigned 1:1 to the 850 mg dose group and the 750 mg dose group. The results confirmed that the clinical efficacy of apatinib (850 mg and 750 mg) in different dose groups was basically the same for advanced HCC with initial treatment and good basic conditions: mTTP and mOS were not significantly different between the two groups (4.2 mo *vs* 3.3 mo, $P > 0.05$; 9.7 mo *vs* 9.8 mo, $P > 0.05$). The DCRs of the two groups were 48.6% and 37.3% ($P > 0.05$), and the ORRs were 8.6% and 0 ($P > 0.05$), respectively. The incidence of adverse events was also similar between these two groups. In terms of safety, the drug-related toxicities in the 850 mg dose group were more than those in the 750 mg dose group, but the differences were not statistically significant, including hand-foot skin reaction (HFSR), elevated aminotransferase, and elevated bilirubin. Grade 3 and above drug-related side effects included hypertension, proteinuria, HFSR, fatigue, and peripheral blood cell reduction. Considering that most patients with liver cancer have basic liver diseases, they recommended that 750 mg qd as dose for subsequent studies.

Kong *et al*[40] retrospectively evaluated the efficacy and safety of apatinib in 22 patients with advanced HCC who were resistant to sorafenib or could not afford sorafenib. Apatinib was administered continuously at 500 mg/d or 250 mg/d with clinical emphasis on TTP, OS, and safety. Until the last follow-up, the median disease

progression time for these 22 patients was 10.4 mo, and 50% of patients survived longer than 11.4 mo. The percentages of patients achieving complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) were 0%, 40.9%, 40.9%, and 18.2%, respectively, and the ORR and DCR were 40.9% and 81.8%, respectively. At the same time, 14 of the 22 cases had decreased alpha-fetoprotein levels, of which seven had fallen by half or more. Adverse events mainly included HFSR (81.8%), diarrhea (77.3%), hypertension (63.6%), fatigue (59.1%), hoarseness (54.5%), and nausea (50%). Grade 3 or 4 drug-related adverse events mainly included hypertension (27.3%), HFSR (13.6%), and thrombocytopenia (9.1%). In view of the side effects of advanced patients and the high-dose treatment, patients receiving low-dose treatment (250 mg/d) had fewer and less adverse events and achieved good responses.

A prospective study by Yu *et al*[41] evaluated the efficacy and safety of apatinib in advanced HCC. A total of 31 patients participated in the study, including four in the intermediate stage and 27 in the advanced stage. The dose was 500 mg/d. According to the first follow-up CT and MRI after 6 wk of treatment, the numbers of patients achieving PR, SD, and PD in 31 patients were 10 (32.3%), 15 (48.4%), and 6 (19.4%). The ORR and DCR were 32.3% and 80.7% respectively. The mPFS was 4.8 mo, and the 6- and 12-mo survival rates were 73.8% and 55.4% respectively. The most common grade 3 adverse effects were hypertension (48.4%), thrombocytopenia (6.5%), and an increase in total bilirubin or transaminase (6.5%). By adjusting drug dosage and symptomatic treatment, all toxic reactions could be controlled.

Liu *et al*[42] retrospectively reviewed the efficacy and safety of apatinib in the treatment of unresectable or recurrent HCC. A total of 32 patients with HCC or intrahepatic bile duct cancer were included in the study[42]. No CR occurred, PR, SD, and PD were observed in 5 (16%), 14 (44%), and 13 (41%) patients, respectively, and DCR was 60%. The mPFS for HCC was 5 mo, and the mPFS for intrahepatic cholangiocarcinoma was 3 mo. The mOS for HCC and bile duct carcinoma were 13 mo and 5 mo, respectively. The most common adverse effects were proteinuria (31%), hypertension (28%), and liver dysfunction (13%).

Zhang *et al*[43] evaluated the efficacy and safety of apatinib for sorafenib refractory advanced hepatitis B virus-associated HCC. A total of 43 patients were retrospectively analyzed[43]. ORR and DCR were 25.6% and 67.4%, respectively. mPFS and mOS were 3 mo and 8 mo, respectively. The 1-year and 2-year survival rates were 34.9% and 9.3%, respectively. The most common toxicities were weight loss, HFSR, and hypertension.

Apatinib shows a therapeutic effect on advanced HCC with lung metastasis[44]. In a retrospective and multicenter study, 61 patients with advanced HCC were enrolled in the study, including 41 patients with lung metastases, three with multiple organ metastases, and 20 with no pulmonary metastases. The main focus was on metastasis specificity and PFS. All patients had a median PFS of 3.37 mo and an ORR of 11.6%. The median mPFS of 41 patients with pulmonary metastases was 5 mo, and the mORR was 22.0%. Compared with patients without lung metastases, patients with only lung metastases had better mPFS (hazard ratio/HR = 0.316), although mORR was similar.

SIDE EFFECTS

In a series of clinical studies of apatinib, common adverse events include hematological toxicity (leukopenia, granulocytopenia, and thrombocytopenia) and non-hematological toxicity (hypertension, proteinuria, HFSR, *etc*.). Among the common important adverse events are hypertension, proteinuria, and HFSR.

In the phase I study of apatinib, the overall incidence of hypertension reached 69.5%, of which grade 3 to 4 reached 6.5%. Hypertension is the most common adverse reaction of anti-angiogenic drugs, especially VEGF/VEGFR inhibitors. Current research suggests that reduction of nitric oxide (NO) and increase of endothelin (ET) are the main causes of hypertension in anti-VEGF treatment[45,46]. Both methods can cause vasodilation dysfunction and strengthen systolic function. In addition, abnormal blood vessel density and reduced capillaries are also the cause of hypertension[47]. In the current treatment plan, besides reducing the drug dose, another effective treatment is the use of antihypertensive drugs.

The overall incidence of proteinuria in the phase I study was 34.8%, and the incidence of grade 3 to 4 was 13%. The occurrence of proteinuria is related to the inhibition of VEGF signaling by apatinib, whereas adequate VEGF is needed to maintain the integrity of glomerular structure and function. In animal experiments, podocyte specific VEGF gene knockout can cause structural and functional changes, which in turn affects glomerular filtration rate and causes proteinuria[48]. Although the

persistence of high blood pressure can cause kidney damage[49], in clinical practice, many patients have proteinuria without hypertension, suggesting that proteinuria caused by apatinib may not be related to hypertension, and the specific mechanism needs further exploration.

The overall incidence of HFSR in phase I clinical studies was 45.6%, and the incidence in grade 3 to 4 was 13%, which can be alleviated by reducing the dose of the drug. Its mechanism is unknown. Possible reasons include: Decreased renewal and dysfunction of endothelial cells; damage to sweat ductal epithelial cells due to inhibition of PDGF and c-Kit; keratinocyte dysfunction due to c-Kit inhibition; and broken balance between vascular and epidermal damage[50,51].

In addition to the common adverse events mentioned above, other adverse events include bleeding, fatigue, diarrhea, infection, dyspnea, hoarseness, skin albinism, and rash. However, most of these events are mild and controllable, and can be relieved with supportive treatment. Remarkably, clinical trials have shown that adverse events caused by apatinib are often associated with better efficacy and longer survival benefits[52].

THE FUTURE OF APATINIB IN HCC

The combination of apatinib with other treatments has yielded interesting results in advanced HCC. In the combination with trans-artery chemo-embolization (TACE), Zhu *et al*[53] reported that after 9 mo of TACE combined with apatinib for advanced HCC, DCR and ORR in the TACE group were 81.82% and 36.36%, and they were 95.45% and 63.64% in the TACE plus apatinib group. The PFS was 11.15 and 16.5 mo, respectively[53]. DCR, ORR, and PFS were significantly improved. There was no significant difference in the incidence of adverse events after embolization between the two groups of patients. However, the incidence of hypertension, HFSR, and proteinuria in the combined group was significantly higher ($P < 0.05$). Adverse effects were alleviated after symptomatic treatment.

Xu *et al*[54] studied the effect of carrelizumab (PD-1 mAb, SHR-1210) and apatinib in the treatment of advanced HCC, gastric cancer, and esophagogastric junction cancer in a phase I clinical study[54]. Of the 16 evaluable HCC patients, eight achieved PR, in whom one was in the apatinib 125 mg cohort and seven received apatinib 250 mg. ORR and DCR were 50.0% and 93.8%, respectively. Patients receiving apatinib had a 6-mo PFS rate of 51.3% and a 9-mo PFS rate of 41%. A phase III study on the combined use of the two drugs is underway (NCT02329860).

CONCLUSION

Apatinib, as a new type of small molecule tyrosine kinase inhibitor, shows high selective affinity for VEGFR-2, blocking its downstream signal transduction. Although there is no sufficient evidence, from the primary research and exploration, apatinib may have potential advantages, such as better ORR, survival benefits, and less toxic and side effects, which is still waiting for further research and confirmation. Combined therapy shows a prominent role by working through different mechanisms and will hold an important position in the future[55]. Apatinib, as an alternative targeted drug, will be likely to have a promising effect in combination therapy. A number of clinical trials of combination therapy including apatinib are currently underway (NCT03793725, NCT03839550, NCT03463876, and NCT03764293). Current research still has certain limitations. Most of the studies are small in size. The mechanisms need further exploration to ensure a higher level of evidence. With the development of basic and clinical research, apatinib alone or in combination with other therapy may benefit more patients with HCC.

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ORIGINAL ARTICLE

Basic Study

Hepatitis B virus detected in paper currencies in a densely populated city of India: A plausible source of horizontal transmission?

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Abstract

BACKGROUND

The recent rise in the incidence of hepatitis B virus (HBV) infections in a densely populated city of eastern India ("mixing vessel" of people of varied socio-economic and immune status) prompted this study. Applying saliva on fingers for enumerating bank notes is a common practice in the Indian subcontinent. Paper notes may be a potential source of "horizontal" transmission of this virus, especially if there are cuts/bruises on the oral mucous membrane or skin.

AIM

To investigate whether paper currencies could be a plausible mode of horizontal transmission of HBV infection.

METHODS

Polymerase chain reactions (PCR) followed by nucleotide sequencing was done for the detection of HBV. Hepatitis B virus surface antigen enzyme-linked immunosorbent assay(HBsAg ELISA) was performed on all HBV deoxyribonucleic acid-positive samples to check the detectability of the virus. Atomic force microscopy (AFM) was carried out for visual confirmation of HBV particles in ultracentrifuged/immunoprecipitated samples from currency paper washings.

RESULTS

HBV-specific PCRs on pellets obtained after ultracentrifugation/immunoprecipitation of the currency paper washings detected potentially intact/viable HBV (genotype D2) in 7.14% of samples (n = 70). AFM gave the

Institute of Chemical Biology Biological Safety Committee. This work involved no use of human subjects or human clinical materials.

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visual confirmation of HBV particles in ultracentrifuged/immunoprecipitated samples from currency paper washings. However, HBV isolates from the currency notes could not be detected by HBsAg ELISA.

CONCLUSION

It is a common practice in the Indian subcontinent to count paper currencies by applying saliva on fingertips. Paper notes may be a potential source of "horizontal" transmission of this virus, especially if there are cuts/bruises on the oral mucous membrane or skin, but it was practically not possible to demonstrate experimentally such transmission. Detection of potentially intact/viable and "occult" HBV from currency poses potential risk of silent transmission of this virus among the general population.

Key Words: Hepatitis B virus; Contamination; Paper currencies; Occult hepatitis B virus; Hepatitis B virus surface antigen enzyme-linked immunosorbent assay; Horizontal transmission

Core Tip: The recent upsurge in hepatitis B virus (HBV) infections in eastern India prompted the search for this virus in low denomination paper notes in this region. Applying saliva on finger tips for enumerating currency notes is a common practice. Thus, paper currencies may be a potential source of "horizontal" HBV transmission, especially if there are cuts/bruises on the oral mucous membrane or skin. We discovered that intact HBV particles are present in about 7.14% of the currencies. Molecular analysis and immunoassays suggested that the circulating HBV are "occult" in nature, hence capable of "silent transmission" in the general population.

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INTRODUCTION

Transaction of paper currency occurs rampantly in exchange of goods and other services. Chances of microbial contamination in the currencies of lower denominations are higher as they are more widespread and exchanged frequently among people irrespective of socio-economic status within a population[1]. While enumerating paper money, people often apply saliva on fingers, a potential means of contaminating the currency notes as well as exposure to microbes already present on the currency notes.

In developing and densely populated countries like India, where people are of diverse immune and hygiene status, handling of currency may lead to transfer of different kinds of microorganisms. Paper currency may be contaminated with droplets from sneezing or coughing and even by touching with contaminated hands[2]. Microbes may also be introduced into the paper notes due to unhygienic habits like keeping currency notes in socks, shoes and pockets, putting them under the carpet or rugs and squeezing them in the hand[3]. Storing these notes in polythene, cotton or leather bags in humid and dark conditions favor the growth of microorganisms on them[4].

Cases of currency contamination with pathogenic microbes have been reported from many countries like Bangladesh, Ghana, Nigeria, United States, Myanmar, Egypt, Nepal and Pakistan[5].

Rhinovirus, rotavirus and influenza virus have been detected on currency notes and coins[5], but till date there had been no reports on detection of hepatitis B virus (HBV) in currency sample. Hepatitis viruses may cause acute or chronic liver disease. The latter condition often leads to cirrhosis and may eventually culminate in liver cancer. Hepatitis viruses may be transmitted *via* the fecal-oral route (hepatitis A and E) or *via*

blood (hepatitis B, C and D).

The recent unusual rise in HBV infection in India[6] prompted the search for HBV in currency notes as they are in circulation among people of varied immune status and may serve as a potential medium of HBV transmission.

MATERIALS AND METHODS

Sample collection and subsequent processing

Paper currency samples, seventy in number, of a single denomination (INR 10) were used for the present study. They were collected during 2016-2017 from hospitals, grocery stores, fish-meat markets and public transport. Five of each such places were chosen; currency samples were collected in duplicate or more, stored in sterile zip lock packs and quickly transported to the laboratory for further processing (Table 1). Two currency samples fresh from the mint, which were not yet into circulation, and two used samples that have been autoclaved were also included in the present study as negative controls. Surfaces of each of the samples were thoroughly washed with 5 mL 1 × phosphate buffered saline (PBS) buffer and each washed solution was stored at 4 °C until used. For the screening of HBV, washed out solutions were ultra-centrifuged at 64000 *g* for 90 min at 4 °C. The resultant pellets were supposed to contain all intact microbes (bacteria, viruses, *etc*.) present in the currency washings, and all extraneous and lighter materials like free nucleic acids should be in the supernatant. The precipitate for each sample, collected at the bottom of the ultracentrifuge tube, was washed thoroughly, resuspended in 1 × PBS buffer and collected in a fresh tube.

None of the treatments to which the paper currency samples were subjected amounted to purposeful act of destroying, willful defacing, disfiguring or mutilation or any other kind of violation of currency handling procedures laid down by the Government of India. Washing of the samples with 1 × PBS buffer did not destroy the currency samples and were still suitable for re-use after washing and subsequent drying in air.

Deoxyribonucleic acid extraction from the test currency samples

Total nucleic acid was extracted from the 1 × PBS buffer wash of the currency samples using High Pure Viral Nucleic acid kit (Roche, Mannheim, Germany). Extracted DNA was quantified by means of Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, United States).

Primer selection, polymerase chain reaction amplification and sequencing

Two sets of primers specific to HBV S gene were used for HBV detection[7]. To amplify HBV S gene, the first round polymerase chain reaction (PCR) was performed with SPL-3 and SPL-2 primers while the nested PCR was performed using the second set of internal primers SPL-4 and SPL-5 (Table 2). The cycling conditions for the first round PCR comprised initial denaturation at 95 °C (5 min) for one cycle followed by 30 cycles of heating at 95 °C (40 s), primer annealing at 55 °C (1.5 min) and extension for 2 min at 72 °C. The final extension was done at 72 °C (10 min) for one cycle followed by hold at 4 °C. The first round PCR product was diluted ten-fold and subjected to the second round nested PCR with SPL-4 & SPL-5 primers. This PCR was programmed as follows: Initial denaturation at 95 °C (10 min) for one cycle followed by 30 cycles of heating at 94 °C (1 min), primer annealing at 55 °C (1 min) and extension at 72 °C (1.5 min). The final extension was carried out at 72 °C for one cycle (10 min).

PCR products were resolved by 1% agarose gel electrophoresis. Bands were observed under ultraviolet light (Carestream Gel Logic 212 Pro, Rochester, NY, United States), and the gel images were recorded. PCR bands of correct size were gel-purified (Qiagen Gel Extraction Kit, Hilden, Germany), eluted in nuclease-free water and subjected to bi-directional DNA sequencing using the same primers used for PCR amplification. All PCRs contained GoTaq® Green Master Mix (M7122, Promega, Madison, WI, United States), 0.2-0.4 μM forward and reverse primers and 100–300 ng DNA.

Nucleotide (nt) sequences, confirmed by bi-directional sequencing of the PCR products were subjected to National Center for Biotechnology Information-Basic Local Alignment Search Tool (BLAST) for determining genetic matches with sequences available in the database. They were then aligned using MEGAX with other closely related sequences identified from the BLAST search. A phylogenetic tree was generated by the Neighbor-Joining method with Bootstrap test (2000 replicates) using MEGAX on 510 nt of S gene (pertaining to nt positions 326-835 as in HBV34 genotype

Table 1 Sites of sample collection

Types of locations of sample collection	Detailed description of the actual sites of collection	Co-ordinates	Distance from central Kolkata[1] (in Km)	Number of currency samples collected (*n* = 70)	Number of samples that are hepatitis B virus-positive
Hospitals	Nil Ratan Sircar Medical College and Hospital	22.5638° N, 88.3690° E	2.6	2	1 HBV-positive (S5)
	SSKM Hospital	22.5396° N, 88.3439° E	4.8	2	-
	Chittaranjan National Cancer Institute (CNCI)	22.5254° N, 88.3465° E	3.7	2	-
	Calcutta National Medical College and Hospital (CNMC)	22.5467° N, 88.3704° E	4.5	3	1HBV-positive (S8)
	KPC Medical College and Hospital	22.4956° N, 88.3706° E	9.3	4	-
Public transport routes (bus): First stop and last stop	230[2](Kamarhati to Alipore Zoo)	22.6847° N, 88.3706° E-22.5248° N, 88.3312° E	13.4 - 5.1	4	-
	47B (Lake town to Super Market, Prince Anwar Shah Road)	22.6070° N, 88.4028° E-22.5015° N, 88.3617° E	19.4-7.5	5	-
	3C/1 (Anandapur to Nagerbazar)	22.5148° N, 88.4098° E-22.6218° N, 88.4180° E	11.5 -19.7	5	-
	45 (Dumdum to Baishnabghata)	22.6471° N, 88.4317° E-22.4729° N, 88.3764° E	22.8-11.4	5	-
	S9 (Jadavpur to Karunamoyee)	22.4956° N, 88.3706° E-22.5851° N, 88.4222° E	8.8 -13.5	5	1 HBV-positive (S6)
Grocery shop	Shyam Bazar	22.5982° N, 88.3687° E	6.2	3	-
	Big Bazaar, Ganguli Bagan	22.4800° N, 88.3757° E	18.2	3	-
	South City Mall	22.5015° N, 88.3617° E	7.5	4	-
	Dunlop	22.6519° N, 88.3786° E	12.4	4	-
	Gariahat	22.5170° N, 88.3658° E	6.7	4	-
Fish-meat market	Howrah	22.5958° N, 88.2636° E	6.1	2	1 HBV-positive (S7)
	Baguihati	22.6107° N, 88.4271° E	18.3	4	-
	Hazra	22.5228° N, 88.3500° E	4.3	4	-
	Jadavpur	22.4956° N, 88.3706° E	8.8	2	1 HBV-positive (S9)
	Gariahat	22.5170° N, 88.3658° E	6.7	3	-

[1]The Indian Museum (22.5579°N, 88.3511°E) is taken as landmark for central Kolkata.
[2]The number indicates the officially designated bus route number. HBV: Hepatitis B virus.

D2 isolate, accession number KC875340, West Bengal, India) in order to characterize the HBV isolates from the currency samples and study their genetic distances with other closely related HBV strains[8].

Quantitative Real time PCR and hepatitis B virus surface antigen detection through enzyme-linked immunosorbent assay

Quantitative real-time PCR was performed on the HBV-positive DNA samples to determine the HBV DNA copy number in the currency samples. A pcDNA3.1 (+) plasmid construct (Invitrogen, Carlsbad, CA, United States) containing the HBVRT-F and HBVRT-R (Table 2) product of HBV genotype D "S" gene cloned into it, was used as the standard for the real-time PCR. Serial dilutions of this recombinant plasmid were used as known standards to calculate the HBV DNA copy number of the currency samples tested. A 98 bp fragment of the "S" gene (pertaining to nt position 379 to 476 as in HBV isolate KC875340) was amplified using the primers HBVRT-F (nt position 379 to 398) and HBVRT-R (nt position 456 to 476). The DNA copy number of each PCR product was determined from its DNA concentration. Four replicates were run for each standard and sample. The real-time PCR mix for each sample contained 10 μL of Luna Universal qPCR Master Mix (New England BioLabs, Ipswich, MA, United States), 1 μL of a mix of primers (10 μM each), 1 μL of sample (< 100 ng DNA) or 2.5 μL of standard and nuclease-free water to a final volume of 20 μL. The thermal cycling conditions in the Quant Studio 5 (Applied Biosystem, Foster City, CA, United States) consisted of initial hold at 95 °C (1 min), followed by 40 cycles of 15 s at 95 °C and 30 s at 58 °C. Fluorescence was monitored during the 55 °C annealing phase. Formation of bands of expected size was further confirmed by agarose gel electrophoresis of the pooled replicates per sample after completion of real-time PCR run.

In order to rule out that the laboratory reagents or equipment were not contaminated with HBV, control nested PCRs were performed with two sets of primers: SPL-3 and SPL-2 as first round primers and SPL-4 and SPL-5 as second round primers, as described previously. Ultracentrifuged pellet suspensions from 25 representative currency samples, including all HBV DNA-positive and remaining HBV DNA-negative samples were tested for the presence of the antigen marker of HBV [*i.e.*, hepatitis B virus surface antigen (HBsAg) through enzyme-linked immunosorbent assay (ELISA)]. The assay was performed using the Monolisa HBsAg Ultra kit (Hercules, CA, Bio-Rad) following manufacturer's instructions. The samples were tested in duplicates, diluted in the ratio of 1:5 in the supplied sample diluent.

Atomic Force Microscopy of ultracentrifuged pellet suspensions

HBV DNA-positive samples that had higher HBV DNA copy number were selected and subjected to atomic force microscopy (AFM) to gather visual evidence for the presence of approximately 42 nm HBV particles (Dane particles)[9].

For this, two representative HBV DNA-positive ultracentrifuged samples and one HBV DNA-negative ultracentrifuged sample (as negative control) were selected. All three samples were put through an identical procedure for AFM sample preparation. Furthermore, immunoprecipitation was performed on the above mentioned three samples. Immunoprecipitation technique was adopted to trap selectively the HBV particles from the ultracentrifuged pellet and to enrich the particles in the available volume for more authentic imaging. For this, Immunoprecipitation Kit-Dynabeads™ Protein G (Invitrogen) was used and the assay was performed following the standard protocol suggested by the manufacturer of the kit. Monoclonal anti-HBV antigen HBsAg (Cat No. SAB4700767, Sigma-Aldrich, St Louis, MO, United States) was used as the trapping antibody for immunoprecipitating the target antigen (*i.e.* HBsAg on HBV virion surface).

The final eluate (antigen-antibody complexes) obtained was treated with 0.2% Triton X-100 in the ratio 1:1 for 30 min followed by pulse sonication to disrupt the virus-antibody complexes. The solution was then centrifuged at 13000 rpm for 5 min at 4 °C. The supernatant was carefully transferred to a fresh centrifuge tube. Both, immunoprecipitated and non-immunoprecipitated samples were diluted in nuclease free water as required, and 5 μL of each diluted sample was applied on freshly cleaved muscovite Ruby mica sheet (ASTM V1 grade) and allowed to air dry. Once the sample was fixed, the mica sheet was put through AFM.

Acoustic-AC mode AFM was performed by means of a Pico plus 5500 AFM (Agilent Technologies, Santa Clara, CA, United States) with a piezo scanner with maximum range of 9 μm. Images were processed by flattening, using Pico view 1.4 version software (Agilent Technologies). Image manipulation was done through Pico Image

Table 2 Oligonucleotide primers used for amplifying target genes in hepatitis B virus

Name	Sequence 5'-3'	Amplicon size (bp)	Reference
SPL-3-F	GCGCGCGCTAGCACCATGGGGARCAYCRYATCRGGA	1652	[7]
SPL-2-R	GCCTTTGCAAGCTTCASACCAATTTATGCCTAC		
SPL-4-F	ACCACAGAGTCTAGACTYGTGGT	1277	
SPL-5-R	GGTCGGAACRRCAGRCGRAGAAG		
HBVRT-F	GTGTCTGCGGCGTTTTATCA	98	This study
HBVRT-R	GACAAACGGGCAACATACCTT		

Advanced version software (Agilent Technologies).

RESULTS

Molecular confirmation of presence of HBV in the currency samples

HBV "S" gene-specific PCRs on these DNA samples revealed the presence of HBV in five out of 70 samples screened (7.14%). PCR products of expected size (*i.e.* 1652 bp for SPL-3-2 and/or 1277 bp for SPL-4-5) were observed in all HBV-positive samples (Figure 1).

The second round 1277 bp bands, obtained in optimum concentration and purity, upon bi-directional sequencing (using SPL-4 and SPL-5 primers) confirmed that the observed PCR products were indeed part of the "S" gene of HBV (Figure 2).

All the HBV isolates from the currency samples were of genotype D2, as evident from their close clustering with other genotype D2 strains in the phylogenetic tree (Figure 3). Only partial S and Pol protein open reading frames were retrievable from the nucleotide sequences obtained for the five HBV-positive samples (GenBank accession numbers: S5: MN158164; S6: MN158165; S7: MN158166; S8: MN158167; S9: MN158168).

It was estimated from quantitative real-time PCR results that the number of HBV copies ranged from (6-10) × 10^8 for sample S5; (2-3) × 10^9 for S6 and S7; (3-4) × 10^5 for S8 and (3-6) × 10^3 for S9. The above copy numbers were estimates of total number of HBV copies present in the entire 5 mL washing from each paper currency sample.

Laboratory reagents (*e.g.*, water for PCR, PCR mix and primers) or equipment (*e.g.*, PCR machines and centrifuges) showed no evidence of HBV-contamination. This was proved by the absence of any visible band in case of control PCRs (water control; primers control) with SPL-3-2 and SPL-4-5 nested PCRs (Figure 1).

All the HBV positive and negative samples tested were found to be negative for HBsAg by ELISA, suggesting that the genotype D2 HBV strains present in the ultracentrifuged pellets were not detectable by Monolisa ELISA. The ELISA performed, passed the qualitative test criteria as per the manufacturer's guidelines. The assay tested each sample in duplicate, and the mean of sample-to-cut off ratio (S/CO) was considered to arrive at the results (data not shown).

Visual confirmation of HBV contamination of currency samples by AFM

When the diluted (1:100) non-immunoprecipitated ultracentrifuged HBV DNA-positive samples were imaged through AFM, clusters of HBV virion-like particles were visible (Figure 4). The diameter of the virus-like particles ranged from 40 to 60 nm (Figure 4).

The HBV DNA- negative sample displayed globular clumps, comprising of globule-like structures much smaller than HBV particles (Figure 5). The topographical demarcation for both the HBV DNA-positive (Figure 4D) and negative samples (Figure 5C) is clearly evident from the images.

AFM imaging of the Triton X-100-treated immunoprecipitated HBV DNA positive-sample validated the aforementioned results (Figure 6). Triton X-100 treatment followed by sonication led to the disruption of the virion clusters into individual particles. Complete/partial disintegration of virus envelope attached to the immunoprecipitating antibody exposed the icosahedral core particle of HBV (Figure 6A, D). The size of the individual virion particle observed was approximately 42 nm in accordance to the diameter of HBV and the number of particles observed

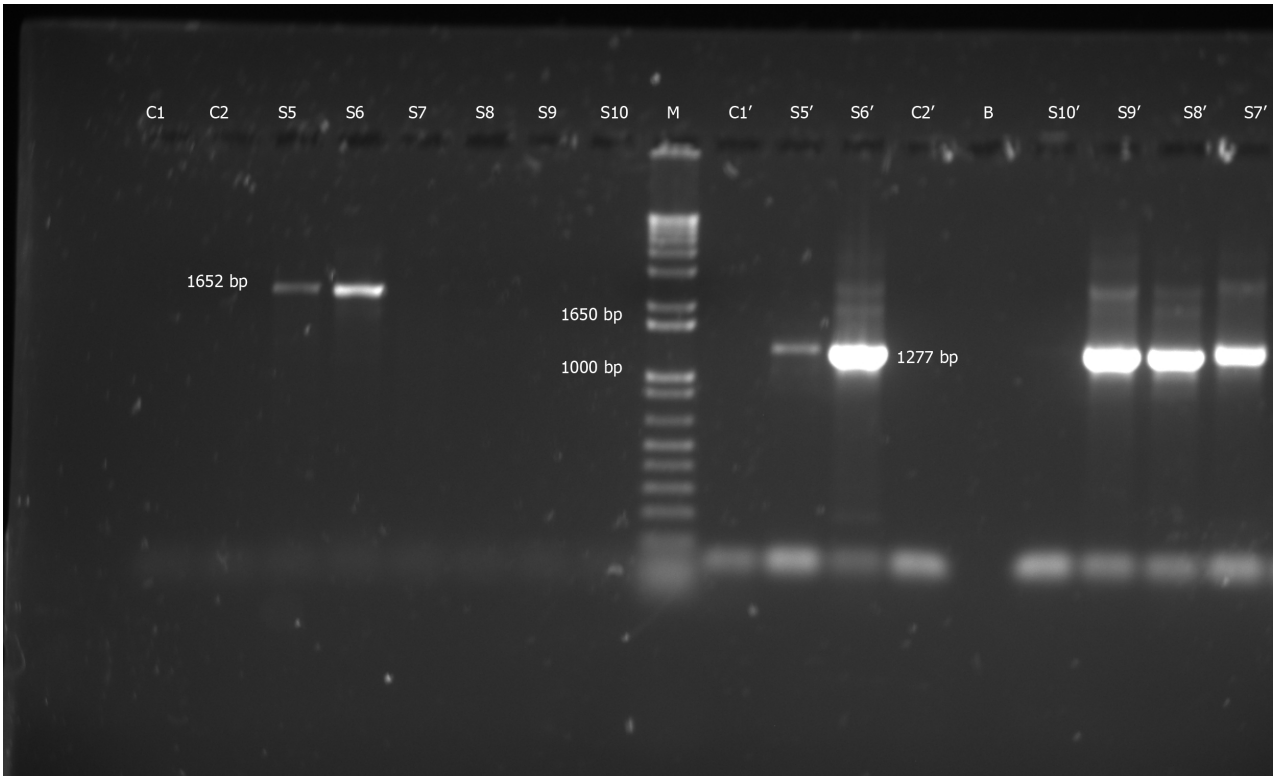


Figure 1 Representative gel electrophoresis on 1.5% agarose gels depicting hepatitis B virus "S" gene-specific polymerase chain reaction products and relevant controls. This proves that none of the lab reagents/equipment were contaminated with hepatitis B virus: Negative control with nuclease-free water used for sample polymerase chain reactions (PCR (C1), negative control for primers SPL-3 & SPL-2, using fresh nuclease-free water (C2), nested SPL-4-5 PCR product with C1 (C1'), SPL-4-5 nested PCR product with C2 (C2'), first round PCR with SPL-3-2 primers yielding 1652 bp band (S5 and S6), SPL-3-2 PCR yielding no bands (S7-S10); second round nested PCRs with SPL-4-5 primers with S5-9 yielding 1277 bp band (S5'-S9'), SPL-4-5 nested PCR yielding no band (S10') and a well left blank (*i.e.* loaded with no sample) (B). M is the molecular weight marker.

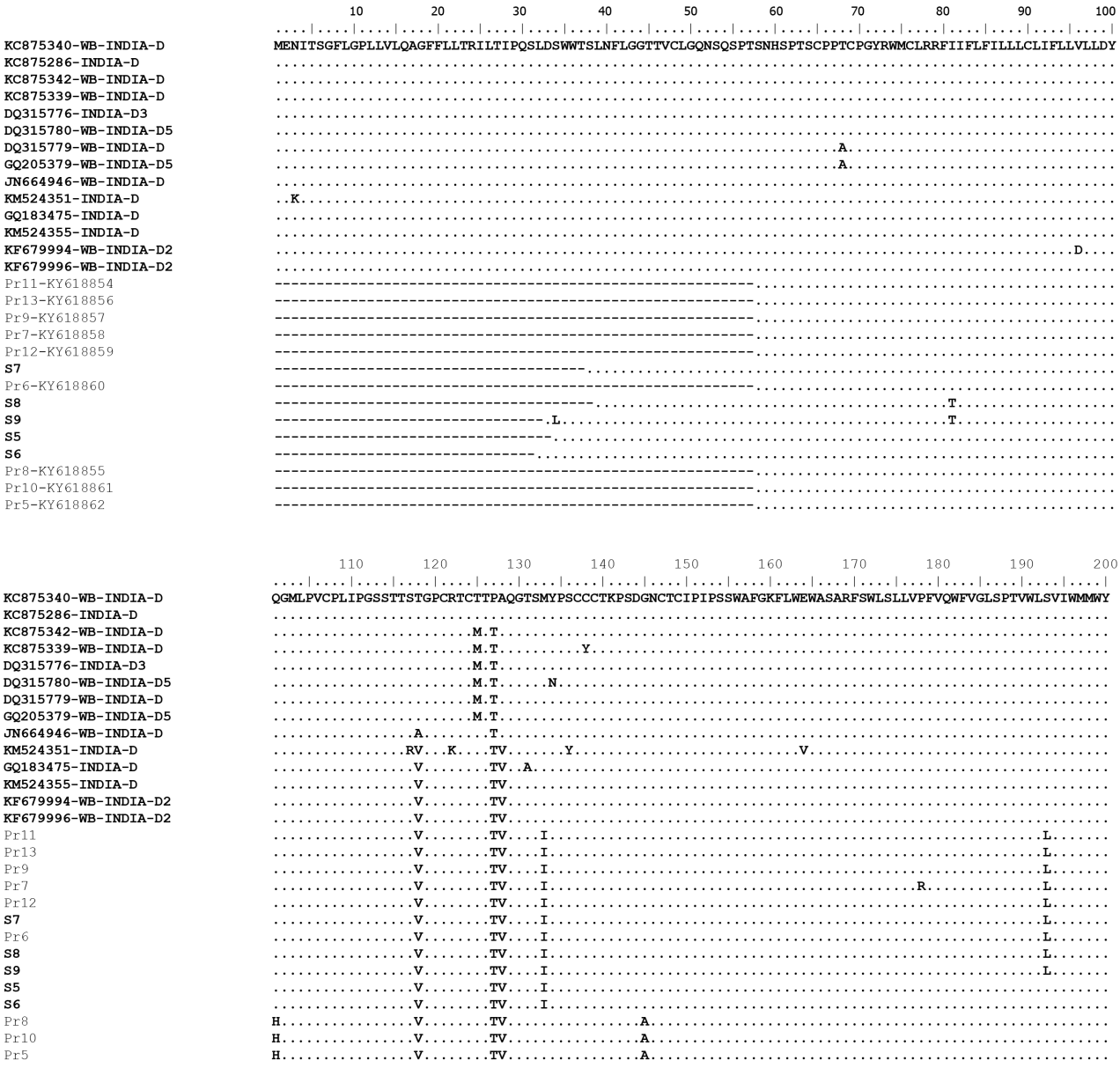
corroborated with the high copy number of HBV DNA found in the samples.

In contrast, the HBV DNA- negative sample, which showed globular structures previously (Figure 5), after being subjected to immunoprecipitation and subsequent Triton X-100 treatment, displayed a clear/vacant field with no globular structures or HBV-like particles visible using AFM (Figure 7A). Even at higher magnification, no such particles could be captured by AFM (Figure 7B).

DISCUSSION

To the best of our knowledge, the present study is the first to detect potentially viable HBV from circulating paper currency samples collected from various parts of Kolkata, a metropolitan city in eastern India. Sequences of the HBV-specific PCR products upon BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 15th February 2020) and phylogenetic analysis closely matched with recently circulating strains of HBV genotype D2 previously isolated from eastern Asia including Kolkata[10-15].

Although the potential role of currency in virus transmission is generally thought to be limited[16], it could still play a role in the spread of communicable viral diseases[17]. HBV, the causative agent of a highly infectious and acute/chronic liver disease, has been reported to retain infectivity when stored at approximately 30 °C for at least 6 mo and withstand drying on a surface for around 7 d when present in blood[18]. It can also remain infectious and survive in environmental surfaces for about a week[19]. This information coupled with the observation of intact and potentially viable HBV on paper currencies from Kolkata area in the present study demands attention because people of this region are of varied immune status and often apply saliva on fingertips to count the paper money. If these people have oral lesions (*e.g.*, mouth ulcers) or broken skin surfaces, it is quite probable that HBV particles on the infected currencies may be transmitted into the human subjects. Concurrent handling of paper currency and household materials may also set the stage for transmission of HBV among different family members[20]. This was further strengthened by reports from India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KC875286-INDIA-D
KC875342-WB-INDIA-D
KC875339-WB-INDIA-D
DQ315776-INDIA-D3
DQ315780-WB-INDIA-D5
DQ315779-WB-INDIA-D
GQ205379-WB-INDIA-D5
JN664946-WB-INDIA-D
KM524351-INDIA-D
GQ183475-INDIA-D
KM524355-INDIA-D
KF679994-WB-INDIA-D2
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KC875340-WB-INDIA-D
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KC875339-WB-INDIA-D
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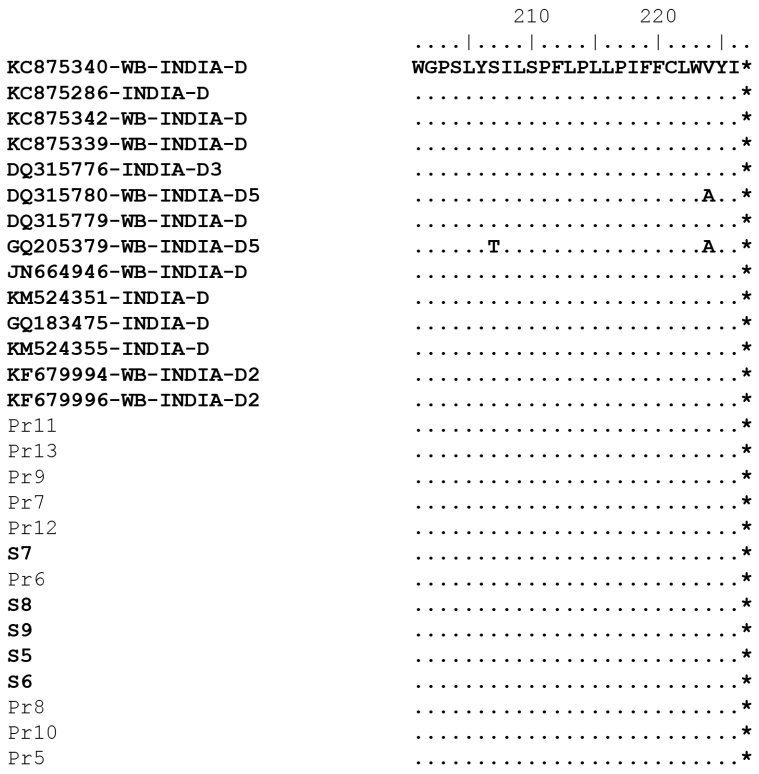

Figure 2 Hepatitis B virus S protein (partial) multiple sequence alignment of the test samples (S5-S9) with hepatitis B virus D2 sequences retrieved from GenBank. The retrieved sequences are identified by their GenBank accession numbers on the figure. Gene sequences pertaining to nucleotide positions 155 to 835 (coding for the full S protein) as in hepatitis B virus 34 genotype D2 isolate (accession number KC875340) are used for comparing with the test samples.

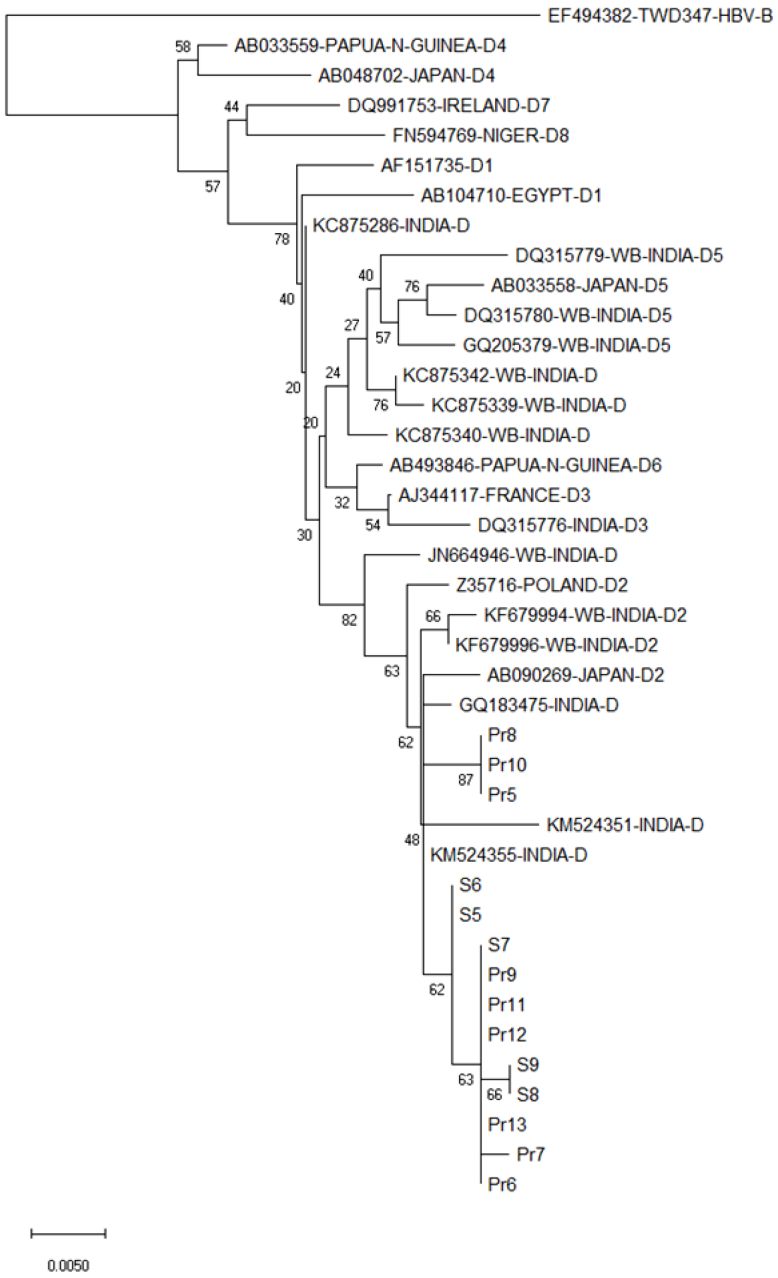

Figure 3 Phylogenetic analysis of the hepatitis B virus S gene (partial) of the hepatitis B virus isolates from the currency notes relative to several other hepatitis B virus isolates of genotype D. The analysis was based on variability in 510 nucleotides (nt), pertaining to nt positions 326-835 as in in hepatitis B virus 34 genotype D2 isolate, accession number KC875340, West Bengal, India. Hepatitis B virus sequences and their place of origin, retrieved from GenBank for comparing with hepatitis B virus sequences in the present study, are identified by their GenBank accession numbers. The numbers shown next to the branches of the phylogenetic tree represent the percentage of replicate trees in which the associated taxa grouped together in the bootstrap test (2000 replicates). The scale bar indicates genetic distance in terms of nucleotide substitution/site/year.

indicating that intra-familial horizontal transmission played a more important role in the spread of HBV infection than the sexual mode of HBV transmission[21,22].

The HBV sequences, identified in the present study, closely matched with HBV genotype D2, widely prevalent in various countries including India[10-12]. All these isolates carried S protein mutations previously reported to impair immune-detection and this explains why these isolates were HBsAg-negative by the enzyme immunoassay[23].

Our findings corroborate well with the fact that HBV genotype D2 is most prevalent in India including Kolkata[24,25]. In fact, the HBV S protein sequences retrieved from the currency samples closely matched at the amino acid level (Figure 2) and nucleotide level (Figure 3) with the HBV sequences, surprisingly detected in blood of *Pityriasis rosea* skin disease patients from Kolkata during 2016, who were screened for HBV in another study in our laboratory. More than 50% of these samples were occult HBV infection (OBI) and carried at least one OBI-signature S protein mutation[23].

HBV spread is silent as most of these infections are going undiagnosed as ELISAs are still the mainstay of virus diagnosis in developing countries like India, and the HBV strains in question are mostly "occult" in nature (*i.e.* not easily detectable by HBsAg-detection ELISAs).

The HBV DNA copy numbers observed in the five HBV-positive currency samples were higher than the 50% minimum infectious dose of even OBI DNA, which had been estimated at 1049 (117-3441) copies[26]. However, the SYBR-green qPCR method used for the HBV DNA quantification is prone to overestimation of the copy numbers as SYBR-green binds to nonspecific double stranded DNA sequences (*e.g.*, primer dimers) besides the target sequence, producing false positive signals[27,28]. In order to cross-check the calculated HBV DNA copy numbers present in entire 5 mL washings from each banknote, the numbers were back-calculated from the number of particles visible in AFM images (Figure 6). This was done with the assumption that HBV particles were evenly distributed in the circular area over which the spherical drop of the sample adsorbed on the mica sheet. The numbers came to be of the order of 10^9 copy numbers (total 5 mL washings from the concerned banknotes), which corroborated with PCR-estimated copy number for the said isolates. Conclusively, the HBV particles present on the currency notes were higher than 50% minimum HBV infectious dose, thereby posing the contaminated currencies as an effective source of HBV transmission.

It was also interesting to observe, in this context, that human saliva, often used for enumerating paper currencies in India, can have significantly high level of HBV contamination even to the level of 10^7/mL virus titre[29,30]. Furthermore, human saliva is often implicated in horizontal mode of HBV transmission besides the common and major routes of HBV transmission, namely, sexual contact, parenteral drug use, transfusion and vertical transmission[31,32].

The HBV genotype D2 strains detected in the currency notes carried several mutations (the trio of T118V/A128V double mutation and P127T mutation; M133I) known to impair immunological detection[23]. We were, indeed, unable to detect S antigen with a method (Bio-Rad's Monolisa ELISA) that is known to use an array of different monoclonal antibodies to reduce the number of S protein mutants that are not detected. Still, it was possible to concentrate these "occult" viruses for microscopy using the monoclonal antibody (SAB4700767), due to the fact that the S protein mutations in these viruses possibly did not affect the binding site of the said Mab. The result of the phylogenetic analysis is not surprising since the HBV genotype and lineage detected is the same that was circulating in the city, and this observation, in fact, supports our finding.

One limitation of our study is that although we think that the popular habit of using saliva in counting paper notes may be related to spread of HBV, we could not rule out several other routes of transmission that could also contribute to the surge of HBV infections in Kolkata in recent years. These include practices such as tattooing, piercing, drugs abuse, sex workers, unregistered medical practices, manicure/pedicure, ritual practices involving needles, knife and other sharp elements and so on[31,32].

Still, it appears that use of saliva in counting currency is a more predominant and generalized habit in the overall Indian population than the aforesaid other practices that expose the population to the risk of HBV transmission. However, many of these factors remain unknown due to the lack of reliable model for virus propagation and experimental infections. Some of these factors include evaluation of infectious dose of the virus by this route, the stability of the virus, the probability of counting money each day (time of exposition) and so on.

We could not demonstrate the "infectivity" of the HBV DNA-positive samples and it was also not feasible to demonstrate experimentally the transmission of HBV *via*

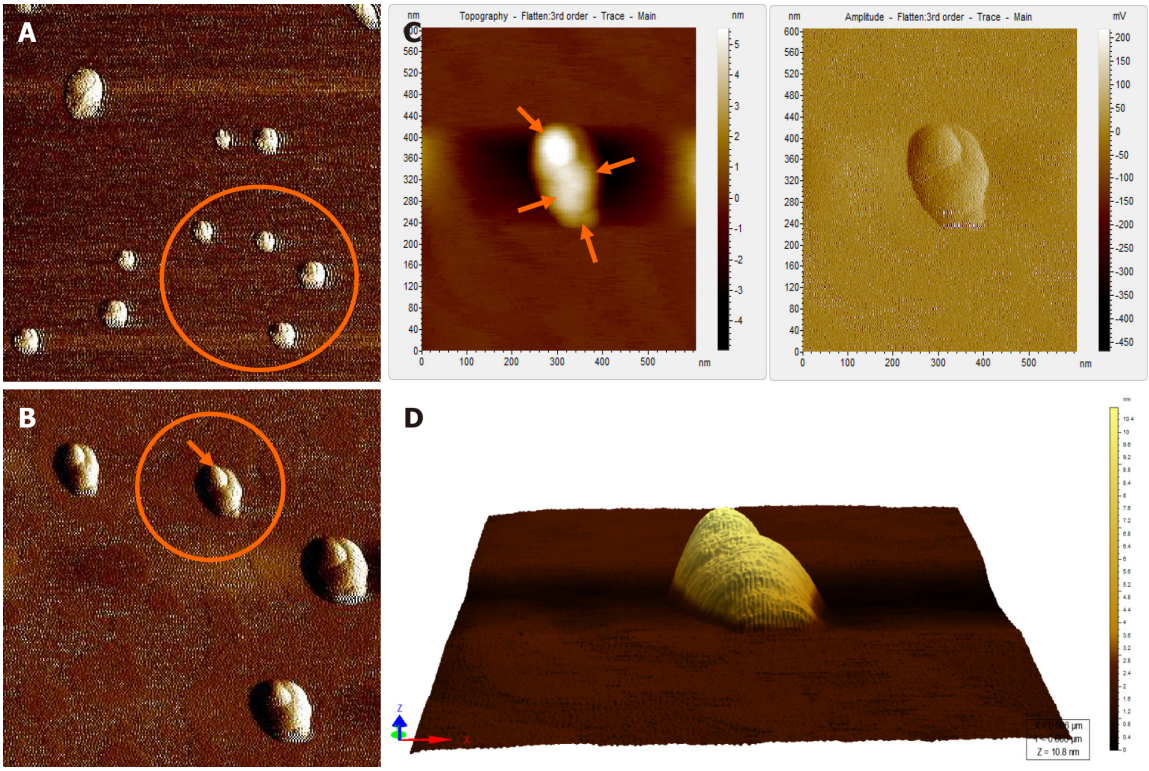

Figure 4 Atomic force microscopy images of hepatitis B virus-positive sample S8. A: Wider field of view for the sample; B: Magnified view of the encircled area marked in Figure 4A; The encircled area illustrates representative cluster of virion particles. The arrow points to a single particle in the cluster; C: Magnified view of encircled area shown in Figure 4B in two different contrasts. The arrows show the individual intact virion particle of approximately 42 nm diameter; D: Three-dimensional (3D) view of the individual clusters seen in Figure 4C.

paper notes. However, detection of intact virions in considerable amount on heavily used currency notes sets up the scenario that if people do contract HBV from the currency samples, they are likely to develop hepatitis B, which is difficult to diagnose by routine ELISA. Thus, there is increased risk of silent spread of the infection in the susceptible population.

Presently, the severe acute respiratory syndrome coronavirus 2 (SARS CoV-2), first reported from Wuhan, China in 2019, has emerged as a pandemic and a major health concern worldwide[33]. It has been stated that there is a high risk of transmission of this virus by touching contaminated dry inanimate surfaces like paper, wood, plastic, metal, steel, glass, ceramic, Teflon and so on. It has been already reported that the various strains of SARS CoV like P9 or GVU6109 can persist on the paper surfaces for 24 h to 5 d at room temperature[34]. Thus, the handling of contaminated paper currencies and enumerating them using saliva can pose a substantial risk of transmission of not only hepatitis B virus, as described in this study, but also SARS CoV-2, thereby contributing to its currently observed rapid spread in densely populated countries like India.

To put into perspective the role of paper currency (with HBV contamination) in virus transmission, we propose a model as follows: Infected saliva to finger to paper notes and then contaminated notes to finger to saliva of susceptible human, supposing that many people count the money in this way.

CONCLUSION

In summary, the presence of highly infectious HBV in commonly circulating currency notes in a populous region, as detected in this study, imposes possible risk of transmission of this pathogen among the general population. This, however, needs further experimental validation as already discussed above. This phenomenon might be contributing to the increasing incidence of HBV infections among the population besides other routes of exposure. Hence, people should be made aware about the unhygienic practices leading to microbial contamination of currencies to reduce the risk of transmission of infectious microbes through currency route.

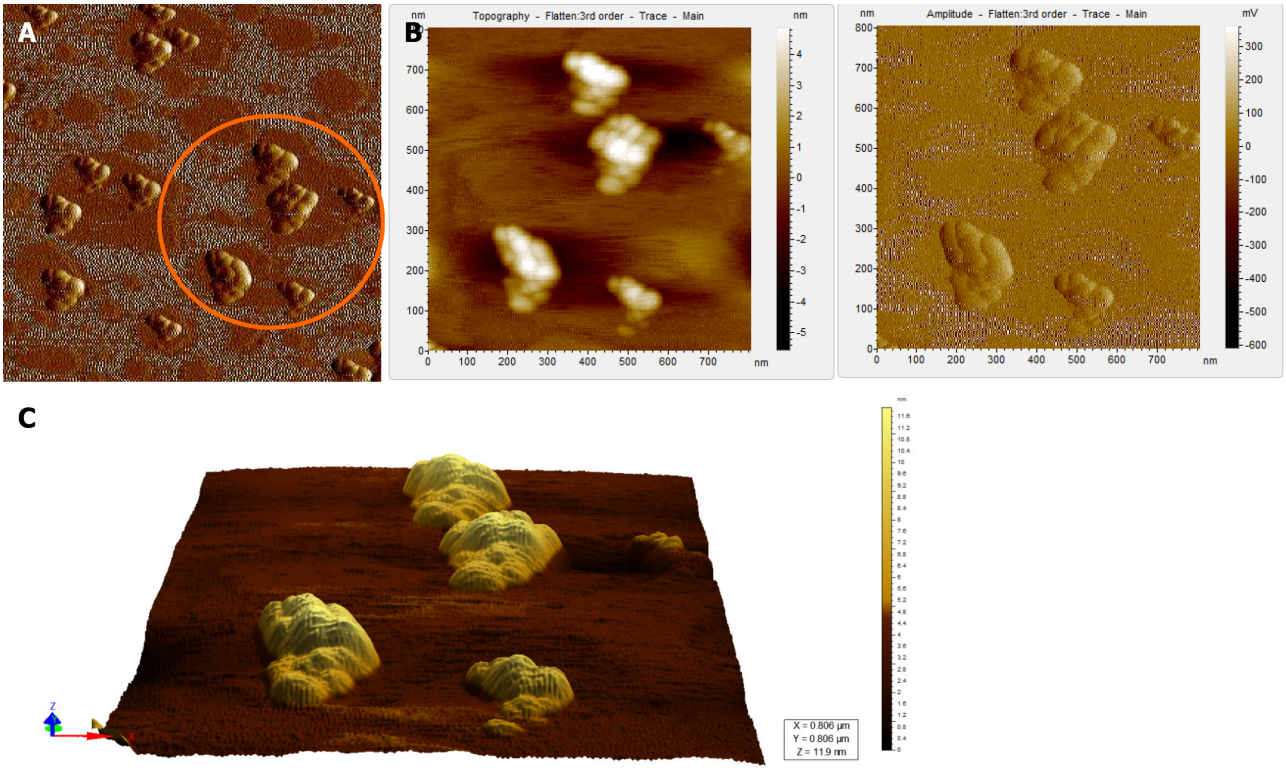

Figure 5 Atomic force microscopy images of a hepatitis B virus-negative sample. A: Wide field of view of clumps of globular structures that look different from that seen in Figure 4A. These clumps share no resemblance with the structure of hepatitis B virus particles; B: Magnified view of the encircled region in Figure 5A in two different contrasts; C: 3D view of the clumps.

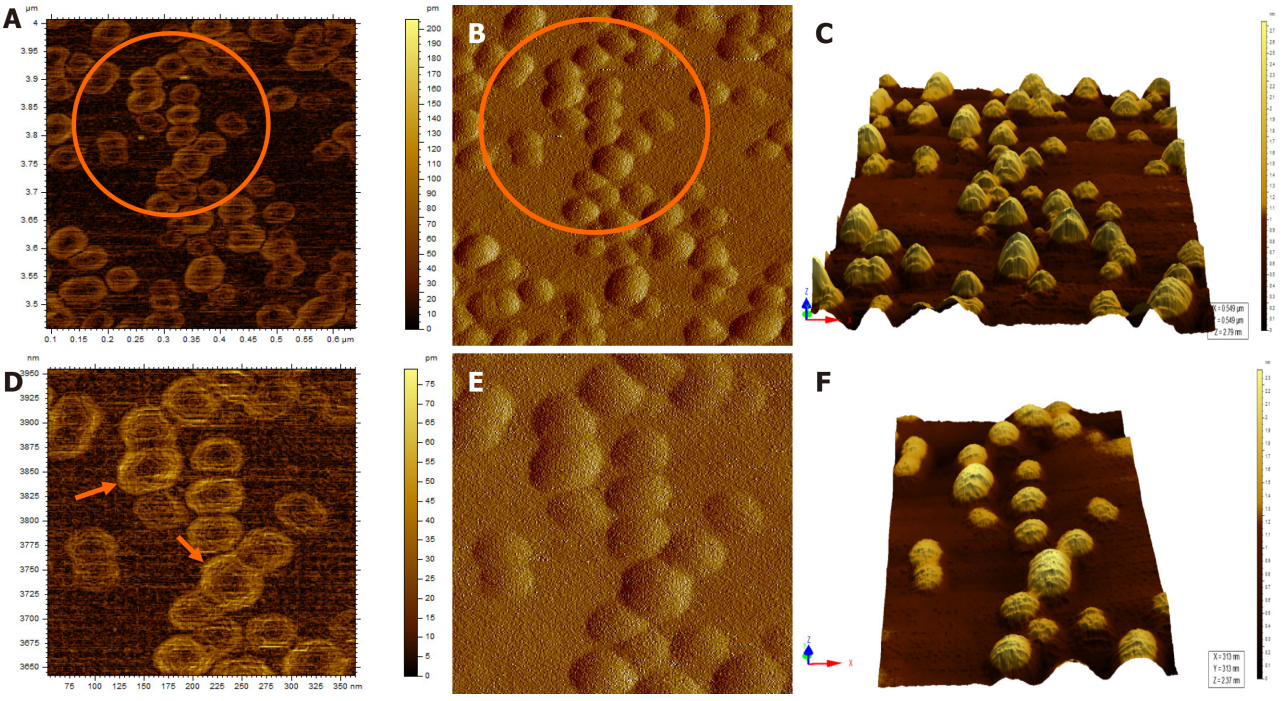

Figure 6 Atomic force microscopy images of sample S8 after immunoprecipitation and Triton X treatment. A and B: Typical icosahedral particles, plausibly hepatitis B virus core particles. Triton X-100 treatment followed by sonication led to partial/complete disintegration of virus envelope, thus exposing the core particles; D and E: Magnified images of the region encircled in Figure 6A and B, respectively. The arrows mark the icosahedral hepatitis B virus core particles; C and F: 3D images of the hepatitis B virus core particles seen in Figure 6B and E, respectively.

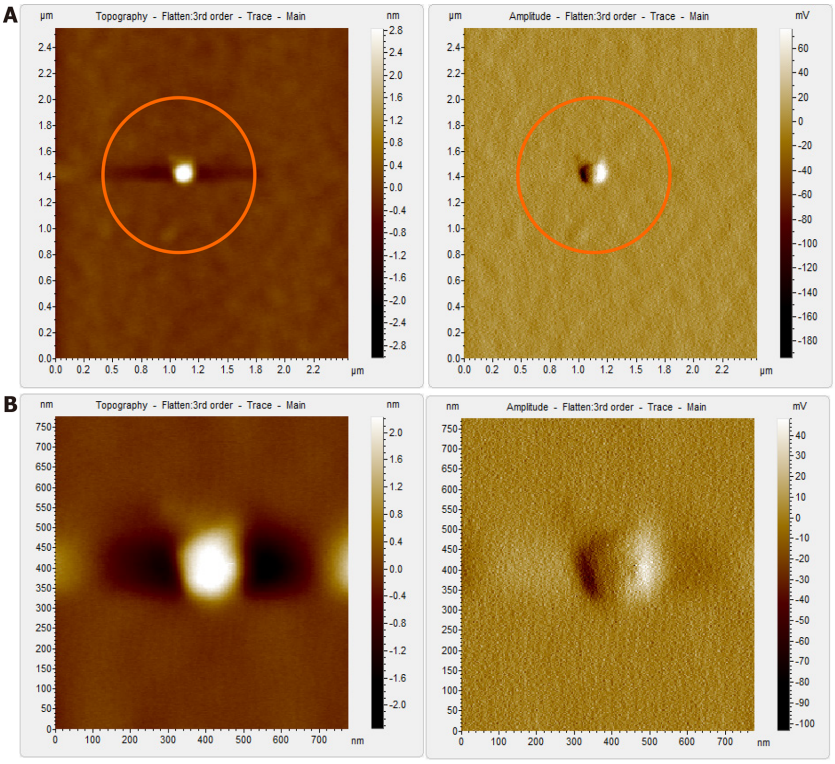

Figure 7 Atomic force microscopy images of hepatitis B virus-deoxyribonucleic acid negative sample, S12 after immunoprecipitation and Triton X treatment. A: Clear field with no previously observed globular structures. Triton X treatment led to the disruption of clumps seen in Figure 5A and was removed during the washing steps; B: Magnified view of the encircled area in Figure 7A, which shows no hepatitis B virus-like particles even at higher magnification.

ARTICLE HIGHLIGHTS

Research background

The recent rise in the incidence of hepatitis B virus (HBV) infections in a densely populated city of eastern India prompted the search. Paper currency is widely used as a mode of transaction for various goods and services irrespective of socio-economic status among the population. Therefore, the chances of microbial contamination specifically in the currencies of lower denominations are higher. The common practice of enumerating currency notes using saliva in Indian subcontinent may be a potential source of horizontal transmission of HBV, especially if there are cuts/bruises on the oral mucous membrane or skin.

Research motivation

The increasing number of cases of HBV infections in eastern India served as the impetus to investigate possible presence of this virus in low denomination paper notes in a densely populated city of India such as Kolkata.

Research objectives

To investigate whether paper currency can serve as a plausible mode of horizontal transmission of HBV infection in areas of high population density.

Research methods

HBV was detected by performing polymerase chain reactions (PCRs) on nucleic acids extracted from ultracentrifuged washings from paper currencies, followed by nucleotide sequencing for the confirmation of the presence of the virus. Hepatitis B virus surface antigen-enzyme-linked immunosorbent assay (HBsAg-ELISA) was carried on HBV DNA-positive samples to check for the detectability of HBV surface antigen. Atomic force microscopy (AFM) was used for visual confirmation of HBV particles in ultracentrifuged/immunoprecipitated samples from currency paper washings.

Research results

Out of all the currency notes screened (n = 70), 7.14% of the samples were found to be contaminated with potentially intact/viable HBV of genotype D2. Atomic force microscopy provided visual confirmation of HBV particles in ultracentrifuged/immunoprecipitated samples from currency paper washings. However, HBV isolates from the currency notes failed to be detected by hepatitis B surface antigen ELISA. Molecular analysis and enzyme immunoassays suggested that the circulating HBV are "occult" in nature (*i.e.* ELISA-negative but DNA-positive).

Research conclusions

Applying saliva on fingers for counting bank notes is a common practice in the Indian subcontinent and many other countries of the world. Paper notes may be a source of "horizontal" transmission of HBV as well as other environmentally stable infectious viruses like severe acute respiratory syndrome coronavirus 2, especially if there are cuts/bruises on the oral mucous membrane or skin. However, it was practically not possible to demonstrate experimentally such transmission. Detection of potentially intact/viable and "occult" HBV on currency notes and in considerable numbers poses potential risk of silent transmission of this virus in densely populated cities like Kolkata.

Research perspectives

Heavily used paper currency may play a potential role in transmission of infectious viruses like HBV. The present study puts forward a model of horizontal HBV transmission from infected saliva to finger to paper currencies and then from contaminated bank notes to finger to saliva of susceptible humans, especially in places where people have the habit of using saliva for counting bank notes.

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ORIGINAL ARTICLE

Case Control Study

PNPLA3 and *TM6SF2* polymorphisms in Brazilian patients with nonalcoholic fatty liver disease

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Abstract

BACKGROUND

Nonalcoholic fatty liver disease (NAFLD) is becoming the most common chronic liver disease worldwide, with significant morbidity associated with nonalcoholic steatohepatitis (NASH). Genome-wide association studies demonstrated that the variants rs738409 C/G in the *PNPLA3* and rs58542926 C/T in the *TM6SF2* genes are determinants of inter-individual and ethnicity-related differences in hepatic fat content and NAFLD progression.

AIM

To investigate *PNPLA3* and *TM6SF2* genotype frequency and their association with NAFLD development and progression in Brazilian patients.

METHODS

This cross-sectional case-control study enrolled 285 individuals from the Gastroenterology and Hepatology clinics at a university hospital in Brazil. The case patients (n = 148) were confirmed to have NAFLD by the identification of hepatic steatosis on ultrasonography and exclusion of other causes of liver disease. According to the clinical protocol, patients underwent liver biopsy when at high risk for NASH and/or advanced fibrosis (n = 65). Steatohepatitis was confirmed in 54 patients. Individuals who did not have biopsy indication or NASH on histology were considered to have simple steatosis (n = 94). The control group (n = 137) was selected among patients that attended the Intestinal Disease clinic and was composed of subjects without abnormalities on abdominal ultrasonography and normal liver biochemical tests. All individuals underwent

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PNPLA3 and *TM6SF2* genotype analysis.

RESULTS

PNPLA3 CC, CG and GG genotype frequencies were 37%, 44% and 19%, respectively, in NAFLD patients and were 58%, 31% and 10% in controls ($P < 0.001$). In a model adjusted for gender, age, body mass index and type 2 diabetes mellitus, the G allele increased the chance of NAFLD (OR = 1.69, 95%CI: 1.21-2.36, $P = 0.002$) and NASH (OR = 3.50, 95%CI: 1.84-6.64, $P < 0.001$). The chance of NASH was even higher with GG homozygosis (OR = 5.53, 95%CI: 2.04-14.92, $P = 0.001$). No association was found between G allele and the features of metabolic syndrome. In histological assessment, *PNPLA3* genotype was not associated with steatosis grade, although GG homozygosis increased the chance of significant NASH activity (OR = 17.11, 95%CI: 1.87-156.25, $P = 0.01$) and fibrosis (OR = 7.42, 95%CI: 1.55-34.47, $P = 0.01$) in the same adjusted model. *TM6SF2* CC, CT and TT genotype frequencies were 83%, 15% and 0.7%, respectively, in NAFLD patients and were 84%, 16% and 0.7% in controls ($P = 0.78$). The T allele presence was not associated with NAFLD or NASH, and was not associated with histological features.

CONCLUSION

PNPLA3 may be involved in susceptibility and progression of NAFLD and NASH in the Brazilian population. More advanced histological liver disease was associated with the G allele. The *TM6SF2* genetic variants were not associated with NAFLD susceptibility and progressive histological forms in the population studied, but further studies are required to confirm these findings.

Key Words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Genetic variation; Single nucleotide polymorphism; Genotype; Brazil; Fibrosis

Core Tip: The rs738409 C/G mutation in the *PNPLA3* gene may be involved in the pathogenesis of nonalcoholic fatty liver disease (NAFLD) in the Brazilian population. We found an association between this polymorphism and higher susceptibility to NAFLD occurrence, disease progression to nonalcoholic steatohepatitis, more severe histological activity scores and the presence of liver fibrosis. The *TM6SF2* gene variants were also evaluated in the Brazilian population, although they were not associated with NAFLD susceptibility and different histological forms.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatocellular accumulation of triglycerides (TG) in the absence of excess alcohol consumption or any other cause of secondary liver steatosis[1,2]. Currently, it is the most common chronic liver disease in Western countries, with an estimated prevalence of 25% globally and 32% in South America[3,4]. NAFLD is strongly associated with the metabolic syndrome [obesity, insulin resistance/type 2 diabetes mellitus (T2DM) and dyslipidemia][4,5] and encompasses a spectrum of liver diseases ranging from simple steatosis (SS), which is usually benign, to nonalcoholic steatohepatitis (NASH), which may lead to liver cirrhosis and hepatocellular carcinoma[6,7]. In fact, liver fibrosis is an independent risk factor of NAFLD severity and liver-related mortality[8,9].

Although highly prevalent, only a minority of NAFLD patients develop significant fibrosis and associated morbidity[10,11]. Thus, NAFLD is considered a complex disorder

in which the disease phenotype results from an interaction between environmental exposure and susceptible polygenic background that comprises multiple independent modifiers[12,13].

In recent years, genome-wide association (GWA) and candidate gene studies have greatly contributed to understanding the genetics of NAFLD and their influence on prognosis[14-16]. In 2008, Romeo *et al*[17] identified that the mutation I148M encoded by the G allele at rs738409 in the gene patatin-like phospholipase domain-containing 3 (*PNPLA3*) is a major determinant of inter-individual and ethnicity-related differences in hepatic fat content. These findings have been confirmed in different populations through GWA[18-20] and candidate gene[21-26] studies, which demonstrated that *PNPLA3* polymorphism was associated with greater susceptibility to NAFLD development and more severe histological and clinical forms.

In 2014, Kozlitina *et al*[27] identified that the E167K (rs58542926 C/T) variant in the *TM6SF2* gene was also associated with increased liver fat content. This polymorphism was later associated with greater susceptibility to NAFLD and advanced histological NASH[28,29]. In this context, there is only one genetic study in Brazilian NAFLD patients[30], and *TM6SF2* polymorphism has not yet been analyzed in this population.

Therefore, in this study, we investigated the association between *PNPLA3* and *TM6SF2* genotypes and clinical parameters of NAFLD, and analyzed the genotype variations as markers of liver histological features in Brazilian adult NAFLD patients. We also investigated the distribution of these genotype variations among Brazilians.

MATERIALS AND METHODS

Subjects and study design

A cross-sectional study was performed at the Outpatient NAFLD Clinic, Medical School of Federal University of Minas Gerais, Belo Horizonte, Brazil. A total of 285 individuals (208 females and 77 males) were enrolled; of which, 148 patients had features of NAFLD (case patients) and 137 were non-NAFLD control subjects.

The case patients were confirmed to have hepatic steatosis by liver ultrasonography (US) according to established criteria[31]; additionally, other causes of liver disease were excluded, including elevated alcohol intake (men, > 30 g/d; women, > 20 g/d), autoimmune disorders (autoimmune hepatitis, primary biliary cholangitis and primary sclerosing cholangitis), hereditary hemochromatosis, alpha-1-antitrypsin deficiency, Wilson disease and hepatitis B or C virus infection. Patients who had decompensated cirrhosis or were taking drugs that induce steatosis were excluded. The NAFLD patients underwent liver biopsy according to the clinical protocol suggested by Chalasani *et al*[2]. Increased risk of NASH and/or advanced fibrosis included the presence of metabolic syndrome or significant fibrosis predicted by noninvasive methods. Subjects who had a liver biopsy showing steatosis, hepatocyte ballooning and lobular inflammation[1] were classified into the NASH group (n = 54) and individuals without an indication for liver biopsy and those who did not fulfill the NASH criteria on biopsy were classified into the SS group (n = 94).

Control subjects were selected from patients attending our institution at the Outpatient Intestinal Disease Clinic whose gender matched the NAFLD patients (97 females and 40 males). Individuals in the control group underwent a standard health examination, liver US and liver biochemistry. They were selected if there was no evidence of fatty liver on US and no liver biochemical abnormalities. Furthermore, the control subjects did not present any of the features of the metabolic syndrome as defined by the International Diabetes Federation criteria[32] and did not abuse alcohol.

Subjects from whom deoxyribonucleic acid (DNA) could be obtained for genotyping *PNPLA3* at rs738409 and *TM6SF2* at rs58542926 were included in the analyses. The case participants and the controls were selected from patients attending our institution from January 2017 to December 2018.

All the investigations performed in this study were conducted in accordance with the guidelines of the 1975 Declaration of Helsinki and its subsequent amendments. Written informed consent was obtained from each subject, and the study was approved by the Ethics Committee of the Federal University of Minas Gerais (CAAE 23610614.0.0000.5149).

Liver biopsy and histopathological evaluation

Liver biopsy was performed in patients with NAFLD who were at increased risk of having NASH and/or advanced fibrosis (*i.e.*, presence of metabolic syndrome or advanced fibrosis indicated by the NAFLD Fibrosis Score[33])[2]. Liver biopsy specimens

were routinely fixed in 40 g/L formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin, Masson trichrome and silver impregnation for reticular fibers analysis. The same liver pathologist, who was blinded to the patients' information, analyzed all the exams. All the biopsies were at least 2 cm in length and contained a minimum of 8 portal tracts. The histological criterion for the diagnosis of NAFLD was macrovesicular fatty deposit in more than 5% of the hepatocytes. The NASH diagnostic criterion was the simultaneous presence of steatosis, ballooning and lobular inflammation in the liver biopsy, according to the Fatty Liver Inhibition of Progression algorithm[1]. Steatosis was graded based on the percentage of hepatocytes containing large and medium-sized intracytoplasmic lipid droplets, on a scale of 0 to 3 (S0: < 5%; S1: 5%-33%, S2: 34%-66%, S3: > 67%). Lobular inflammation was defined as a focus of 2 or more inflammatory cells within the lobule organized either as microgranulomas or located within the sinusoids. Foci were counted at × 20 magnification (grade 0: none; 1: ≤ 2 foci per lobule; 2: > 2 foci per lobule). Hepatocyte ballooning was graded from 0 to 2 (0: Normal hepatocytes with cuboidal shape, sharp angles and pink eosinophilic cytoplasm; 1: Presence of clusters of hepatocytes with a rounded shape and pale cytoplasm; 2: As for grade 1, associated with at least one enlarged ballooned hepatocyte). Severity of fibrosis was scored according to the Pathology Committee of the NASH Clinical Research Network method[34] and was expressed on a 4-point scale, as follows: 0, none; 1, perivenular and/or perisinusoidal fibrosis in zone 3; 2, combined pericellular and portal fibrosis; 3, septal/bridging fibrosis; 4, cirrhosis. The grade of NASH activity (A) was calculated by the addition of the grades of ballooning and lobular inflammation (from A0 to A4). Based on the Steatosis, Activity, and Fibrosis score evaluation[35], NASH was also classified histologically into mild (A ≤ 2 and F ≤ 2) and significant (A > 2 and/or F > 2).

Clinical and laboratory evaluation

Patient weight and height were measured using a calibrated scale after removing shoes and heavy clothing, if any. Body mass index (BMI) was calculated as the ratio of weight (in kilograms)/height (in square meters). Waist circumference was measured at the mid-level between the lower rib and the iliac crest[36]. Venous blood samples were obtained from the subjects after an overnight fast (12 h) to measure plasma glucose, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyltranspeptidase, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol (LDL), TG, total bilirubin, platelets, and ferritin concentrations. Serum vitamin D and fasting insulin levels were obtained only from the NAFLD patients. Homeostasis Model Assessment was used to evaluate insulin resistance and was calculated as fasting serum insulin (μU/mL) × fasting plasma glucose (mmol/L)/22.5[37]. All biochemical parameters were measured in a conventional automated analyzer. A blood sample was also obtained for DNA extraction and genotyping.

DNA preparation and single nucleotide polymorphism genotyping

Genomic DNA was extracted using the high salt method[38]. A predesigned TaqMan® probe (Applied Biosystems, Foster City, CA, United States) was purchased for genotyping rs738409 (C_7241_10) and rs58542926 (C_89463510_20). Genotyping was performed by real-time PCR in allelic discrimination mode, using the Strategene® Mx3005 equipment (MxPro QPCR System, 2007 Software, La Jolla, CA, United States). PCR protocols were carried out according to the TaqMan® Genotyping Master Mix manufacturer's instructions (Applied Biosystems, Foster City, CA, United States). The success rates of these assays were > 99%.

Statistical analysis

Statistical analyses were performed using SPSS 23.0 software (IBM, United States). Data are expressed as mean ± SD for normally distributed continuous variables, as median and interquartile range when the distribution was skewed, or as number and percentage for qualitative variables. Continuous variables distribution was assessed by the Shapiro-Wilk test. The Hardy-Weinberg equilibrium was checked for all individual single nucleotide polymorphisms (SNPs)[39]. The Student's *t*-test or a non-parametric test, *i.e.*, Mann-Whitney U-test were used to compare quantitative data, as appropriate. χ^2 test or Fisher's exact tests were used for comparison of categorical data. All tests were two-tailed and *P* values < 0.05 were considered significant. Variables associated with a *P* value < 0.20 in univariate analysis were included in multivariate analysis.

Multinomial binary logistic regression analysis was performed to detect the effect of a SNP mutation on liver histology. For association analysis, the *PNPLA3* rs738409 and

the *TM6SF2* rs58542926 variants were coded in an additive and dominant genetic model. The model fit was assessed by the Hosmer-Lemeshow test.

RESULTS

Patient characteristics

Clinical features, anthropometric variables and laboratory findings of the 148 NAFLD patients and 137 controls are shown in Table 1.

Comparing the NAFLD group with the controls, there were no significant differences regarding gender and age distributions. NAFLD patients showed a significantly higher frequency of the metabolic syndrome components–T2DM, arterial hypertension and dyslipidemia–and significantly higher values of BMI. Fasting glucose, TG, AST, ALT, ALP and gamma glutamyltranspeptidase serum concentrations were also significantly higher in the NAFLD subjects (Table 1).

Liver biopsy was performed in 65 of the NAFLD patients (43.9%). Based on histological findings, 54 (36.5%) individuals (7 with cirrhosis) were included in the NASH group. The SS group included 94 (63.5%) NAFLD subjects without a clinical indication for histological examination (*i.e.* patients without metabolic syndrome and/or advanced fibrosis by noninvasive methods) and those without NASH on biopsy. Clinical and demographic characteristics of the NASH and SS patients are depicted in Table 2.

A significantly higher proportion of subjects in the NASH group had T2DM or impaired fasting glucose (IFG) when compared to the SS group, but there was no difference regarding the frequency of arterial hypertension, dyslipidemia or BMI. AST, ALT and ferritin serum concentration levels were significantly higher in the NASH group than in the SS group (Table 2). Fibrosis was detected in 35 of the 65 biopsies: F1 = 18, F2 = 5, F3 = 5, and F4 = 7.

Association between clinical and metabolic characteristics and the PNPLA3 variant

The *PNPLA3* gene was successfully amplified by real-time PCR in all NAFLD patients and controls. The minor allele frequency (MAF) of *PNPLA3* rs738409 in overall patients was 34%, and the genotype frequencies were CC 47%, CG 38% and GG 15%. The Hardy-Weinberg equilibrium was demonstrated for the selected SNP of *PNPLA3* in the NAFLD ($P = 0.26$) and the control ($P = 0.07$) groups.

The C and G allele frequencies in the NAFLD patients were 59% and 41%, respectively, and were 74% and 26% among the controls. *PNPLA3* genotypes are described in Table 3; their frequencies were significantly different between the NAFLD patients and the controls ($P < 0.001$).

The chance of NAFLD was increased by the presence of the G allele [odds ratio (OR) = 2.37, 95%CI: 1.47-3.82; $P < 0.001$], even after adjustment for age, gender, BMI and T2DM/IFG (OR = 1.69, 95%CI: 1.21-2.36; $P = 0.002$) (Table 4). This association was even stronger when CC homozygotes were compared with GG homozygotes (OR = 3.13, 95%CI: 1.49-6.57; $P = 0.003$).

In the NAFLD group, no association was found between PNPLA3 CC *vs* CC + CG genotypes concerning BMI ($P = 0.421$), waist circumference ($P = 0.641$), FG ($P = 0.795$), high-density lipoprotein cholesterol ($P = 0.723$), low-density lipoprotein ($P = 0.614$) and TG ($P = 0.269$). On the other hand, serum AST ($P < 0.001$) and ALT ($P = 0.002$) were associated with the presence of rs738409 G allele at *PNPLA3*.

Association between histological features and the PNPLA3 variant

The NAFLD group was assessed for the association between histological features including steatosis, ballooning, inflammation and fibrosis, and the *PNPLA3* genotype. Although the presence of hepatic steatosis on US was associated with the G variant rs738409, we found no association between the histological grade of steatosis and the presence of the G allele (Figure 1B). The severity of both lobular inflammation and hepatocellular ballooning was associated with the *PNPLA3* variant G allele ($P < 0.001$).

The prevalence of NASH was 50% in the NAFLD subjects with the CC genotype (10/20), 77% in those with the CG genotype (23/30), and 100% in those with the GG genotype (15/15) ($P < 0.001$) (Figure 1A). G allele presence increased the chance of NASH (OR = 2.21, 95%CI: 1.04-4.71, $P = 0.039$), especially after adjusted for age, gender, BMI and T2DM (OR = 3.50, 95%CI: 1.84-6.64, $P < 0.001$). This chance was even higher when we analyzed homozygosis GG *vs* CC (OR = 6.07, 95%CI: 2.06-17.81, $P = 0.001$), even after the same adjusted model (OR = 5.53, 95%CI: 2.04-14.92, $P = 0.001$) (Table 4).

Table 1 Clinical and demographic characteristics of nonalcoholic fatty liver disease patients and controls

Variables	NAFLD, *n* = 148	Controls, *n* = 137	*P* value
Female	111 (75.0%)	97 (70.8%)	0.426
Age (yr)	57 (46.3-63)	55 (43 61.5)	0.610
BMI (kg/m^2)	32.8 ± 4.8	25.4 ± 3.3	< 0.0001
Hypertension	98 (66.2%)	24 (17.5%)	< 0.0001
Diabetes mellitus	69 (46.6%)	12 (8.8%)	< 0.0001
Dyslipidemia	108 (73.0%)	19 (13.5%)	< 0.0001
Fasting glucose (mg/dL)	101 (90-124.8)	88 (83-98)	< 0.0001
AST (U/L)	34 (25.5-56.5)	24 (20.3-29)	< 0.0001
ALT (U/L)	38 (28-70.5)	29.5 (23-38)	< 0.0001
GGT (U/L)	53 (29-116)	28 (18.5-55.5)	< 0.0001
ALP (U/L)	97 (74-116.8)	75 (58.3-83.8)	< 0.0001
Total bilirubin (mg/dL)	0.6 (0.5-0.9)	0.7 (0.5-0.8)	0.438
Triglycerides (mg/dL)	162.5 (110-205.3)	126.5 (93-176)	0.006
Total cholesterol (mg/dL)	195.8 ± 41.5	179.9 ± 47.1	0.006
VLDL (mg/dL)	32 (22-42.3)	24.5 (17.3-33.8)	0.001
HDL (mg/dL)	48 (39.5-54)	47 (40.5-60.8)	0.404
LDL (mg/dL)	107.5 (89.5-137.9)	96 (67.8-117.5)	0.009
Platelets (10^9/L)	219.4 ± 81.8	254.6 ± 82.4	0.439
Ferritin (ng/mL)	89 (42.7-168.5)	32 (32-32)	0.349
D vitamin (ng/mL)	22 (19-27.7)	26.95 (20.5-32.5)	0.139

The data are expressed as *n* (%), or as mean ± SD for normally distributed variables and as median (interquartile range) when distribution of the variable was skewed. NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; AST: Serum aspartate aminotransferase; VLDL: Very low-density lipoprotein; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; LDL: Low-density lipoprotein cholesterol.

The chance of significant NASH activity (A > 2) was higher in homozygosis GG (OR = 17.11; 95%CI: 1.87-156.25; *P* = 0.012) in multivariate analysis. Based on the Steatosis, Activity, and Fibrosis score, significant NASH was associated with *PNPLA3* GG genotype (Figure 1D).

The presence of fibrosis was observed in 7 of the 20 (35%) patients who underwent liver biopsy with the CC genotype; in 16 of 30 (53%) with the CG genotype, and in 12 of 15 (80%) with the GG genotype (*P* = 0.01) (Figure 1F). Presence of the G allele increased the chance of liver fibrosis in a model adjusted for age, gender, BMI and T2DM/IFG (OR = 3.05; 95%CI: 1.01-9.17; *P* = 0.04). The GG homozygosis increased the chance of fibrosis (OR = 7.42; 95%CI: 1.55-35.47; *P* = 0.01) after the same adjusted model (Table 5).

Association between the TM6SF2 variant, clinical characteristics and histological features

The *TM6SF2* rs58542926 genotypes were confirmed to be in Hardy–Weinberg equilibrium with a MAF in the overall NAFLD cohort of 8%. The genotype frequencies were CC 84%; CT 15%; and TT 1%.

TM6SF2 genotype distribution among NAFLD and controls are described in Table 6. There was no significant difference in the allele distribution between cases and controls (*P* = 0.78). The frequencies of T alleles were 9% in the controls and 8% in NAFLD patients. The presence of T alleles was not associated with NAFLD or NASH in univariate or multivariate analysis. *TM6SF2* T alleles were more frequent in the NASH group (22%) than in the SS group (12%), but without statistical significance (*P* = 0.89).

Finally, there was no association between the gene *TM6SF2* rs58542926 and liver steatosis (*P* = 0.62), ballooning (*P* = 0.14), lobular inflammation (*P* = 0.99) and fibrosis (

Table 2 Clinical and demographic characteristics of the nonalcoholic steatohepatitis and simple steatosis population

Variables	NASH, *n* = 54	Simple steatosis, *n* = 94	*P* value
Female	44 (81.5%)	67 (71.3%)	0.169
Age (yr)	59 (45.8-63)	57 (47.8-64)	0.935
BMI (kg/m^2)	32.7 ± 4.8	32.8 ± 4.8	0.968
WC	106.6 (100-114)	105.3 (96-113.8)	0.306
Arterial hypertension	39 (72.2%)	59 (62.8%)	0.886
Diabetes mellitus	35 (64.8%)	34 (36.2%)	0.007
Dyslipidemia	45 (83.3%)	63 (67.0%)	0.248
Fasting insulin	16.5 (11.9-31)	14 (8-18)	0.055
Fasting glucose (mg/dL)	112.5 (91-151.5)	98 (90-112)	0.019
AST (U/L)	53.5 (31.7-69)	30 (23-38)	< 0.001
ALT (U/L)	61 (36-79.3)	33 (24-49)	< 0.001
GGT (U/L)	87 (51.7-164.3)	39 (27-73.3)	< 0.001
ALP (U/L)	99 (78-127.5)	88 (72-116)	0.075
Total bilirubin (mg/dL)	0.59 (0.5-0.9)	0.6 (0.5-0.9)	0.986
Triglycerides (mg/dL)	162 (113-227.8)	163 (109.25-201)	0.544
Total cholesterol (mg/dL)	196.4 ± 39.9	195.4 ± 42.7	0.793
VLDL (mg/dL)	32 (22.8-45.3)	32 (22-40.8)	0.440
HDL (mg/dL)	46 (37.8-53)	48 (40-55)	0.432
LDL (mg/dL)	108.5 (89-138.4)	107 (88.5-137.4)	0.793
Platelets (10^9/L)	184 ± 67.4	308 ± 1.4	0.053
Ferritin (ng/mL)	109 (56.75-267.5)	69 (37.2-143.8)	0.019
D vitamin (ng/mL)	21.1 (17.55-25.9)	23.4 (19-28)	0.086
HOMA-IR	5.26 (2.97-8.8)	3.36 (1.77-4.7)	0.330

The data are expressed as *n* (%), or as mean ± SD for normally distributed variables and as median (interquartile range) when distribution of the variable was skewed. NASH: Nonalcoholic steatohepatitis; BMI: Body mass index; AST: Serum aspartate aminotransferase; VLDL: Very low-density lipoprotein; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; LDL: Low-density lipoprotein cholesterol.

Table 3 *PNPLA3* rs738409 genotype frequencies in nonalcoholic fatty liver disease patients and controls

	Genotype frequency % (*n*)			*P* value
	CC	CG	GG	
NAFLD	37.2 (55)	43.9 (65)	18.9 (28)	< 0.001
Controls	58.4 (80)	31.4 (43)	10.2 (14)	

Data were analyzed by the Chi-square test. NAFLD: Nonalcoholic fatty liver disease.

P = 0.89).

DISCUSSION

This study demonstrated that the *PNPLA3* genotype is associated with NAFLD susceptibility and different clinical forms. The presence of the G allele was associated with NAFLD occurrence when compared to the controls, and with NASH when compared to SS, in addition it was associated with higher NASH histological activity

Table 4 Association between the *PNPLA3* rs738409 genotype and nonalcoholic fatty liver disease or nonalcoholic steatohepatitis, adjusted for age, gender, body mass index and diabetes

Genotypes	OR	95%CI	*P* value
NAFLD *vs* Control			
CC *vs* CG/GG	1.69	1.21–2.36	0.002
CC *vs* GG	3.13	1.49–6.57	0.003
NASH *vs* SS			
CC *vs* CG/GG	3.50	1.84–6.64	< 0.001
CC *vs* GG	5.53	2.04-14.92	0.001

NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

Table 5 Association of the *PNPLA3* rs738409 genotype and activity of nonalcoholic steatohepatitis and fibrosis, adjusted for age, gender, body mass index and diabetes, evaluated by multivariate binary logistic regression analysis

Genotypes	OR	95%CI	*P* value
Activity of NASH (A > 2 *vs* A < 2)			
CC *vs* CG/GG	0.65	0.22-1.88	0.433
CC *vs* GG	17.11	1.87 - 156.25	0.012
Fibrosis (presence *vs* absence)			
CC *vs* CG/GG	3.05	1.01-9.17	0.046
CC *vs* GG	7.42	1.55-35.47	0.012

NASH: Nonalcoholic steatohepatitis.

Table 6 *TM6SF2* rs58542926 genotype frequencies in nonalcoholic fatty liver disease patients and controls

	Genotype frequency % (*n*)			*P* value
	CC	CT	TT	
NAFLD	83.2 (125)	14.9 (22)	0.7 (1)	0.78
Controls	84.5 (114)	16.1 (22)	0.7 (1)	

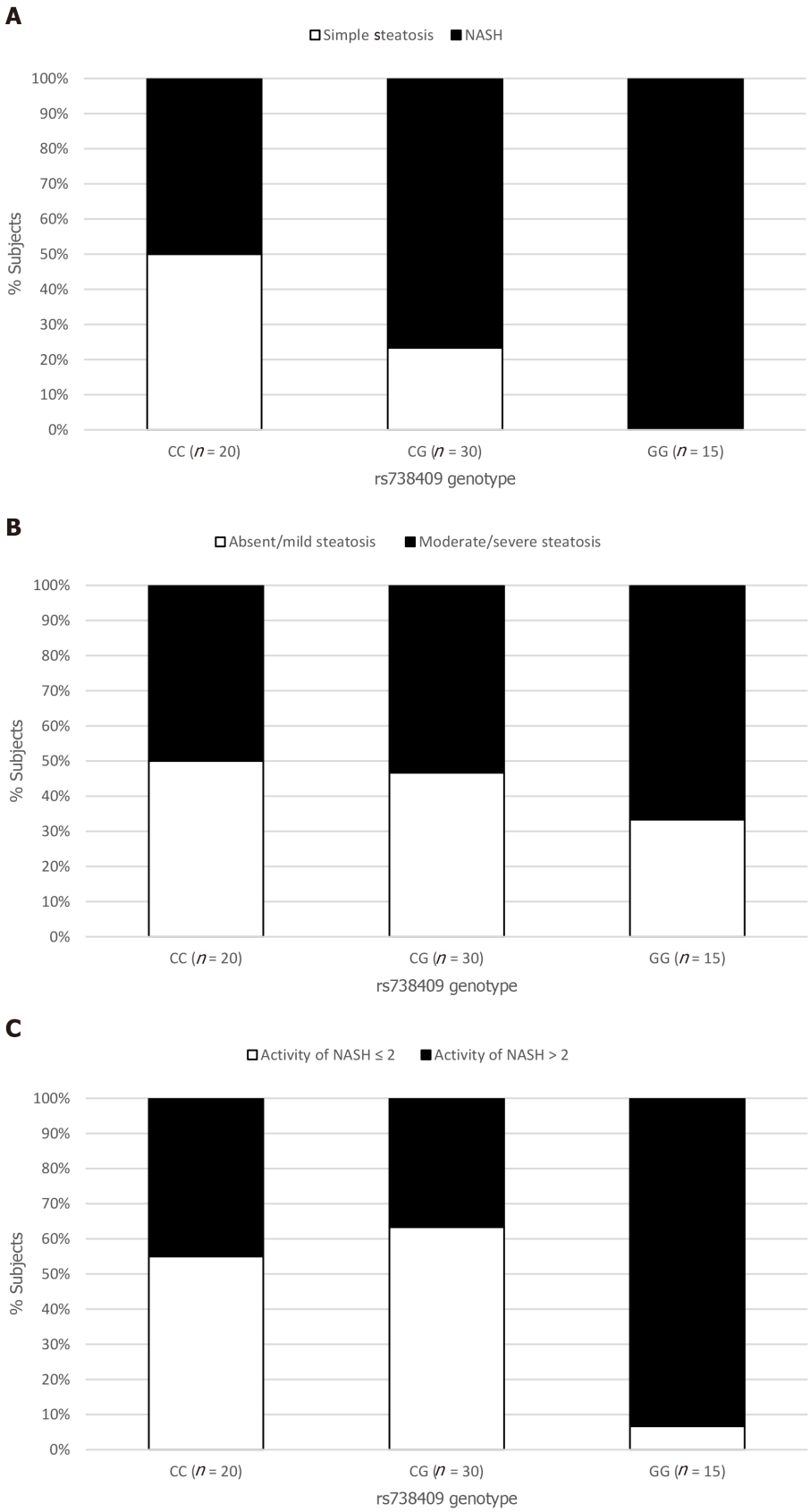
NAFLD: Nonalcoholic fatty liver disease.

score and the presence of fibrosis in individuals with histopathological assessment. The *TM6SF2* genotype frequency was analyzed for the first time in a Brazilian population, and we did not observe any significant difference in the variant gene distribution between NAFLD patients and controls, or between NASH and SS subjects.

The first GWA study in NAFLD identified a single highly significant association between increased hepatic TG content and the *PNPLA3*[17]. Subsequent studies demonstrated that this variant was also associated with the progression of NAFLD in different ethnic populations around the world[21,25,26,41,42].

In the Brazilian population, there are only two studies on NAFLD and *PNPLA3* genotypes[30,43]. Machado *et al*[43] investigated the gene polymorphism in T2DM individuals and Mazo *et al*[30] evaluated the genetic variation in NAFLD subjects compared to controls. The *TM6SF2* polymorphisms were assessed in the investigation by Mazo *et al*[30]; however, they were not in Hardy-Weinberg equilibrium in the NAFLD group, which precluded this analysis. Therefore, the present study contributes to the *PNPLA3* genotype study in Brazil and is the first study to document the distribution of *TM6SF2* alleles and genotypes in Brazilian NAFLD patients.

In this sample, the frequency of the minor (G) allele at *PNPLA3* rs738409 was higher (34%) than the frequency reported in European (23%) and African (15%) individuals,


A
Simple steatosis
NASH
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% Subjects
CC (n = 20)
CG (n = 30)
GG (n = 15)
rs738409 genotype
B
Absent/mild steatosis
Moderate/severe steatosis
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% Subjects
CC (n = 20)
CG (n = 30)
GG (n = 15)
rs738409 genotype
C
Activity of NASH ≤ 2
Activity of NASH > 2
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% Subjects
CC (n = 20)
CG (n = 30)
GG (n = 15)
rs738409 genotype

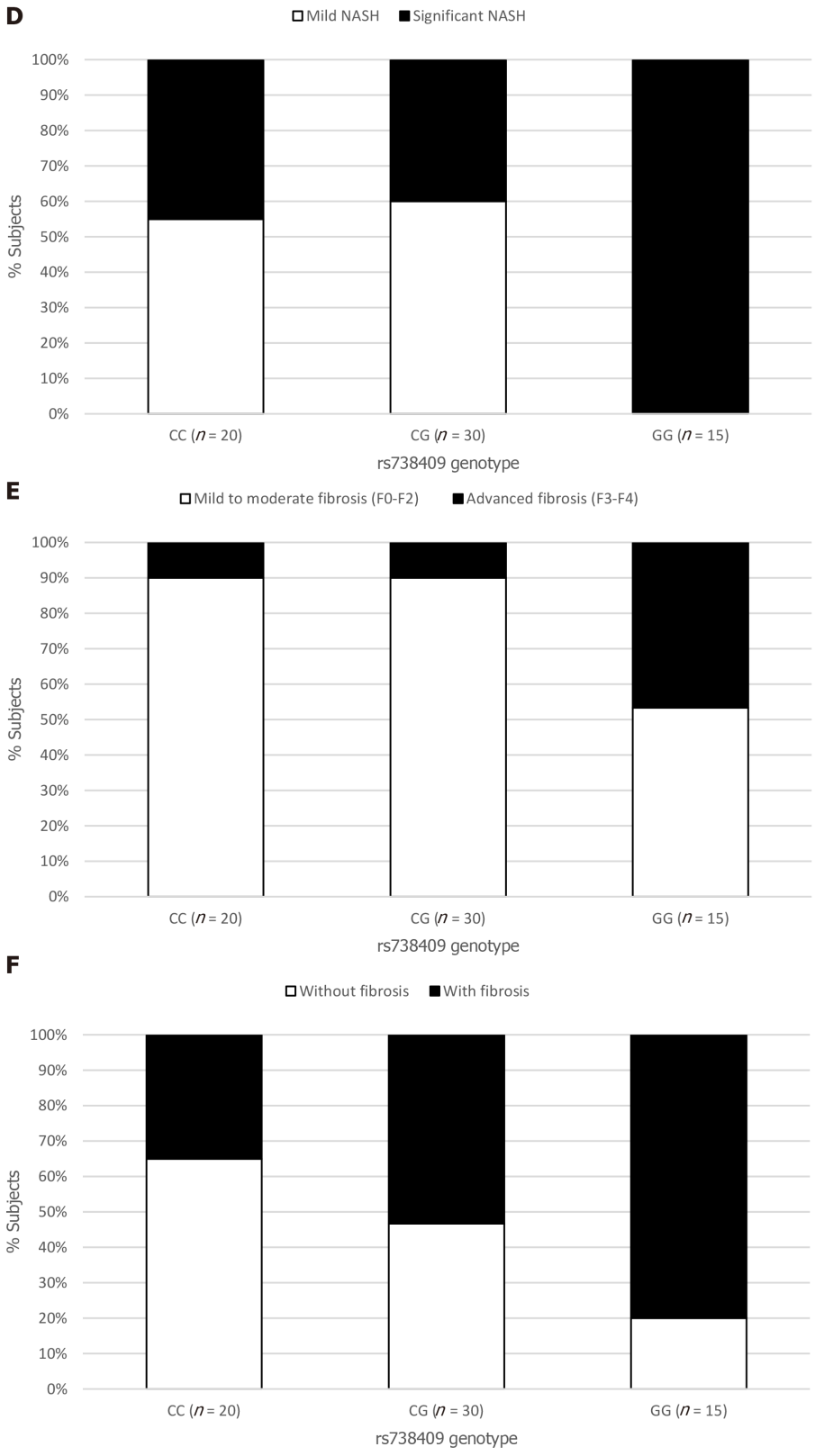


Figure 1 Relationship between the rs738409 genotype and the histological parameters. A: Prevalence of nonalcoholic steatohepatitis (NASH) P = 0.001; B: Prevalence of absent (S = 0) and mild (S = 1) steatosis or moderate (S = 2) and severe (S = 3) steatosis (P = 0.099); C: Prevalence of mild (A ≤ 2) or significant (A > 2) activity of NASH (P = 0.013); D: Prevalence of mild (A ≤ 2 and F ≤ 2) or significant (A > 2 and/or F > 2) grade of NASH based on the Steatosis, Activity, and Fibrosis score evaluation (P = 0.003); E: Prevalence of mild to moderate fibrosis (F0-F2) or advanced fibrosis (F3-F4) (P = 0.001); and F: Prevalence of fibrosis occurrence (P = 0.010). NASH: Nonalcoholic steatohepatitis.

lower than observed in American subjects (45%), and similar to that found in East Asian individuals (35%). The observed differences in the MAF for rs738409 between different racial groups are in agreement with the differences in the risk of hepatic

steatosis[44].

Findings from this study confirmed the association between *PNPLA3* and liver fat content, and showed that the rs738409 G allele increases the chance of NAFLD in Brazilian patients by about 1.6-fold, regardless of metabolic syndrome features. Furthermore, GG homozygosis seems to have an even stronger association with liver fat.

NAFLD is currently considered the hepatic manifestation of the metabolic syndrome[45]. In a recent meta-analysis, most of the included studies showed lack of association between *PNPLA3* genotypes and BMI, fasting glucose levels and Homeostasis Model Assessment[21]. In the study by Machado *et al*[43], analysis of the associations between *PNPLA3* genotypes and the metabolic syndrome components in a T2DM population demonstrated that the G allele was only associated with better glycemic control. In the current study, we found no association between anthropometric and metabolic parameters with the *PNPLA3* gene variant, which confirms previous results.

The presence of the G allele was not only associated with NAFLD occurrence, but also with the NASH phenotype in patients who underwent liver biopsy. This finding was different to a previous Brazilian analysis[30], in which NASH occurrence was not associated with the presence of the G allele in NAFLD individuals. This could be attributed to the fact that the prior Brazilian study enrolled a small number of SS individuals (n = 34) and the SS: NASH proportion was 1.0: 6.3, whereas in our study it was 1.7: 1.0.

Interestingly, no significant association between rs738409 and steatosis grade was found in our study. However, the grade of NASH histological activity and the presence of fibrosis were associated with the *PNPLA3* genotype. In fact, our data support that more aggressive disease with higher fibrosis scores was associated with rs738409 variation–subjects with higher activity scores (A > 2) were 17 times more likely to be GG homozygous than to be homozygous for the C allele; and subjects with liver fibrosis were 7.4-fold more likely to be GG homozygous than to be CC. Lack of significant association between histological steatosis grade and rs738409 genotype was observed in at least two other case-control studies[46,47]; and an association between *PNPLA3* and inflammation activity and fibrosis in NAFLD has also been found in other studies[21,23,25]. Our study, however, was limited by the fact that only 44% of the patients underwent liver biopsy.

As stated, the *TM6SF2* rs58542926 genotypes were described in this study for the first time in Brazilians. The MAF (8%) was similar to that observed in a Northern European sample (7%)[48], but lower than that observed in other European samples (12% and 13%)[28,29]. Different to that described in other studies[27,29,41,42], we did not find significant differences regarding the *TM6SF2* genotypes between NAFLD patients and controls, or between NASH and SS individuals. This finding suggests that in the Brazilian population, the genetic variants of rs58542926 *TM6SF2* may have a distinct influence on NAFLD than that observed in other populations, which is understandable, as Brazilian NAFLD patients have been reported as an admixed population presenting genetic ancestry contributions from European (48.8%), African (41.7%) and Amerindian (9.5%)[49]. Besides that, as *TM6SF2* MAF is less frequent in the general population, larger samples may be required to confirm this finding.

CONCLUSION

In conclusion, we observed that *PNPLA3* may be involved in the progression of NAFLD in the Brazilian population. Individuals who had histopathological assessment and more advanced liver disease were more likely to carry the G allele. The *TM6SF2* genetic variants were not associated with NAFLD susceptibility and severity in the population studied, although further studies with larger samples are required to confirm these findings.

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in Western countries and encompasses a spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). Although highly prevalent, only a minority of

NAFLD patients develop significant fibrosis. Thus, NAFLD is considered a complex disorder in which the disease phenotype results from an interaction between environmental exposure and a susceptible polygenic background. Polymorphisms of *PNPLA3* and *TM6SF2* genes have been associated with greater susceptibility to NAFLD development in previous studies.

Research motivation

There is only one genetic study in Brazilian NAFLD patients, and *TM6SF2* polymorphism has not yet been analyzed in this population. As Brazilian NAFLD subjects have been reported as an admixed population presenting diverse genetic ancestry contributions, it is relevant to study the associations between various genotypes and fatty liver disease progression.

Research objectives

We aimed to investigate the association between *PNPLA3* and *TM6SF2* genotypes and clinical parameters of NAFLD, and analyzed the genotype variations as markers of liver histological features in adult Brazilian NAFLD patients. We also investigated the distribution of these genotype variations among Brazilians.

Research methods

This cross-sectional study enrolled 285 individuals, of which 148 patients had features of NAFLD (case patients) and 137 were non-NAFLD control subjects. NAFLD was diagnosed based on hepatic steatosis by liver ultrasonography and exclusion of other causes of liver disease. Patients who had decompensated cirrhosis or were taking drugs that induce steatosis were excluded. From the total of NAFLD patients, 65 underwent liver biopsy according to the clinical protocol of increased risk for NASH and/or advanced fibrosis. DNA was obtained for genotyping *PNPLA3* at rs738409 and *TM6SF2* at rs58542926.

Research results

PNPLA3 CC, CG and GG genotype frequencies were 37%, 44% and 19%, respectively, in NAFLD patients and were 58%, 31% and 10% in controls ($P < 0.001$). In a model adjusted for gender, age, body mass index and type 2 diabetes mellitus, the G allele increased the chance of NAFLD (OR = 1.69, 95%CI: 1.21-2.36, $P = 0.002$) and NASH (OR = 3.50, 95%CI: 1.84-6.64, $P < 0.001$). The chance of NASH was even higher with GG homozygosis (OR = 5.53, 95%CI: 2.04-14.92, $P = 0.001$). No association was found between G allele and the features of metabolic syndrome. In the histological assessment, *PNPLA3* genotype was not associated with steatosis grade, although GG homozygosis increased the chance of significant NASH activity (OR = 17.11, 95%CI: 1.87-156.25, $P = 0.01$) and fibrosis (OR = 7.42, 95%CI: 1.55-34.47, $P = 0.01$) in the same adjusted model. *TM6SF2* CC, CT and TT genotype frequencies were 83%, 15% and 0.7%, respectively, in NAFLD patients and were 84%, 16% and 0.7% in controls ($P = 0.78$). Presence of the T allele was not associated with NAFLD or NASH, or with histological features.

Research conclusions

PNPLA3 may be involved in the susceptibility and progression of NAFLD and NASH in the Brazilian population. More advanced histological liver disease was associated with the G allele. The *TM6SF2* genetic variants were not associated with NAFLD susceptibility and progressive histological forms in the population studied.

Research perspectives

The description of variant genotypes distribution in NAFLD Brazilian patients contributes to a better understanding of the disease clinical characteristics and atypical features in this population. As the *TM6SF2* polymorphism is less frequent in the general population, investigations with larger sample are needed. Further studies may investigate additional particular components of fatty liver disease in Brazil. The role of genotyping assessment for risk stratification is still uncertain.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Impact of sarcopenia on mortality in patients undergoing liver re-transplantation

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Abstract

BACKGROUND

Sarcopenia, which is a loss of skeletal muscle mass, has been reported to increase post-transplant mortality and morbidity in patients undergoing the first liver transplant. Cross-sectional imaging modalities typically determine sarcopenia in patients with cirrhosis by measuring core abdominal musculatures. However, there is limited evidence for sarcopenia related outcomes in patients undergoing liver re-transplantation (re-OLT).

AIM

To evaluate the risk of mortality in patients with pre-existing sarcopenia following liver re-OLT.

interest related to the manuscript.

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METHODS

This is a retrospective study of all adult patients who had undergone a liver re-OLT at the University of Nebraska Medical Center from January 1, 2007 to January 1, 2017. We divided patients into sarcopenia and no sarcopenia groups. "TeraRecon AquariusNet 4.4.12.194" software was used to evaluate computed tomography or magnetic resonance imaging of the patients done within one year prior to their re-OLT, to calculate the Psoas muscle area at L3-L4 intervertebral disc. We defined cutoffs for sarcopenia as < 1561 mm^2 for males and < 1464 mm^2 for females. The primary outcome was to compare 90 d, one, and 5-year survival rates. We also compared complications after re-OLT, length of stay, and re-admission within 30 d. Survival analysis was performed with Kaplan-Meier survival analysis. Continuous variables were evaluated with Wilcoxon rank-sum tests. Categorical variables were evaluated with Fisher's exact tests.

RESULTS

Fifty-seven patients were included, 32 males: 25 females, median age 50 years. Two patients were excluded due to incomplete information. Overall, 47% (26) of patients who underwent re-OLT had sarcopenia. Females were found to have significantly more sarcopenia than males (73% *vs* 17%, $P < 0.001$). Median model for end stage liver disease at re-OLT was 28 in both sarcopenia and no sarcopenia groups. Patients in the no sarcopenia group had a trend of longer median time between the first and second transplant (36.5 mo *vs* 16.7 mo). Biological markers, outcome parameters, and survival at 90 d, 1 and 5 years, were similar between the two groups. Sarcopenia in re-OLT at our center was noted to be twice as common (47%) as historically reported in patients undergoing primary liver transplantation.

CONCLUSION

Overall survival and outcome parameters were no different in those with and without the evidence of sarcopenia after re-OLT.

Key Words: Sarcopenia; Liver transplantation; Mortality; Re-transplantation; Psoas muscle index

Core Tip: There is a limited data on outcomes among patients with sarcopenia undergoing re-transplantation of the liver. A retrospective study of 57 patients who underwent re-transplantation showed 47% of patients had sarcopenia by Psoas muscle index at the level of L3-L4 Intervertebral disc prior to re-transplantation. Biological markers, outcome parameters, and survival at 90 d, 1 and 5 years, were similar between the two groups. Sarcopenia in re-transplantation was noted to be twice as common as historically reported in patients undergoing primary liver transplantation.

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INTRODUCTION

The European Working Group on Sarcopenia in older people defined sarcopenia as the progressive and generalized loss of skeletal muscle mass and strength, resulting in an increased risk of poor quality of life and death[1]. Due to the alterations in protein turnover and metabolic changes in liver disease, sarcopenia is a common complication of cirrhosis[2]. In patients with cirrhosis, sarcopenia can be identified using cross-sectional imaging modalities such as computed tomography (CT) or magnetic

resonance imaging (MRI) to measure skeletal muscle area of core abdominal muscles[3]. Skeletal muscle measurements are obtained at the level of the third-fourth lumbar vertebrae as measurements at this level give an accurate estimation of total body skeletal muscle mass[4]. Psoas muscle area (PMA) is most commonly measured as it can be easily evaluated by imaging and is not susceptible to compression by ascites[5]. Sarcopenia is diagnosed when PMA is less than 1561 mm^2 in men and less than 1464 mm^2 in women with cirrhosis[3].

Sarcopenia has been noted in 22%-65% of cirrhotic patients and is associated with increased waiting list mortality in liver transplant patients, with studies reporting at least a 2-fold higher risk of mortality in sarcopenic patients compared to those without sarcopenia[3,4,6-8]. Sarcopenia is also associated with higher mortality after primary liver transplantation, with one-year survival rates following primary liver transplant ranging from 50%-64% for sarcopenic patients compared with 85%-94% for non-sarcopenic patients[3,9,10]. Graft survival is also affected by sarcopenia, and patients without sarcopenia experience significantly higher one-year graft survival rates than do patients with sarcopenia (90.1% and 77.9%, respectively)[11].

For liver transplant recipients with graft failure, re-transplantation (re-OLT) is the only effective treatment option[12]. The United Network for Organ Sharing reported that the graft failure rate for orthotopic liver transplant (OLT) recipients in the United States in 2016 was 7.3% and 9.8% at six months and one year, respectively[13]. Similarly, recent studies have found the incidence of re-OLT among OLT recipients to be 4%-17%[13-20]. While survival rates following primary liver transplantation continue to improve, overall survival following re-OLT remains 10% to 20% lower than after primary OLT[13,18].

Given the limited number of organs available for transplant, it is essential to determine factors that may be associated with increased mortality after liver re-transplantation. While sarcopenia has been associated with worse outcomes, and a higher risk of mortality following primary OLT, the effect of sarcopenia on re-OLT has not been addressed. Interestingly, studies have shown that sarcopenia can be reversible, with 28% of patients experiencing resolution of sarcopenia following primary OLT[21]. However, the incidence of sarcopenia prior to re-OLT is not well reported. This study was undertaken to examine the relationship between sarcopenia and re-OLT and to determine whether sarcopenia prior to re-OLT increases the risk of mortality following re-OLT.

MATERIALS AND METHODS

Study design

This is a retrospective study of the patients who had liver re-OLT at University of Nebraska Medical Center (UNMC) between 01/01/07 and 01/01/2017. The study was approved by Institutional Review Board (IRB) of UNMC (IRB number# 236-17-EP). Two experienced staff body radiologists retrospectively evaluated abdominal CTs or MRIs of these patients, which were obtained within one year prior to their liver re-transplantation. "TeraRecon Aquarius Net 4.4.12.194" software (Foster City, CA, USA) was utilized to calculate the 2D PMA of each psoas muscle, measured in a semiautomatic fashion on a single axial image at L3-L4 intervertebral disc level. The sum of bilateral PMAs was recorded (Figure 1). Based on PMA, patients were divided into sarcopenia *vs* no sarcopenia group. Sarcopenia was defined as PMA < 1561 mm^2 for males and PMA < 1464 mm^2 for females[3].

Study subjects

Inclusion criteria were: (1) Age > 18 years old; and (2) Patients who had liver re-OLT between 01/01/07 - 01/01/2017 and had CT or MRI of the abdomen and pelvis done within one year prior to their liver re-OLT were included. Exclusion criteria were: (1) Patients who did not meet the inclusion criteria; (2) Pregnant females; and (3) Incomplete medical record information.

Study outcomes and statistical analysis

The primary outcome was to compare 90 d, one, and 5-year survival rates between the two groups (sarcopenia *vs* no sarcopenia). Secondary outcomes: Hospital length of stay (LOS), intensive care unit (ICU) LOS, readmission within 30 d, need for dialysis, pulmonary complications, primary non-function (PNF), acute rejection episodes, arterial and biliary complications between patients who had sarcopenia *vs* no sarcopenia. Pulmonary complications are conditions such as pneumonia, pleural

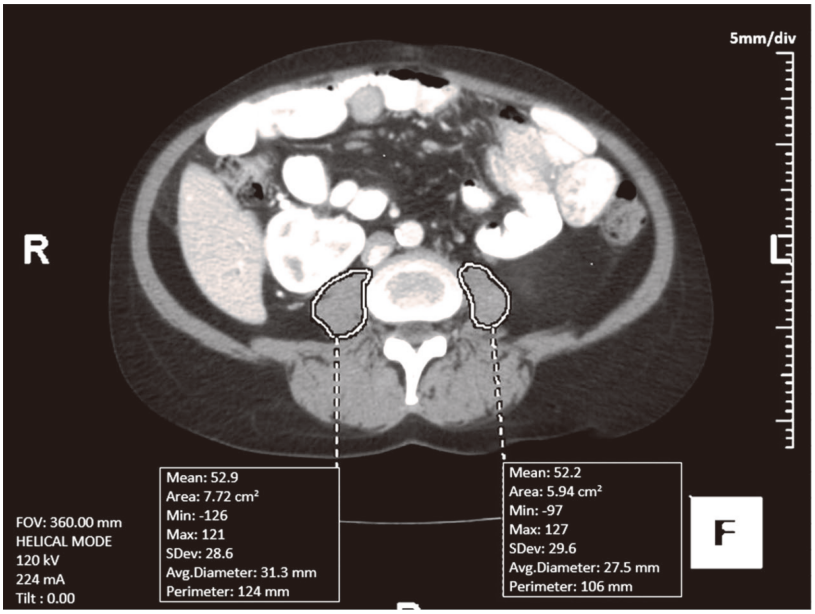

Figure 1 The 2D Psoas muscle area of each psoas muscle, measured in a semiautomatic fashion on a single axial image at L3-L4 intervertebral disc level.

effusions, or respiratory failure requiring supplement oxygen. PNF was defined as an irreversible graft failure requiring emergent liver retransplantation during the first 10 d of the transplantation. Acute rejection episodes were biopsy proven. Arterial complications were defined mainly as conditions such as hepatic artery thrombosis and stenosis. Biliary complications were defined as conditions such as biloma formation, cholangitis, anastomotic strictures and anastomotic leak. Potential confounding variables were also evaluated between those with and without sarcopenia including age, sex, body mass index, model for end stage liver disease (MELD), the time between the first and second transplants, and several biomarkers such as albumin, platelet counts, creatinine, International Normalized Ratio (INR), sodium, and presence of ascites. Comparison of baseline characteristics were done on sarcopenia and no sarcopenia groups to minimize selection bias. Survival analysis was conducted with Kaplan-Meier survival curve. Wilcoxon rank-sum tests were used to evaluate continuous variables, while Fisher's exact tests were used to compare categorical variables. *P* values of less than 0.05 were considered significant. All statistical analyses were conducted using STATA version 14 (The Stata Corp, College Station, TX, United States). The statistical methods of this study were reviewed by Harlan Sayles from Department of Biostatistics, College of Public Health, UNMC.

RESULTS

A total of 57 patients were included in our study; 32 (56%) were males and 25 (44%) were females. The overall median age in our study was 50 years. Two patients were excluded from the no sarcopenia group due to incomplete information. Overall, 47% (26) of patients underwent re-OLT had sarcopenia and 53% (29) of patients had no evidence of sarcopenia.

Demographics and other potential confounding variables

In our study, female gender was found to be significantly associated with sarcopenia with $P < 0.001$ (73% females *vs* 17% females in no sarcopenia group). Median MELD at re-OLT was 28 in both the groups. Patients in the no sarcopenia group had a trend of longer median time between the first and second transplant as compared to patients in the sarcopenia group, however this was not significant (36.5 mo *vs* 16.7 mo, $P = 0.34$). Biological markers including albumin, platelets, creatinine, INR and sodium were similar in both the groups (Table 1). There was no difference noted in regard to the presence of ascites in patients with sarcopenia *vs* no sarcopenia group (81% *vs* 62%, $P = 0.13$)

Table 1 Demographics and biochemical variables in our patient population

Potential confounder	*n*	Sarcopenia [Median (IQR) or *n* (%)]	No sarcopenia [Median (IQR) or *n* (%)]	*P* value
Age (yr)	55	49.5 (34, 59)	50 (34, 59)	0.906
Male sex	55	7 (27)	25 (86)	< 0.001
BMI	55	24 (23, 29)	24.7 (21.7, 30.9)	0.781
MELD1	55	28 (24, 33)	28 (24, 33)	0.846
Time between 1st and 2nd OLT (mo)	55	16.7 (5.2, 108.1)	36.5 (7.4, 121.4)	0.337
Albumin	55	2.6 (2.1, 3.3)	2.7 (2.4, 3.1)	0.685
Platelets	55	100 (75, 189)	127 (79, 189)	0.544
Cr	55	1.24 (0.87, 1.76)	1.55 (1.26, 2.01)	0.149
INR	55	1.3 (1.1, 2.1)	1.5 (1.1, 1.8)	0.892
Na	55	136 (132, 139)	136 (134, 138)	0.716
Ascites	55	21 (81)	18 (62)	0.127

IQR: Interquartile range; BMI: Body mass index; Cr: Creatinine; MELD: Model for end-stage liver disease; OLT: Orthotopic liver transplantation; INR: International normalized ratio; Na: Sodium.

Primary and secondary outcomes

The survival rates between sarcopenia and no sarcopenia group at 90 d (88% *vs* 90%), 1-year (76% *vs* 81%) and 5-year (59% *vs* 68%) were similar. No statistical difference was noted in terms of mortality. Kaplan-Meier survival estimates were performed at 90 d, one, and 5-years between the two groups depicting no difference in the survival (Figure 2). The median ICU LOS, readmission to ICU, number of days intubated, need for dialysis and readmissions within 30 d were comparable between the two groups. The median total hospital LOS in no sarcopenia group was higher (21 d) as compared to sarcopenia group (14 d), but this was not statistically significant. The sarcopenia group had trend of higher pulmonary complications as compared to no sarcopenia group (38% *vs* 18%, $P = 0.1$). The post-transplant graft complications in terms of PNF, acute rejections, arterial and biliary complications were similar between the two groups (Table 2).

DISCUSSION

Due to the high prevalence of sarcopenia in patients with cirrhosis and its association with adverse outcomes, it is important to identify patients with sarcopenia prior to liver transplantation. While multiple modalities have been used to diagnose sarcopenia, the current gold standard is to use CT and/or MRI imaging to estimate muscle mass[1]. These imaging modalities are preferred as they provide higher accuracy in quantifying muscle and fat as compared to other methods such as dual-energy X-ray absorptiometry or bio impedance analysis[5]. While earlier studies used the third lumbar skeletal muscle index (L3) to identify sarcopenia, recent studies have shown that PMA gives a more accurate estimation of total body skeletal muscle mass as it is not affected by ascites or hepatomegaly[3,5]. PMA has also been shown to be strongly associated with post-transplant mortality[9]. For these reasons, we used PMA obtained from CT and/or MRI images to diagnose sarcopenia in our study population.

Studies have shown that up to 17% of cirrhotic patients who undergo liver transplantation will require re-OLT due to graft failure. The most common indications for liver re-OLT reported in the literature include PNF, hepatic artery thrombosis, arterial and biliary complications, recurrence of disease and acute or chronic rejection. The median time interval from primary transplantation to re-OLT ranged from 8 d to 557 d in these studies[13,14,16-19]. Similar to previous studies, patients in our study underwent re-OLT for PNF, acute rejection, hepatic artery thrombosis, arterial and biliary complications. The incidence of these complications was similar in patients with sarcopenia compared to those without. Compared to previous studies, our study population without sarcopenia had longer median time from primary transplantation

Table 2 Associations between sarcopenia status and outcomes

Outcome	*n*	Sarcopenia Median (IQR) or *n* (%)]	No Sarcopenia Median (IQR) or *n* (%)]	*P* value
Post-op survival for 90 d	55	23 (88)	26 (90)	1.000
Post-op survival for 1 yr	52	19 (76)	22 (81)	0.740
Post-op survival for 5 yr	41	13 (59)	13 (68)	0.746
ICU length of stay (d)	55	3 (2, 4)	2 (2, 5)	0.696
Readmission to ICU	54	4 (15)	4 (14)	1.000
Total length of stay (d)	55	13.5 (10, 27)	21 (9, 28)	0.661
Intubation days	55	1.5 (1, 3)	1 (1, 3)	0.484
Pulmonary complications	54	10 (38)	5 (18)	0.131
Need for dialysis	54	6 (23)	7 (25)	1.000
Readmission within 30 d	55	13 (50)	13 (45)	0.790
PNF	55	2 (8)	0 (0)	0.219
Acute rejection	55	2 (8)	4 (14)	0.672
Arterial complications	55	6 (23)	5 (17)	0.739
Biliary complications	55	8 (31)	10 (34)	1.000

IQR: Interquartile range; ICU: Intensive care unit; PNF: Primary non-function.

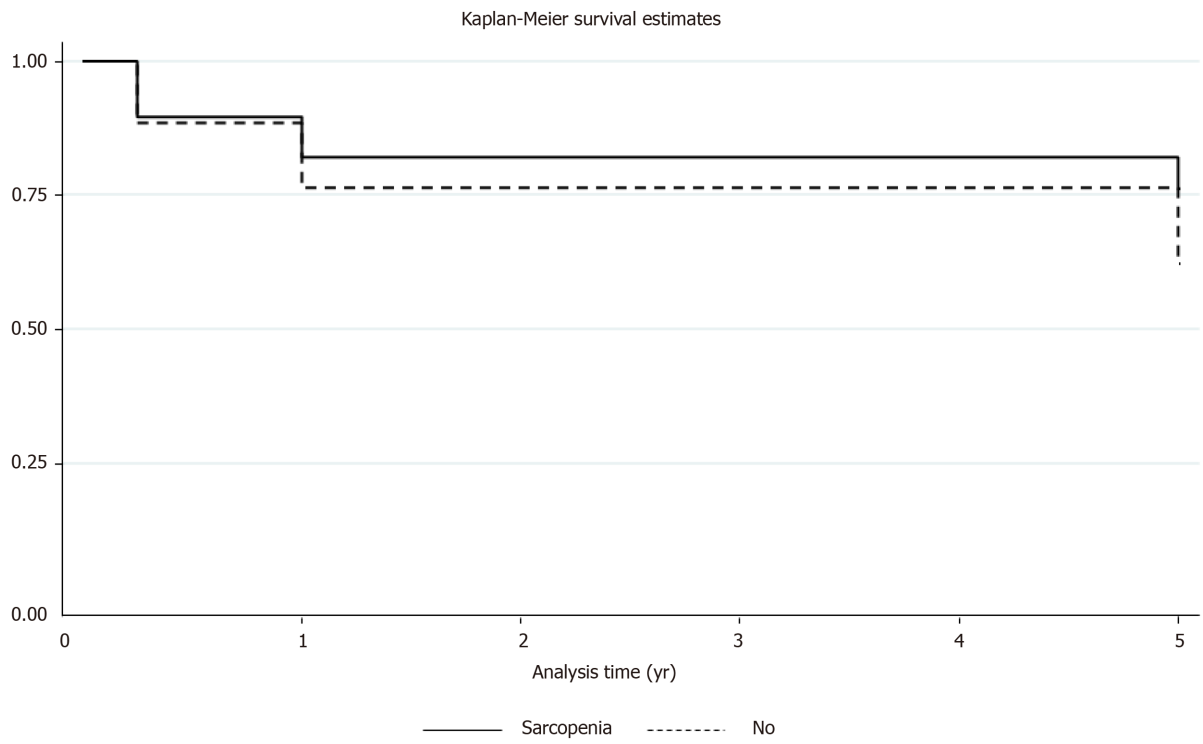

Figure 2 Kaplan-Meier analysis showings 90-d, 1-year and 5-year survival analysis in patients with sarcopenia status.

to re-OLT compared to sarcopenic patients (36.5 mo *vs* 16.7 mo). Although clinically, this was an important finding, but this difference was not statistically significant (P = 0.34). Unlike previous studies which have shown that sarcopenia is more prevalent in males, we found female gender to be significantly associated with sarcopenia in our study[7,21].

Sarcopenia has been well studied in patients undergoing primary liver transplantation but the effects of sarcopenia on patients who require re-OLT remain relatively unknown. Given that many of the clinical consequences of cirrhosis reverse

with liver transplantation, it might be expected that sarcopenia also improves following transplantation. Montano *et al*[21] studied sarcopenia in cirrhotic patients undergoing liver transplantation and found that 28% of patients experienced resolution of sarcopenia following transplant. Other studies, however, found that the incidence of sarcopenia increased after transplant[5,8]. In a study by Tsien *et al*[8], 94% of patients with pre-transplant sarcopenia had persistent sarcopenia after transplant, and sarcopenia developed in 44% of patients who did not have sarcopenia prior to OLT. Likewise, Jeon *et al*[5] found the prevalence of sarcopenia after liver transplant to be 46%, an increase from 36% prior to transplant. Similarly, we found the incidence of sarcopenia at our center to be 47%, suggesting that sarcopenia persists after initial transplant. Potential mechanisms for this include ongoing muscle loss due to immunosuppression regimens as well as prolonged hospital courses and sedentary lifestyle following transplant[22].

The adverse effects of sarcopenia on cirrhotic patients undergoing liver transplantation are well documented in the literature. Patients with sarcopenia experience longer hospital stays as well as longer stays in the ICU[3,11,21]. They also have a higher frequency of bacterial infections and post-operative sepsis[11,21,23]. Infections which occur in sarcopenic patients are also more likely to be severe[24]. Interestingly, we found no difference in total hospital LOS or ICU LOS after re-OLT in patients with sarcopenia compared to those without sarcopenia. We did note higher rates of pulmonary complications in patients with sarcopenia, however this difference was not statistically significant. Most importantly, we found no significant difference in the overall survival at 90 d, one and 5-year between patients with and without evidence of sarcopenia. This finding was unexpected given that sarcopenia has been associated with both higher waiting list mortality and post-transplant mortality[4]. Tandon *et al*[7] reported that survival rates for patients on the waiting list were 16% higher at one year and 19% higher at three years for non-sarcopenic patients compared to those with sarcopenia. Furthermore, Englesbe *et al*[9] found that total psoas area significantly affected post liver transplant mortality and the risk of mortality increased as psoas area decreased. In that study, one-year survival after liver transplant was 49.7% for the sarcopenic group and 87% for the non-sarcopenic group.

As 4%-17% of liver transplant recipients will require liver re-OLT, understanding the impact of sarcopenia on these patients is becoming increasingly important[13-20]. However, few studies have examined sarcopenia in this population and the effects of sarcopenia on mortality remain unknown. While we did not find any significant difference in mortality between sarcopenic and non-sarcopenic patients, this may be explained by our small population size.

Limitations

This is a single center retrospective study with small sample size of 55 patients (2 patients were excluded). The sarcopenia status of the patients prior to their first liver transplant was unknown.

CONCLUSION

Sarcopenia in re-OLT at our center was noted to be twice as common (47%) as historically reported in patients undergoing primary transplantation. Sarcopenia was more common in females. Our study population with sarcopenia had shorter median time from primary transplantation to re-OLT compared to the patients without sarcopenia. However, in re-OLT patient's overall survival and other outcome parameters were no different in those with and without evidence of sarcopenia. Further, multicenter studies are needed to validate our findings.

ARTICLE HIGHLIGHTS

Research background

Sarcopenia, which is a loss of skeletal muscle mass, has been reported to increase post-transplant mortality and morbidity in patients undergoing the first liver transplant. Cross-sectional imaging modalities typically determine sarcopenia in patients with cirrhosis by measuring core abdominal musculatures.

Research motivation

Identification of sarcopenia is becoming more prevalent in the initial liver transplantation. However, there is limited evidence for sarcopenia related outcomes in patients undergoing liver re-transplantation (re-OLT).

Research objectives

The study aimed to evaluate the risk of mortality in patients with pre-existing sarcopenia following liver re-OLT.

Research methods

This is a retrospective study of patients who had undergone a liver re-OLT. The presence of sarcopenia was determined by the Psoas Muscle Area on cross-sectional imaging. The primary outcome was to compare 90 d, one, and 5-year survival rates between sarcopenia and no sarcopenia group.

Research results

Overall, 47% of patients who underwent re-OLT had sarcopenia. Biological markers, outcome parameters, and survival at 90 d, 1 and 5 years, were similar between the two groups. Sarcopenia in re-OLT at our center was noted to be twice as common as historically reported in patients undergoing primary liver transplantation.

Research conclusions

Overall survival and outcome parameters were no different in those with and without the evidence of sarcopenia after re-OLT.

Research perspectives

Although sarcopenia has been shown to predict the outcomes after the first liver transplantation, our study did not show the difference in survival and outcome parameters between sarcopenia and non-sarcopenia groups. Besides, sarcopenia was more prevalent in patients undergoing re-OLT compared to initial transplantation. Larger prospective studies are needed to assess the impact of sarcopenia in patients undergoing re-OLT.

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ORIGINAL ARTICLE

Retrospective Study

Increased incidence of and microbiologic changes in pyogenic liver abscesses in the Mexican population

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán.

Informed consent statement: Patients were not required to give

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Abstract

BACKGROUND

Pyogenic liver abscess (PLA) is a rare disease with an estimated incidence that varies widely across the globe, being as high as 115.4/100000 habitants in Taiwan and as low as 1.1-1.2/100000 habitants in Europe and Canada. Even though there are multiple microorganisms capable of producing an abscess in the liver, including *Entamoeba histolytica*, fungi, and viruses, most abscesses are derived from bacterial infections. The epidemiology of PLA in Mexico is currently unknown.

AIM

To describe the clinical, demographic and microbiologic characteristics of PLA in Mexico.

METHODS

This is a retrospective study carried out in two centers, and included patients seen between 2006 and 2018 with the diagnosis of pyogenic abscess. We collected demographic, clinical, and microbiological information, treatment, complications, and outcomes. A logistic regression analysis was used to determine the association between different variables and mortality rates.

RESULTS

informed consent because the data were obtained from medical charts. This was a retrospective study.

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A total of 345 patients were included in this study. 233 (67.5%) had confirmed PLA, 133 (30%) patients had no positive culture and negative serology and 9 (2.5%) had mixed abscesses. The mean age was 50 years (ranging from 16-97 years) and 63% were female. 65% of the patients had positive cultures for Extended Spectrum Beta-Lactamases (ESBL)-*Escherichia coli* and *Klebsiella pneumoniae*. Cefotaxime was administered in 60% of cases. The most common sources of infection were ascending cholangitis and cholecystitis in 34 (10%) and 31 (9%), respectively. The median length of hospital stay was 14 d. 165 patients underwent percutaneous catheter drainage. The inpatient mortality rate was 63%. Immunocompromised state [OR 3.9, 95%CI: 1.42-10.46], ESBL- *Escherichia coli* [OR 6.7, 95%CI: 2.7-16.2] and *Klebsiella pneumoniae* [OR 4-8, 95%CI: 1.6-14.4] predicted inpatient mortality by multivariate analysis.

CONCLUSION

The prevalence of PLA is increasing in Mexico and has a very high mortality rate. ESBL-*Escherichia coli* and *Klebsiella pneumoniae* are the most common microorganisms causing PLA and are independent predictors of inpatient mortality.

Key Words: Liver abscess; Pyogenic; Mexican population; Epidemiology; Complications; Outcomes; Mortality

Core Tip: In this retrospective study we investigated the clinical, demographic, and microbiologic characteristics of pyogenic liver abscess (PLA) in Mexico. We found that the prevalence of PLA in Mexico is increasing and had a very high mortality rate (63%) in our study. Our data also indicated that the presence of ESBL-*Escherichia coli* and *Klebsiella pneumoniae* and an immunocompromised state were independent predictors of high-risk mortality with an adjusted OR of 6.7, 4.8 and 3.9, respectively.

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INTRODUCTION

Although there are multiple microorganisms capable of producing an abscess in the liver, including *Entamoeba histolytica*, fungi, and viruses, most abscesses are derived from bacterial infections[1,2]. Pyogenic liver abscess (PLA) is a rare disease with an estimated incidence that varies widely across the globe, being as high as 115.4/100000 inhabitants in Taiwan and as low as 1.1-1.2/100000 inhabitants in Europe and Canada[1,2].

There has been a shift in the etiology of PLAs over the years. Previously, intraabdominal infections such as complicated appendicitis or diverticulitis with portal bacterial seeding have represented the most frequent causes[1], whereas currently, biliary tree diseases such as cholecystitis, choledocholithiasis, tumoral obstruction, strictures, congenital malformations, and hepatobiliary instrumentation are the most common etiologies[1-3]. With regard to bacterial isolates, before 1980, the most commonly reported agents were *Escherichia coli, Enterobacteriaceae*, anaerobes, and other members of the intestinal flora[1,2,4,5], but in recent years, some highly virulent strains of *Klebsiella pneumoniae* have been isolated in Taiwan and mainly in Singapore[6], where many series have been reported. There are also new cases in the United States and Europe[1].

In Mexico, the epidemiology of PLAs is unknown, with some small case series reporting up to 41 cases from all over the country with a predominance of *Escherichia*

coli and a lower reported incidence[7]. The aim of this study was to describe the current clinical, demographic, and microbiologic characteristics of PLAs in two high-volume centers in Mexico.

MATERIALS AND METHODS

This was a retrospective study that included patients who were seen between 2006 and 2018 at two referral centers in Mexico City: The Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ) and the Hospital General Dr. Manuel Gea González. Eligible patients were those with a diagnosis of liver abscess by imaging, and individuals with an unspecified type of abscess with negative amoeba serology, a mixed-type abscess defined by positive bacterial culture and positive amoeba serology and positive bacterial culture-confirmed PLAs were included. We excluded patients aged < 18 years and those with incomplete information; with confirmed amoebic abscesses, defined as those with positive serology and an absence of positive bacterial cultures; those in whom *Entamoeba histolytica* was isolated; and those in whom fungi were isolated. Patients with infected liver cysts and infected hydatid cysts were also excluded.

We collected demographic, clinical, and microbiological information from each patient's medical chart, including age, sex, symptoms, laboratory results, radiological characteristics, microbiological isolates, type of treatment, complications, length of hospital stay, and outcomes. Due to small numbers of specific cases, immunocompromise was defined as the presence of positive human immunodeficiency virus (HIV) status, underlying malignancy, or the presence of autoimmune disease. For the statistical analysis, data are presented as the means ± SD or frequencies and percentages, according to their distribution. Numeric and categorical variables were compared using the chi-squared test. To study the association between the different variables and mortality rates, we used a logistic regression model. Variables of interest plus variables with a *P* value less than 0.1 in univariate analysis were included in a multivariate regression analysis. Logistic regression using significant variables was applied with the Omnibus test and Hosmer-Lemeshow test to explain mortality (utilizing R2 Nagelkerke). Analyses were conducted with Stata version 12 (Stata Corp. LP, College Station, TX, United States).

Treatment protocol

All patients underwent abdominal ultrasound, computed tomography (CT) scanning or magnetic resonance imaging (MRI) studies. Patients with PLAs underwent at least one set of blood cultures before the administration of parenteral empiric broad-spectrum antibiotic agents in accordance with local antimicrobial resistance surveillance data.

The duration of parenteral antibiotic agents, the switch to oral formulations, and the total duration of therapy were decided by the individual physician and guided by clinical, biochemical, and radiologic responses.

The decision on percutaneous catheter drainage (PCD) depended on the clinical response to medical treatment and the size of the PLA. PCD was performed using CT guidance by interventional radiologists *via* the placement of a 10–12F pigtail catheter *in situ*. PCD was performed when any of the following criteria were met: (1) Size of the PLA > 4 cm, solitary or dominant; (2) Hemodynamic instability or the need for inotropic support upon admission; and (3) Failure of antibiotic therapy for PLA.

Definitions

A giant PLA (GPLA) was defined as an abscess greater than or equal to 10 cm in diameter.

A multiloculated abscess was defined as an abscess with 2 or more septations within its cavity.

Nonresponse to interventional radiology-assisted drainage was defined as patients having persistent signs of sepsis after 5 d of intravenous (IV) antibiotics and PCD, thus requiring escalation of therapy.

Indications for surgery

Percutaneous drainage failure; patients with ongoing sepsis after antibiotics and PCD or patients with difficult-to-access sites such as the liver dome; abscesses not amenable to percutaneous drainage, such as multiloculated abscesses or those with thick purulent material that could not be aspirated; ruptured abscesses; and underlying

causes requiring surgical removal, such as in the case of a foreign body.

RESULTS

Within the database, 404 patients were documented between both centers: 175 in the General Hospital and 229 in the INCMNSZ. Of these patients, 58 had amebic abscesses confirmed by *Entamoeba histolytica* serology, and only 1 patient developed a fungal abscess secondary to *Candida albicans*; those patients were excluded from the study, and a total of 345 patients were included.

Demographic, clinical and laboratory results

We included 345 patients: 233 (67.5%) had a confirmed PLA, 133 (30%) were patients with no positive cultures and negative serology, and 9 (2.6%) had mixed abscesses. The mean age was 50 years (ranging from 16-97 years), and 217 patients (63%) were female. The initial presentation included fever, fatigue, right upper quadrant pain, and diarrhea in 293 (85%), 167 (48%), 259 (75%), and 52 (15%) patients, respectively. The mean time between the onset of symptoms and medical consultation was 22 d. The most frequent comorbidities were type 2 diabetes mellitus (T2DM), hypertension, underlying malignancy, and biliary tree lesions, which were found in 87 (25%), 32 (9.2%), 21 (6%), and 16 (4.6%) patients, respectively.

The most relevant laboratory findings were leukocytosis with an elevated neutrophil count, and among the liver biochemistry alterations, a mean total bilirubin level of 2.24 mg/dL and a mean alkaline phosphatase level of 256.37 U/L were observed; the rest of the clinical and analytical data are summarized in Table 1.

Microbiological results

Abscess samples were obtained from direct-needle aspiration; 65% of the patients had positive cultures, and the most commonly isolated microorganisms were ESBL-*Escherichia coli* and *Klebsiella pneumoniae*, corresponding to 16% and 8%, respectively. In 59 cases, the culture isolated 2 different bacteria, and in 50 cases, more than two microorganisms were isolated. Figure 1 shows the frequency of the different bacteria cultured.

The antibiotic therapy administered varied considerably among the patients, with different approaches for each case; initial empiric treatment included cefotaxime in 60% cases, and in 90% of cases, it included a drug combination, most commonly with metronidazole. Interestingly, more than 90% of the cases required a combination with more than 1 drug; again, the previously mentioned third-generation cephalosporin was the most commonly used drug. Of those patients exposed to multiple regimens, at least 6 of them were exposed to six drug regimens, and 2 of them needed a seventh drug, of which vancomycin and cefuroxime were prescribed. In 82% of the cases, these regimens lasted for more than 14 d, with a median prescription time of 19 d (2-65 d).

The most common causes were ascending cholangitis and cholecystitis in 34 (10%) and 31 (9%) patients, respectively, but in 40% of cases, no precipitating event was identified. Figure 2 shows the distribution of the different sources of infection with ascending cholangitis and cholecystitis, both being the most common precipitating events.

Imaging results

A total of 50.72% (n = 175) of the studied population had an abdominal ultrasound that initially suggested a diagnosis of PLA; for morphological diagnosis, a CT scan was performed in 320 patients, and MRI was carried out in just 14% of the cases for diagnosis confirmation.

The number of abscesses per case was quantified with the help of imaging techniques. In 51% of the cases (n = 178) only one documentable lesion was observed, 25% had at least 2 abscesses, and in 9% multiple abscesses in the liver parenchyma were identified.

86.7% of the lesions were located in the right lobe of the liver with segments VII, VI, and VIII being the most frequently affected in 32%, 28%, and 22% of the cases, respectively.

Hospitalization, therapeutic and outcome results

The median length of hospital stay was 14 d (1-53 d), 45 patients required intensive care unit admission with a median stay of 1 d (1-30 d), and most patients required regular inpatient beds for a median of 13 d (1-53 d).

Table 1 Clinical and laboratory results of patients with pyogenic liver abscess

BMI mean ± SD	27 ± 5
BMI classification	Patients, *n* (%)
Normal	140 (41)
Overweight	141 (41)
Obesity class 1	43 (12)
Obesity class 2	12 (3)
Obesity class 3	9 (3)
Sign/Symptom	
Fever	293 (85)
Right upper quadrant pain	259 (75)
Fatigue	167 (48)
Diarrhea	52 (15)
Laboratory findings	mean ± SD
Hemoglobin, g/dL	11.7 ± 2.48
Leucocyte count, × 10^3/μL	15.52 ± 10.03
Neutrophil percentage, %	48.48 ± 31.89
Lymphocyte percentage, %	9.73 ± 11.46
Platelet count, K/μL	315 ± 180.4
INR	1.27 ± 0.51
Glucose, mg/dL	130 ± 68.12
Creatinine, mg/dL	1.14 ± 0.83
Sodium, mmol/L	132.3 ± 17.45
Total bilirubin, mg/dL	2.24 ± 2.91
Albumin, g/dL	2.63 ± 2.05
AST, U/L	59.3 ± 73.23
ALT, U/L	57.6 ± 82.09
GGT, U/L	133.5 ± 208.43
AP, U/L	256.37 ± 219.30
Lesion size	*n* (%)
< 5 cm	54 (16)
5-10 cm	138 (40)
> 10 cm	28 (8)
< 500 mL	77 (22)
≥ 500 mL	48 (14)

This table shows the demographic and clinical characteristics. The most relevant laboratory finding among the liver biochemistry alterations was leukocytosis with an elevated neutrophil count. BMI: Body mass index; INR: International normalized ratio; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transferase; AP: Alkaline phosphatase.

In terms of source control, 165 patients underwent PCD, 64 underwent percutaneous needle aspiration (PNA), 48 underwent open drainage (OD), and 32 underwent laparoscopic drainage (LD). Twenty-four patients required surgical therapy after an incomplete response to interventional radiology-assisted drainage. Of the 345 patients, at least 66% (n = 229) required interventional radiology therapy. The two types of registered procedures were PNA and PCD. A total of 72% patients underwent PCD, and the remaining 28% of patients received PNA.

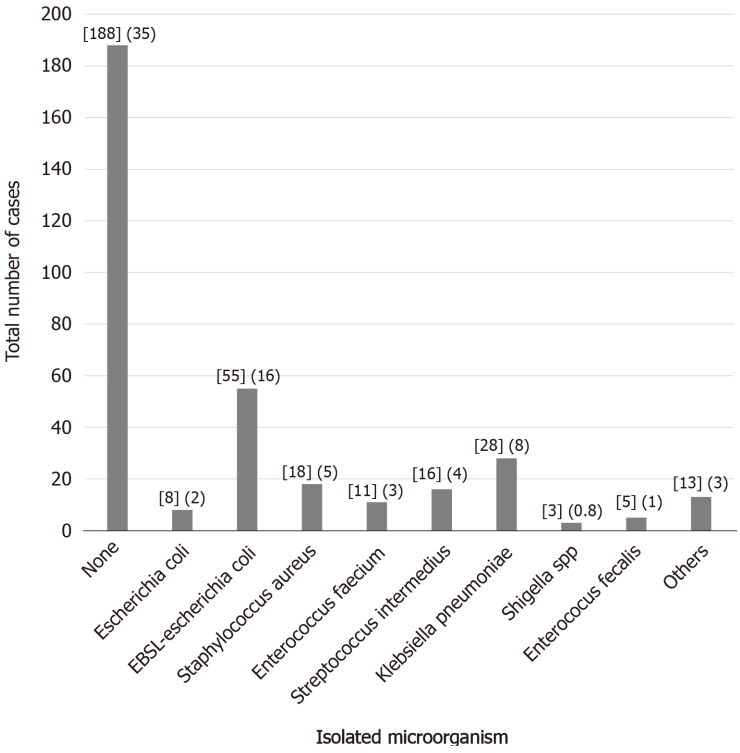

Figure 1 Distribution of isolated microorganisms in patients with pyogenic liver abscess *n* (%). The figure shows the frequency of the different bacteria cultured. The majority of cases did not have microorganism isolation in the cultures.

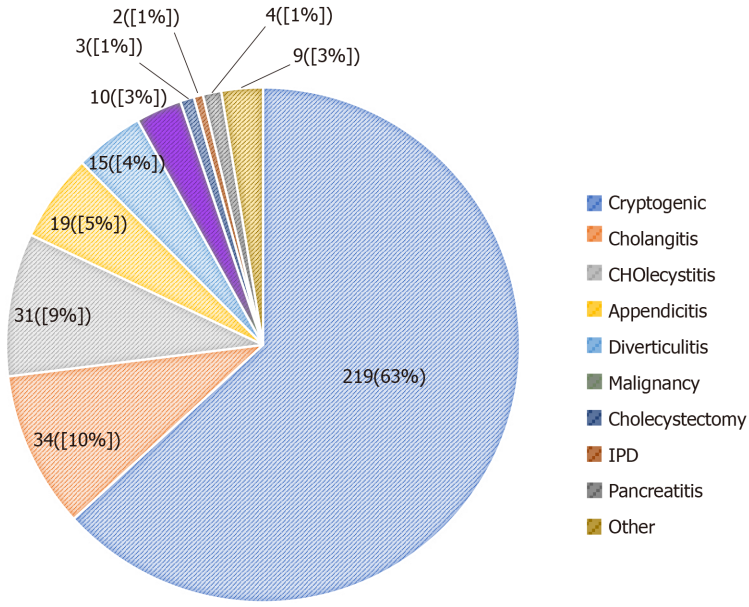

Figure 2 Source of infection in patients with pyogenic liver abscess. The figure shows the distribution of the different sources of infection, with ascending cholangitis and cholecystitis both being the most common precipitating events.

The other type of documented procedure was surgical OD or LD of the abscess. Only 22% of patients demanded a surgical intervention, of whom 48 (60%) corresponded to OD and the remaining 40% to LD. Fifty-five of those patients underwent direct surgical therapy, and only 24 who were previously exposed to interventional radiology procedures still required surgery.

A total of 120 (35%) patients had complications during their hospitalization; the most frequent complications were septic shock in 50 patients and sepsis in 18 (Third International Consensus Definitions for Sepsis and Septic Shock)[8]. Less frequent complications were biliary fistula, ruptured abscess, pneumonia, empyema, and

myocardial infarction.

The inpatient mortality rate attributed to the abscess or its complications was 63%. In univariate analysis, variables associated with mortality were age, body mass index (BMI), T2DM, the presence of immunosuppression, ESBL-*Escherichia coli*, and septic shock, as shown in Table 2. In multivariate analysis, age and BMI were not significant, but the presence of immunocompromise, ESBL-*Escherichia coli*, and *Klebsiella pneumoniae* explained 15.8% of the mortality, with ESBL-*Escherichia coli* being the most critical component (adjusted OR 6.7), as shown in Table 3.

Follow-up

Repeat radiologic imaging (ultrasonography or CT) was performed for all patients at two- to three-week intervals to evaluate the response to treatment. Parenteral antibiotic agents were converted to oral formulations based on clinical progress and were guided by bacteriology results and down-trending inflammatory markers. Antibiotic agents were discontinued at clinical and near-radiologic resolution (absence or significant reduction in size of abscess on imaging).

DISCUSSION

In our study, the median age of the patients was 51 years, and the male to female ratio was 1:1.7, with a female predominance. Among the reported cases from the INCMNSZ by Téllez-Zenteno, the mean age was 52 ± 14 years, which was similar to the age in our series, but an inverse result in the sex ratio was observed, with a male predominance[7]. On the other hand, European series, such as the one published by Serraino *et al*[5], reported an average age of 65.4 years with a male predominance. According to the world literature, the peak incidence of PLA is found in the same age groups as those in our study but with a typical male predominance[1].

A total of 85% of our patients reported fever during their initial presentation, followed by 48% with right upper quadrant pain, both of which are the most commonly documented signs and symptoms in many of the world case series and in our previous series[5-10], but the clinical picture shows many nonspecific findings, such as jaundice, pleural effusion, anorexia, nausea and vomiting[1,2].

As previously mentioned, the etiology of PLA has suffered a shift from the expansion of intraabdominal infection to biliary tree-derived diseases. Nonetheless, 63% of the cases did not have any documentable cause of their PLA on their medical records after evaluating the pathological history, trauma, and possible postoperative complications; performing CT and cholangioresonance to evaluate the biliary tree as well as endoscopic retrograde cholangiopancreatography in the indicated cases; and searching for bacteremia from any source of sepsis and foreign bodies.

According to the series by Téllez-Zenteno, 53% of patients had an unidentified source of infection in contrast to most recent series, which report between 25% and 40% of cryptogenic PLA cases[5,7,11,12]. The reason for the high cryptogenic PLA count is not apparent but might be derived from previous exposure to antibiotics, delayed medical attention, and increased prevalence of risk factors such as T2DM regardless of this phenomenon seen worldwide[13,14]. Among the patients with an underlying cause, both cholangitis and cholecystitis accounted for 19% of the cases, and other series have reported similar findings with 20% up to 55% depending on the consulted literature[5,9,15,16]. According to the Mexican series, biliary tract diseases were present in 24% of the cases, which is analogous to the most recent findings[7].

A total of 58% of our patients reported at least one medical comorbidity, in which T2DM was present in 25% of the patients, akin to what is described in Mexican, European and United States series[5,7,15].

As already stated, *Escherichia coli* is the most important pathogen causing PLA in the world literature and even in the present and previous Mexican case series[1,2,6]. Even so, ESBL-*Escherichia coli* represented a majority of the isolated microorganisms in this series, where the increase compared to the series by Téllez-Zenteno might be explained by the widespread expansion of endoscopic procedures such as biliary stenting, thus promoting the risk of bacterial bile colonization[17]. Of outstanding importance is the absolute increase in *Klebsiella pneumoniae* cases compared to 20 years ago (n = 5 *vs* n = 28) due to emerging concern regarding a certain strain of this microorganism as it has been an increasing cause of PLA. There has been a steady increase in the number of reports on this particular pathogen in cryptogenic PLA not only in Asian but also in Western countries[18-20]; this variant has been associated with an abundant production of capsular material along with other heightened intracellular

Table 2 Univariate analysis of high-risk mortality variables in patients with pyogenic liver abscess

High-risk variable	Univariate				
	Dead	Alive	Total	OR (95%CI)	*P* value
Age					
	52.7 ± 16.7	48.7 ± 16.9			0.02
BMI					
	26.2 ± 4.4	27.4 ± 5.3			0.017
T2DM[2]					
Yes	52	35	87	0.8	0.48
No	165	93	258		
Immunocompromise[1]					
Yes	30	5	35	3.9	< 0.01
No	187	123	310		
E. coli					
Yes	3	5	8	0.3	0.13
No	214	123	337		
ESBL[2]-*Escherichia coli*					
Yes	49	6	55	5.9	< 0.01
No	168	122	290		
Klebsiella pneumoniae					
Yes	24	4	28	3.9	< 0.01
No	193	124	317		
Septic shock					
Yes	31	19	50	1	< 0.89
No	186	109	295		
Total	217	128	345		

In univariate analysis, the variables associated with mortality were age, BMI, T2DM, the presence of immunosuppression, ESBL-*Escherichia coli*, and septic shock.

[1]Includes HIV-positive patients and those with underlying malignancy and autoimmune conditions.

[2]Extended-spectrum beta-lactamase. BMI: Body mass index; T2DM: Type 2 diabetes mellitus.

functions that result in a hypermucoviscous phenotype[1,2,18]. Cryptogenic PLAs associated with these *Klebsiella pneumoniae* strains are thought to be due to colonization of the GI tract with secondary invasion of the intestinal mucosa and portal venous blood, but the true pathogenesis is not completely understood [1,2,20]. Shelat *et al*[21] demonstrated that despite demographic and clinical differences, the overall outcomes of culture-negative pyogenic liver abscess (CNPLA) patients were equivalent to those of Klebsiella-positive PLA (KPPLA) patients: Hospital stay (15.7 d *vs* 16.8 d, $P = 0.397$) and overall 30-d mortality (14.0% *vs* 11.0%, $P = 0.292$) in the CNPLA and KPPLA groups, respectively. Hence, it is justified to treat these patients with empirical antibiotics targeting *Klebsiella pneumoniae*.

Intravenous antibiotic therapy was the therapeutic mainstay. According to the WSES guidelines on intra-abdominal infections, initial antibiotic therapy is typically empirical in nature because a patient with abdominal sepsis requires immediate treatment, and microbiological data (culture and susceptibility results) can require up to 48-72 h before they are available for a more detailed analysis. The selection of appropriate empiric antibiotic therapy is critical for preventing unnecessary morbidity and mortality[22].

In our study, the majority of patients were treated with third-generation cephalosporins ± metronidazole as the combination of choice, which is related to the

Table 3 Multivariate analysis of high-risk mortality variables in patients with pyogenic liver abscess

High-risk variable	Multivariate analysis		
	Adjusted OR	95%CI	*P* value
Immunocompromise			
Yes	3.9	1.42-10.46	< 0.01
No			
ESBL[1]-*Escherichia coli*			
Yes	6.7	2.7-16.2	< 0.01
No			
Klebsiella pneumoniae			
Yes	4.8	1.6-14.4	< 0.01
No			

In multivariate analysis, the presence of immunocompromise, *EBSL-Escherichia coli* and *Klebsiella pneumoniae* explained 15.8% of the mortality.
[1]Extended-spectrum beta-lactamase.

local flora being represented by sensitive gram-negative enterobacteria and anaerobes. The prevalence of multidrug-resistant (MDR) organisms seems to be ever-rising secondarily due to endoscopic and surgical interventions predisposing patients to recurrent biliary infections and exposure to broad-spectrum antibiotics[23].

Infections caused by resistant gram-negative bacteria, such as *Klebsiella pneumoniae*, are rapidly emerging as a major source of multidrug-resistant infections worldwide and are the most common causative pathogen of gas-forming PLA (GFPLA). GFPLA represents a small proportion of PLA with a reported incidence of 7%-24%, and it has traditionally been associated with high mortality (25.7% to 37.1%). In one study, Chan *et al*[24] reported no significant differences between GFLPA and non-GFLPA in the need for percutaneous drainage [26/36 (72.2%) *vs* 47/72 (65.3%), respectively, $P = 0.467$] and in-hospital all-cause death [4/36 (11.1%) *vs* 7 (9.7%), $P = 0.822$].

The median treatment duration in this study was 19 d, with more than 80% of the patients having received at least 14 d of antibiotic therapy, which, according to the world literature, should be at least 2-6 wk[1,13,17].

Although no general consensus has been published, a small PLA (3-5 cm) is suitable for antibiotic therapy alone, while larger diameter abscesses might need drainage[25]. PCD can be performed when the PLA is > 4 cm, which is arbitrary and is based on the mathematical principles of sphere volumes. It becomes obvious that with increments in diameter from 4 to 5 cm, the volume of spheres doubles from 33 to 65 cc. The 4 cm size also allows for the satisfactory placement of pigtail drains and hence seems to be a logical cut-off value for PCD[6].

PNA or PCD was the interventional radiology method used to drain the PLA, with the latter being the most commonly used in our series due to its high efficacy. PNA is less expensive and more straightforward and does not require catheter care, and its potential complications and multiple abscess cavities can be aspirated but require multiple punctures; additionally, there is a high failure rate with viscous pus or greater cavities. On the other hand, PCD is a high-success rate therapy but might cause discomfort, pain, or cellulitis at the insertion point[26].

The other therapeutic approach to PLA is surgical drainage, either open or laparoscopic. PLA rupture, PNA/PCD failure, anatomic inaccessibility through percutaneous means, and multiloculated and coexisting pathology requiring surgery mandate a more invasive strategy[27]. In a retrospective study by Ahmed *et al*[28], 39 patients with GPLA were safely treated with IV antibiotics and PCD, and the authors concluded that lesion size was not a contraindication for PCD.

In our series, 22% of the patients underwent OD or LD; 70% of the patients underwent surgery as the primary therapy for the abscess, and the remaining 30% underwent salvage therapy when percutaneous methods failed. Some authors suggest that surgery should be the first-line treatment in patients with a high mortality risk, particularly in critically ill patients[29]. Tan *et al*[30] published a retrospective study comparing LD and percutaneous methods and failed to achieve clinical superiority of LD despite the theoretical benefits of laparoscopic surgery; the authors still

recommend LD due to its lower tendency for developing complications due to sepsis and the possibility to wash the peritoneal cavity with ease[31]. Although in our study the ratio of OD *vs* LD was 3:2, some series have reported that both approaches are valid and safe in patients able to withstand the physiological stress of surgery and LD, offering advantages for the postoperative recovery of GI function and hospital stay[30].

Abdominal ultrasound is a safe and noninvasive method for evaluating the liver parenchyma and usually shows a hypoechoic mass (or masses), but its echogenicity is variable[1,32]. Therefore, another imaging method is usually needed; in our series, a CT scan was the primary confirmatory method, with a contrast-enhanced technique being used in 85% of the cases and with the remaining patients undergoing magnetic resonance for confirmation. Classic literature describes that PLAs are usually located in the right lobe, which is associated with dominant portal blood input to this anatomic region[1]; our patients' primary lesions were located in segments VII, VI, and VII, which correlates with frequently described findings.

Laboratory findings are usually nonspecific for PLAs but are on par with the inflammatory status of the patient; leukocytosis with an elevated PMN count, hypoalbuminemia, elevated transaminases and alkaline phosphatase, and hyperglycemia were among the most common findings in this study, which are in accordance with other case series[5,33,34].

Complications related to PLA are not uncommon, and rates can be as high as 72%, with bacteremia, empyema, septic shock, and metastatic infection being the most frequent[2]. Relating to our own series, the most frequent complications were septic shock and sepsis, both of which are frequently mentioned in the United States, European or Chinese series[11,12,15]. Septic shock has been associated with excess mortality in patients with PLAs, so identifying which factors relate to its development is a matter of interest. Cho *et al*[35] described that older age and malignancy were associated with 3.0- and 2.1-fold higher rates of septic shock, as well as elevated procalcitonin levels[36].

The mortality rates due to PLA have varied over the years; previous data have described that patients developing septic shock can reach a mortality rate of 19%[5], but more recent case series mention in-hospital mortality rates of up to 10%[5,7,13,28]. Interestingly, we demonstrated an extraordinarily high in-hospital mortality rate of 63% due to abscess-related complications; this could be due to many factors, including the advanced age of the studied population; the fact that both centers are tertiary care centers where patients from the whole country are referred, thus delaying hospitalization and appropriate antibiotic treatment; the presence of ESBL-*Escherichia coli* and *Klebsiella pneumoniae* related to PLA; and the high incidence of septic shock in the study population as well as hospital-acquired infections. Comorbidities also played an important role in the high mortality rate in the series because at least 60% of T2DM patients and 85% of patients with other causes of immunosuppression (HIV infection, underlying malignancy, and autoimmune diseases) impacted the increased mortality.

In our study, T2DM and the development of septic shock did not achieve statistical significance, but as expected, advanced age and higher BMI were high-risk characteristics. The presence of both ESBL-*Escherichia coli* and *Klebsiella pneumoniae* demonstrated adjusted ORs of 6.7 and 4.8, respectively, thus being the most critical high-risk characteristics related to mortality in this study, as well as the presence of compromised immune function, which in turn had a 4-fold greater risk of death. Through logistic regression, these 3 variables explained at least 16% of the mortality in our study, with other factors, such as the previously mentioned factors, playing an important role and could be a focal point for further studies.

We consider it important to highlight the measures aimed at reducing mortality, including clinical suspicion and early diagnosis, the availability of imaging studies such as CT, and deciding on a timely intervention for radiology and surgery in difficult to manage cases.

CONCLUSION

Even though demographic characteristics have remained almost constant in the past decade, an increased prevalence in both referral centers has been noted. More than half of the cases were cryptogenic PLA, which correlates with global epidemiology and a steady shift to a predominant *Klebsiella pneumoniae*-related abscess. Biliary tree disease was the most common source of infection with ESBL-*Escherichia coli* and *Klebsiella pneumoniae* being the most common microorganisms, both of which together with an

immunocompromised state were independent predictors of high mortality risk.

ARTICLE HIGHLIGHTS

Research background

In Mexico, the epidemiology of pyogenic liver abscess (PLA) is unknown, with some small case series. This study recruited patients from two high-level hospitals in Mexico.

Research motivation

PLA is a rare medical condition that has had a change in its etiology in recent years; however, more studies are needed to determine the epidemiology of PLA in Mexico.

Research objectives

The aim of this study was to describe the current clinical, demographic, and microbiologic characteristics of PLAs in two high-volume centers in Mexico. These data will allow us to understand the behavior of this disease in Mexico.

Research methods

This is a retrospective analysis of patients with PLA from two high-level hospitals in Mexico. A chart review was performed to evaluate the clinical, demographic and microbiologic characteristics of PLA. A multivariate analysis was performed to identify independent risk factors associated with mortality.

Research results

The main isolated microorganisms were ESBL-*Escherichia coli* and *Klebsiella pneumoniae*. The inpatient mortality rate was 63%. In multivariate analysis, immunocompromised state, ESBL-*Escherichia coli*, and *Klebsiella pneumoniae* were independent predictors of high risk mortality

Research conclusions

An increased prevalence in both referral centers has been noted. The mortality rate was significantly higher compared to previously reported rates worldwide, reaching 63%. There was a steady shift to a predominant *Klebsiella pneumoniae*-related abscess.

Research perspectives

As a retrospective review, our study is limited. Prospective studies that monitor the mortality rate are required, which in this study was high compared to that reported in other series.

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ORIGINAL ARTICLE

Observational Study

Malnutrition and non-compliance to nutritional recommendations in patients with cirrhosis are associated with a lower survival

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Author contributions: Crisan D, Procopet B, Stefanescu H and Crisan N had key contributions in the conception and design of the study and have provided a critical revision of the manuscript for important intellectual content; Epure A, Suciu A, Fodor A and Mois E have performed the nutritional assessment; have applied the nutritional recommendations adherence questionnaires and worked on data acquisition; Crisan D, Procopet B and Craciun R have contributed to analysis and interpretation of the data and drafting the manuscript; all authors gave their final approval of the version to be submitted and agree to be accountable for all aspects of the work.

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Abstract

BACKGROUND

Malnutrition is frequently encountered in patients with cirrhosis and appears to significantly impact their prognosis. While evaluating the burden of malnutrition in cirrhosis is gathering momentum, as suggested by multiple recently published reports, there is still a persistent scarcity of solid data in the field, especially with regards to the role of nutritional interventions.

AIM

To assess the prevalence of malnutrition in patients with advanced cirrhosis and to evaluate its impact on survival.

METHODS

One hundred and one consecutive patients with advanced cirrhosis were screened for malnutrition using the Subjective Global Assessment (SGA) criteria and the mid-arm circumference (MAC). Malnutrition was defined as SGA class B and C and MAC < 10th percentile. All patients were interviewed regarding their food intake using an adapted questionnaire. Subsequently, total energy intake was

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Informed consent statement: Participants were informed about the plan about our study details. And if they agreed to participate in, written informed consent was obtained from each participant prior to their inclusion in the study.

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calculated and further subdivided in main nutrients. The data were then compared to the available recommendations at the time of analysis to assess adherence.

RESULTS

54/79 patients (68.4%) in the decompensated group had malnutrition, while only 3/22 patients (13.6%) were malnourished in the compensated group. After a median follow-up time of 27 mo (0-53), the overall mortality was 70%. Survival was significantly lower among patients with malnutrition. The mortality rates were 50% at 1 year and 63% at 2 years for the patients with malnutrition, compared to 21% at 1 year and 30% at 2 years for patients without malnutrition (P = 0.01). On multivariate analysis, the factors independently associated with mortality were age, creatinine level and adherence to the protein intake recommendations. The mortality was lower in patients with the appropriate protein intake: 8% at 1 year and 28% at 2 years in the adherent group, compared to 47% at 1 year and 56% at 2 years in the non-adherent group.

CONCLUSION

The prevalence of malnutrition is high among patients with advanced cirrhosis and might be related in part to a low adherence to nutritional recommendations, especially with regards to protein intake.

Key Words: Malnutrition; Decompensated cirrhosis; Survival; Subjective global assessment; Protein intake

Core Tip: It is already known that the patients having cirrhosis can be affected by malnutrition and this status can impact the survival. This article studied the prevalence of malnutrition in advanced stages of cirrhosis, and its influence on survival. Our results showed that the prevalence of malnutrition is high in patients with advanced cirrhosis and is related in part to a low adherence to nutritional recommendations. Appropriate protein intake could increase the survival.

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INTRODUCTION

Poor nutritional status has a high prevalence in patients with advanced liver disease and has significant influence on prognosis[1]. At the initial development of the Child-Turcotte score, the nutritional status was included in the evaluation of patients submitted to porto-caval shunting surgery and the score had significant prognostic relevance[2]. Currently, the Child-Pugh-Turcotte score is still one of the most utilized systems to stage cirrhosis and to assess the prognosis of these patients. Malnutrition has proved to be independently correlated with survival and the addition of mid-arm muscle circumference (MAMC) and triceps skinfold thickness (TST) to the Child-Pugh score increases the prognostic accuracy[3].

The mechanism of malnutrition in cirrhosis is multifactorial, including lower nutrient intake (loss of appetite, impaired digestion/absorption or low protein diet), hypercatabolic status and decreased liver protein synthesis[4]. However, data regarding the evaluation of lifestyle and nutritional intake in patients with advanced liver disease and their adherence to nutritional recommendations is limited.

Even if malnutrition is more prevalent in advanced stages of cirrhosis, the assessment of nutritional status in these patients may be difficult mainly because of sodium-water retention. Therefore, tracking weight change or body mass index most

likely does not accurately reflect the nutritional status of a patient. The latest available guidelines at the time of data collection were the 2006 ESPEN (European Society for Parenteral and Enteral Nutrition) guidelines, which recommended the use of Subjective Global Assessment (SGA) or anthropometry to identify those at risk for malnutrition in patients with cirrhosis[5]. These recommendations were reinforced in the 2019 revised edition of the guidelines[6]. As the anthropometric parameters should not be influenced by ascites or peripheral oedema and, MAMC or mid-arm circumference (MAC) and TST can be confidently used. However, the applicability of these parameters in decompensated cirrhotic patients has not been extensively validated. In order to avoid malnutrition and its negative consequences for patients with cirrhosis, the aforementioned guidelines recommend a daily energy intake of 30–35 kcal/kgBW/d (125–146 kJ/kgBW/d) and a protein intake of 1.2–1.5 g/kgBW/d[6]. However, the adherence to these recommend-ations is not known.

The aims of this study were: (1) To assess the adherence of patients with advanced cirrhosis to nutritional recommendations; (2) To evaluate the prevalence of malnutrition in patients with cirrhosis stratified according to their clinical stage (compensated or decompensated); and (3) To assess the influence of malnutrition and adherence to nutritional recommendation on survival.

MATERIALS AND METHODS

This is a prospective observational study that included 101 consecutive patients with cirrhosis of any aetiology hospitalized in our tertiary Health Care Centre. Sixty-four patients who were recruited while being hospitalized for clinical decompensation in the inpatient ward between the 1st of March and the 30th of June 2013 were considered for inclusion. Patients were included in the decompensated cirrhosis group based on the presence of ascites (and related complications: Spontaneous bacterial peritonitis, hepatic hydrothorax), portal hypertension related bleeding (variceal bleeding), diagnosis of acute on-chronic liver failure, overt hepatic encephalopathy (grade 2 to 4 in West Haven scale) or other specified events (kidney dysfunction, hepato-renal syndrome, bacterial infections and cardiopulmonary complications of portal hypertension). All consecutive patients presented to our centre during the study period were considered primarily eligible for inclusion. Eleven patients did not consent and were not included. Furthermore, a cohort of 37 consecutive patients with liver cirrhosis, completing the regular follow-up in June 2013 in the outpatient ward, was included. Among them, 15 (40.5%) had previous decompensation and were included in the decompensated group and 12 patients (32.4%) had early stage of hepatocellular carcinoma (BCLC-0 and A) previously treated by percutaneous radiofrequency ablation and, at the moment of inclusion, were in the follow-up stages. There was no significant difference between patients with or without HCC in the control group and none of the patients with HCC had previous decompensation events. All patients that agreed to participate signed an informed consent and the Ethical Committee of our University approved the protocol. The present study was designed with respect to the ethical guidelines issued by the 2000 revision (Edinburgh) of the 1975 Declaration of Helsinki. The flowchart of patient enrolment is illustrated in Figure 1.

The diagnosis of cirrhosis was based on specific findings in the patient's medical history, clinical examination, laboratory findings and imaging examinations. Physical examination including anthropometric measurements, blood tests, abdominal ultrasonography and a complete nutritional evaluation were done at the inclusion of all patients.

All included patients together with the closest family members were questioned about their detailed alimentary intake in the last 2 wk and were asked to approximate the food habits in the last 3 mo. For this purpose, the National Health and Nutrition Examination Survey (NHANES) Food Questionnaire (https://wwwn.cdc.gov/nchs/nhanes/) was modified and adapted to the general food habits for our country (we eliminated questions referring to some foods that are rarely consumed in our country). Apart from the reduction of the number of questions we also reduced the period of assessment to 3 mo and, thus, some questions regarding seasonal and out-seasonal consumption of some seasonal foods were either merged or eliminated. Finally, the questionnaire comprised 80 questions and was based on the approximation of the quantity of main foods in the last 3 mo. The mean time for the application of the questionnaire was 30-40 min. The list of principal foods and the calculation of the energy and principal nutrients (proteins, lipids, carbohydrates and

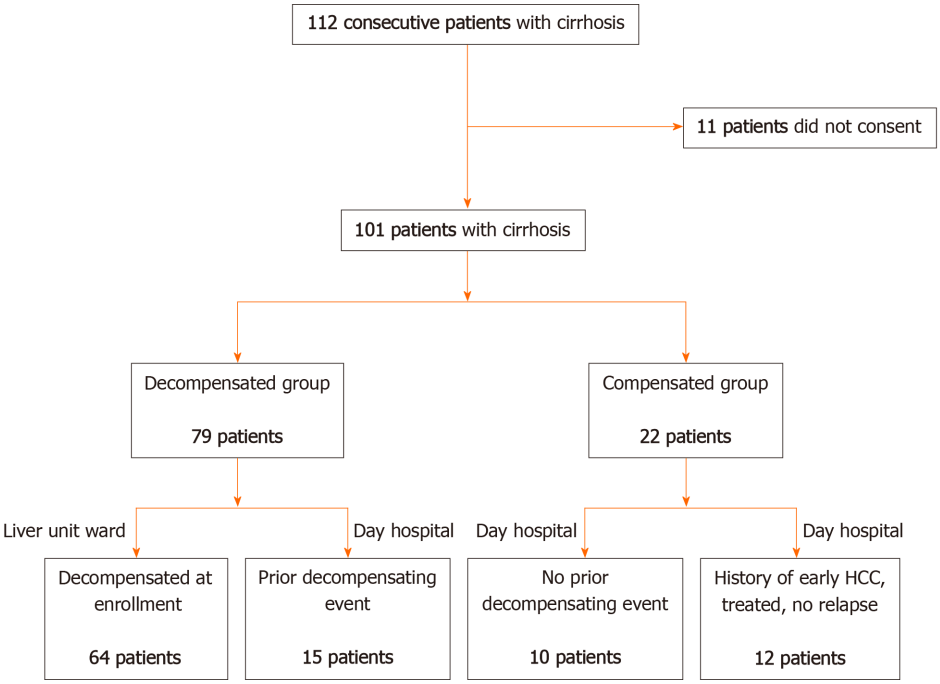

Figure 1 Patient enrollment algorithm.

salt) intake was made using the "Healthy alimentation guidelines" published by the National Ministry of Health on their official website. These guidelines include lists of principal foods and their main nutrients content expressed by 100g. Therefore, for each patient the food intake was calculated and expressed as: kcal/kg body weight/d; protein g/kgBW/d, lipid g/kgBW/d, carbohydrates g/kgBW/d intake and salt g/kgBW/d intake.

The food intake was compared with the 2019 ESPEN (European Society for Parenteral and Enteral Nutrition) recommendations for patients with liver cirrhosis (30–35 kcal/kgBW/d energy daily intake and a protein intake of 1.2–1.5 g/kgBW/d)[6]. Patients were considered adherent if their caloric or protein intake was within the proposed recommendations.

The nutritional status evaluation was based on SGA assessment and MAC. Because of the lack of a specific measuring tool, TST was not possible and, therefore, MAMC was also unavailable.

SGA was assessed according to the Detsky *et al*[7] protocol, which included the history of the weight curve in the last 6 mo and during the last 2 wk, dietary intake, gastro-intestinal symptoms, functional capacity and clinical examination, including the evaluation of subcutaneous fat, muscle wasting, oedema and ascites. In this particular clinical scenario, the presence of ascites and oedema was considered as a sign of decompensation rather than a sign of malnutrition. According to the SGA evaluation the patients were classified as: Well-nourished SGA-A, mild to moderate malnutrition SGA-B and severe malnutrition SGA-C. In decompensated patients the SGA ranking was completed by consensus between the nutritionist and the hepatologist using adapted recommendations[8].

MAC was measured in the non-dominant arm at the midway between the acromion and olecranon process. Malnutrition was defined as a MAC < 10th percentile and severe malnutrition if MAC < 5th percentile using as a reference values adapted for age and gender from the Bishop's study[9].

Because not all the cohort was prospectively followed-up in our centre after discharge, data regarding survival at 1st August 2017 and date of death of the included population were obtained from the National Population Register.

The study complies with the STROBE guidelines.

Statistical analysis

Data was expressed as mean ± SD or median and range and qualitative variables as frequencies. The *t*-student or Mann-Whitney tests were used for quantitative variables for the comparison between groups and the Chi-square and Fisher's exact tests were used for qualitative data where appropriate. For multivariate analysis, a binary logistic

regression using the backward LR model was used in order to determine the parameters associated with decompensation and malnutrition. Concordance between SGA and MAC regarding malnutrition diagnosis was evaluated by the weighted (two categories) kappa method. Concordance coefficient (k) was graded by the scale proposed by Landis and Koch[10]: 0%-10% = poor, 10%-20% = slight, 21%-40% = fair, 41%-60% = moderate, 61%–80% = substantial and 81%-100% = almost perfect.

For survival analysis, the cox regression was used to identify independent risk predictors (OR 95%CI) for death. Only variables significantly associated with the judgment criteria in the univariate analysis were considered for the multivariate analysis. For multivariate analysis, a cox regression using the backward LR model was used in order to determine the parameters associated with death. Actuarial rates of survival were calculated using the Kaplan–Meier plots and compared by the Log rank test. $P < 0.05$ was considered as the level of significance. The statistical review of the study was performed by a biomedical statistician. Statistical analysis was performed using the SPSS software version 20 (SPSS Inc. Chicago, IL, United States).

RESULTS

Among the 79 patients included in the decompensated group, 42 (53.2%) were male and the mean age was 61.3 ± 10. There were no differences between groups regarding age and gender. The most prevalent aetiology in the decompensated group was alcohol-related liver disease – 32 patients (51.9%), while in the compensated group viral cirrhosis was predominant – 16 patients (72.7%). The differences between the two groups were statistically significant ($P = 0.005$). Fifty patients (63.2%) from the decompensated group were on beta-blocker treatment for primary or secondary prophylaxis of variceal bleeding. The full baseline characteristics of the included population are listed in Table 1.

Ascites was the most frequent decompensation event - 59 (74.6%) patients, followed by hepatic encephalopathy ($n = 29$; 36.7% of patients), and variceal bleeding ($n = 20$; 25.3%).

Overall, the adherence to the current nutritional guidelines for cirrhosis was extremely low. Only 21 (20.8%) patients had the recommended alimentary intake of 30-35 kcal/kgBW/d and 57 (56.4%) patients had a suboptimal energy intake (<30 kcal/kgBW/d). Regarding the proportion of protein intake, only 26 (25.7%) patients had the recommended amount of protein (1.2-1.5 g/kgBW/d), while the rest had either superior (40%) or lower (34.3%) protein intake. When we analysed the patients from the compensated group, we observed that 11 out of 22 patients (50%) had <30 kcal/kgBW/d energy intake.

Because the majority of the patients had ascites as the main decompensation event, we also evaluated the salt intake and compared it with the actual recommendation[11] of 4.6-6.9 g/d salt intake in patients with ascites, namely a "no added salt diet". Among all patients, only 26 (25.7%) had a salt intake < 6.9 g/d and 7 (6.9%) < 4.6 g/d, which demonstrated a very poor adherence to the "no added salt diet". In the decompensated group, only 20 (25.3%) patients had a salt intake < 6.9 g/d.

When comparing patients adherent or not to the no added salt diet (< 6.9 g/d) regarding the food intake, those adherent to the diet had significantly lower protein, lipid, carbohydrates and caloric intake (Supplementary Table 1).

When comparing decompensated to the compensated group, there was no difference regarding the main nutrients intake (Table 2).

According to the SGA classification, in the decompensated group, 54 (68.4%) patients had malnutrition and, among them, 19 (24.1%) had severe malnutrition (SGC-C). In the compensated group, only 3 (13.6%) were malnourished and no patient had severe malnutrition. In comparison, six (40%) out of 15 patients with prior history of decompensation admitted to or day hospital had malnutrition according to the SGA criteria ($P = 0.06$). Using the MAC criteria, in the decompensated group, 48 (60.8%) had malnutrition, whereas in the compensated group, 6 patients (27.3%) were malnourished. Details about nutritional markers are found in Table 2. Regarding the malnutrition diagnosis, there was only a slight-fair agreement between SGA and MAC (k = 0.261, $P = 0.009$).

As expected, comparing decompensated and compensated groups in univariate analysis (Table 1), variables related to liver function [Child-Pugh and model for end-stage liver disease (MELD) scores, international normalized ratio (INR), albumin, serum bilirubin, serum sodium] and variables related to the nutritional status (MAC and SGA score, cholesterol level) were associated with current or prior history of liver

Table 1 Baseline characteristics

Variable*n* (%) or mean ± SD	Decompensated, *n* = 79	Compensated, *n* = 22	*P* value
Gender M/F	42 (53.2)/ 37 (46.8)	11 (50)/ 11 (50)	NS
Age	61.3 ± 10	64.5 ± 10.9	NS
Child-Pugh A/B/C	8(10.1%)/32(40.5%)/39(49.4%)	19(86.4%)/3(13.6%)/0(0%)	< 0.001
Aetiology			0.005
Viral	30 (38.0)	16 (72.7)	
Alcohol	32 (51.9)	3 (13.6)	
Other	5 (10.1)	3 (13.6)	
Platelet count (10^9/L)	122.9 ± 86.9	123.1 ± 60.9	NS
INR	1.82 ± 0.53	1.27 ± 0.25	< 0.001
Total bilirubin (mg/dL)	5.4 ± 6.9	1.2 ± 0.7	0.006
Albumin (g/L)	3.1 ± 0.6	3.9 ± 0.7	< 0.001
AST (U/L)	83.3 ± 67.4	56.6 ± 29.5	0.08
ALT (U/L)	42.3 ± 42.7	46.6 ± 31.2	NS
Haemoglobin (g/dL)	10.9 ± 3.9	12.7 ± 2.2	0.05
Na (mEq/L)	134.5 ± 7.0	139.9 ± 3.3	< 0.001
Creatinine (mg/dL)	1.19 ± 0.88	0.77 ± 0.19	< 0.001
Cholesterol (mg/dL)	120.5 ± 51.4	154.7 ± 39.1	0.01
Triglycerides (mg/dL)	80.9 ± 42.6	78.1 ± 22.5	NS
Child Pugh score	9.6 ± 2.6	5.8 ± 1.2	< 0.001
MELD score	19.2 ± 7.6	11.2 ± 5.6	< 0.001

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; INR: International normalized ratio; MELD: Model for end-stage liver disease; NS: Not significant.

disease decompensation. These findings were further confirmed in multivariate analysis, where decompensation was independently associated with the MELD score (HR 1.43; 95%CI: 1.16-1.76) and the malnutrition diagnosis based on SGA (HR 7.15; 95%CI: 1.63-31.29).

In the univariate analysis, the variables associated with malnutrition were: Child-Pugh score, MELD score, haemoglobin levels, INR, albumin, total protein levels, cholesterol levels, sodium level and gender.

In the multivariate analysis, the variables independently associated with malnutrition were: Lower cholesterol levels (HR 0.97, 95%CI: 0.95-0.98, P = 0.001), lower sodium (HR = 0.71, 95%CI: 0.67-0.93, P = 0.005) and male sex (HR = 0.24, 95%CI: 0.05-1.02, P = 0.054).

Neither patients with or without malnutrition demonstrated a better adherence to an energy intake recommendation: 10 (17.5%) in malnourished patients and 11 (25%) well-nourished patients. However, the percentage of patients adherent to the recommended protein intake (1.2-1.5 g/kgBW/d) tended to be lower in malnourished patients, 11 (19%) malnourished patients *vs* 15 (34%) patients with normal nutritional values (P = 0.09).

Seventy-one (70%) patients died after the median time of 27 mo (0-53). Five patients (7.8%) died within one month after the inclusion. Among them, 41 (57.7%) had < 30 kcal/kgBW/d and 25 (35%) had < 1.2 g/kgBW/d protein intake.

The survival is lower in patients with malnutrition when the SGA definition is used: 50% at 1 year and 63% at 2 years of patient with malnutrition died *vs* 21% at 1 year and 30% at 2 years patients without malnutrition (P = 0.01, Figure 2). However, using the MAC < 10^{th} criteria for malnutrition definition, there is no difference in survival (Supplementary Figure 1).

According to the multivariate analysis (Table 3), the factors independently associated with death are age, creatinine level and the adherence to the protein intake

Table 2 Comparison between decompensated and compensated patients regarding the nutritional status and the principal nutrients intake, *n* (%)

Variable	Decompensated, *n* = 79	Compensated, *n* = 22	*P* value
MAC (cm)	25.8 ± 3.9	27.9 ± 2.9	0.02
MAC < 10th	48 (60.8)	6 (27.3)	0.005
MAC < 5th	30 (38.0)	4 (18.2)	0.08
SGA			< 0.001
A (normal)	25 (31.6)	19 (86.4)	
B (mild-moderate)	35 (44.3)	3 (13.6)	
C (severe)	19 (24.1)	0 (0)	
BMI	26.6 ± 4.7	27.1 ± 3.3	NS
Kilocalories/d	2080 ± 419	2200 ± 601	NS
Kcal/kg/d	29 ± 8	30.1 ± 9.2	NS
Proteins (g/kg/d)	1.4 ± 0.4	1.4 ± 0.5	NS
Proteins 1.2-1.5 g/kg/d	20 (25.3)	6 (27.3)	NS
Proteins < 1.2 g/kg/d	27 (34.2)	6 (27.3)	NS
Salt (mmol/d)[1]	147 ± 4.2	147 ± 4.8	NS
< 120mmol/d	20 (25.3)	6 (27.3)	NS

[1]The current recommendation for no added salt diet in patients with ascites is 4.6-6.9 g/d (80-120 mmol/d); NS: Not significant; MAC: Mid-arm circumference; SGA: Subjective Global Assessment; BMI: Body mass index.

Table 3 Univariate and multivariate analysis of factors associated with mortality

Variable	Univariate analysis; OR (95%CI)	*P* value	Multivariate analysis[1]; OR (95%CI)	*P* value
Age	1.03 (1.01-1.06)	0.004	1.03 (1.00-1.06)	0.002
Child-Pugh score	1.13 (1.04-1.23)	0.002	1.09 (0.98-1.20)	0.09
MELD score	1.06 (1.03-1.09)	< 0.001		
Presence if malnutrition[2]	1.82 (1.12-2.94)	0.01		
Creatinine (mg/dL)	1.94 (1.46-2.57)	< 0.001	2.13 (1.31-3.45)	0.03
INR	1.60 (1.05-2.43)	0.02		
Albumin (g/L)	0.45 (0.29-0.68)	0.01		
Cholesterol (mg/dL)	0.99 (0.98-0.99)	0.02		
Serum sodium (mEq/L)	0.94 (0.90-0.98)	0.003		
Protein intake 1.2-1.5 g/kg/d[3]	0.51 (0.28-0.90)	0.02	0.40 (0.20-0.77)	0.007

[1]In order to avoid collinearity between repeating variables, in model were included: Age, Child-Pugh score, presence of malnutrition, creatinine, cholesterol, serum sodium and protein intake.
[2]According to Subjective Global Assessment criteria.
[3]Adherence to protein intake recommendation was analysed. INR: International normalized ratio; MELD: Model for End-Stage Liver Disease.

recommendation (as a protective factor). When the adherence to this recommendation was analysed with the Kaplan-Meier curves the results showed that adherent patients have a significantly better survival. The mortality rate was 8% at 1 year and 28% at 2 years in the adherent group *vs* 47% at 1 year and 56% at 2 years in the non-adherent group (P = 0.01, Figure 3).

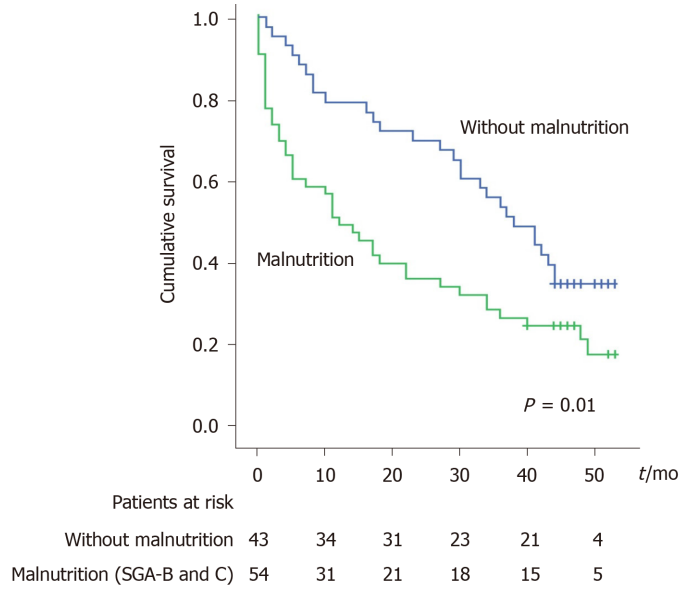

Figure 2 Kaplan-Meier curves of survival according to the presence of malnutrition based on Subjective Global Assessment criteria (malnourished–blue line; well nourished-green line). SAG: Subjective Global Assessment.

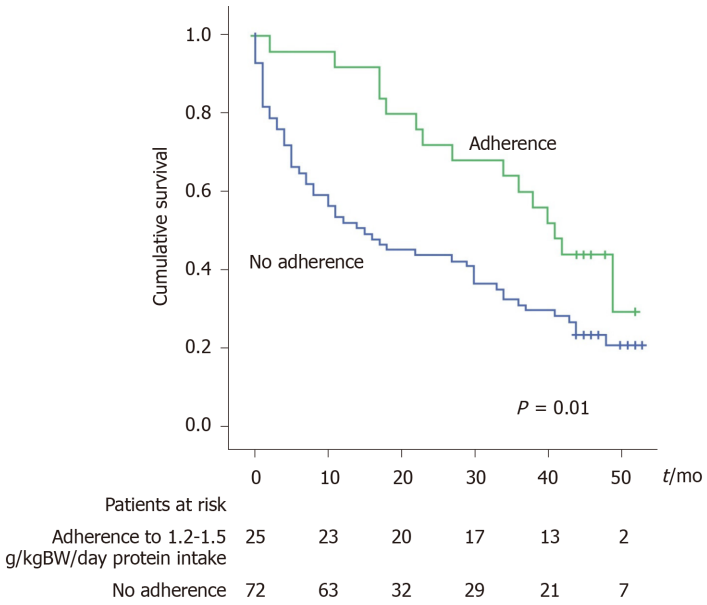

Figure 3 Kaplan-Meier curves of survival according to the adherence of protein intake recommendation of 1.2-1.5 g/kg/d (patients adhered to protein intake recommendation - green line *vs* non-adherent – blue line).

DISCUSSION

The presence of malnutrition is very prevalent in patients with cirrhosis and very relevant to the prognosis of these patients before and after liver transplantation[12,13]. In the present study, we confirmed that malnutrition has a high prevalence among patients with advanced cirrhosis, especially in the decompensated group, and is independently associated with decompensation. The low adherence to the protein intake recommendations is independently associated with a lower survival. A possible explanation for high prevalence of malnutrition is the bad adherence to nutritional recommendation. Indeed, we found that 56.4% of the patients had a suboptimal energy intake (< 30 kcal/kgBW/d) and 33% had a suboptimal protein intake (< 1.2 g/kgBW/d), even in the fully compensated phase of the disease. Low caloric intake in patients with advanced cirrhosis was confirmed also by other reports, which found that 80% of patients Child-Pugh C and 52% in Child-Pugh B had < 30 kcal/kgBW/d intake[14]. Interestingly, in fully compensated patients, even if the prevalence of

malnutrition is much lower than re-compensated and decompensated patients, 50% had a lower energy intake than recommended. Therefore, probably one of the main mechanisms of malnutrition in cirrhosis is a lower nutrient's intake. More efforts should be made to identify patients with inappropriate adherence to nutritional recommendation and to correct the nutrition earlier, before malnutrition and decompensation occur. Among other factors that may contribute to malnutrition, the decrease in liver function probably has an important role since malnutrition is associated with higher MELD scores, bilirubin and INR and lower albumin levels.

One of the major concerns is the low adherence to the no added salt diet in our advanced cirrhosis subgroup (92% of the patients with decompensation had ascites). The first step in the treatment of sodium retention in cirrhosis is salt restriction to a level between 4.6 and 6.9 g/d[11]. Of all patients, only 25% had a salt intake < 6.9 g/d. Recently, other group confirmed these findings in an outpatient cohort with ascites, closely followed within a "Care Management Program" aiming to better manage patients with cirrhosis[15]. In their cohort, only 30% of the patients followed a low salt diet, in spite of almost half of the patients believing that they were adherent to salt consumption recommendations. Moreover, the adherent patients had a lower caloric intake. In our cohort, we also found that patients with no added salt diet had significantly lower energetic (calories/kg/d), protein, lipid and carbohydrates intake. All these data sustain the hypothesis that the no added salt diet may contribute to the loss of appetite and a lower food intake, consequently further deteriorating the nutritional status.

One of the most difficult issues is to establish which is the most appropriate method to diagnose malnutrition in cirrhosis. At the time of data collection, ESPEN recommended the use of SGA as well as anthropometric measurements for the diagnosis of malnutrition in patients with cirrhosis[5]. However, there was no distinction between different stages of cirrhosis (compensated *vs* decompensated) for these recommendations and none of the methods was considered as standard. In our cohort, the concordance between SGA and MAC was only slightly significant. The best concordance was in the compensated group. In the decompensated group, using SGA criteria, 75% of the patients had malnutrition whereas using MAC < 10th only 64% were malnourished. In the compensated group, when using SGA, 24% patients had malnutrition whereas for MAC < 10th, 35% of the patients were malnourished. In light of these results, we may conclude that SGA and MAC do not have the same applicability in different stages of cirrhosis. However, in the absence of a standard method for malnutrition diagnosis, it is difficult to conclude which of the methods has the best performance. In a study of 50 compensated cirrhotic patients, the handgrip test, another validated method to assess nutritional status based on muscular strength, had a superior predictive performance compared to SGA with regards decompensation within one year[16]. In this cohort, 28% patients had malnutrition according to SGA and 63% according to the handgrip test. However, the rate of decompensation at one year is very high (42%) and the authors do not report sufficient details regarding the variables related to liver function. In another study, using MAMC < 5th the prevalence of malnutrition was 45% in Child-Pugh C patients and 25% in Child-Pugh A[14]. All these divergent findings suggest that probably none of these methods is very well adapted to cirrhotic patients. By including weight curve, dietary intake and presence of ascites and oedema among the diagnostic criteria, SGA overestimates the prevalence of malnutrition in the decompensated group since all these variables are influenced by the liver function. At the same time, for some anthropometric parameters that are not influenced so much by sodium- water retention, the standard population for generating the percentiles and analysing the results was a historical American cohort of healthy subjects[9]. Probably, the best way to assess the nutritional status in cirrhosis is to apply different methods and look for concordance and interpret according to the clinical stage of the disease.

There was an independent association between adherence to protein intake recommendation (1.2-1.5 g/kg/d) and a better survival. Although it was historically thought that patients with advanced cirrhosis should follow a protein-restricted diet due to the risk of hepatic encephalopathy, it has long-been proven that the amount of protein intake does not influence the course of hepatic encephalopathy[17]. In our study we found that 74% of the patients had a lower protein intake than ESPEN's recommendations. These findings are reproducing the results of a large Canadian cohort of patients on liver transplantation list where only 24% had appropriate protein intake[18]. The group also found that a lower protein intake is associated with transplant waiting list mortality. Low protein intake could be the cause of muscular depletion that was also associated with high mortality[19]. Although survival was lower in patients with malnutrition (based on SGA criteria, Figure 1), in multivariate analysis

the presence of malnutrition was not independently associated with survival, losing its significance in the face of variables related to liver and kidney functions. Probably, the small sample size precludes obtaining a strong conclusion regarding the relation between the presence of malnutrition and survival. Moreover, in advanced stages of disease the presence of complications related to portal hypertension or liver failure weights more in the prognosis of these patients.

There are some limitations in our study. Undoubtedly, we are aware of the low number of patients, which did not allow a more detailed subgroup analysis (in different decompensation scenarios or aetiologies) as well as it hindered the discriminant significance of our multivariate analysis. Secondly, we are well aware of the possible errors in the patient's reporting of food intake during the application of our questionnaire. Moreover, in our study we did not assess the impact of previously dietary counselling and its efficiency. We tried to overcome this form of subjectivity in assessment by also interviewing family members and by using a food questionnaire adapted to the alimentary habits of our country. Third, the food questionnaire adapted from NHANES Survey Food Questionnaire had no previous validation. Nevertheless, our questionnaire maintains the structure of the NHANES Survey Food Questionnaire, which is a well validated instrument. Fourth, in the analysis of the adherence of the low salt diet we did not use the 24 h natriuresis for the objective assessment of adherence and. However, given a cautious approach with emphasis on the core trend, rather than an adamant focus on specifics, the key findings should withstand scrutiny.

Correlations with other validated tools could further expand the knowledge on the topic. Amino acid intake profile, as well as branched-chained amino acids blood-levels, body composition assessment and imaging studies for the evaluation of sarcopenia were not analysed. Metrics developed and thoroughly validated after the design of our study, such as skeletal muscle area, skeletal muscle index or muscle radiation attenuation[20] could certainly add valuable insights. Furthermore, validation of our results on other cohorts and in comparison to the aforementioned tools could add strength to our findings.

CONCLUSION

In conclusion, given the main findings of this study, namely that prevalence of malnutrition is high in patients with cirrhosis and that adherence to the nutritional recommendations in this population is very low, intensive efforts should be initiated to identify patients at risk for malnutrition (dietary assessment) and to correct their dietary habits as early as possible. Appropriate protein intake should be strongly recommended because this may improve survival, with a low risk to benefit ratio.

ARTICLE HIGHLIGHTS

Research background

While often understated when compared to other complications of chronic liver disease, malnutrition appears to be a silent but key contributor to survival and quality of life in patients with cirrhosis. Although the field is currently gathering momentum, the available data are still scarce and there is a dire need for standardized evaluation and therapeutic approach.

Research motivation

The focus of our research was to assess the real life impact of malnutrition on survival in a group of cirrhotic patients and to observe whether adherence to current nutritional recommendations alters their outcome.

Research objectives

The aims of the current research were to determine the prevalence of malnutrition in a consecutive series of cirrhotic patients and to determine its impact on survival. Furthermore, we wanted to evaluate whether adherence to current nutritional recommendations improves their outcome. By answering these clinical questions, we tried to set a working baseline, hoping to provide a solid starting point for future research in the field.

Research methods

Malnutrition was assessed using the Subjective Global Assessment criteria and the mid-arm circumference. These are easy-to-use, cost efficient, bedside methods with extensive prior validation and standardization. Furthermore, dietary habits were evaluated using a comprehensive food intake questionnaire adapted to the specifics of our culture. Total energy and main nutrient intake were calculated based on their response. Patients were followed-up for a median of 27 mo and factors associated with their prognosis were accounted for in uni- and multivariate analysis.

Research results

Malnutrition was highly prevalent in patients with cirrhosis and a current or prior decompensating event (68.4%). In comparison, only 13.6% of patients with no history of decompensation were malnourished ($P < 0.001$). While the overall mortality in our whole group was 70% after a median follow-up of 27 mo, patients with malnutrition had a significantly worse outcome: 50% mortality at 1 year and 63% at 2 years for the patients with malnutrition, compared to 21% at 1 year and 30% at 2 years for patients without malnutrition ($P = 0.01$). On multivariate analysis, adherence to nutritional recommendations was associated with a better prognosis.

Research conclusions

Our results reflect the important burden of malnutrition in patients with advanced liver disease, especially in the setting of a decompensating event. Consequently, a more attentive approach to nutrition should complement pharmacologic and interventional therapy in patients with cirrhosis, as it appears to have a significant impact on survival.

Research perspectives

Further research should try to translate more basic research findings into clinical practice, while clinical studies should try to provide solid grounding for guideline recommendations. In this light, there is a dire need for large scale high-quality, multicentric studies on easy-to-use, non-invasive and cost-efficient methods to screen for and grade malnutrition. Not least, dietary habits of patients with advanced liver disease should be thoroughly examined, in order to provide realistic, easy to follow nutritional recommendation in order to increase adherence.

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ORIGINAL ARTICLE

Observational Study

Effect of treating chronic hepatitis C with direct-acting antivirals on extrahepatic cutaneous manifestations

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Author contributions: El Kassas M and Salah EM conceptualized the idea and design; all authors participated in, and supervised the patients' treatment and follow-up; Hegazy OMO drafted the manuscript; Salah EM revised and edited the manuscript; all authors revised and approved the final version of the manuscript.

Institutional review board statement: All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Research Ethics Committee (REC) for human subject research at the Faculty of Medicine, Helwan University (Serial: 1-2018) on 22 August 2018.

Informed consent statement: Informed consent was obtained from all participants included in the study.

Conflict-of-interest statement:

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Abstract

BACKGROUND

Hepatitis C virus (HCV) is a disease with a significant global impact, affecting approximately 2%-2.5% of the world's population. New direct-acting antivirals (DAAs) have been introduced over the past few years with great success in viral eradication. The association of chronic HCV infection with a wide spectrum of cutaneous manifestations has been widely reported in the literature.

AIM

To assess the effect of treating HCV with DAAs on the extrahepatic cutaneous manifestations of HCV.

METHODS

This prospective observational study included 1039 HCV positive Egyptian patients who were eligible to receive DAAs. A total of 30 patients were diagnosed with extrahepatic cutaneous manifestations and fulfilled the inclusion criteria of the study. Of these patients, 6 had classic lichen planus, 8 were diagnosed with psoriasis vulgaris and 16 had pruritus. All patients received DAAs from October 2018 to July 2019 in the form of a three-month course of sofosbuvir/daclatasvir combination. Patients with lichen planus or psoriasis were dermoscopically evaluated before treatment and 6 mo after treatment, while patients with hepatic pruritus were assessed using the 12-Item Pruritus Severity Scale over the same period.

RESULTS

All patients with psoriasis showed significant improvement in all psoriatic plaques, and all patients with hepatic pruritus scored 0 on the 12-Item Pruritus Severity Scale indicating total improvement of pruritus. In addition, four of six

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patients with lichen planus showed complete improvement.

CONCLUSION
Treatment of HCV with DAAs was significantly effective in improving virus-related extrahepatic cutaneous manifestations.

Key Words: Directly acting antivirals; Extrahepatic manifestations; Hepatitis C virus; Lichen planus; Pruritus; Cutaneous manifestations

Core Tip: In this study, we investigated the impact of hepatitis C virus (HCV) clearance using direct-acting antivirals (DAAs) on the dermatological extrahepatic manifestations of HCV. To our knowledge, this is the largest cohort of patients with cutaneous manifestations of HCV to be treated in the literature (30 patients). In addition, we used dermoscopy for the first time in this study to better evaluate the response of cutaneous diseases to DAAs.

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INTRODUCTION

Hepatitis C virus (HCV) is one of the human hepatotropic viruses that infects around 71 million individuals globally[1]. Spontaneous clearance of the virus fails in nearly half of cases and acute infection progresses to chronic hepatitis C, and further progression of the disease could lead to cirrhosis, and hepatocellular carcinoma (HCC)[2]. Egypt was considered a country with a high prevalence of HCV. After recognizing the magnitude of this problem, the Egyptian National Committee for Control of Viral Hepatitis (NCCVH), established specialized treatment centers for the evaluation and management of viral hepatitis[3-7]. Treatment of chronic HCV showed a significant shift after the introduction of direct-acting antivirals (DAAs), which proved a great success with an acceptable safety profile compared to the previous standard of care treatment (pegylated interferon and ribavirin)[8-10]. Many treatment regimens have been considered for the management of HCV, which is usually a combination of two or more DAAs classes[11-13].

Being a hepatotropic and lymphotropic virus, HCV does not only cause hepatic manifestations, it also leads to a significant number of extra-hepatic manifestations. Around 74% of patients with HCV will show HCV-related extrahepatic manifestations in their lifetime[14]. The development of HCV-related extrahepatic manifestations most likely involves autoimmune mechanisms. This theory is supported by the appearance of autoimmune features, such as palpable purpura, complex lymphoproliferative disorders (*e.g.*, lymphomas), and immune complex deposit diseases that cause local and/or systemic complications[14,15]. Among the extrahepatic manifestations, dermatologic manifestations significantly add to the morbidity of the disease[16]. The association of chronic HCV infection with a broad spectrum of cutaneous manifestations has been widely reported in the literature, with varying strengths of epidemiological association. In registry-based studies, approximately 17% of HCV patients have at least one skin manifestation, which can be induced directly or indirectly by chronic HCV infection[17]. Dermatosis disorders are known to be linked to HCV infection including mixed cryoglobulinemia, lichen planus, porphyria cutanea tarda, and necrolytic acral erythema[18]. Besides these dermatoses, HCV can also be associated with autoimmune cutaneous diseases. Vitiligo is one of the autoimmune cutaneous diseases reported in studies to have a similar prevalence in patients with HCV infection and in the healthy population with vitiligo[19]. Psoriasis can also be found in association with HCV infection. This was confirmed by studies that found

anti-HCV antibodies in psoriatic patients as well as HCV-RNA in the lesions of patients with psoriasis and HCV infection[20]. Pruritus is also a common dermatologic manifestation that is recognized as an early sign of chronic HCV infection[21,22]. This study aimed to assess the effect of treating HCV with DAAs on the extrahepatic cutaneous manifestations of HCV.

MATERIALS AND METHODS

This prospective observational study included 1039 HCV patients, who were recruited from New Cairo Viral Hepatitis Treatment Center, one of the specialized treatment centers for HCV management, affiliated to the Egyptian NCCVH[7], from October 2018 to July 2019. These patients received HCV antiviral therapy with sofosbuvir/daclatasvir (SOF/DAC) combination regimen for 12 wk and were followed-up until assessment of virological response 12 wk after treatment cessation. Patients were treated with DAAs according to the standardized protocol for HCV treatment issued by the NCCVH. The main exclusion criteria were: Child-Turcotte-Pugh (CTP) class C, hemoglobin level below 10 g/dL, platelet count less than 50000/mm^3, diagnosed with HCC 6 mo following a successful intervention, extrahepatic malignancy after 2 years of cure, co-infection with hepatitis B or HIV, pregnancy or inability to use effective contraception, and hypersensitivity to any of the treatment medications[5]. In addition to the previous contraindication, we excluded patients who received dermatological treatment for their cutaneous manifestations, patients with renal failure, patients with autoimmune diseases, and those who received simeprevir as it can induce skin lesions as a complication[9].

A total of 30 patients were diagnosed with extrahepatic cutaneous manifestations and fulfilled the study inclusion criteria: Six patients were diagnosed with classic lichen planus, eight patients with psoriasis vulgaris, and sixteen patients had hepatic pruritus. A full medical history was obtained for these patients, followed by a clinical examination, and a complete liver biochemical profile. All subjects were tested for HCV viremia by polymerase chain reaction, and hepatitis B surface antigen was determined to exclude chronic HBV infection. Kidney function tests were also performed to exclude patients with renal failure. In addition an antinuclear antibody test was carried out to exclude patients with autoimmune hepatitis.

A dermatological assessment was performed by photographing skin lesions (in cases with lichen planus lesions and psoriatic plaques), before and after antiviral treatment. Dermoscopic photography of the lichen planus lesions and psoriatic plaques was performed before receiving and after completing treatment using a Dermlite DL4 3Gen. dermoscope. Patients with hepatic pruritus were evaluated before and after treatment using the 12-Item Pruritus, Severity Scale developed and validated by Reich and colleagues[23].

Descriptive statistics

Data were collected, revised, coded, and entered into the SPSS version 26 software. Qualitative data are presented as numbers and percentages, while quantitative data are presented as mean, standard deviations and ranges. Comparisons between two groups with qualitative data were carried out using the Chi-square test was used instead of the Chi-square test when the expected count in any cell was found less than 5. Comparisons between two independent groups with quantitative data and parametric distribution was performed using the independent *t*-test. Comparisons between more than two groups with parametric distribution was performed using One Way Analysis of Variance. Pearson correlation coefficients were used to assess the relationship between two studied parameters in the same group. The receiver operating characteristic curve was used to assess the cut-off point with the best sensitivity, specificity, positive predictive value, and negative predictive value. The confidence interval was set to 95% and the margin of error accepted was set to 5%. Thus, the significance of the following P values was considered as follows: $P > 0.05$: Non significant, $P < 0.05$: Significant, and $P < 0.01$: Highly significant.

RESULTS

Thirty patients were included in the study. The mean age was 45.67 years and ranged from 24 to 60 years. Sixteen of the 30 recruited subjects were female. All patients

received the SOF/DAC regimen, and all reached SVR12.

Cutaneous manifestations associated with chronic HCV infection were observed in all 30 recruited subjects and consisted of hepatic pruritus in 16 patients, lichen planus in 6 patients and psoriasis vulgaris in 8 patients. Age and sex distribution of the studied patients are shown in Table 1.

Lichen planus

Four of six patients with lichen planus showed complete improvement of lesions following treatment, while no improvement was seen in the remaining two patients. The impact of HCV treatment on lichen planus is shown in Table 2. Figure 1 shows lichen planus papules on the hand of a patient before and after receiving DAAs, with dermoscopic images of lichen planus papules before and after treatment for HCV.

Psoriasis

Eight patients in this study had psoriasis, and all patients showed complete improvement of psoriatic plaques 12 wk after finishing treatment. The impact of HCV treatment on psoriasis is shown in Table 3. Figure 2 shows psoriatic plaques on the back and thigh of a patient before and after receiving DAAs for HCV, in addition to dermoscopic images of psoriatic plaques before and after treatment.

Hepatic pruritus

Sixteen patients in this study complained of pruritus. All patients with pruritus showed complete improvement and reported total relief of pruritus according to the 12-Item Pruritus Severity Scale. According to this scale, the mean score before treatment was 9.94 ± 1.61, with a range of 7-12. Complete disappearance of pruritus was reported with a score of zero in all patients at the follow-up visit.

DISCUSSION

The association of chronic HCV infection with a wide spectrum of cutaneous manifestations has been widely reported in the literature, with varying strengths of epidemiological association. In registry-based studies, approximately 17% of HCV patients showed at least one skin manifestation, which was induced directly or indirectly by chronic HCV infection[17]. This study was an observational prospective hospital-based study, which included 30 HCV positive patients with associated extrahepatic cutaneous manifestations. All cutaneous lesions were assessed clinically and dermoscopically before receiving treatment and during the follow-up visit after six months of treatment. To our knowledge, this is the first study to assesses and follow-up HCV patients with extrahepatic cutaneous manifestations using a dermatoscope. Out of 1039 HCV patients who were referred to receive antiviral therapy, only those with dermatological manifestations were included in the study (30 patients). Of these 30 patients, six had cutaneous lichen planus, eight had psoriasis vulgaris, and sixteen had hepatic pruritus. Some dermatological disorders which are more frequent and are closely related to HCV such as porphyria cutanea tarda and necrolytic acral erythema were not observed. This could be explained by the pathognomonic nature of disorders such as HCV, and hence most of the patients with these disorders were diagnosed with HCV infection and treated early in the HCV treatment project which started in Egypt in 2015.

All patients with hepatic pruritus in this study reported complete resolution after finishing antiviral therapy. Although pruritus is often associated with HCV infection, the exclusion of all other causes of pruritus either dermatological or systemic, and its disappearance after viral clearance could confirm this association, and raise the suspicion of pruritus as an extrahepatic manifestation of HCV infection.

All patients with psoriasis showed total resolution of all psoriatic plaques. The results obtained by Enomoto *et al*[24], were concordant with our results, as they reported a male patient with a nine-year history of refractory psoriasis, with a Psoriasis Area and Severity Index (PASI) score of 3.8. The patient's symptoms and signs gradually resolved after a 12-wk course of oral, fixed-dose ledipasvir–sofosbuvir. The same results were also confirmed in a report from Egypt, where the authors described an 18-year-old male with psoriasis who received sofosbuvir and ribavirin and showed a sustained virologic response and the disappearance of skin lesions without the use of topical or systemic treatments for psoriasis six months after the end of treatment[25]. This was also in agreement with a published report of a patient with refractory psoriasis. The patient with HCV infection was treated with daclatasvir and

Table 1 Age and gender distribution in each group, *n* (%)

		Lichen planus group, *n* = 6	Psoriasis group, *n* = 8	Pruritus group, *n* = 16	Test value	*P* value	Significance
Gender	Female	3 (50.0)	4 (50.0)	9 (56.2)	0.117[1]	0.943	NS
	Male	3 (50.0)	4 (50.0)	7 (43.8)			
Age	mean ± SD	42.67 ± 5.85	53.00 ± 7.01	43.13 ± 14.34	2.203[2]	0.130	NS
	Range	35-51	40-60	24-60			

[1]Here we used the Chi Square test as a test of significance as gender is a qualitative variable with three groups compared.
[2]Here we used the ANOVA test as a test of significance as age is a quantitative variable with three groups compared. NS: Non significant ($P > 0.05$).

Table 2 Results in patients with lichen planus

Lichen planus	First visit, *n* (%)	Second visit, *n* (%)	Chi-square test		
			χ^2	*P* value	Significance
Absent	0 (0)	4 (66.7)	6.000	0.014	S
Present	6 (100)	2 (33.3)			

$P > 0.05$: Non significant; $P < 0.05$: Significant; $P < 0.01$: Highly significant.

Table 3 Results in patients with psoriasis

Psoriasis	First visit, *n* (%)	Second visit, *n* (%)	Chi-square test		
			χ^2	*P* value	Significance
Absent	0 (0)	8 (100)	16.000	0.000	HS
Present	8 (100)	0 (0)			

$P > 0.05$: Non significant; $P < 0.05$: Significant; $P < 0.01$: Highly significant.

asunaprevir combination, and his PASI score decreased from 3.4 to 0[26].

Four of the six patients diagnosed with lichen planus in the present study had a complete cure, while the remaining two patients showed no improvement in their lesions. Ansari *et al*[27] in 2017, supported these study findings in a 55-year-old HCV positive male patient with associated lichen planus. The patient received HCV treatment with ledipasvir-sofosbuvir, which led to a marked improvement in cutaneous lesions.

The main limitation in our study was the small number of recruited patients (30 subjects), who were enrolled after screening 1039 HCV patients over a 7-mo period. This relatively small number could be explained by the fact that most cases with HCV-related dermatological manifestations were discovered and treated at earlier stages of the HCV treatment project in Egypt. As this was a pilot study, it was the first time that a dermoscope was used to evaluate the response of extrahepatic cutaneous manifestations to HCV treatment with DAAs. Further studies with a larger number of patients and more diverse dermatological lesions are warranted to confirm our findings.

CONCLUSION

In conclusion, treatment of HCV infection with DAAs was effective in all patients with hepatic pruritus and psoriasis, and in most patients with lichen planus. These findings confirm the association between HCV infection and the extrahepatic dermatological manifestations of this virus. Based on our results, treatment of HCV infection in patients with extrahepatic dermatological manifestations is highly recommended.

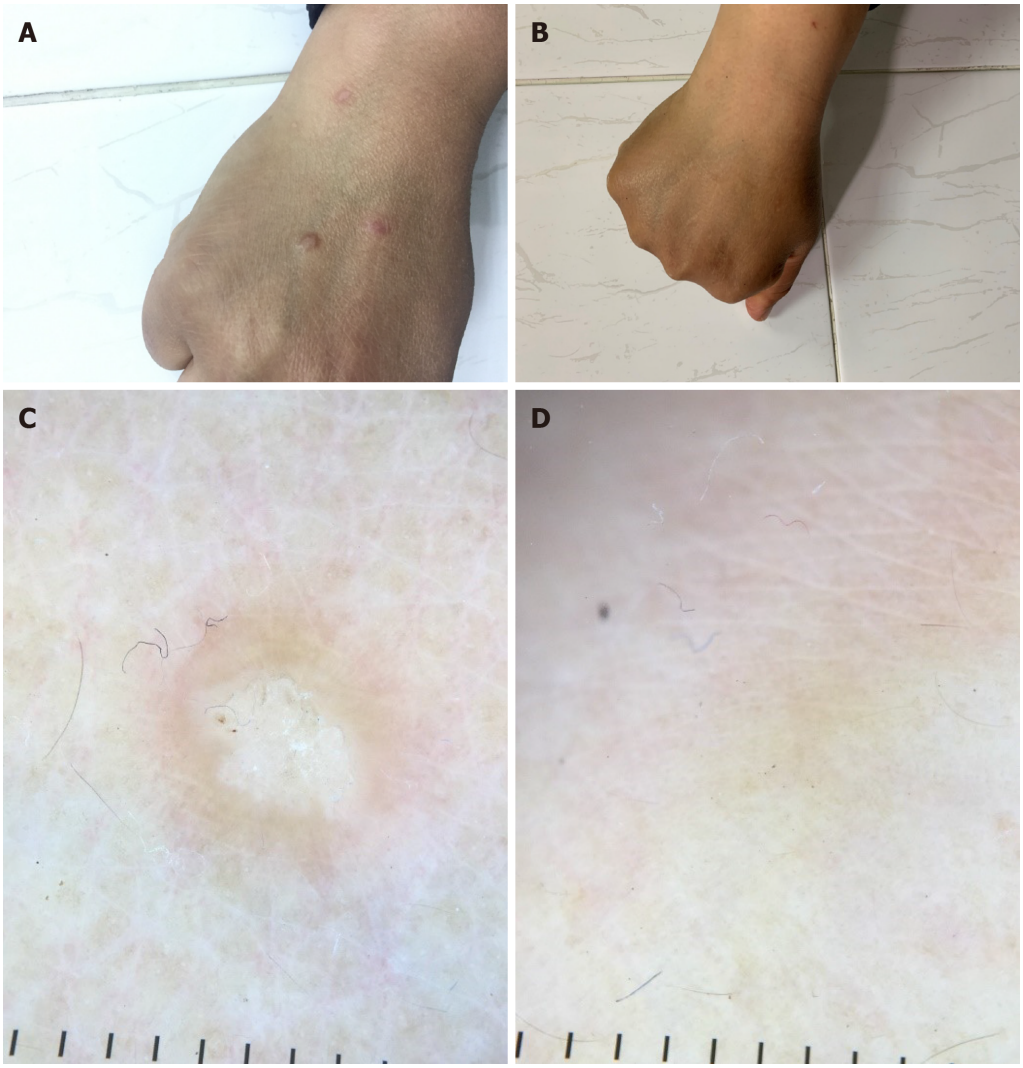

Figure 1 Shows lichen planus papules on the hand of a patient (A) before and (B) after receiving direct-acting antivirals as treatment for hepatitis C virus infection. Dermoscopic image of the lichen planus papules (C) before and (D) after receiving direct-acting antivirals as treatment for hepatitis C virus infection.

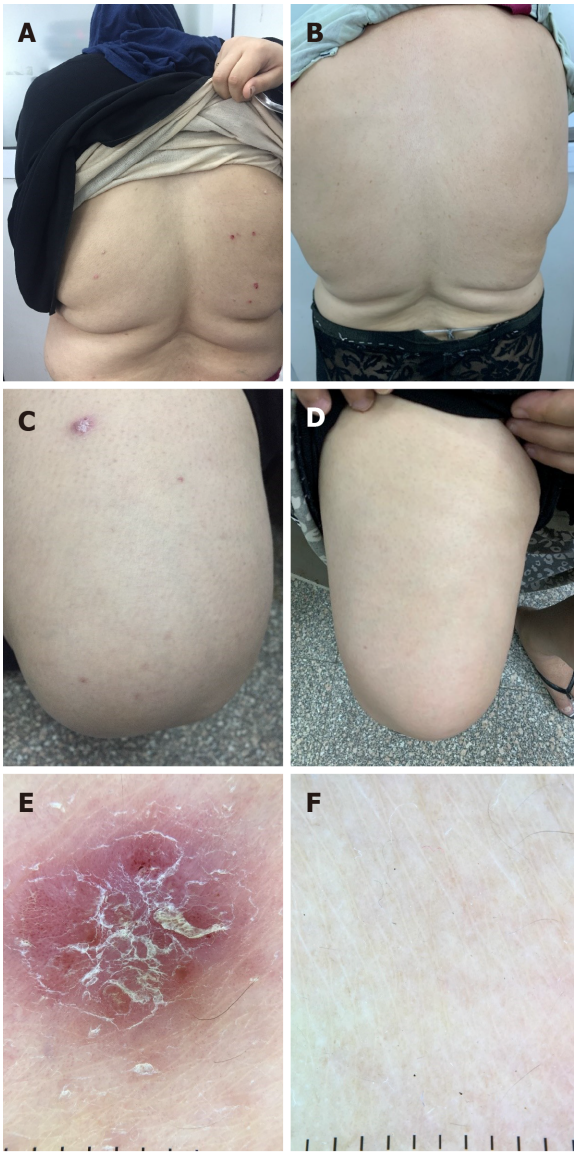

Figure 2 Shows psoriatic plaques on the back of a patient (A) before and (B) after receiving direct-acting antivirals as treatment for hepatitis C virus infection and on thighs (C) before and (D) after receiving treatment. Dermoscopic image of the psoriatic plaques (E) before and (F) after receiving direct-acting antivirals as treatment for hepatitis C virus infection.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV) is a disease with a significant global impact, affecting approximately 2%-2.5% of the world's population. New direct-acting antivirals (DAAs) have been introduced over the past few years leading to successful viral eradication.

Research motivation

The association of chronic HCV infection with a wide spectrum of cutaneous manifestations has been widely reported in the literature.

Research objectives

This study aimed to assess the effect of treating HCV with DAAs on the extrahepatic cutaneous manifestations of HCV.

Research methods

A prospective observational study included HCV positive Egyptian patients who were eligible to receive DAAs. Patients with lichen planus or psoriasis were

dermoscopically evaluated before treatment and 6 mo after treatment, while patients with hepatic pruritus were assessed using the 12-Item Pruritus Severity Scale over the same period. All patients received DAAs from October 2018 to July 2019 in the form of a three-month course of sofosbuvir/daclatasvir combination.

Research results

A total of 30 from 1039 patients eligible for antiviral treatment were diagnosed with extrahepatic cutaneous manifestations and fulfilled the inclusion criteria of this study. Of these 30 patients, 6 patients had classic lichen planus, 8 patients had psoriasis vulgaris and 16 had hepatic pruritus. All patients with psoriasis showed significant improvement of all psoriatic plaques, and all patients with hepatic pruritus scored 0 on the 12-Item Pruritus Severity Scale indicating total improvement of pruritus. In addition, four of six patients with lichen planus showed complete improvement.

Research conclusions

Treatment of HCV with DAAs was effective in improving HCV-related extrahepatic cutaneous manifestations.

Research perspectives

Further studies with a larger number of patients and more diverse dermatological lesions are warranted to confirm our findings.

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ORIGINAL ARTICLE

Observational Study

Novel markers of endothelial dysfunction in hepatitis C virus-related cirrhosis: More than a mere prediction of esophageal varices

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Abstract

BACKGROUND

Hepatitis C virus (HCV) infection may affect lipid metabolism by enhancing the circulating levels of inflammatory cytokines, together with its impact on endothelial function.

AIM

To evaluate the potential correlation of changes in lipid profile, carotid intima-media thickness (CIMT), and ankle-brachial index with the severity of fibrosis, grades of esophageal varices (EVs), and fibrosis indices.

METHODS

The study included 240 subjects who were divided into 3 groups; group 1 (n = 90, HCV-related cirrhotic patients with EVs), group 2 (n = 90, HCV-related cirrhotic patients without EVs), and group 3 (n = 60, served as the healthy control group). All patients underwent routine laboratory tests, including a lipid profile assay. Low-density lipoproteins (LDL)/platelet count and platelet/splenic diameter ratios were calculated. Abdominal ultrasonography, CIMT by carotid Doppler, bedside ankle-brachial index (ABI), liver stiffness measurement, and upper gastrointestinal endoscopy were performed.

RESULTS

Multivariate logistic regression revealed that very-low-density lipoprotein (VLDL) (β = 0.988, odds ratio 2.5, P = 0.001), LDL/platelet count ratio (β = 1.178, odds ratio 3.24, P = 0.001), CIMT (β = 1.37, odds ratio 3.9, P = 0.001), and ABI (β = 2.3, odds ratio 5.9, P = 0.001) were the key variables associated with significant fibrosis, EVs and endothelial dysfunction. CIMT and LDL/platelet count ratio were predictive of advanced fibrosis and EVs at cutoff values of 1.1 mm and 1

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mm, respectively, with an area under the curve (AUC) of 0.966 and 0.960 (P = 0.001), while VLDL and ABI at a cutoff of 16.5 mg/dL and 0.94 were predictive of advanced fibrosis and EVs with an AUC of 0.891 and 0.823, respectively (P = 0.001).

CONCLUSION
CIMT, ABI, VLDL, LDL/platelet count ratio are good non-invasive predictors of advanced fibrosis, presence of EVs, and endothelial dysfunction in liver cirrhosis.

Key Words: Lipid profile; Liver cirrhosis; Esophageal varices; Carotid-intima media thickness; Ankle-brachial index

Core Tip: Hepatitis C virus (HCV) infection may affect lipid metabolism by enhancing the circulating levels of inflammatory cytokines. HCV may induce endothelial dysfunction. Carotid intima-media thickness, low-density lipoproteins (LDL)/platelet count ratio, ankle-brachial index, and very LDL were predictive of advanced fibrosis, esophageal varices, and endothelial dysfunction at cutoff values of 1.1 mm, 1, 0.94, and 16.5 mg/dL, respectively. Novel markers provide information beyond the previous traditional markers such as platelet count, FIB-4, and platelet/splenic diameter ratio with additional data regarding endothelial dysfunction and subclinical atherosclerosis.

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INTRODUCTION

Hepatitis C virus (HCV) infection represents a major public health burden with an estimated global prevalence of 2.8% resulting in more than 185 million infected patients[1]. Prompt recognition of vascular changes in patients with significant liver fibrosis is required for directing therapy and follow-up against both cardiovascular disease and esophageal varices (EVs).

Chronic HCV infection is associated with a chronic inflammatory state leading to a disproportion of pro-inflammatory/anti-inflammatory cytokines ratio. Also, lipid abnormalities, insulin resistance (IR), and increased risk of atherosclerosis have been described in chronic HCV[2,3].

HCV may trigger atherosclerosis through the production of intracellular adhesion molecules, anti-endothelial antibodies, oxidative stress generation, and IR[4,5]. Increased carotid intima-media thickness (CIMT) represents an initial ultrasonographic sign of atherosclerosis that can be easily evaluated at the bedside. Previously, HCV RNA was isolated from carotid plaques from patients infected with HCV[6,7].

HCV is a hybrid molecule composed of viral and lipoprotein components (lipoviroparticles); the latter resemble very low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs)[8], and conjugate with cell surface receptors mainly scavenger receptor class B member 1 protein and the LDL receptor[9], the latter binds to either the lipoprotein component of the viral particle or the hyper-variable region 1 of glycoprotein E2[10].

HCV infection causes a defect in intrahepatic cholesterol synthesis due to its employment in viral replication, with a later decrease in the available cholesterol for peripheral delivery *via* VLDL and this stimulates more expression of LDL receptors and an increase in LDL uptake by the liver accounting for the decreased serum LDL; therefore, sustained virological response (SVR) may cause a rebound increase in lipid levels[11]. VLDL level may be low in advanced liver diseases due to decreased synthesis and therefore it can be used as a marker of advanced fibrosis[12,13].

In addition to predicting EVs and fibrosis severity, the objective of this study was to

determine predictive markers of vascular changes and endothelial dysfunction in HCV-related cirrhosis.

MATERIALS AND METHODS

Study aim

Evaluation of the association of changes in lipid profile, CIMT and ankle-brachial index (ABI) with the severity of fibrosis, grades of EVs, and fibrosis indices.

Study design

This cross-sectional case-control study was conducted in the Gastroenterology and Hepatology Clinic, Department of Internal Medicine, Faculty of Medicine, Zagazig University Hospitals during the period from November 2018 to December 2019. The research protocol (IRB-255-2018) was accepted by the Zagazig Faculty of Medicine Ethical Committee.

All procedures were carried out under the Zagazig University's ethical principles and in compliance with the Helsinki declaration and its more recent modifications. Informed consent was obtained from each patient who participated in the study.

Patient population

The study included chronic HCV infected patients (n = 180) and 60 healthy subjects as a control group, all were matched for age, sex, and body mass index.Diagnosis of liver cirrhosis was based on clinical, laboratory, ultrasonographic, and FibroScan features. Eligible patients obtained a diagnosis of liver cirrhosis secondary to HCV infection proven by a positive anti-HCV test and HCV-RNA in serum, and were divided into 2 groups: Group 1 which included 90 cirrhotic patients complicated with EVs and group 2 which included 90 cirrhotic patients without EVs. Group 3 included the control subjects.

Exclusion criteria

Smoking, obesity, patients with other diseases that may alter serum lipid levels such as diabetes, non-alcoholic fatty liver disease or chronic alcohol consumption, any patients who had previously received anti-viral therapy for HCV or had been cured of HCV which may induce a rebound increase in serum lipids, lipid-lowering medications, recipients of solid organ transplantation and patients who had refused to participate in the study.

Laboratory analysis

Routine laboratory tests were performed including liver and kidney function tests, full blood count, and coagulation profile. LDL-C was calculated using the Friedewald formula: LDL-C (mg/dL) = Total cholesterol–(HDL-C)–(triglycerides/5)[14]. The LDL/platelet count ratio was also calculated. VLDL cholesterol was estimated by dividing the TGs/5if the TGs were lower than 450 mg/dL[15].

Abdominal ultrasonography

The ultrasonographic features of liver cirrhosis or the presence of ascites were documented. Criteria for portal hypertension were defined as portal vein diameter greater than 13 mm, splenic bipolar diameter greater than 130 mm or the presence of portal venous collaterals[16].

Carotid artery intima-media thickness

Common carotid arteries were evaluated on both sides by an experienced radiologist who was blinded to clinical data using B-mode duplex ultrasound with a 7.5MHz linear probe (Siemens G60®). CIMT was measured from the intima lumen interface to the media adventitia interface; with a value > 0.9 mm considered abnormal. Three measures were obtained on either side; the mean CIMT was defined as the mean right and left CIMT[17].

Non-invasive documentation of liver fibrosis

Liver stiffness measurement: Liver stiffness measurement was performed by an experienced physician who was blinded to the clinical data of the patients using FibroScan®. Fibrosis stages F0-1; F2; F3 and F4 or cirrhosis were defined by the spectrum of liver stiffness values 2.5-7, 7-9.5, 9.5-12.5 and > 12.5 kPa, respectively[18].

Traditional non-invasive tools

Fibrosis index-4: This was calculated by the following equation using 4 factors: [age (year)×aspartate aminotransferase (U/L)]/[platelet count (PLT) (10^9/L)]×[alanine aminotransferase (U/L)]. A fibrosis index-4 (FIB-4) score < 1.45 displayed a negative predictive value of 90% for advanced fibrosis, however, a FIB-4 > 3.25 had a 97% specificity and a positive predictive value of 65% for advanced fibrosis[19].

PLT/splenic diameter ratio: The PLT/splenic diameter (PLT/SD) ratio was calculated by dividing the number of platelets (μL) by the maximum bipolar diameter of the spleen in millimeters, detected by abdominal ultrasound.

Upper gastrointestinalendoscopy

EVs were diagnosed and graded from grade I to grade IV, using the Paquet grading system[20].

Assessment of endothelial dysfunction

Endothelial dysfunction can be evaluated by flow-mediated dilatation in the brachial artery using high-resolution ultrasound or simply by bedside ABI which is considered an easy and cost-effective method for assessing endothelial dysfunction[21].The patient remained in the supine position for 5 min and the blood pressure cuff was applied to the arm and lower calf, then the stethoscope was used to measure the systolic pressure in the brachial and dorsalis pedis arteries of both sides. ABI was calculated by dividing the highest value of pressure in the dorsalis pedis arteries by the highest brachial pressure. The normal ABI is over 1 and the cutoff value to diagnose peripheral arterial disease is ≤ 0.90 at rest with a sensitivity of 95% and specificity of 100%, values > 1.40 suggest arteriosclerosis mainly seen in diabetes or chronic renal failure[22].

Statistical analysis

All data were statistically analyzed using SPSS 20 for Windows (SPSS Inc., Chicago, IL, United States). Quantitative data were expressed as the mean ± SD. Qualitative data were expressed as absolute frequencies (number) and (percentage). The *F* test was performed to compare between more than two groups of normally distributed variables. LSD with Bonferroni correction was applied to detect the difference between groups.

Spearman's correlation coefficient (*r*) was performed for ordinal variables and Pearson correlation for continuous variables. Logistic regression analysis was performed by forwarding selection to identify variables independently associated with advanced fibrosis and endothelial dysfunction. All variables with $P < 0.05$ were considered statistically significant.

Receiver operating characteristic curves were plotted and the area under the curve (AUC) was calculated, the performance of the cutoff value was judged by calculation of Youden's J value; values near 1 indicated good performance (J = sensitivity + specificity – 1). Sensitivity/specificity and positive/negative predictive values for the non-invasive diagnosis of fibrosis, EVs, and endothelial dysfunction were assessed considering liver stiffness as the reference for stages of fibrosis.

RESULTS

Basic demographic, clinical, laboratory and endoscopic findings

The current study included 240 subjects, and the baseline demographic, laboratory, and endoscopic findings of all subjects are summarized in Table 1. A highly significant statistical difference regarding serum transaminases and platelet count was found among the groups with the key significant difference between group 1 (cirrhosis with EVs) and group 2 (cirrhosis without EVs), and group 1 (cirrhosis with EVs) and group 3 (control group) ($P = 0.001$ and 0.001, respectively).

The total cholesterol and LDL levels were significantly lower in group 1 with a significant difference between groups 1 & 2, and 1 & 3 ($P = 0.001$ and 0.001, respectively).

VLDL was significantly lower in group 1 with a significant difference between groups 1 & 2, and 1 & 3 ($P = 0.001$ and 0.001, respectively) and between group 2 & 3 ($P = 0.001$). Also, the LDL/platelet count ratio was significantly higher in group 1 with a significant difference between groups 1 & 2, and 1 & 3 ($P = 0.001$ and 0.001, respectively).

Table 1 Baseline laboratory and endoscopic findings of the enrolled patients

Variable	Liver cirrhosis with EVs	Liver cirrhosis without EVs	Control group	*P* value[1]
n	90	90	60	-
Sex (M/F)	62/28	60/30	40/20	0.4
Age (yr)	49.6 ± 8.4	47.7 ± 9.7	46 ± 6.7	0.32
BMI (kg/m^2)	26.3 ± 2.1	25.8 ± 1.3	26.6 ± 0.9	0.21
ABI	0.94 ± 0.09	1.08 ± 0.13	1.16 ± 0.11	0.001
AST (IU/L)	53.7 ± 10.2	43.7 ± 5.6	31.1 ± 10.8	0.001
ALT (IU/L)	47.2 ± 7.8	36.2 ± 8.5	30.6 ± 7.7	0.001
Platelet count × 10^3	79.2 ± 21.2	189.3 ± 43.2	197.7 ± 18.7	0.001
Total cholesterol (mg/dL)	177.6 ± 18.8	199.2 ± 27.4	210.4 ± 12.6	0.01
HDL (mg/dL)	39.5 ± 4.3	44.6 ± 7.1	35.4 ± 5.3	0.004
TGs (mg/dL)	135.7 ± 14.4	168.8 ± 12.8	180.8 ± 8.4	0.01
VLDL (mg/dL)	16.6 ± 4.3	26.5 ± 5.3	25 ± 5.7	0.001
LDL (mg/dL)	105.8 ± 14.5	128.9 ± 20.6	138.9 ± 16.5	0.03
FIB-4	4.97 ± 1.8	1.54 ± 0.47	1.51 ± 0.3	0.001
Platelets/SD ratio	449 ± 167	1375 ± 380	1702 ± 238	0.001
LDL/platelet count ratio	1.41 ± 0.4	0.71 ± 0.21	0.75 ± 0.09	0.001
Child-Pugh class (*n*)	A (26), B (46), C (18)	A (88), B (2)	Non-cirrhotic	-
Endoscopy	-	-	-	-
EVs	-	-	-	-
Grade I-II, *n* (%)	32 (35.5)	-	-	-
Grade III, *n* (%)	42 (46.7)	-	-	-
Grade IV, *n* (%)	16 (17.8)	-	-	-
[2]Fundal varix, *n* (%)	12 (13.3)	-	-	-

[1]*P* value less than 0.05 is considered significant.
[2]Fundal varices were not isolated but associated with G I esophageal varices (EV) (*n* = 2), G II EV (*n* = 6), G III-IV EV (*n* = 4).
BMI: Body mass index; ABI: Ankle-brachial index; LDL: Low-density lipoproteins; HDL: High-density lipoproteins; VLDL: Very low-density lipoproteins; TGs: Triglycerides; EVs: Esophageal varices; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; SD: Splenic diameter; FIB-4: Fibrosis index-4.

FIB-4 values were significantly higher in group 1 with a significant difference between group 1 & 2, and 1 & 3 (*P* = 0.001 and 0.001, respectively) as presented in Table 1. In addition, ABI was significantly lower in group 1 when compared with group 2 and 3 (Table 1).

Radiological findings

Carotid-intima media thickness was significantly higher in group 1 when compared with group 2 and 3. These results are presented in Figure 1 and Table 2. SD and FibroScan readings (kPa) were significantly higher in cirrhotic patients with EVs with a significant difference between group 1 & 2, and 1 & 3 (*P* = 0.001 and 0.001), however, the PLT/SD ratio was significantly lower in the same group as presented in Table 2. The cutoff value of the PLT/SD ratio associated with advanced fibrosis and presence of EVs was 872 with a sensitivity of 100%, specificity of 94%, AUC of 0.991, *P* = 0.001, and Youden's J value of 0.94.

Liver stiffness significantly correlated with platelet count (*r* = -0.615, *P* = 0.001), VLDL (*r* = -0.619, *P* = 0.001), SD (*r* = 0.534, *P* = 0.001), FIB-4 (*r* = 0.588, *P* = 0.001), CIMT (0.712, *P* = 0.001), LDL/PLT ratio (*r* = 0.677, *P* = 0.001), and ABI (*r* = -0.546, *P* = 0.001).

Table 2 Ultrasonographic and liver stiffness values of the enrolled patients

Variable	Liver cirrhosis with EVs	Liver cirrhosis without EVs	Control group	*P* value
Splenic diameter	182.9 ± 21.4	141 ± 12.8	111.2 ± 9.7	0.003
Platelets/SD ratio	449 ± 166	1374.9 ± 380	1701 ± 239	0.001
FibroScan (kPa)	24.4 ± 6.02	14.8 ± 1.9	5.2 ± 0.82	0.001
CIMT (mm)	1.2 ± 0.13	0.69 ± 0.14	0.62 ± 0.11	0.001

CIMT: Carotid-intima media thickness; EVs: Esophageal varices; SD: Splenic diameter.

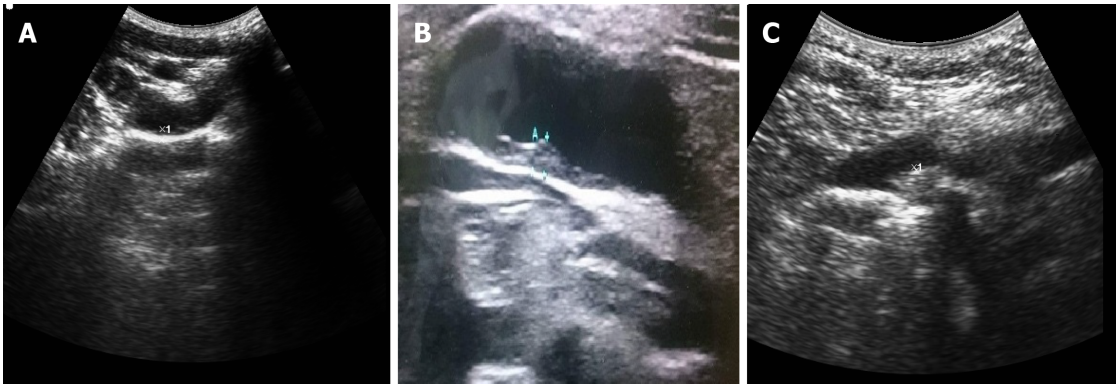

Figure 1 Carotid intima-media thickness in the study groups. A: Carotid-intima media thickness (CIMT) in a cirrhotic patient without esophageal varices (EVs) (0.72 mm); B: CIMT in a cirrhotic patient with EVs (1.42 mm); C: CIMT in a healthy control subject (0.6 mm).

Correlation of the new non-invasive markers to traditional tools

An increasingly significant direct correlation was detected between the LDL/PLT ratio, LS (r = 0.677, P = 0.001) and FIB4 (r = 0.763, P = 0.001). On the other hand, a high significant negative correlation was detected between VLDL and LS (r = -0.769, P = 0.001) as well as FIB4 (r = -0.533, P = 0.001) as shown in Figure 2 and Supplementary Figure 1.

Cutoff values of the new non-invasive markers for diagnostic performance for fibrosis severity, EVs and endothelial dysfunction in cirrhotic patients

Multivariate logistic regression was carried out to identify variables independently associated with fibrosis severity, EVs and endothelial dysfunction and revealed that VLDL (β = 0.988, odds ratio 2.5, P = 0.001), LDL/PLT ratio (β = 1.178, odds ratio 3.24, P = 0.001), CIMT (β = 1.37, odds ratio 3.9, P = 0.001), and ABI (β = 2.3, odds ratio 5.9, P = 0.001) were independently associated with fibrosis severity.

CIMT and LDL/PLT ratio were predictive of advanced fibrosis, EVs and endothelial dysfunction at cutoff values 1.1 mm and 1, respectively, with an AUC of 0.966 and 0.960, 95%CI(0.913-1 and 0.916-1), sensitivity of 86.9% and 94%, specificity of 95% and 82% as presented in Figure 3, and Youden's J value = 0.819 and 0.76, respectively.

VLDL and ABI cutoff values predictive of advanced fibrosis, EVs and endothelial dysfunction were 16.5 mg/dL and 0.94 with an AUC of 0.891 and 0.823, sensitivity of 74.1% and 90.5%, and specificity of 100% and 92%, and corresponding to Youden's J value = 0.741 and 0.82, respectively, as presented in Figure 4.

When patients were categorized based on the determined cutoff values of VLDL, CIMT, ABI, and the LDL/platelet ratio; they displayed an extremely significant discriminating ability for lipid profile, platelet count, SD, FIB-4 and the PLT/SD ratio as presented in Table 3.

CIMT > 1.1 mm, LDL/PLT ratio > 1, and VLDL < 16.5 were associated with lower ABI values and in this way endothelial dysfunction (P = 0.001) when compared with other non-invasive tools. Also, CIMT > 1.1 mm, LDL/PLT ratio > 1, ABI < 0.94 and VLDL < 16.5 showed comparable results for liver stiffness and were more efficient in identifying EVs (P = 0.001) and equally large EVs (P = 0.048) when compared with traditional tools such as FIB-4 and the platelets/SD ratio as shown in Table 4.

Table 3 Discriminating ability of the novel markers in the studied groups

Variable	VLDL			CIMT			LDL/platelet ratio			ABI		
Cutoff value	Below 16.5	Above 16.5	*P* value	Below 1.1	Above 1.1	*P* value	Below 1	Above 1	*P* value	Below 0.94	Above 0.94	*P* value
n	76	164	-	96	144	-	98	142	-	70	170	-
Sex (M/F)	56/20	106/58	0.2	64/32	98/46	0.86	66/32	96/46	0.83	47/23	115/55	0.45
Age (yr)	50.3 ± 10	48.5 ± 8.3	0.3	49.6 ± 8.6	48.7 ± 9	0.56	49.8 ± 8.8	48 ± 9	0.78	51.5 ± 8.9	48.2 ± 7.4	0.2
AST (IU/L)	46 ± 11.6	38.4 ± 12.1	0.01	47.5 ± 11.8	34.9 ± 8.4	0.01	52 ± 11	35 ± 9	0.001	49 ± 12	38 ± 11	0.03
ALT (IU/L)	45 ± 8.4	37.5 ± 8.5	0.03	46 ± 8	35.4 ± 7	0.03	45 ± 7.8	34 ± 5.6	0.02	43 ± 8	32 ± 8	0.047
Platelet count × 10^3	83.6 ± 47	165.5 ± 62.2	0.001	82.5 ± 41.4	182 ± 40	0.001	81 ± 26	188 ± 41	0.001	87 ± 23	160 ± 61	0.001
Total cholesterol	166 ± 21	198 ± 24	0.02	170 ± 18	200.5 ± 26	0.034	168 ± 26	197 ± 28	0.04	173 ± 27	196 ± 25	0.023
Triglycerides (mg/dL)	135.7 ± 15	164 ± 19	0.03	137 ± 15	168 ± 15	0.032	140 ± 19	168 ± 16	0.045	143 ± 18	162 ± 24	0.01
VLDL (mg/dL)	14.6 ± 1.5	26.2 ± 5.6	0.001	17 ± 4.9	28 ± 7	0.001	15 ± 4	32 ± 6	0.001	17 ± 5	25 ± 12	0.03
LDL (mg/dL)	106 ± 15	126 ± 21	0.001	102 ± 13	130 ± 20	0.023	110 ± 17	122 ± 20	0.06	109 ± 19	129 ± 22	0.021
HDL (mg/dL)	40 ± 4	43 ± 8	0.89	39 ± 5	42 ± 8.2	0.45	40 ± 5	42 ± 8	0.82	39 ± 4	42 ± 8	0.34
FIB-4	5.03 ± 1.8	2.2 ± 1.6	0.001	5 ± 2	1.7 ± 0.6	0.001	4.9 ± 1.9	1.6 ± 0.5	0.001	4.2 ± 2	2.4 ± 1.9	0.01
LDL/platelet count ratio	1.5 ± 0.48	0.84 ± 0.3	0.001	1.4 ± 0.4	0.75 ± 0.21	0.001	1.5 ± 0.3	0.7 ± 0.15	0.001	1.24 ± 0.21	0.91 ± 0.34	0.001S
Splenic diameter (mm)	186.3 ± 25.4	141.5 ± 24	0.001	185.4 ± 20	133 ± 17	0.001	181 ± 24	122 ± 11	0.001	171 ± 29	136 ± 13	0.001
Platelets/SD	492.6 ± 76.2	1237.7 ± 502	0.001	463.3 ± 43	1400 ± 410	0.001	471 ± 213	1440 ± 402	0.001	678 ± 298	1173 ± 554	0.001

ABI: Ankle-brachial index; LDL: Low-density lipoproteins; HDL: High-density lipoproteins; VLDL: Very low-density lipoproteins; CIMT: Carotid-intima media thickness; SD: Splenic diameter; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; FIB-4: Fibrosis index-4.

DISCUSSION

Compensated cirrhosis may be difficult to differentiate from chronic hepatitis[23,24]. Accurate assessment of the fibrosis stage requires screening for complications mainly EVs to commence appropriate treatment[25].

Tools to estimate the extent and severity of liver fibrosis may be invasive such as liver biopsy, or non-invasive such as serological tests and imaging[26]. Liver biopsy is the best available standard modality of reference, but it has some limits and it is refused by most patients[27,28].

Could endothelial dysfunction be linked to histological severity and EVs development in HCV-related cirrhosis? This was the research question in the current

Table 4 Performance of the novel markers when compared to the traditional tools

Cutoff value	PLT/SD ratio above 872	FIB-4 above 3.25	ABI below 0.94	LDL/PLT ratio above 1	CIMT above 1.1	VLDL below 16.5	*P* value
n	90	90	70	98	96	76	
LS (kPa)	24.4 ± 6.2	26. 5 ± 5.2	22 ± 8.2	23 ± 7	24.6 ± 6.7	25.3 ± 6.3	$F = 1.87$, $P = 0.117$
ABI	1.08 ± 0.09	1.09 ± 0.1	0.887 ± 0.04	0.93 ± 0.1	0.88 ± 0.1	0.96 ± 0.08	$F = 30$, $P = 0.001$
Endoscopy	-	-	-	-	-	-	-
EVs, *n* (%)	82/90 (91.1)	56/90 (62.2)	65/70 (92.9)	92/98 (94)	90/96 (93.8)	68/76 (89.5)	$P = 0.001$
Grade I-II, *n* (%)	26/32 (81.3)	14/32 (35)	20/32 (62.5)	24/32 (75)	24/32 (75)	10/32 (31.3)	$\chi2 = 13.3$, $P = 0.009$
Grade III, *n* (%)	32/42 (76.2)	30/42 (71.4)	30/42 (71.4)	42/42 (100)	40/42 (95)	34/42 (81)	$\chi2 = 7.67$, $P = 0.104$
Grade IV, *n* (%)	14/16 (87.5)	6/16 (37.5)	9/16 (56.3)	16/16 (100)	16/16 (100)	12/16 (75)	$\chi2 = 9.55$, $P = 0.048$
Fundal varix, *n* (%)	10/12 (83.3)	6/12 (50)	6/12 (50)	10/12 (83)	10/12 (83)	12/12 (100)	$\chi2 = 3.47$, $P = 0.48$

LS: Liver stiffness; ABI: Ankle-brachial index; LDL: Low-density lipoproteins; VLDL: Very low-density lipoproteins; EVs: Esophageal varices; CIMT: Carotid-intima media thickness; PLT: Platelets; SD: Splenic diameter; FIB-4: Fibrosis index-4.

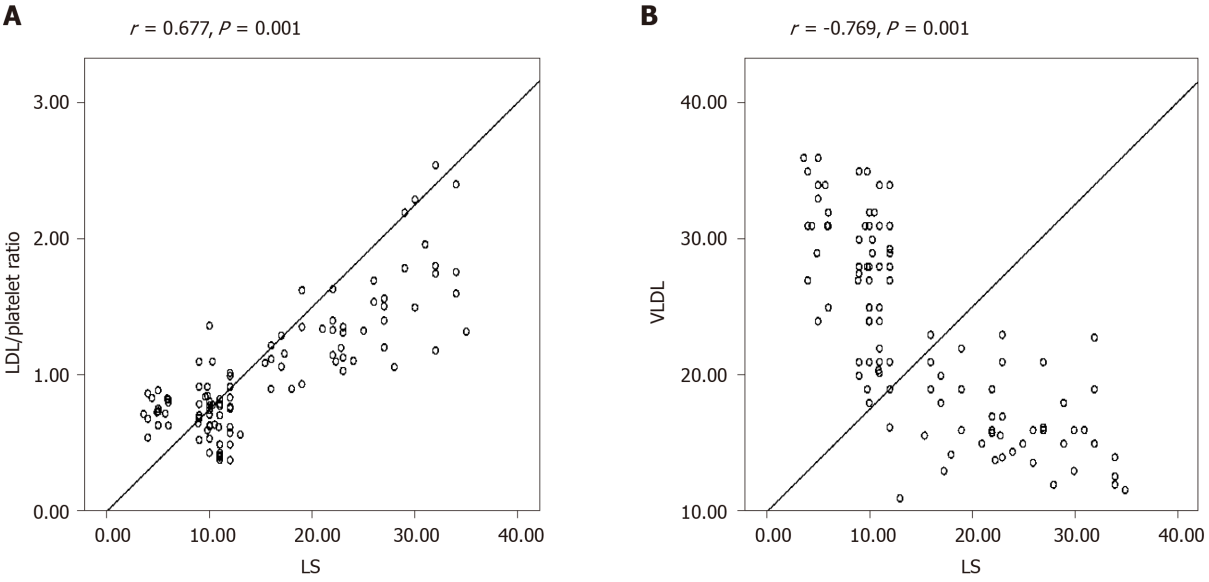

Figure 2 Correlation of liver stiffness with low-density lipoproteins/platelet count ratio (A) and very low-density lipoproteins (B). LS: Liver stiffness; LDL: Low-density lipoproteins; VLDL: Very low-density lipoproteins.

study, which revealed that cirrhotic patients with and without EVs experienced significantly more reduced levels of total cholesterol, triglycerides, LDL, VLDL and several studies determined the changes in the lipid profile of patients with chronic liver disease and correlated them with the severity of liver disease. Abbasi *et al*[29] stated that serum cholesterol and triglycerides levels were inversely proportional to the histological severity. Ghadir *et al*[30] and Boemeke *et al*[31] observed a significant decrease in LDL, triglyceride, VLDL, and total cholesterol in cirrhotic patients compared with controls.

In advanced cirrhosis, IR is more common and associated with dyslipidemia with enhanced systemic inflammation[32,33]. There are no available reports that link lipid profile changes, CIMT, and ABI as non-invasive tools to the presence and size of EVs.

The current study is the first to describe the capability of predicting the presence and grading of EVs using lipid profile, CIMT, and ABI and to link the emergence of

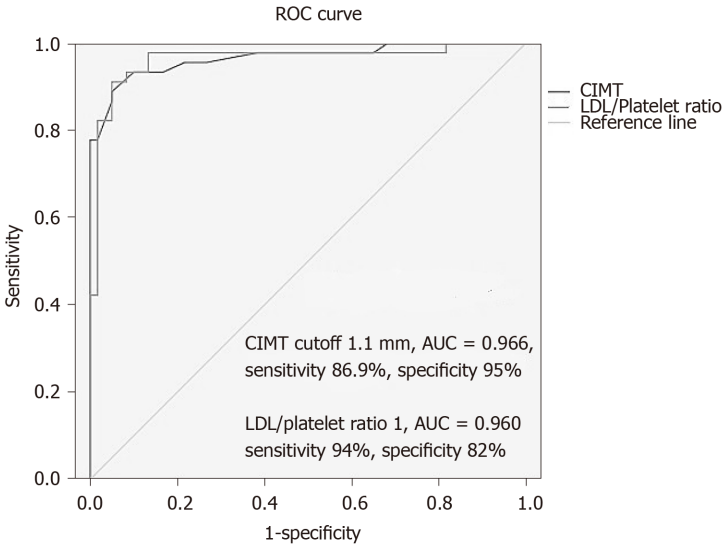

Figure 3 Receiver operating characteristic curve to detect cutoff values of carotid-intima media thickness and low-density lipoproteins/platelet count ratio. LDL: Low-density lipoproteins; CIMT: Carotid-intima media thickness; AUC: Area under the curve; ROC: Receiver operating characteristic.

EVs with underlying endothelial dysfunction that may increase morbidity through an added cardiovascular risk.

In a study which enrolled patients with HCV and liver cirrhosis, the CIMT and epicardial fat thickness were significantly increased in the cirrhotic and non-cirrhotic HCV groups when compared with the control group, and CIMT and epicardial fat thickness were significantly increased with the progression of Child's class, spleen span, portal vein diameter and negatively associated with PLT[34].

Another study showed that CIMT was significantly higher in HCV-positive patients (1.04) than in HCV-negative patients (0.71) with more frequent plaque formation and therefore chronic HCV was an independent risk factor for stroke[35]. Patients with chronic liver disease and cirrhosis have more elevated risks of acute coronary syndrome and peripheral arterial disease than those without chronic liver disease and cirrhosis[36,37] .

In another study conducted in patients with HCV-related liver cirrhosis without a previous history of cerebrovascular disease, cardiac and peripheral vascular diseases, a decrease in the brachial-ankle pulse wave velocity was reported to be directly in proportion to the severity of cirrhosis ($F = 4.90$, $P < 0.05$)[38].

Due to the proven lipid changes in cirrhotic patients and thrombocytopenia as a well-known non-invasive predictor of liver cirrhosis and EVs; a new promising non-invasive predictor (LDL/PLT ratio) was calculated, and our results confirmed that this ratio was significantly higher in cirrhotic patients complicated with EVs ($P < 0.001$).

Using multivariate logistic regression to detect variables independently associated with significant fibrosis, EVs, and endothelial dysfunction; CIMT, VLDL, LDL/PLT ratio, and ABI were the most significant variables.

CIMT > 1.1 mm, LDL/PLT ratio > 1,VLDL < 16.5 mg/d, and ABI < 0.94 were significantly associated with higher liver stiffness values, FIB4, SD and increased incidence of larger varices ($P < 0.001$) with a significant discriminating ability for the degree of liver stiffness and grades of EVs with the advantage of providing information on endothelial dysfunction assessed by ABI when they were compared with the traditional non-invasive tools such as FIB-4 and PLT/SD ratio.

The limitation of the current study is that it was conducted in a single center and specific cardiac investigations should be performed to diagnose cardiovascular disease risk such as electrocardiography, echocardiography and coronary CT angiography which need to be conducted in high risk cirrhotic patients in future studies.

CONCLUSION

In conclusion, based on the current results, this may offer the chance for these markers to serve as non-invasive predictors of cirrhosis, EVs and endothelial dysfunction in

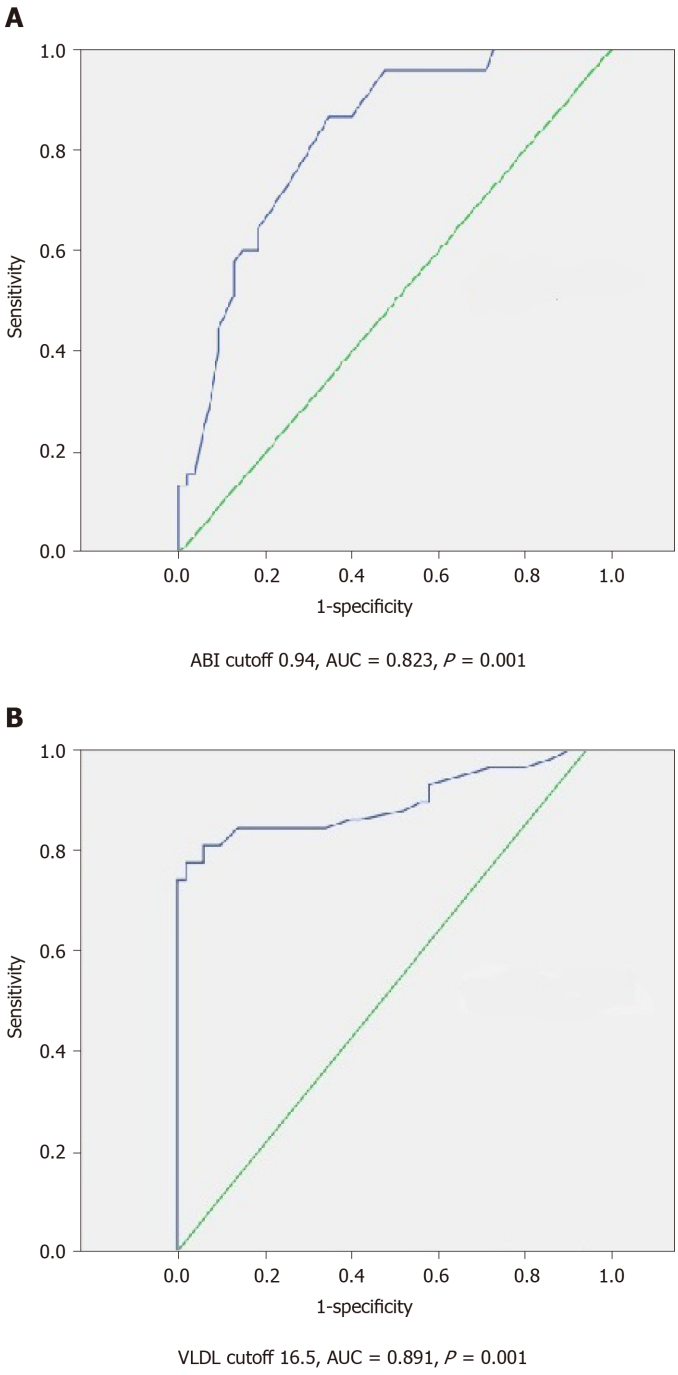

Figure 4 Receiver operating characteristic curve to detect cutoff value of ankle-brachial index (A) and very low-density lipoprotein (B).
ABI: Ankle-brachial index; VLDL: Very low-density lipoprotein; AUC: Area under the curve.

this category of patients. The study raised an issue worthy of research, as patients with advanced fibrosis and larger varices had higher CIMT and lower ABI; therefore, placing these patients at an increased cardiovascular risk added to the risk of variceal bleeding; thus, prevention and treatment should be discussed in other studies.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV) infection may affect lipid metabolism by enhancing the circulating levels of inflammatory cytokines. HCV may induce endothelial dysfunction.

Research motivation

We believe that there is a potential correlation between the changes in lipid profile, carotid intima-media thickness (CIMT) and ankle-brachial index with the severity of fibrosis, grades of esophageal varices (EVs), and fibrosis indices.

Research objectives

To identify predictive markers of vascular changes and endothelial dysfunction in HCV-related cirrhosis

Research methods

HCV infected cirrhotic patients with and without EVs were evaluated by routine laboratory tests, including lipid profile assay, abdominal ultrasonography, carotid intima-media thickness (CIMT) by carotid Doppler, bedside ankle-brachial index (ABI), liver stiffness measurement, and upper gastrointestinal endoscopy and compared to the healthy control group. Logistic regression analysis was performed to identify variables independently associated with advanced fibrosis and endothelial dysfunction.

Research results

CIMT, low-density lipoproteins (LDL)/platelet ratio, ABI, and very LDL (VLDL) were predictive of advanced fibrosis, EVs and endothelial dysfunction. They were effective at cutoff values of 1.1 mm, 1, 0.94, and 16.5 mg/dL, respectively.

Research conclusions

CIMT, ABI, VLDL, and LDL/platelet count ratio are good non-invasive predictors of advanced fibrosis, presence of EVs, and endothelial dysfunction in liver cirrhosis.

Research perspectives

The proposed markers serve as non-invasive predictors of cirrhosis, EVs and endothelial dysfunction, and patients with advanced fibrosis and larger varices had higher CIMT and lower ABI consequently, they bear an increased cardiovascular risk added to the risk of variceal bleeding. The study was designed and validated in a single-center. External, prospective validation is required to determine the widespread applicability and utility of this model.

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CASE REPORT

Kratom induced severe cholestatic liver injury histologically mimicking primary biliary cholangitis: A case report

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Informed consent statement: Informed written consent was obtained from the patient for publication of this report and any accompanying images.

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Abstract

BACKGROUND

Kratom is a psychoactive substance that is isolated from the plant *Mitragyna speciosa*. The leaves can be chewed fresh or dried, smoked, or infused similar to herbal teas. The plant leaves have been used by natives of Southeast Asia for centuries. The substance has been used for its stimulant activity at low doses, and as an opium substitute at higher doses due to a morphine like effect.

CASE SUMMARY

A 37-year-old female with a history of depression and obesity (body mass index: 32) presented to emergency room with a week-long history of nausea, decreased appetite, fatigue, and two days of jaundice. On admission bilirubin was markedly elevated. Her condition was thought to be due to consumption of Kratom 2 wk before onset of symptoms. Liver biopsy showed changes mimicking primary biliary cholangitis. Patient's symptoms and jaundice improved quickly.

CONCLUSION

The use of Kratom has been on the rise in recent years across the United States and Europe. Several case reports have associated adverse health impact of

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Kratom-containing products including death due to its ability to alter levels of consciousness. Only a few case reports have highlighted the hepatotoxic effects of Kratom. Even fewer reports exist describing the detailed histopathological changes.

Key Words: Case report; Kratom; Cholestasis; Liver injury; *Mitragyna speciosa*; Cholangitis; Substance induced injury

Core Tip: Kratom induced liver injury is an important differential diagnosis for physicians to consider in any patient presenting with acute liver injury. As observed in our patient, this manifestation of Kratom consumption may occur even at low doses. Further, this case report demonstrates that a thorough history is essential for an accurate and timely diagnosis. Patients may consider dietary and herb supplements to be natural and risk-free products, not realizing the potential for harm. In addition to asking their patients about consumption of any supplements, it is imperative that physicians update themselves so as to be able to discuss the benefits and risks, and counsel their patients effectively. Identifying use of supplements helps in early diagnosis and treatment, while also preventing future harm. From the pathology perspective, biliary changes associated with Kratom injury can mimic primary biliary cholangitis.

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INTRODUCTION

Acute liver failure is a severe condition which may rapidly become fatal[1]. In the United States, a significant number of these cases occur due to drug-induced liver injury[2]. Apart from prescribed medications, consumption of herbal and dietary supplements also plays an important role in causing liver injury[2]. We present here a case of a 37-year-old female, with drug induced liver injury mimicking as antimitochondrial antibody (AMA) negative primary biliary cholangitis (PBC), secondary to consumption of an herbal supplement, Kratom. Derived from the leaves of *Mitragyna speciosa*, a plant found in Southeast Asia and Africa, this supplement is used for its stimulant properties, as a substitute for opioids, and to help opioid withdrawal symptoms[3]. People in the United States report using this supplement predominantly for pain relief, and also report increased levels of energy and focus with consumption[4]. Our patient learned of this herbal supplement from a friend and reported consuming it in order to boost her energy levels. Although uncommon, this herbal supplement is associated with the risk of hepatotoxicity, making it imperative for physicians to be aware of its harmful effects and caution their patients against its use[4].

CASE PRESENTATION

Chief complaints

A 37-year-old female with a history of depression and obesity (body mass index: 32) presented to emergency room with a week-long history of nausea, decreased appetite, fatigue, and two days of jaundice.

History of present illness

Four days prior to admission, she noticed that her stools were becoming tanner and

eventually turned white. She also reported that her urine was dark. Two days prior to admission the patient noticed jaundice and scleral icterus which prompted her to seek treatment. Her only home medication was venlafaxine, which she had been taking for several years. She has no history of alcohol abuse.

On further questioning about new medications or supplement use, the patient reported using an herbal supplement containing Kratom two weeks prior to the onset of her symptoms. She used the supplement for the first time in her life. Encouraged by a friend to use the supplement to "boost energy levels," she believed it was safe because it was "all natural". She consumed approximately three grams in total over the course of three days in the form of powder (which she dissolved in water) and tablets. During her hospitalization, the patient's liver enzymes continued to rise. On day 3 of her hospitalization, a liver biopsy was performed.

History of past illness

History of depression and obesity.

Physical examination

Unremarkable except jaundice.

Laboratory examinations

On admission, the patient had markedly elevated liver enzymes (Table 1). Other basic laboratory findings including blood count, basic metabolic panel, coagulation panel, serum thyroid stimulating hormone and antinuclear antibody were within normal range. Viral and autoimmune hepatitis studies were normal. Ceruloplasmin level was also normal. AMA was negative.

Imaging examinations

An abdominal ultrasound showed diffuse increased echogenicity of the liver with normal liver size and contour suggests diffuse hepatic steatosis (Figure 1). No intrahepatic or common biliary duct dilation or gall stones seen. An abdominal computed tomography scan showed similar findings. Magnetic resonance cholangiopancreatography did not demonstrate any further abnormalities.

FINAL DIAGNOSIS

Kratom induced severe liver injury histologically mimicking PBC.

TREATMENT

The patient was started on prednisone 40 mg daily. Advised to avoid any new medications or over the counter products with the potential risk of liver injury till her liver enzymes normalized.

OUTCOME AND FOLLOW-UP

The patient was discharged on day 5 of hospitalization and follow up was arranged with gastroenterology clinic. The patient had liver enzymes checked six days after discharge; her symptoms and liver enzymes showed marked improvement. Steroids were stopped. Patient was lost to further lab follow up with gastroenterology clinic but reported feeling back to her normal on follow up phone call 2-wk post hospitalization.

DISCUSSION

The herbal supplement Kratom is a psychoactive substance derived from the leaves of *Mitragyna speciosa*, a plant native to Southeast Asia[3]. Its leaves are used for a variety of purposes, such as pain relief, enhancing energy levels, substituting opioids, managing opioid withdrawal[3,4]. The psychoactive compounds of Kratom, mitragynine, and 7-hydroxymitragynine may also result in altered consciousness, particularly at high doses of consumption[3]. As a result, Kratom is a controlled drug in several countries

Table 1 Patient's liver function labs from the day after admission until the day of hospital discharge

	HD1	HD2	HD3	HD4	HD5	Six days post- discharge	Normal values
Total bilirubin (mg/dL)	10.3	12.0	14.5	17.2	19.5	5.7	< 1.0
Alkaline phosphatase (U/L)	672	677	744	817	839	507	50-160
ALT (U/L)	578	585	600	608	591	323	0-30
AST (U/L)	455	461	437	401	385	101	0-40

HD: Hospital day; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

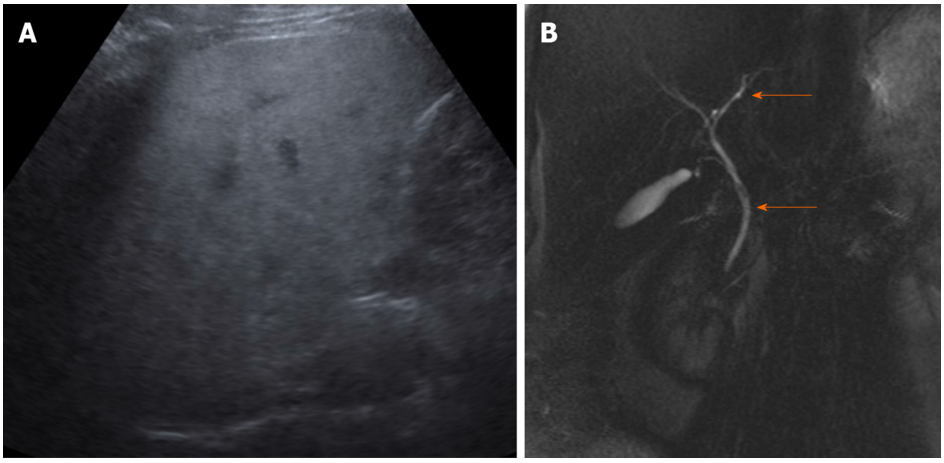

Figure 1 The abdominal ultrasound. A: Diffuse fatty infiltration of liver; B: Normal common bile duct and intrahepatic biliary ducts (orange arrows) in magnetic resonance cholangiopancreatography 3D image.

and illegal in several others[5]. The Drug Enforcement Administration of the United States considers it a Drug and Chemical of Concern[6]. While Kratom products are legal in most parts of the United States, a few states and cities have banned them[4]. With concerns regarding its safety, the Food and Drug Administration warns consumers against the use of these products[4,7].

Further, studies have shown that patients reporting Kratom use may present with confusion, lethargy, irritability, agitation, nausea and vomiting, tachycardia, hypertension or in severe cases, bradycardia, seizures, increased bilirubin, renal failure and even coma[4]. Further, Kratom is also reported to exert effects similar to opioids, such as sedation, hypnosis, nausea, stupor and respiratory depression[3,4,8]. In addition to this, the Food and Drug Administration has recalled Kratom supplements due to contamination with Salmonella[9].

Although Kratom induced liver injury is described, reports with detailed description of histopathological changes are rare which are mentioned below in Table 2. Rapid clinical and liver enzyme improvement supports the diagnosis of Kratom induced liver injury.

An interesting aspect of our case is the pathological features of liver injury (Figure 2). The zone 3 cholestasis was felt to reflect drug effect; zone 3 cholestasis is a common finding in cholestatic drug reactions but not a feature of early stage PBC. The lymphocytic cholangitis, a typical feature of PBC and unusual medication-injury finding, raised initial concern for underlying early stage PBC. This is of lesser concern given her negative antinuclear antibody and AMA status, trend toward rapid resolution of liver enzymes, and a case report by Aldyab *et al*[10] in 2019 that reported a case of kratom toxicity with granulomatous cholangitis (another form of florid duct lesion) that mimicked PBC. This was from a 40-year-old female presenting with liver injury after Kratom use who initially perceived to have AMA-negative PBC but diagnosed with Kratom induced liver injury after rapid normalization of liver enzymes.

In the first published case report of intrahepatic cholestasis due to consumption of Kratom, the patient's laboratory results showed a peak bilirubin of 29.3 mg/dL with prolonged elimination based on known half-life of the drug and analysis of urine

Table 2 Kratom-induced hepatotoxicity with review of literature in patients with liver biopsy

Ref.	Age, sex	Clinical findings	Form, amount, duration of Kratom consumed	Peak bilirubin (mg/dL)	Disease pattern	Radiological findings	Histological findings
Kapp *et al*[11]	25, M	Abdominal pain, brown urine, jaundice, pruritus	Powder, 1 to 2 teaspoon twice a day and increased to 4-6 teaspoon over 2 wk (1 teaspoon approximately 2-3 g)	Direct bilirubin 29.3	Cholestatic (increased bilirubin, AST, ALT, ALP)	USG, CT-hepatic steatosis	Cholestatic injury, no hepatocellular damage, canalicular cholestasis
Drago *et al*[14]	23, M	Jaundice, pale stool, brown urine for 4 d	Powder, 85 g total over 6 wk	Direct bilirubin 5.8	Cholestatic (increased bilirubin, AST, ALT, ALP)	USG, CT-normal	Cholestatic liver injury
Bernier *et al*[15]	41, F	Jaundice, diarrhea, pruritus	Form not available, 1 teaspoon twice daily for 1 wk	Direct bilirubin 15	Cholestatic (increased bilirubin, AST, ALT, ALP)	-	Intralobular bile duct destruction with cholestatic overload
Shah *et al*[16]	30, F	Abdominal pain, jaundice, dark urine, pruritus	Tea containing Kratom, dose not available	Direct bilirubin 18	Cholestatic (increased bilirubin, AST, ALT, ALP)	MRI-normal, ERCP–no bile duct obstruction	Intrahepatic cholestasis
Riverso *et al*[13]	38, M	Dark urine, light stools, fever	Not available	Total bilirubin 5.6	Cholestatic (increased bilirubin, AST, ALT, ALP)	USG-normal	Acute cholestatic injury, mild bile duct injury, portal inflammation
Mackenzie *et al*[17] and De Francesco *et al*[18]	27, M	Vomiting, epigastric pain, diarrhea with associated heavy alcohol intake	Powder, 3-4 teaspoon multiple times weekly for several wk	Total bilirubin 11.2	Cholestatic (increased bilirubin, AST, ALT, ALP)	-	Widespread hepatocellular necrosis with extracellular cholestasis
Fernandes *et al*[12]	52, M	Mild fatigue, jaundice	Crushed leaves with water, 1 teaspoon (approximately 1.5 g) once or twice a day for 2 mo	Total bilirubin 28.9	Cholestatic (increased bilirubin, ALP; slightly increased AST, ALT)	MRI - normal	Canalicular cholestasis, bile duct injury, hepatic lobule injury, mixed inflammation in portal tracts
Aldyab *et al*[10]	40, F	Abdominal pain, fever	Form not available, once a week for 1 mo	Total bilirubin 5.1	Mixed cholestatic and hepatocellular (increased bilirubin, AST, ALT, ALP)	CT, MRCP–mild, nonspecific periportal edema	Granulomatous duct injury
Pronesti *et al*[19]	30, M	Dark urine and pale stool for 1 wk, scleral icterus for 1 d	Powder with water, for 4-6 wk	Total bilirubin 5.7, direct bilirubin 4.5	Cholestatic (increased bilirubin, AST, ALT, ALP)	USG–coarse hepatic echotexture	Hepatocellular and canalicular cholestasis with inflammation and focal prominent eosinophils. No fibrosis
LiverTox case 6972[20]	25, M	Abdominal pain, fever, jaundice, dark urine, pruritus	Powder, for 23 d	Total bilirubin 22.4	Mixed Hepatocellular and cholestatic (increased bilirubin, AST, ALT, ALP)	USG, CT–gall bladder wall thickening with increased perihepatic lymph nodes	Cholestatic injury with mild necrosis and inflammation

M: Male; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; USG: Ultrasonography; CT: Computed tomography; F: Female; MRI: Magnetic resonance imaging; ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography.

samples[11]. It was speculated that this could be due to the patient's underlying steatohepatitis[11]. Similarly, our patient also had steatohepatitis observed on imaging and pathology, which could explain why even the relatively low doses of Kratom used by our patient compared to other cases discussed in the literature led to such profound liver injury.

As Kratom use appears to be on the rise in the United States, physicians need to be aware of its potential for adverse effects[4,9]. This case highlights how even small

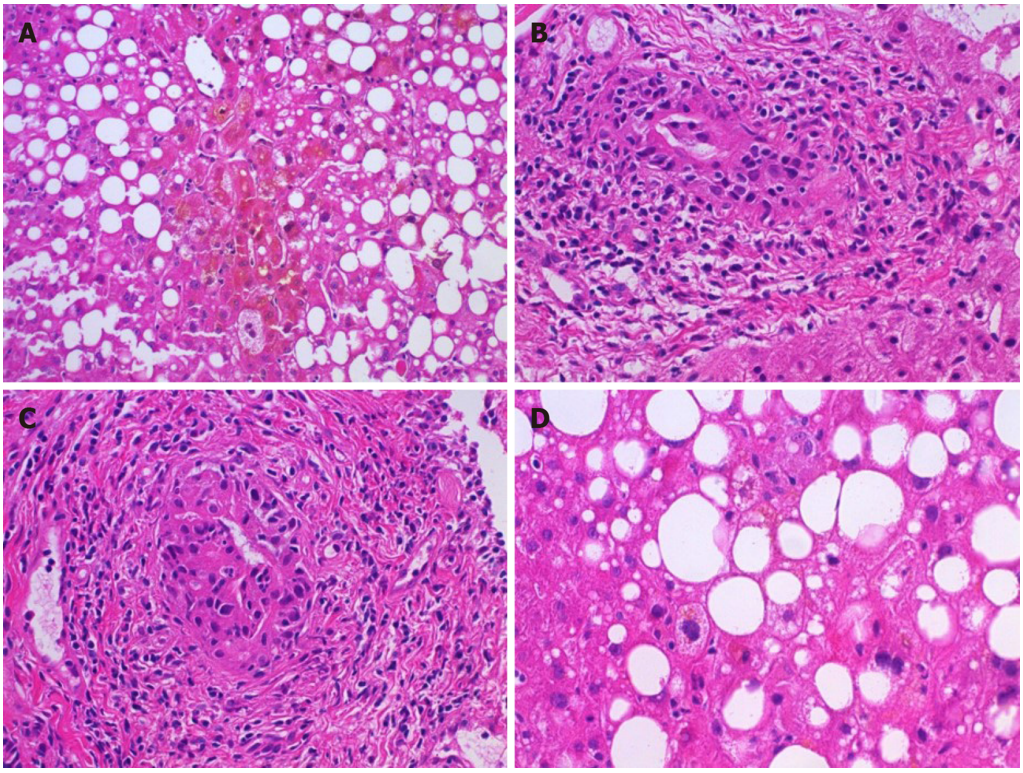

Figure 2 Histopathological findings. A: Centrilobular areas showed well defined cholestasis; B and C: Portal tracts showed moderate chronic inflammation and brisk lymphocytic-predominant bile duct injury; D: Background liver showed steatohepatitis which was felt to most likely be due to underlying obesity-related non-alcoholic fatty liver disease.

amounts of the supplement can be hepatotoxic. Physicians need to be able to discuss safety concerns of over-the-counter supplements with their patients. Moreover, the rising use of supplements and recreational substances can pose diagnostic challenges for clinicians when their use is not reported. Medical providers always need to consider the use of supplements when patients present with possible drug-induced liver injury.

CONCLUSION

Kratom induced liver injury is an important differential diagnosis for physicians to consider in any patient presenting with acute liver injury. As observed in our patient, this manifestation of Kratom consumption may occur even at low doses. Further, this case report demonstrates that a thorough history is essential for an accurate and timely diagnosis. Patients may consider dietary and herb supplements to be natural and risk-free products, not realizing the potential for harm. In addition to asking their patients about the consumption of any supplements, it is imperative that physicians update themselves so as to be able to discuss the benefits and risks and counsel their patients effectively. Identifying use of supplements helps in early diagnosis and treatment, while also preventing future harm. From the pathology perspective, biliary changes associated with Kratom injury can mimic PBC.

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CASE REPORT

Severe COVID-19 after liver transplantation, surviving the pitfalls of learning on-the-go: Three case reports

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Author contributions: Alconchel F, Cascales-Campos PA and Ramírez P designed this article; Alconchel F, Pons JA, Martínez M, Valiente-Campos J, Martínez-Alarcón L, Ortiz ML and Gajownik U collected a substantial portion of data; Alconchel F, Cascales-Campos PA, Parrilla P, Robles R, Sánchez-Bueno F, Moreno S and Ramírez P analysised and interpreted of data; Alconchel F, Cascales-Campos PA and Ramírez P drafted of the manuscript; Parrilla P, Robles R, Sánchez-Bueno F, Moreno S and Ramírez P revised of the manuscript's important intellectual content critically; Parrilla P and Ramírez P supervised; Alconchel F, Cascales-

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Abstract

BACKGROUND

The novel coronavirus 2019 (COVID-19) pandemic has dramatically transformed the care of the liver transplant patient. In patients who are immunosuppressed and with multiple comorbidities, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has been associated with increased severity and mortality. The main objective of this report is to communicate our experience in the therapeutic management of SARS-CoV-2 infection in 3 liver transplant patients. Secondly, we stress the management and investigation of the contagious spreading into a liver transplant ward.

CASE SUMMARY

Campos PA, Pons JA, Martínez M, Valiente-Campos J, Gajownik U, Ortiz ML, Parrila P, Robles R, Sánchez-Bueno F, Moreno S and Ramírez approved of the final version to be published.

Informed consent statement: Informed written consent was obtained from the patient for publication of this report and any accompanying images.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

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The patients were two women (aged 61 years and 62 years) and one man (aged 68 years), all of them having recently received a liver transplant. All three patients required intensive care unit admission and invasive mechanical ventilation. Two of them progressed severely until death. The other one, who received tocilizumab, had a good recovery. In the outbreak, the wife of one of the patients and four healthcare professionals involved in their care were also infected.

CONCLUSION
We illustrate in detail the evolution of a nosocomial COVID-19 outbreak in a liver transplant ward. We believe that these findings will contribute to a better understanding of the natural history of the disease and will improve the treatment of the liver transplant patient with COVID-19.

Key Words: Liver transplantation; COVID-19; SARS-CoV-2; Cross infection; Nosocomial infection; Case report

Core Tip: In patients who are immunosuppressed and with multiple comorbidities, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has been associated with increased severity and mortality. We report our experience in the therapeutic management of SARS-CoV-2 infection in 3 liver transplant patients and stress the management and investigation of a contagious spreading into a liver transplant ward.

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INTRODUCTION

After the first cases of coronavirus-2 pneumonia (SARS-CoV-2) were detected in early December 2019 in Wuhan (Hubei, China)[1,2] a pandemic has overtaken hundreds of countries[3]. The main active sources are currently located in Europe and the United States[4]. This medical emergency has tested global healthcare systems, which have established strategic changes and protocols to prioritize healthcare and avoid overloading. The frequency of liver transplantation operations has been seriously affected. Transplant programs depend on the availability of donors, the vast majority of whom are deceased, and medical personnel normally oversee these programs in intensive care units, but these facilities are currently overcrowded. The result of these conditions has been a dramatic decrease in activity in all transplant groups around the world. In Spain, the world leader in organ donation, surgeons had access to the livers of about 100 deceased donors per week during the 3-month period before the detection of the first case of novel coronavirus (COVID-19); this number has since dropped dramatically to a level of only 15 donors per week[5,6].

In an effort to contain the pandemic, drastic community measures of social confinement and distancing have been established, and these measures extend to the healthcare environment, enhancing telematic activities for the ambulatory management of patients. At the in-hospital level, most of the preventive measures are aimed at preventing the spread of infection by healthcare professionals during the care of COVID-19 patients. The impact of nosocomial infection by COVID-19 has warranted little attention and could be especially relevant to transplant recipients during their hospitalization.

The main objective of our work is to communicate our experience in the therapeutic management of severe SARS-CoV-2 infection in three liver transplant patients who required invasive mechanical ventilation, two of whom had an infection of nosocomial

origin. We also analysed some lessons learned from this experience.

CASE PRESENTATION

Since the detection of the first case of COVID-19 in our region on March 8, 2020, and until May 31st, 2020, a total of twelve liver transplant patients have been hospitalized in our liver transplant unit.

Chief complaints

Case 1: Sixty-one-year-old woman with a liver transplant in September 2019 for cryptogenic cirrhosis. In early March, she was admitted for diarrhea, and a few days later she developed acute respiratory failure, and heart failure. A first RT-PCR of SARS-CoV-2 from throat and pharyngeal swabs was negative but became positive three days later after a second RT-PCR was conducted due to high clinical suspicion (Figure 1). Treatment with hydroxychloroquine and lopinavir/ritonavir was then initiated, adjusting the tacrolimus levels, but the patient suffered progressive clinical and analytical worsening, with the need for invasive mechanical ventilation, associated with pulmonary superinfection by *Enterococcus faecalis* and *Enterococcus faecium* detected in bronchoalveolar lavage fluid. Finally, the patient developed shock with multisystem failure and died in the third week of hospitalization.

Case 2: Sixty-eight-year-old male, transplanted on March 4, 2020, by non-alcoholic steatohepatitis. During the immediate post-transplant period, he was diagnosed with a biliary stricture and was treated endoscopically. His wife, the primary caregiver, tested positive for SARS-CoV-2 *via* RT-PCR from pharyngeal swabs on March 18, 2020, after reporting slightly compatible symptoms. All staff in contact with her, including the patient himself (who was initially negative), were evaluated with RT-PCR. Of a total of 40 people tested, one hepatologist was positive for SARS-CoV-2; this physician was in contact with all patients admitted at that time. Four days later, the patient, without symptoms, was discharged. Two days after discharge, the patient was readmitted for fever and cough, and the RT-PCR of SARS-CoV-2 was positive (Figure 1). Early treatment with hydroxychloroquine and azithromycin was initiated, adjusting the doses of mycophenolic acid and tacrolimus. Seven days after the positive result, the patient was admitted to the intensive care unit due to deterioration of respiratory function requiring invasive mechanical ventilation and treatment with tocilizumab. The patient progressed satisfactorily to home discharge and asymptomatic, but still with a positive RT-PCR of SARS-CoV-2 two months later.

Case 3: Sixty-two-year-old woman who received a liver transplant in February 2019 secondary to primary biliary cholangitis and was discharged in the first week of March 2020 after an episode of constitutional syndrome. On April 6, 2020, she was readmitted with fever, dyspnea, and diarrhea, with a RT-PCR positive for SARS-CoV-2. Forty-eight hours later, the patient progressively deteriorated, requiring admission to intensive care unit with invasive mechanical ventilation, and was treated with tocilizumab in addition to hydroxychloroquine, azithromycin, and methylprednisolone. Mycophenolic acid was suspended, and doses of tacrolimus were reduced to the minimum necessary. After four days of invasive mechanical ventilation, extubation was performed. In spite of the measures adopted, the patient evolved severely. Two months after the onset of the outbreak, and still with a positive RT-PCR of SARS-CoV-2, she developed a tracheoesophageal fistula. An esophageal prosthesis and an extracorporeal venovenous membrane oxygenation (vv-ECMO) were placed. Forty-five days after the first positive RT-PCR of SARS-CoV-2 the virus was negative in the RT-PCR of the bronchoalveolar lavage. Unfortunately, the patient died eighty days after the onset of the outbreak in our liver transplant unit.

History of present illness

A cluster of three patients who temporarily coincided in the hospitalization ward developed a SARS-CoV-2 [reverse transcription polymerase chain reaction (RT-PCR) throat swab] infection. Given the use of anonymous clinical data and the observational approach of our paper, our work was exempt from approval from an ethics' board. Table 1 shows the main clinical features of these three patients. It is important to note that the wife of case 2, one hepatologist on the transplant team, and three nurses in the ward were also infected with the SARS-CoV-2 virus.

In our transplant unit, the outbreak of SARS-CoV-2 began on March 18. After a

Table 1 Details of the three cases reported

	Case 1	Case 2	Case 3
Age (yr)	61	68	62
Sex	Female	Male	Female
LT indication	Cryptogenic cirrhosis	NASH	Primary biliary cholangitis
Date of liver transplant	September 7, 2019	March 3, 2020	February 13, 2019
Immunosuppression (per day)	Tacrolimus 5 mg and prednisone 5 mg	Tacrolimus 7 mg, mycophenolic acid 2000 mg, prednisone 20 mg	Tacrolimus 3 mg, mycophenolic acid 2000 mg, prednisone 5 mg
Blood concentration of tacrolimus (before COVID-19)	7 ng/mL	7.5 ng/mL	5.2 ng/mL
Allograft function (before COVID-19)	Normal	Normal	Increased GGT and ALP
Comorbidities	Hypothyroidism	Diabetes, hypertension, stroke	Hypertension
Laboratory test[1]:			
PaO_2: FiO_2 ratio (while IMV)	237 (76-376)	367 (337-385)	256 (133-329)
White-cell count (× 10^3/UL)	4.50 (2.38-9.87)	6.61 (1.8-17.4)	15.71 (7.02-36.14)
Lymphocyte count (× 10^3 /UL)	0.30 (0-0.65)	0.325 (0.2-1.02)	1 (0.54-2.01)
Platelet count (× 10^3/UL)	9.5 (3-38)	113.5 (37-372)	290 (158-406)
Hemoglobin (g/DL)	8.7 (6.5-9.4)	9.2 (7-11.8)	10.4 (7.6-12.9)
Il-6 (pg/mL)	599 (400-799)	558 (192-1000)	54 (50-286)
C-reactive protein (mg/DL)	10.3 (8.2-18.3)	4 (0.4-13.3)	1.8 (0.7-4.6)
Procalcitonin (ng/mL)	2.2 (1.1-7.8)	0.25 (0.12-0.43)	0.28 (0.17-0.75)
Ferritin (ng/mL)	5338 (814-9862)	1262 (392-2095)	2047 (1360-2297)
Lactate dehydrogenase (U/L)	452 (209-649)	265 (161-378)	399 (165-646)
Aspartate aminotransferase (U/L)	126 (29-466)	14 (9-36)	30 (16-44)
Alanine aminotransferase (U/L)	89 (29-197)	21 (8-31)	24 (5-98)
Total bilirubin (mg/DL)	1.2 (0.3-2.65)	0.56 (0.39-1.23)	1.82 (0.21-4)
Creatine kinase (U/L)	14 (10-18)	13 (7-75)	29 (29-36)
Creatinine (mg/DL)	0.69 (0.45-1.07)	1.22 (1.04-1.93)	0.89 (0.54-2.65)
D-dimer (ng/mL)	1073 (565-1825)	1347 (620 - 3431)	283 (153-648)
Sodium (meq/L)	137 (128-141)	139 (136 - 163)	141 (135-145)
Potassium (meq/L)	4.6 (2.9-5.8)	4 (3.3-4.9)	4 (3.2-5.2)
Chloride (meq/L)	102 (97-105)	103 (100-111)	107 (99-112)
RT-PCR of SARS-CoV-2	Negative on day 3; positive on day 6	Negative on day 8; positive on days 13, 36, 42, 47, 54, 65 and 79	Negative on days 14, 72 and 75; positive on days 26, 42 and 55
Radiologic findings	Bilateral pneumonia, pleural effusion	Bilateral pneumonia, peripheral ground-glass opacity, pleural effusion	Bilateral pneumonia, peripheral ground-glass opacity
Treatment	HCQ (200 mg daily), azithromycin (250 mg daily), LPV/r (one dose 400/100 mg), vancomycin (1 g daily)	HCQ (200 mg daily), azithromycin (250 mg daily), tocilizumab (one dose 8 mg/kg), methylprednisolone (180 mg three doses)	HCQ (200 mg daily), azithromycin (250 mg daily), tocilizumab (one dose 8 mg/kg), methylprednisolone (60 mg daily), vv-ECMO
Immunosuppressant dose reduction	Yes (low dose of tacrolimus)	Yes (mycophenolic acid suspended and low dose of tacrolimus)	Yes (mycophenolic acid suspended)
Rejection during or after COVID-19	No	No	Yes
Complications	Secondary *Enterococcus faecalis*	Asymptomatic intra-abdominal	Tracheoesophageal fistula

	(BAL culture) lung infection	collection

[1]Expressed as the median and range, of the analytical values since the RT-PCR of SARS-CoV-2 was positive until present. ALP: Alkaline phosphatase; BAL: Bronchoalveolar lavage; GGT: Gamma-glutamyltransferase; HCQ: Hydroxychloroquine; ICU: Intensive care unit; IMV: Invasive mechanical ventilation; LPV/r: Lopinavir/ritonavir; NASH: Non-alcoholic steatohepatitis; NE: Norepinephrine; RT-PCR: Reverse transcription polymerase chain reaction; vv-ECMO: Venovenous extracorporeal membrane oxygenation.

positive RT-PCR result in case 1, the wife of case 2 also tested positive after reporting a fever and neck pain. Without delay, we conducted an exhaustive investigation on all the members of the unit (25 nurses and assistants, two cleaning staff, one warden, and nine physicians), with one nursing assistant testing positive for SARS-CoV-2 (hospital admission for ten days for pneumonia, without the need for intensive care unit), two nurses testing positive (one with a mild symptoms and negative RT-PCR at one month, and the other asymptomatic and negative at 13 d), and a hepatologist testing positive (negative at 15 d, asymptomatic and no admission).

Physical examination

The remaining healthcare personnel who tested negative for SARS-CoV-2 were placed in preventive home confinement for 14 d, with negative RT-PCR determinations of SARS-CoV-2 thereafter. In addition, the hospital ward was closed for complete disinfection.

Further diagnostic work-up

Figure 2 shows the epidemiological timeline of the three positive liver transplant recipients as well as the four contacts in the ward (one doctor, one assistant, and two nurses) who tested positive for SARS-CoV-2.

FINAL DIAGNOSIS

The final diagnosis of the presented cases is severe COVID-19 after liver transplantation.

TREATMENT

Case 1 was treated with Hydroxychloroquine (HCQ, 200 mg daily), azithromycin (250 mg daily), lopinavir/ritonavir (one dose 400/100 mg) and vancomycin (1 g daily). The patient in case 2, underwent a treatment that included HCQ (200 mg daily), azithromycin (250 mg daily), tocilizumab (one dose 8 mg/kg) and methylprednisolone (180 mg three doses). In the case of patient 3, however, the outcome was more severe and required the use of a vv-ECMO in addition to HCQ (200 mg daily), azithromycin (250 mg daily), tocilizumab (one dose 8 mg/kg) and methylprednisolone (60 mg daily) (Table 1 and Figure 1).

OUTCOME AND FOLLOW-UP

All three patients required intensive care unit admission and invasive mechanical ventilation (Figure 1). Two of them (cases 1 and 3) progressed severely until death. The other one (case 2), who received tocilizumab, had a good recovery. In the outbreak, the wife of one of the patients and four healthcare professionals involved in their care were also infected (Figure 2).

DISCUSSION

The most appropriate management for transplant recipients who develop COVID-19 and the impact of the infection on this population are not well known. In a previous publication on a population of 111 liver transplant patients with more than ten years of evolution and residents in lombardy (the epicentre of the pandemic in Italy), three

Figure 1 Clinical evolution of each case in a chronological perspective. A: (Case 1) Although the first reverse transcription polymerase chain reaction of severe acute respiratory syndrome coronavirus 2 was negative for the first few days, dyspnea became worse requiring intensive care unit admission. A single dose of lopinavir/ritonavir was administered on day 7; B: (Case 2) A dose of tocilizumab was administered on day 33. The patient improved progressively until he was discharged home; and C: (Case 3) A dose of tocilizumab was administered on day 28. The patient suffered a progressive worsening. A tracheoesophageal fistula was detected and an oesophageal prosthesis was placed. In addition, a venovenous extracorporeal membrane oxygenation was implemented to improve the patient's oxygenation. BAL: Bronchoalveolar lavage; ICU: Intensive care unit; IMV: Invasive mechanical ventilation; LPV/r: Lopinavir/ritonavir; NIV: Non-invasive ventilation; RT-PCR: Reverse transcription polymerase chain reaction; vv-ECMO: Venovenous extracorporeal membrane oxygenation.

deaths were reported due to COVID-19[7]. The three liver transplant patients were all male and over 65 years old, with a body mass index greater than 28 kg/m^2 and with cardiovascular risk factors. The occurrence of co-morbidities such as older age, obesity, diabetes mellitus, use of anti-hypertensive drugs, and other cardiovascular risk factors have been associated with more severe clinical manifestations in the general population[8]. There is no consensus regarding the optimal management of immunosuppressive treatment, and most groups suggest not to modify the immunosuppression strategies in asymptomatic or mild infections, but the experience reported in the literature in severe and critical conditions is variable, and some authors with whom we agree defend the decrease of immunosuppression[9].

In the physiopathology of SARS-CoV-2, the liver appears to be a susceptible organ[10] and liver damage occurs according to three mechanisms: (1) Direct cytotoxicity of the virus itself; (2) Indirect damage from autoimmune aetiology; and (3) Hepatotoxicity of drugs used in the management of the infection (remdesivir, tocilizumab, chloroquine and its derivatives, and azithromycin, among others)[11]. Typical clinical manifestations of SARS-CoV-2 infection include fever, cough, and respiratory distress. The presence of gastrointestinal symptoms at the onset of the picture has been previously reported[12-15] and, along with the fever, were the initial manifestations in cases 1 and 3 of our series. In this initial phase, the RT-PCR determinations of SARS-CoV-2 were negative in both cases, and the patients presented gastrointestinal and non-respiratory symptoms. This pattern of symptoms was similar to that reported in a renal transplant patient with COVID-19[16,17].

The clinical situation worsened rapidly in case 1, evolving rapidly towards a fatal outcome. In this patient, elevated levels of C-reactive protein and procalcitonin were detected, as well as severe lymphopenia[18]. In addition, the presence of *Enterococcus faecium* and *Enterococcus faecalis* was found in the culture of the bronchoalveolar flush, which, in the context of SARS-CoV-2 pneumonia (Figure 1A), undoubtedly triggered the clinical course toward shock and the death of the patient. Although some authors support the hypothesis that SARS-CoV-2 may cause true sepsis of viral origin by direct attack of the virus[19], this theory is not proven in practice, and we believe that that this theory does not explain by itself the fulminant evolution of this patient. Nevertheless, the clinical evolution was favourable in case 2, in which the patient was treated with hydroxychloroquine and tocilizumab. In case 1, we opted for the use of a lopinavir/ritonavir combination, although we do not know if this was correct, but the dose of tacrolimus was closely monitored due to the interaction of these drugs through the inhibition of cytochrome P450 family 3 subfamily A[20,21]. Several studies have shown that patients infected with SARS-CoV-2 can develop a hyperinflammatory state which leads to an acute respiratory distress syndrome [acute respiratory distress syndrome (ARDS)[22]]. During ARDS pathogenesis, a "cytokine storm" including IL-6, tumor necrosis factor alpha, and IL-12 is released[23]. These data suggest, that a blockage of pro-inflammatory pathways could be a therapeutic alternative in the management of patients with severe COVID-19. Tocilizumab is a monoclonal antibody that specifically binds to the IL-6 receptor and inhibits signal transduction mediated by the binding of this receptor to its ligand. In two of our patients, tocilizumab was administered early, even before the need for invasive mechanical ventilation, achieving favorable outcomes. In contrast, in case 1, where tocilizumab was not administered, a situation of shock and multiorgan failure was triggered, resulting in the death of the patient. These findings would be in consonance with what was previously published regarding ARDS and suggest that tocilizumab could be an effective treatment for severe patients with COVID-19.

The liver transplant population has several specific peculiarities. It has been described that, after infection, more than 50% of patients with a liver transplant develop severe forms of the disease[8]. In addition, the time for virus detection tests among these patients to become negative (clearance) is longer than that among the general population, and positive RT-PCRs of SARS-CoV-2 have been described in transplant patients beyond 53 d from the first positive test[24], which carries a potentially higher risk of contagion and the need for a longer period of isolation[25].

The vast majority of measures to prevent infection in the population are focused on non-hospital settings (such as confinement and telemedicine). In hospitals, these measures are preferably designed to prevent the transmission of COVID-19 from patients to healthcare personnel, where the use of personal protective equipment is mandatory. Furthermore, in the case of patients with liver disease, additional measures must be taken. Xiao *et al*[26] suggest, for example, that the communication between patients and medical staff should be done online and each patient taken care of by one attending doctor and one nurse exclusively.

Preventive measures should begin as soon as the recipient is admitted, including the

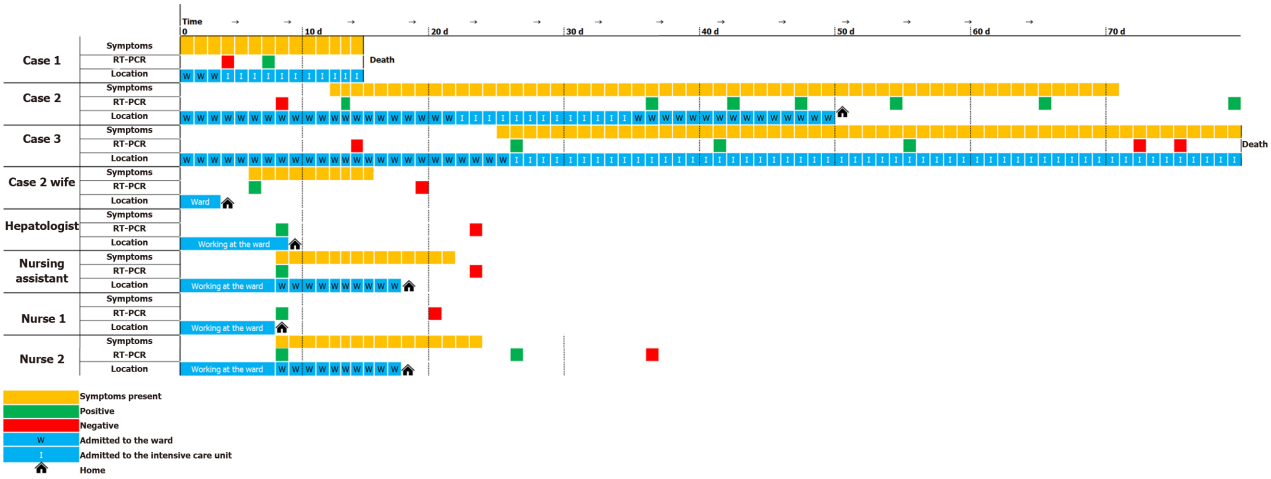

Figure 2 Epidemiological evolution of severe acute respiratory syndrome coronavirus 2 outbreak in our transplant division, according to the presence or absence of symptoms, the positivity of the reverse transcription polymerase chain reaction, and the patients' locations each day from symptoms onset. In orange, when suggestive symptomatology of novel coronavirus 2019 was present. In green, when the reverse transcription polymerase chain reaction of severe acute respiratory syndrome coronavirus 2 was positive. In red, when reverse transcription polymerase chain reaction was performed but was negative. W: Admitted to the ward; I: Admitted to the intensive care unit.

existence of a specific safety circuit until the result of the RT-PCR is known. For example, two of our patients who were candidates for transplant in the last week have tested positive on the day of the transplant but had no symptoms, and the donor grafts were therefore transplanted to other recipients. In addition to community transmission (case 1), nosocomial transmission of the virus must also be considered (cases 2 and 3). Once a case of nosocomial transmission is discovered, special measures should be applied not only to ward staff but also to all ward patients and facilities. In our experience after case 2, the entire ward was evacuated, and a procedure of disinfection and a quarantine of the premises and of all healthcare staff who had worked on the ward were undertaken.

Another problem in relation to the in-hospital management of SARS-CoV-2–infected transplant patients resides in the discordance between the positivity of RT-PCR and the symptoms suggestive of COVID-19, indicating a high-risk window of infection[27]. As other published works have examined[28], in the outbreak that took place in our unit, there was a period in which healthcare professionals and companions who were asymptomatic carriers of SARS-CoV-2 concurred in space and time with other patients and healthcare professionals who did not have the virus. These circumstances favored the propagation of the virus until the first positive case was detected and the necessary measures were taken.

A final, equally important aspect is the real impact of the COVID-19 pandemic on organ donation, transplant policies, and waiting list mortality, which altogether constitute the so-called "indirect mortality" from SARS-CoV-2. Most countries have implemented emergency policies to prevent contagion, ranging from issuing systematic screening tests for SARS-CoV-2 in all donors and recipients, limiting donation to far from the hospital where the graft will be implanted, restricting liver transplant activity only in acute liver failure or critical patients[29,30], and implementing telemedicine in outpatient follow-up. In these circumstances, increases in both mortality among those on the waiting list and in the number of drop-outs due to clinical worsening or tumour progression (indirect deaths from COVID-19) are to be expected. In fact, in Spain, organ donation and transplantation have decreased dramatically. Before the declaration of the state of alarm on March 13, 2020, there were 7.2 donors and 16 transplants per day on average, but since that date, the rates have fallen to an average of 1.2 donors and 2.1 transplants per day[5].

CONCLUSION

Therefore, there are several lessons learned from our experience. Firstly, early administration of anti-IL-6 monoclonal antibodies could be beneficial in slowing down the cytokine storm in critically ill patients with COVID-19. Secondly, the disease

prodrome in two patients were the gastrointestinal and not the respiratory symptoms. Finally, COVID-19 is highly contagious, so drastic preventive measures and exhaustive epidemiological investigations must be conducted in the case of clinical suspected disease in the ward, even if the RT-PCR of SARS-CoV-2 has been tested negative.

Many uncertainties persist in relation to the diagnosis, treatment, and management of COVID-19 in liver transplant patients. It is certain that we will learn more about the disease and be able to treat it more effectively in the coming months. In the meantime, we are walking blind, and we must rely on our scarce previous experience, on our intuition, and on the oldest methodology in medicine: Trial and error.

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LETTER TO THE EDITOR

Role of platelet-albumin-bilirubin score in predicting re-bleeding after band ligation for acute variceal hemorrhage

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Abstract

Platelet-albumin-bilirubin (PALBI) score was proposed by Roayaie *et al* with modification of previously studied albumin-bilirubin score to include platelet as an indicator of portal hypertension in 2015. Predictive value of this score was recently tested by Elshaarawy *et al* for re-bleeding in patients presenting with acute variceal hemorrhage. We did a similar study at our center (n = 170) to look at incidence of re-bleeding after band ligation defined as drop in 2 units of hemoglobin and witnessed melena or hematemesis within 2 wk of the procedure. We calculated PALBI scores for all patients based on lab values prior to the procedure. Of 25.3% had re-bleeding episodes, area under receiver operating characteristic curve for PALBI as predictor of re-bleeding was 0.601 (95% confidence interval: 0.502-0.699). PALBI score showed moderate accuracy at predicting re-bleeding in our population.

Key Words: Cirrhosis; Band ligation; Portal hypertension; Ascites; Platelet-albumin-bilirubin; Model of end stage liver disease

Core Tip: Platelet-albumin-bilirubin score showed moderate accuracy in predicting re-bleeding after band ligation in patients presenting with acute variceal hemorrhage.

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TO THE EDITOR

We read with great interest article by Elshaarawy *et al*[1] regarding the role of platelet-albumin-bilirubin (PALBI) score in predicting re-bleeding and in-patient mortality for patients presenting with acute variceal hemorrhage[1]. The authors found that area under receiver operating characteristic (AUROC) for PALBI with outcome of re-bleeding was 0.794. This was higher than Child-Turcot-Pugh (CTP), Model of End Stage Liver Disease and Albumin-Bilirubin (ALBI) scores, which were 0.681, 0.74 and 0.766, respectively. PALBI score was proposed by Roayaie *et al*[2] with modification of previously proposed ALBI score to include platelet as an indicator of portal hypertension in 2015[2]. It has been studied as a predictor of liver transplant outcomes[3], rate of decompensation in compensated cirrhosis[4], outcomes of locoregional treatment for liver cancer[5] and now for re-bleeding after acute variceal hemorrhage.

We did a similar study at our center and calculated the PALBI score to validate this data. Our study comprised of 170 patients with a diagnosis of cirrhosis who presented with acute variceal hemorrhage and underwent esophageal variceal band ligation from 2017 to 2018. Of our patients, 18.8% were CTP-A, 48.2% CTP-B and 32.9% CTP-C. In comparison, Elshaarawy *et al*[1] had 4.5% CTP-A, 29.2% CTP-B and 66.8% CTP-C patients. Our outcome of interest was re-bleeding with the definition proposed by *Baveno VI*: Drop in two units of hemoglobin along with hematemesis or melena observed clinically within 2 wk of the procedure. 25.3% had re-bleeding in our population based on this definition. 12.1%, 22.6% and 64.3% of our patients qualified for PALBI category 1 (score ≤ -2.53), 2 (score > -2.53 and ≤ -2.09) and 3 (score >-2.09) respectively.

AUROC for PALBI score in predicting re-bleeding was calculated to be 0.601 (95% confidence interval: 0.502-0.699) and the curve is shown in Figure 1. This indicates moderate quality at best of the PALBI score in predicting re-bleeding after band ligation. This is lower than the reported AUROC by Elshaarawy *et al*[1] by 24.3%. 4.5% of re-bleeders in our cohort belonged to PALBI category 1, 28.9% to PALBI category 2 and 28.7% to PALBI category 3. Rates of re-bleeding in each category are shown in Figure 2.

We found PALBI score to be relatively less accurate than reported by Elshaarawy *et al*[1] in predicting re-bleeding after band ligation. This discrepancy can be due to differences in the size and characteristics of patient population and definition of the outcome. We only included patients who underwent band ligation for acute variceal hemorrhage from esophageal varices. In contrast, only 51.7% of Elshaarawy *et al*[1] underwent band ligation alone as treatment for variceal hemorrhage. Their outcome of interest was re-bleeding within 5 d, but we evaluated for re-bleeding within 2 wk following the procedure. Both studies were limited by retrospective design, small number of patients and data from a single institution.

In conclusion, PALBI score is a promising tool for predicting re-bleeding after initial presentation with acute variceal hemorrhage. More data is needed to validate its use in clinical settings post band ligation procedure.

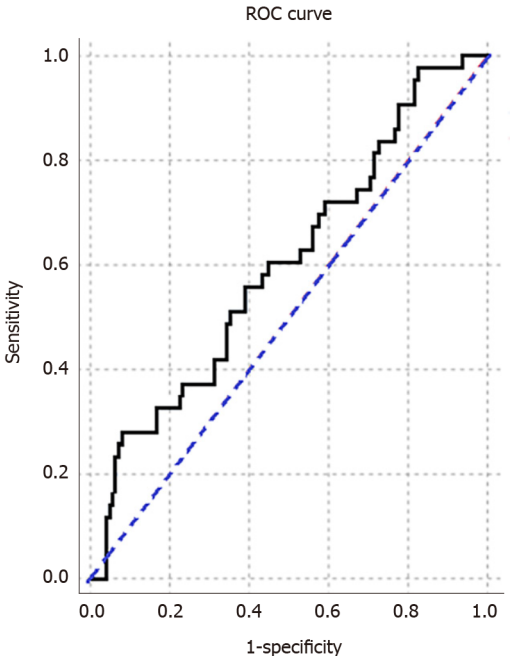

Figure 1 Receiver operating characteristic curve for platelet-albumin-bilirubin score and occurrence of re-bleeding. ROC: Receiver operating characteristic.

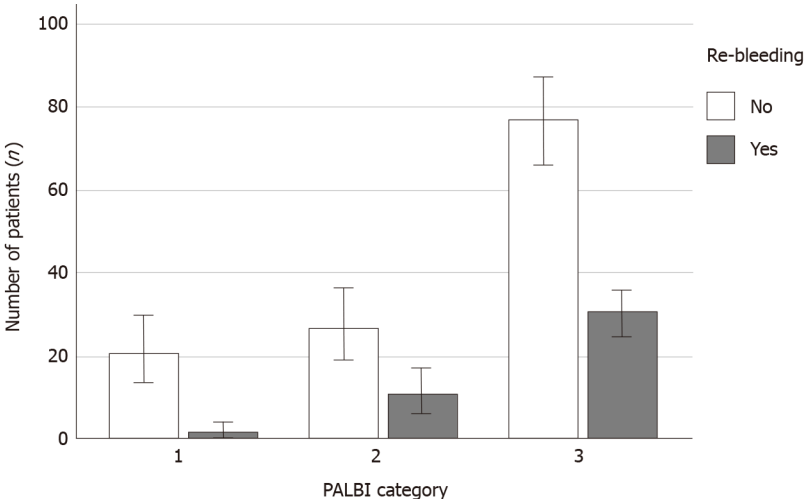

Figure 2 Comparison of different platelet-albumin-bilirubin categories with regards to number of patients who had re-bleeding. PALBI: platelet-albumin-bilirubin.

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