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ABOUT COVER

Editorial Board member of *World Journal of Hepatology*, Dr. Aurelio Sonzogni is Senior Pathologist at Papa Giovanni XXII Hospital in Bergamo, Italy, a tertiary care institution focused on care of terminal organ dysfunction, mainly kidney and liver. Having received his MD from University of Milan in 1985, Dr. Sonzogni undertook his postgraduate training in Surgical Pathology at Milan University, receiving his Master's degree in 1988. After serving as Assistant Pathologist since 1987, he rose to Senior Pathologist in 1992 in the Gastroenterology and Liver Pathology Section of Bergamo Hospital and has held the position since. His ongoing research interests are digestive, liver and kidney non-neoplastic diseases, particularly involving transplantation pathology and pediatric pathology.

AIMS AND SCOPE

The primary aim of *World Journal of Hepatology* (*WJH*, *World J Hepatol*) is to provide scholars and readers from various fields of hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJH mainly publishes articles reporting research results and findings obtained in the field of hepatology and covering a wide range of topics including chronic cholestatic liver diseases, cirrhosis and its complications, clinical alcoholic liver disease, drug induced liver disease autoimmune, fatty liver disease, genetic and pediatric liver diseases, hepatocellular carcinoma, hepatic stellate cells and fibrosis, liver immunology, liver regeneration, hepatic surgery, liver transplantation, biliary tract pathophysiology, non-invasive markers of liver fibrosis, viral hepatitis.

INDEXING/ABSTRACTING

The *WJH* is now abstracted and indexed in PubMed, PubMed Central, Emerging Sources Citation Index (Web of Science), Scopus, China National Knowledge Infrastructure (CNKI), China Science and Technology Journal Database (CSTJ), and Superstar Journals Database. The *WJH*'s CiteScore for 2019 is 5.8 and Scopus CiteScore rank 2019: Hepatology is 22/61.

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EDITORIAL

Meeting report of the editorial board meeting for *World Journal of Hepatology* 2021

Li Ma, Xiang Li

ORCID number: Li Ma 0000-0002-2903-5704; Xiang Li 0000-0002-3585-4159.

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Li Ma, Xiang Li, Editorial Office, Baishideng Publishing Group Inc, Pleasanton, CA 94566, United States

Corresponding author: Li Ma, PhD, Vice Editor-in-Chief, Editorial Office, Baishideng Publishing Group Inc, 7041 Koll Center Parkway, Suite 160, Pleasanton, CA 94566, United States. l.ma@wjgnet.com

Abstract

The 2021 online editorial board meeting of the *World Journal of Hepatology (WJH)* was held on January 16, 2021. Xiang Li, Director of Production Office on behalf of the Baishideng Publishing Group, organized the meeting. Three Editors-in-Chiefs (EiCs) and 15 Baishideng Publishing Group staff attended the meeting. The meeting goal was to brief EiCs on journal performance and gather ideas for journal development in 2021. In 2020, *WJH* published 204 articles, a 20% increase compared to 2019, authors were from 32 countries and regions, and the average citation per article was three times. However, attracting high quality original article submissions remains a challenge. The EiCs provided feedback and suggestions centered on four topics: (1) Improve journal quality by building editorial; (2) Improve board engagement by establishing a clear policy and consistent internal communications; (3) Improve peer review quality and efficiency; and (4) Refine the current journal marketing strategy to increase visibility and discoverability.

Key Words: *World Journal of Hepatology*; Baishideng; Editorial board meeting; Journal development

Core Tip: The 2021 *World Journal of Hepatology* editorial board meeting was held on January 16, 2021. The meeting goal was to brief board members on journal performance and gather ideas for journal development in 2021. The discussion focused on (1) improving journal quality by building editorial; (2) improving board engagement by establishing a clear policy and consistent communications; (3) improving peer review quality and efficiency; and (4) refining current journal marketing strategy.

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INTRODUCTION

Every year *World Journal of Hepatology* (*WJH*) editorial office organizes the editorial board meeting at the American Association for the Study of Liver Disease annual meeting. Due to the coronavirus disease 2019 pandemic, the 2021 meeting moved to online format. *WJH* editorial office hosted the meeting on January 16, 2021 to review journal performance in 2020 and identify strategies to further *WJH*'s mission, which is to publish high impact research in the field of hepatology. The meeting was moderated by Dr. Li Ma, Company Vice-Editor-in-Chief. The first part of the meeting consisted of presentations on journal status review and plans for 2021, and the second part consisted of open discussions with Editors-in-Chiefs (EiCs) for their feedback and suggestions.

ATTENDEES

This online meeting brought together three EiCs: Namely Dr. Ke-Qin Hu, Dr. Koo Jeong Kang, Dr. Nikolaos Pyrsopoulos, and 15 Editors (Li Ma, Xiang Li, Jin-Lei Wang, Ze-Mao Gong, Ya-Juan Ma, Jia-Ping Yan, Yun-XiaoJian Wu, Dong-Mei Wang, Jia-Ru Fan, Chen-Chen Gao, Le Zhang, Ji-Hong Liu, Yu-Jie Ma, Yan-Liang Zhang, Li-Li Wang) from Baishideng Publishing Group Inc (Figure 1).

REPORTS

WJH year in review 2020

Xiang Li began the meeting by offering an overview of *WJH*'s journal statistics, status quo of editorial board, challenges journal faces, and update from publisher. She showed in 2020 that *WJH* published 204 papers, an 20% increase compared to 2019 (181 papers). The 2020 editorial board consists of 195 members from 45 countries and regions. The top three countries are China, Italy and United States. Forty-four percent of Editorial board members reviewed at least one manuscript in 2020. She highlighted that the main challenge is to attract high quality, high impact original research submissions. Xiang Li finally highlighted new features Baishideng Publishing Group has launched to serve better the authors, including open peer review, shortened peer review time by using artificial intelligence empowered search techniques and post publication promotion by marketing articles to targeted audiences[1].

Dr. Ma presented the journal's 2021 priorities: (1) Commissioning and publishing high impact original articles in the important areas of hepatology; (2) Encouraging editorial board members to recommend and submit to the journal; and (3) Improving the overall quality and relevance of *WJH*.

OPEN DISCUSSION FROM EDITOR-IN-CHIEFS FOCUSED ON FOUR MAIN TOPICS

Peer review

Dr. Hu inquired about the low response rate of peer review invitations; he commented that the current key word matching search using the in-house database is suboptimal. He presented the following possible solutions: (1) Allow authors to suggest reviewers; editor should cross check if suggested reviewers are suitable candidates; and (2) Prioritize editorial board member as peer reviewers. Dr. Ma addressed the above question that the current peer reviewer search strategy offers a fair and unbiased review process, but there is room for improvement. Dr. Pyrsopoulos added that it is also worth improving peer review quality when poor language is used by the peer

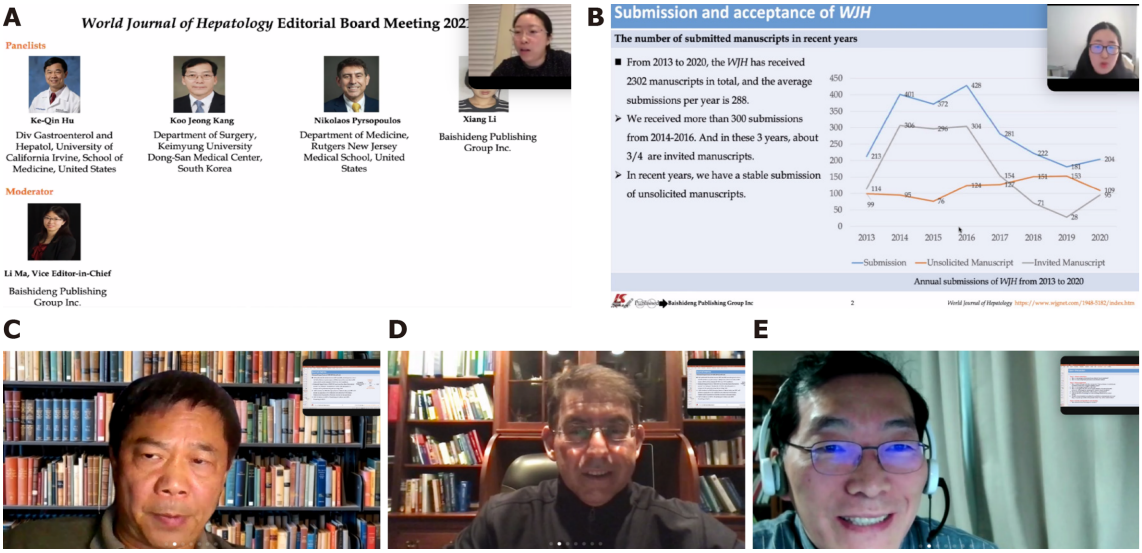

Figure 1 2021 *World Journal of Hepatology* editorial board meeting presenters. A: Dr. Li Ma; B: Xiang Li; C: Dr. Ke-Qin Hu; D: Dr. Nikolaos Pyrsopoulos; E: Dr. Koo Jeong Kang.

reviewer, and the editorial office should contact the reviewer for professional and constructive comment.

Journal quality

Dr. Hu pointed out that *WJH* should aim to build up a strong editorial and suggested that the editorial office should group upcoming manuscripts in topics or inform EiCs in advance so they can help group the topics. EiCs can assist with writing or help finding suitable authors to write an editorial or commentary. Dr. Pyrsopoulos brought up the topic of how to handle better invited manuscript rejection, as authors may feel disappointed when an invited article is rejected. On a different note, he suggested the importance of tracking the evolution of journal citations to monitor journal health.

Maintain an active editorial board

Statistics presented by Xiang Li showed that about 50% of editorial board members are inactive and that they did not review a paper for the journal in 2020. Both Dr. Hu and Dr. Pyrsopoulos suggest that the Editorial Office should send a kind reminder to these editors about the duties and commitments. In addition, internal metrics should be set to monitor editorial board member activities. Along with the above points, a "Dashboard" should be created to show editorial board member their statistics, including the number of invitations sent, response time, time taken to review and review quality grading by the handling editor. Orientation should be prepared for editorial board members to familiarize them with journal history, policy and peer review best practice. In addition, a quarterly editorial board member newsletter can inform everyone about journal news, initiatives and policy updates. Lastly, but most importantly, there should be a recognition mechanism for those editorial board members who contribute to journal growth by submitting their research or performing peer review.

Dr. Kang reminded everyone that a high-quality and active editorial board is more crucial to journal growth. He inquired about the percentage of surgeons serving on the board and surgery related papers published in *WJH* and proposed increasing liver surgeon representation. Dr. Pyrsopoulos emphasized the advantages of having a large editorial board to increase submissions.

Journal marketing

Dr. Pyrsopoulos and Dr. Hu gave a very comprehensive overview on how to increase journal visibility. They commented that the editorial office needs to think about how to market and position the journal. Research online advertising channels, such as social media, increase the discoverability in search engines. Offline channels include society conferences (American Association for the Study of Liver Disease, European Association for the Study of the Liver, Asian Pacific Association for the Study of the Liver), setting up a booth to meet and connect with authors and donating and

supporting the conference.

ACTIONS AND FUTURE PLANS FROM THE EDITORIAL OFFICE

First, the *WJH* editorial office would like to collaborate with EiCs to select two editorial worthy articles in 2021 Q1 and Q2 and to coordinate a suitable candidate to write editorial.

Second, research suitable venues online (website advertising) and offline (leading Hepatology meetings) should be utilized to promote the journal. A volunteer editorial board member will need to be recruited to help manage content of a dedicated *WJH* twitter account.

Third, editorial board member engagement needs to improve and editorial board member review rate should increase from 44% to 70%. An annual editorial board member activity "dashboard" email that contains review number, speed and reviewed article status would be helpful. Thank you letters should be sent to recognize their valuable time. It is important to send bi-annual editorial board newsletters to convey journal news, internal information exchange, publisher update, *etc.*

Fourth, EiCs should collaborate to identify and address author submission pain points in order to improve author publishing experiences.

Fifth, liver surgery editorial board member representation should be expanded and experts from this field should be invited to submit articles.

CONCLUSION

All EiCs expressed commitment and enthusiasm to help the journal grow. Dr. Hu is committed to moving the journal to higher ranking and improving journal quality, and he is interested to provide guidance to make the submission system more streamlined and user friendly. Dr. Pyrsopoulos and Dr. Kang said that they will continue to contribute their research and encourage their peers to contribute; they will work together with the editorial office to have *WJH* recognized by leading databases. They also expressed wishes to continue the in person editorial board meeting when possible at future American Association for the Study of Liver Diseases annual meeting, as it is a great way to catch up with other members and the editorial office. Dr. Li Ma thanked the EiCs for their contributions in the past years[2-6], enlightening ideas and time to attend the meeting. The next online editorial board meeting is tentatively set for June 2021. All editorial members are welcome to attend, stay tuned for more details!

ACKNOWLEDGEMENTS

WJH editorial office thanks all the EiCs for their leadership, guidance and contributions to the journal growth.

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REVIEW

Extrahepatic cholangiocarcinoma: Current status of endoscopic approach and additional therapies

Alina Ioana Tantau, Alina Mandrutiu, Anamaria Pop, Roxana Delia Zaharie, Dana Crisan, Carmen Monica Preda, Marcel Tantau, Voicu Mercea

ORCID number: Alina Ioana Tantau 0000-0001-5975-0779; Alina Mandrutiu 0000-0001-5321-8853; Anamaria Pop 0000-0002-4884-8145; Roxana Delia Zaharie 0000-0002-8622-3787; Dana Crisan 0000-0002-1627-9670; Carmen Monica Preda 0000-0002-2675-7971; Marcel Tantau 0000-0002-7749-0007; Voicu Mercea 0000-0001-7593-2142.

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Alina Ioana Tantau, Department of Internal Medicine and Gastroenterology, "Iuliu Hatieganu" University of Medicine and Pharmacy, 4th Medical Clinic, Cluj-Napoca 400012, Cluj, Romania

Alina Mandrutiu, Anamaria Pop, Department of Gastroenterology and Hepatology, Gastroenterology and Hepatology Medical Center, Cluj-Napoca 400132, Cluj, Romania

Roxana Delia Zaharie, Department of Gastroenterology, "Prof. Dr. Octavian Fodor" Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca 400162, Cluj, Romania

Roxana Delia Zaharie, Department of Gastroenterology, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca 400012, Cluj, Romania

Dana Crisan, Internal Medicine Department, Cluj-Napoca Internal Medicine Department, "Iuliu Hatieganu" University of Medicine and Pharmacy, 5th Medical Clinic, Cluj-Napoca 400012, Cluj, Romania

Carmen Monica Preda, Department of Gastroenterology and Hepatology, Clinic Fundeni Institute, "Carol Davila" University of Medicine and Pharmacy, Bucharest 22328, Romania

Marcel Tantau, Voicu Mercea, Department of Internal Medicine and Gastroenterology, "Prof. Dr. Octavian Fodor" Regional Institute of Gastroenterology and Hepatology, Cluj-Napoca 400162, Cluj, Romania

Marcel Tantau, Voicu Mercea, Department of Internal Medicine and Gastroenterology, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca 400012, Cluj, Romania

Corresponding author: Roxana Delia Zaharie, MD, PhD, Assistant Lecturer, Department of Gastroenterology, "Prof. Dr. Octavian Fodor" Regional Institute of Gastroenterology and Hepatology, 19-21 Croitorilor Street, Cluj-Napoca 400162, Cluj, Romania.
endoscopict.correspondence@gmail.com

Abstract

The prognosis of patients with advanced or unresectable extrahepatic cholangiocarcinoma is poor. More than 50% of patients with jaundice are inoperable at the time of first diagnosis. Endoscopic treatment in patients with obstructive jaundice ensures bile duct drainage in preoperative or palliative settings. Relief of symptoms (pain, pruritus, jaundice) and improvement in quality of life are the aims of palliative therapy. Stent implantation by endoscopic

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retrograde cholangiopancreatography is generally preferred for long-term palliation. There is a vast variety of plastic and metal stents, covered or uncovered. The stent choice depends on the expected length of survival, quality of life, costs and physician expertise. This review will provide the framework for the endoscopic minimally invasive therapy in extrahepatic cholangiocarcinoma. Moreover, additional therapies, such as brachytherapy, photodynamic therapy, radiofrequency ablation, chemotherapy, molecular-targeted therapy and/or immunotherapy by the endoscopic approach, are the nonsurgical methods associated with survival improvement rate and/or local symptom palliation.

Key Words: Cholangiocarcinoma; Endoscopic drainage; Endoscopic retrograde cholangiopancreatography; Photodynamic therapy; Radiofrequency ablation; Brachytherapy

Core Tip: Cholangiocarcinoma is an aggressive tumor with a poor prognosis mainly due to its late diagnosis. The development of new minimally invasive techniques provides these patients a chance to relieve symptoms and attain a better quality of life. We herein discuss the palliation of obstructive jaundice by radiofrequency ablation, photodynamic therapy and brachytherapy in advanced extrahepatic cholangiocarcinoma.

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INTRODUCTION

Cholangiocarcinomas (CCAs) have a very high mortality rate worldwide[1,2]. Diagnosis is challenging and delayed in many cases due to the common asymptomatic clinical behavior of early-stage disease, the lack of a standardized screening protocol for early-stage disease and the limitations inherent to using CA19-9 as a cancer marker[3].The ability to achieve a definite cytopathological or histopathological diagnosis in patients with suspected CCA ranges widely in the literature from 26% to 80%[4-8]. Magnetic resonance imaging plus magnetic resonance cholangiopancreatography is the preferred imaging modality as it can assess resectability and tumor extent with a high accuracy[9-15]. Endoscopic ultrasound (EUS) and fine needle aspiration guided by EUS is a useful technique in the diagnosis and staging of CCA (Figure 1 and 2) and should always be taken into consideration for CCA clinical management. For patients with obstructive jaundice, in particular, intraductal ultrasonography has been suggested for the assessment of bile duct strictures and local tumor staging[16] (Figure 3).

CCAs are divided into three types: Intrahepatic CCA, distal CCA (dCCA) and perihilar CCAs (pCCA) or Klatskin tumors. The majority of CCAs are pCCAs (60%-75% of cases). dCCA is present in 15%to 25% of cases, and intrahepatic CCA accounts for 5%to 15% of cases[17-20].

Surgery is the only curative treatment for extrahepatic CCA with the goal of R0 resection. Unfortunately, only a minority of patients (approximately 35%) have early stage disease and are candidates for this curative treatment option[21].Furthermore, only a few patients with pCCA are candidates for liver transplantation following neoadjuvant chemotherapy[22].

More than 50% of patients with jaundice are reportedly inoperable at the time of first diagnosis. Locally advanced, unresectable CCA cases include patients with macroscopic residual disease following resection, locally advanced, categorically unresectable disease at presentation or locally recurrent disease after potentially curative treatment. Prognosis of these patients is poor with a median survival time of < 6 mo[23]. Relief of symptoms (pain, pruritus, jaundice) and improvement in quality of

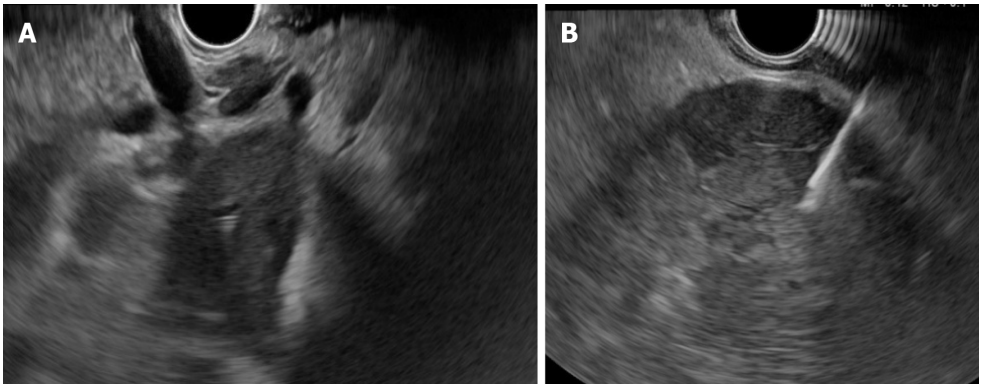

Figure 1 Endoscopic ultrasound for liver evaluation. A: Hilium view of the liver tumoral mass; B: Fine needle aspiration guided endoscopic ultrasound for left lobe intrahepatic cholangiocarcinoma.

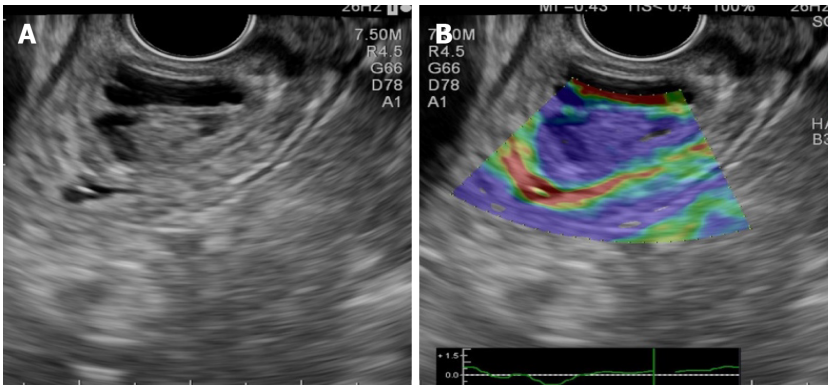

Figure 2 Endoscopic ultrasound of the distal common bile duct. A: Small non-invasive tumoral mass (distal cholangiocarcinoma); B: Elastography: Blue color of the tumor (hard stiffness).

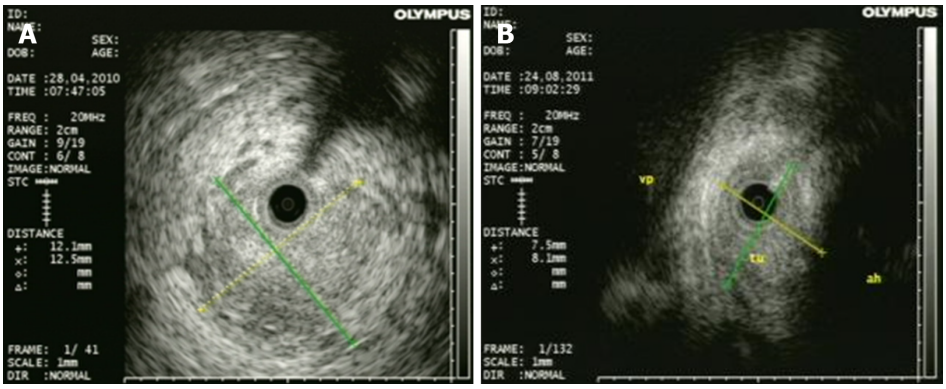

Figure 3 Intraductal ultrasonography for diagnosis of cholangiocarcinoma. A: Large tumoral mass with invasion of surrounding tissue; B: Infiltrative cholangiocarcinoma in proximity of the hepatic artery.

life are the aims of palliative therapy.

Each subtype of CCA has different clinical management[24]. Therefore, an individualized approach is mandatory for pCCA or dCCA. In patients with extrahepatic CCA who are not candidates for surgery or liver transplantation, consideration should be given to enrollment in a clinical trial, particularly those evaluating targeted therapy[25].

Additional treatment measures in locally advanced extrahepatic CCA may include the following: Stenting, radiofrequency ablation (RFA), photodynamic therapy (PDT), radiation therapy, chemotherapy, molecular-targeted therapy and/or immune-therapy[25].

Preoperative or palliative biliary drainage using stents are two main approaches for

extrahepatic CCA[26]. Stents can be placed *via* endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangiography to relieve biliary obstruction. Stenting may relieve the jaundice and pruritus and improve the quality of life[26]. In ERCP, a unilateral or bilateral plastic or metallic stent can be used[23,24,26].

RFA and PDT are effective in restoring biliary drainage and improving quality of life in patients with nonresectable disseminated extrahepatic CCA[23,27]. Local radiotherapy combined with metallic stent placement is a new and efficient method in advanced extrahepatic CCA[28]. Several clinical trials are evaluating the effect of specific molecular agents targeting various signaling pathways in advanced extrahepatic CCA[25]. Our proposal is to highlight the utility and the efficiency of different endoscopic techniques and additional measures in extrahepatic CCA.

PALLIATION OF OBSTRUCTIVE JAUNDICE

Endoscopic treatment of CCA with obstructive jaundice ensures bile duct drainage in preoperative or palliative settings[23,26]. Endoscopic procedures are the preferred palliative treatment options for patients with advanced or unresectable CCA. In patients with advanced pCCA, endoscopic biliary drainage *via* ERCP is more difficult than those with dCCA[23,25,26]. If the transpapillary approach failed, then other procedures can be considered: Percutaneous transhepatic biliary drainage (PTBD), endoscopic ultrasound-guided biliary drainage (EUS-BD) or hepatico-gastrostomy or locoregional therapies including transluminal PDT and RFA[27].

Preoperative biliary drainage

There is some controversy in the literature as to how preoperative biliary drainage should be accomplished prior to laparotomy for patients with obstructive jaundice[29-30]. In a European multicenter study, Gouma *et al*[31] showed that the postoperative outcomes in patients with pCCA who underwent surgery and preoperative biliary drainage were not improved. However, the rate of mortality was lower in patients who received en bloc right hepatectomy. In dCCA, preoperative bile duct drainage is not always necessary unless neoadjuvant chemotherapy is planned and might be associated with an increased risk of cholangitis and postoperative infectious complications[32].

Acute cholangitis, sepsis, bilirubin > 10-15 mg/dL, scheduled neoadjuvant therapy and the need for extensive hepatic resection are indications for preoperative biliary drainage. The goal is to reduce peri- and postoperative complications[23,24,26,29]. Cholestasis, liver dysfunction and biliary cirrhosis can develop rapidly with unrelieved obstruction and may influence postoperative morbidity and mortality after surgery[23-26,33]. The definitive operation is deferring until bilirubin levels are less than 2 to 3 mg/dL[33].

Some centers prefer preoperative biliary decompression in order to decrease the total bilirubin level to under 3 mg/dL, whereas others recommend resection in patients without biliary drainage. In our center the decision to perform preoperative biliary drainage is made in the setting of a multidisciplinary team, and it is not generally recommended unless severe liver dysfunction is suspected.

It should be taken into account that the stent may induce different artifacts in subsequent images. Therefore, previous high-quality imaging is required (computed tomography, magnetic resonance imaging, magnetic resonance cholangiopancreatography, endoscopic ultrasound and intraductal ultrasonography) to assess the tumor resectability[23-26,33]. The biliary stent may be a hindrance for the surgeon to find the proximal tumor extent. Resection of pCCA always requires a concomitant major liver resection. Liver segments that will remain after surgery should be drained sufficiently with a plastic stent to improve postoperative liver function and regeneration[29].

There are different data regarding the benefits of preoperative biliary drainage in jaundice patients with pCCA without absolute indications for biliary drainage[29]. The most recent studies concluded that routine biliary drainage does not impart any advantage because it does not improve the morbidity or mortality of patients with resected pCCA[31,34,35]. A recent meta-analysis and a systematic review showed that preoperative biliary drainage have not changed the incidence of postoperative complications, hospitalization time, R0 or survival rate. However, in jaundice patients, preoperative biliary drainage decreased postoperative mortality[36].

In dCCA, a European multicenter study did not find any differences regarding mortality rate in patients with preoperative biliary drainage[37]. Moreover, in a recent

retrospective study, preoperative endoscopic biliary drainage was associated with a decrease in the survival rate[38].

In PTBD, some studies reported that catheter tract recurrence rates were up to 6%[32], and the median time of recurrence was months. Furthermore, the technical success rate regarding the decrease of biliary level is higher with the endoscopic approach than with PTBD[39,40]. In a recent randomized prospective study, the risk of cholangitis in patients who underwent surgery was higher in the PTBD group compared with the endoscopic biliary drainage group (59% *vs* 37%) (P = 0.1)[41].

PTBD is no longer recommended for preoperative biliary drainage in patients with extrahepatic CCA, and an endoscopic approach is currently preferred[24,26]. The risk of endoscopic plastic stent occlusion is 60%. Therefore, there are several groups of experts who recommend preoperative nasobiliary drainage. Kawashima *et al*[42] compared preoperative nasobiliary drainage with endobiliary stenting drainage in 164 patients with pCCA. They found a longer stent patency and a lower risk of cholangitis in the nasobiliary group than the endobiliary stenting group.

Palliative biliary drainage

The relief of symptoms (pain, pruritus, jaundice) and improvement in quality of life are the goals of palliative therapy. Radiotherapy, PDT, RFA, local ablation and embolization are nonsurgical local therapies that can prolong the time to local failure (in patients with macroscopically positive margins) or to palliate local symptoms, pain or jaundice (in patients with unresectable or recurrent disease).

In patients with pCCA and dCCA who are not suitable for surgery or liver transplantation, the guidelines recommend endoscopic bile duct drainage as the first approach[23,24,26,34]. In patients with a good performance status an additional treatment, such as chemotherapy, radiotherapy, molecularly targeted therapy and/or immunotherapy, is recommended[25].

Stenting

Stent implantation by ERCP should be the standard procedure[24-26] (Figure 4 and 5). Placement of a stent is generally preferred for long-term palliation. This approach has similar successful palliation and survival rates and less morbidity compared with the surgical approach[43]. The endoscopic drainage with one or more stents is technically possible in 70% to 100% of cases. The extent of decompression that is necessary to restore sufficient bile flow while avoiding the risk of bacterial cholangitis, the optimal approach to placement of the stents and the use of plastic or metal uncovered/covered stents are the major issues of biliary endoscopic stenting[44].

The goal of palliative drainage is to drain more than one half of the biliary tree, although it has been shown that the jaundice may be clinically improved if only a quarter of the liver is drained[45]. A target stenting using previous superior imaging methods is preferred[44]. In cases of cholangitis, drainage of all suspected infected intrahepatic segmental branches should be performed[24].

In complex and difficult cases a multimodality biliary drainage (transpapillary drainage in combination with PTBD) should be considered[44]. Rendezvous technique, anterograde PTBD and transluminal stenting through the stomach, duodenum or jejunum walls are the procedures using EUS-BD in these cases. This approach can be performed even when a passage of a wire through a biliary stricture is not possible[46]. In a meta-analysis conducted by Leng *et al*[47], the technical success rate of PTBD varied from 60% to 90% and the morbidity rate from 18%to 67%. In some difficult cases, an external drainage has been required. Therefore, the quality of life of these patients is decreased. EUS-BD technical success varied from 70% to 100%, and the rate of complications was up to 77%[48,49]. A few comparative studies are available[50-54] (Table 1). The technical success rates are similar in most studies with a higher incidence of complications for PTBD than EUS-BD[50-54].

Unilateral or bilateral endoscopic stenting

In most cases, unilateral stent placement should be adequate for biliary drainage *via* ERCP because only 25% to 30% of the liver needs to be drained to relieve jaundice[54-56]. However, unilateral drainage alone may not relieve jaundice completely and may increase the risk of cholangitis due to contrast medium injection into undrained bile ducts[45]. Unilateral stenting is technically easier and less expensive than bilateral stenting with reintervention for stent dysfunction also being considerably easier[45]. In our practice, we prefer to place a unilateral self-expandable metallic stent (SEMS) in order to provide good efficacy of biliary drainage with minimum risk of cholangitis. In clinical practice, many endoscopists prefer to place bilateral stents (plastic or metal) in

Table 1 Success rate and complications for percutaneous transhepatic biliary drainage, endoscopic ultrasound-guided biliary drainage, endoscopic retrograde cholangiopancreatography and self-expandable metal stent

Ref.	Procedure	Patients, *n*	Technical success %	Morbidity %
Artifon *et al*[50], 2012	PTBD, EUS-BD	12, 13	100, 100	25.00, 15.30
Bapaye *et al*[51], 2013	PTBD, EUS-BD	26, 25	46.00, 92.00	46.00, 20.00
Khashab *et al*[52], 2015	PTBD, EUS-BD	51, 22	100, 86.40	39.20, 18.20
Dhir *et al*[53], 2015	ERCP SEMS, EUS-BD	104, 104	94.23, 93.26	8.65, 8.65

ERCP: Endoscopic retrograde cholangiopancreatography; EUS-BD: Endoscopic ultrasound-guided biliary drainage; PTBD: Percutaneous transhepatic biliary drainage; SEMS: Self-expandable metal stent.

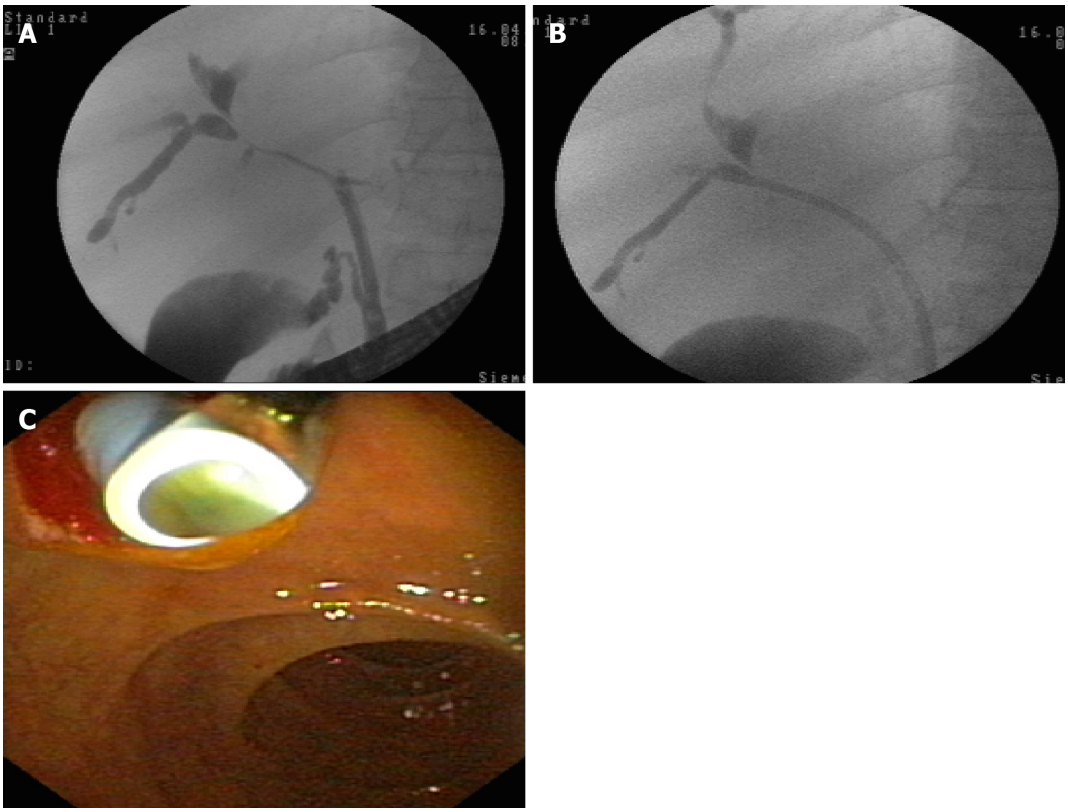

Figure 4 Endoscopic retrograde cholangiopancreatography. Radiologic and endoscopic view. A: Bismuth IV cholangiocarcinoma of the hilum; B: Endoscopic stenting with plastic stent in place; C: Endoscopic view of plastic stent at the level of the papilla.

an attempt to maximize biliary drainage and to prevent cholangitis.

Previous studies have demonstrated that bilateral stenting is associated with longer stent patency compared to unilateral stenting[57,58]. In a recent multicenter prospective randomized study conducted by Lee *et al*[59], the same survival rate in patients with bilateral SEMS biliary drainage but with a longer stent patency *vs* unilateral SEMS biliary stenting were shown. No significant difference between unilateral and bilateral SEMS regarding the technical success or complications was shown[59]. These results highlighted the superiority of bilateral stenting. However, several study results have similarly supported the superiority of unilateral stenting[54-56,60].

In a recent meta-analysis involving 782 patients, bilateral biliary drainage had a lower re-intervention rate compared to unilateral drainage in patients with pCCA with no significant difference in technical success and early or late complication rates[61].

Plastic stents or SEMS

Endoscopic biliary drainage can be performed using plastic or SEMS. There are a variety of plastic and metal stents, covered or uncovered. While some studies showed benefits of metallic stents regarding the successful drainage and early complication

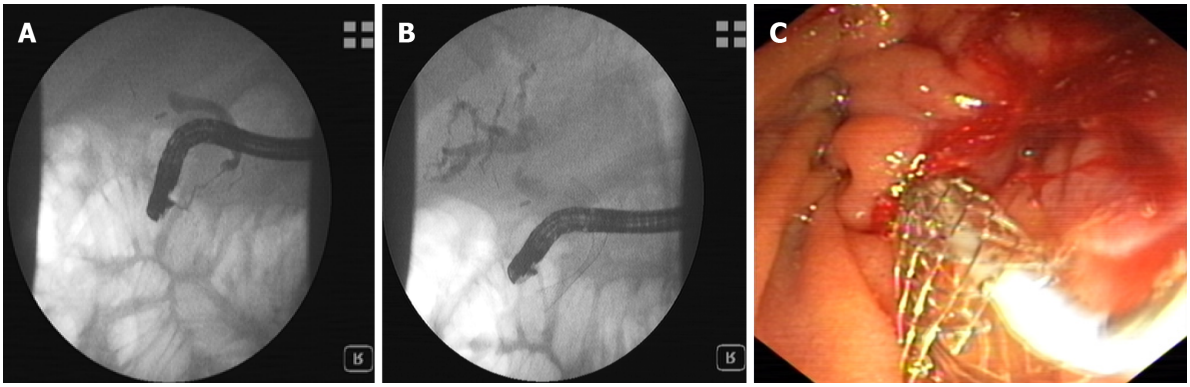

Figure 5 Endoscopic retrograde cholangiopancreatography. Radiologic and endoscopic view. A: Bismuth IV cholangiocarcinoma of the hilum; B: Endoscopic stenting with metallic stent in place; C: Endoscopic view of metallic stent at the level of the papilla.

rate, stent patency and survival rate[55-59,62], a systematic review concluded that neither stent type offered a survival advantage[63]. The decision to use one *vs* another should be guided by the expected length of survival, quality of life, costs and physician expertise. Usually, SEMS should be considered for patients with a life expectancy of longer than 3 mo[44]. The results of different meta-analyses that compared SEMS with plastic stents for endoscopic drainage of distal malignant biliary obstruction are illustrated in Table 2[62-66].

Plastic (polyethylene) stents are inexpensive, effective and easily removable or exchangeable[38-44]. The major disadvantage is a higher rate of occlusion by sludge and/or bacterial biofilm with cholangitis development and necessity of multiple ERCPs[60,62-66]. Instead, metal stents have a longer patency (approximately 8-12 mo *vs* 2-5 mo for plastic stents)[61-66], higher costs and may not be removable. The high occlusion rate of plastic stents (average 42%) can be reduced by changing the stents every 3-6 mo[60,62-66]. Another way is to wait for a complication before changing the stent because many patients will die before the stents will obstruct. The preferred approach for patients who are expected to live beyond a few months is to replace the plastic stent with a metal one as soon as is feasible[44].

In dCCA, uncovered SEMS are used in patients with an intact gallbladder[26]. For patients who have undergone prior cholecystectomy, the choice of a covered *vs* uncovered SEMS is individualized given the location and geometry of the stenosis. Patients with extrinsic compression may be adequately treated with an uncovered SEMS, while those with intrinsic and/or papillary tumors may benefit from a covered SEMS in an attempt to minimize tumor ingrowth[26,67,68]. The patency rates are not higher for covered stents despite showing significantly less tumor ingrowth. Tumor overgrowth and stent obstruction by debris and biliary sludge are associated with a low patency rate for uncovered SEMS[68]. Covered SEMS should be used for pCCA. Deployment may inadvertently result in the occlusion of a major hepatic duct[24,26,44,68].

The stent in stent technique (Y stenting) and the side-by-side technique (Figure 6) are two endoscopic techniques for biliary drainage in CCA. By using the Y stent technique, Hwang *et al*[69] demonstrated an 86.7% technical success rate and a 100% functional success rate regardless of the stent type. For side-by-side stenting technique in pCCA, Lee *et al*[70] reported a 91% technical success rate and a 100% functional success rate with no statistically significant difference between stent patency and median survival of the 8-mm and 10-mm groups.

The reported rate of stent dysfunction following pCCA biliary drainage was 45%-57% due to tumor ingrowth, tumor overgrowth or stent migration[55-58]. Given the fact that SEMS may be successfully revised in the majority of cases and that the second SEMS have a higher patency compared with plastic stents, it seems that SEMS are the best choice in cases of SEMS dysfunction[55-59].

Guidelines recommend prophylactic antibiotics in patients with plastic or metal stents for long-term palliation of obstructive jaundice after the first episode of cholangitis[24,26,44]. In 5%-10% of cases, endoscopic biliary drainage by ERCP will fail or will be incomplete[54-69]. In this case, multimodality drainage should be considered[24,26,44].

Percutaneous vs endoscopic approach

Several studies have shown a higher rate of successful palliation of jaundice and lower

Table 2 Meta-analyses comparing self-expandable metal stents with plastic stents for the endoscopic drainage of distal malignant biliary obstruction

Ref.	Studies included	Patients, *n*	Procedures	Results
Almadi *et al*[62], 2017	20	1713	Endoscopic or percutaneous palliative biliary drainage with plastic stent *vs* SEMS	Stent patency 4.45 mo (95%CI: 0.31-8.59) in favor of SEMS; Overall survival 0.67 (95%CI: 0.66-1.99), no difference
Moole *et al*[33], 2017	11	947	Endoscopic palliative biliary drainage with plastic stent *vs* SEMS	Stent occlusion OR 0.48 (95%CI: 0.34-0.67) in favor of SEMS; Overall survival/time to death: (1) SEMS, 157.3 d (95%CI: 148.9-165.6), (2) Plastic, 120.6 d (95%CI: 114.3-126.9), P = 0.0024
Zorrón Pu *et al*[64], 2015	13	1133	Endoscopic palliative biliary drainage with plastic stent *vs* SEMS	Stent dysfunction, RD -0.26 (95%CI: -0.32 to -0.20) in favor of SEMS; Survival longer in the SEMS group (187 d *vs* 162 d, P < 0.0001)
Sawas *et al*[65], 2015	19	1989	Endoscopic or percutaneous palliative biliary drainage with plastic stent *vs* SEMS	Stent occlusion, HR 0.42 (95%CI: 0.27-0.64) in favor of SEMS; 30-d survival, HR 0.82 (95%CI: 0.45-1.48), no difference
Hong *et al*[66], 2013	10	785	Endoscopic palliative biliary drainage with plastic stent *vs* SEMS	Stent patency, HR 0.37 (95%CI: 0.28-0.48) in favor of SEMS; Survival, HR 0.81 (95%CI: 0.68-0.96) in favor of SEMS

CI: Confidence interval; HR: Hazard ratio; OR: Odds ratio; RCT: Randomized controlled trial; RD: Risk difference; SEMS: Self-expandable metal stent; WMD: Weighted mean difference.

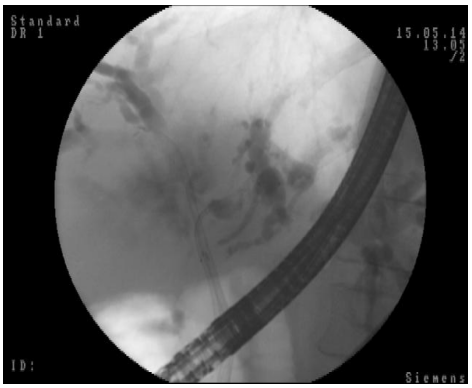

Figure 6 Endoscopic retrograde cholangiopancreatography. Radiologic view. Side-by-side technique (metallic stents in both intrahepatic ducts).

rates of cholangitis in the percutaneous approach rather than the endoscopic approach of biliary drainage in patients with malignant hilar obstruction (pCCA/gallbladder cancer)[71-73]. Bile leaks and bleeding are more frequent and morbidity and mortality are higher than the endoscopic approach[73]. Percutaneous stents are usually left to open drainage externally from the body and are less comfortable for the patient. Another technique is the combination of ERCP with percutaneous drainage.

EUS-BD: EUS-BD has been proposed as an effective alternative for PTBD after failed ERCP[74-80]. The use of EUS-BD is feasible for a left system drainage procedure in patients with advanced CCA who failed transpapillary drainage[74-80]. For extrahepatic CCA, the procedure of choice is EUS-guided hepatico-gastrostomy, which allows left system access only. It is less invasive given that it affords a more accurate control as well as more access sites to the bile duct than the classical alternatives of PTBD or surgery[77]. After the identification of the biliary duct, the technique consists of puncturing and dilatation by EUS with stent placement across the bile duct into the digestive lumen. Literature data showed a 94.0% per-protocol success rate and a 90.2% intention-to-treat basis success rate[75-81].

Peritoneal bile leakage and cholangitis are the most frequent complications[75-81]. Early migration or the clogging of the plastic stents may lead to cholangitis[76]. Bile peritonitis and biloma are more frequent in transmural SEMS placement[77,80]. However, most complications are mild and can be conservatively treated[81]. By combining an uncovered metal stent with a covered metal stent inside, the risk of leakage is minimized. The uncovered stent is initially deployed to provide anchorage and prevent migration. The covered stent is inserted coaxially and dropped in the first

stent. A fully covered SEMS[77] or a double pig-tail stent through the expanded SEMS may be used to prevent stent migration[78].

The advantages of EUS-guided hepatico-gastrostomy over rendezvous or anterograde stent insertion are particularly relevant in patients with prior duodenal or biliary SEMS who experience recurrent biliary obstruction[79,81]. Dhir *et al*[82] compared ERCP-guided biliary drainage with EUS-guided approach in patients with malignant distal obstruction who required SEMS placement. They found that the short-term outcome of EUS-BD is comparable to that of ERCP. Postprocedural pancreatitis rates were higher in the ERCP group[82,83]. Clinical efficacy of a novel technique of EUS-BD for right intrahepatic bile duct obstruction was evaluated[84,85]. Most of the studies have only shown the role of EUS-BD in distal biliary obstruction, and the utility of EUS-BD for pCCA is limited. Recent studies have reported the efficacy of EUS-BD in a setting of failed ERCP for biliary drainage in proximal malignant obstruction[86,87].

Kongkam *et al*[88] proposed a new concept of a combination of ERCP and EUS-BD for biliary drainage in pCCA as a primary biliary drainage method whereby ERCP with a single SEMS is placed into either the right or the left intrahepatic bile duct. In cases of failure of all interventional options, surgical bypass should be considered as the last rescue procedure. It is typically only performed during an unsuccessful attempt at resection, or it may be necessary in jaundice patients in whom stenting is not possible due to tumor location[1,6,7,18].

ROLE OF CHOLANGIOSCOPY

Peroral cholangioscopy (POC) allowing direct visualization of the biliary tract with targeted biopsy of suspicious lesions is a useful diagnostic procedure in the evaluation of biliary strictures (Figure 7). A recent study[89] showed that POC use for the assessment of intraductal spread in potentially resectable pCCA can accurately detect and can change surgical management. In the future, preoperative staging of CCAs should combine radiological with endoscopic (*i.e.* POC evaluation) in order to optimize surgical results.

Another study[90] compared the performance characteristics of single-operator cholangioscopy-guided biopsies and transpapillary biopsies with standard sampling techniques for the detection of CCA. It showed that single-operator cholangioscopy-guided and transpapillary biopsies improved sensitivity for the detection of CCAs in combination with other ERCP-based techniques compared to brush cytology alone. However, it seemed that these modalities did not significantly improve the sensitivity for the detection of malignancy in primary sclerosing cholangitis.

A very recent publication[91] evaluated a newly developed POC classification system by comparing classified lesions with histological and genetic findings. Thirty biopsies were analyzed from 11 patients with biliary tract cancer who underwent POC. An original classification of POC findings was made based on the biliary surface's form (F factor, 4 grades) and vessel structure (V factor, 3 grades). Histological malignancy rate increased with increasing F- and V-factor scores. The system was validated by comparing it to the histological diagnosis and genetic mutation analysis in simultaneously biopsied specimens. F-V classification is the first reported system to quantify and classify biliary tract cancer based on POC findings.

RADIOFREQUENCY ABLATION

Percutaneous image-guided RFA is a potential "new tool" for the endobiliary treatment of pCCA[92]. After selective intrahepatic duct cannulation, the 0.035-inch guidewire is placed across the stricture point. The lesion is identified during cholangiography. After the previous sphincterotomy, the RFA is performed using a specific catheter. It is mandatory for all of tumor area to be caught during the procedure. The coagulated tissue will be removed using a balloon probe, and a stent will be inserted[93]. There are only a few studies regarding the successful therapy with intraductal RFA for pCCA[94,95]. A recent study[95] including 65 patients with unresectable extrahepatic CCA showed that the mean survival time was significantly greater among those who underwent RFA plus stenting compared with stenting alone (13 mo *vs* 8 mo). At 12 mo, the survival rate was 63% in the RFA group compared with 12% in the stenting-only group. Stent patency was also longer in the RFA group (7 mo *vs* 3 mo). The adverse event rate did not differ significantly between groups (6% and 9%).

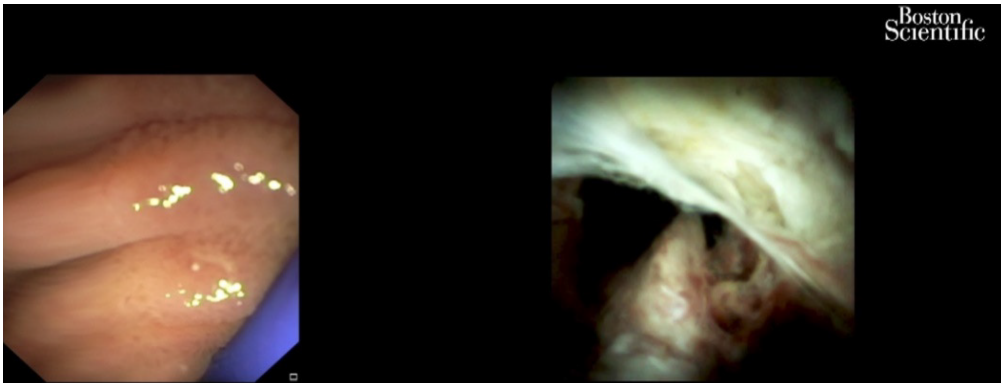

Figure 7 Cholangioscopy: Hilum malignant obstruction.

These results are overlapping with those of a meta-analysis, which was comprised of 505 patients and evaluated the effectiveness of biliary stent placement with RFA on stent patency and patient survival[27]. The pooled weighted mean difference in stent patency was 50.6 d, favoring patients receiving RFA and an improved survival in patients treated with RFA. RFA was associated with a higher risk of postprocedural abdominal pain. There was no significant difference between the RFA and stent placement-only groups with regard to the risk of cholangitis, acute cholecystitis, pancreatitis and hemobilia[27].

A prospective open-label multicenter study included 12 patients with histologically proven endobiliary adenoma remnant (ductal extent < 20 mm) after endoscopic papillectomy for ampullary tumor. RFA was performed during ERCP with biliary ± pancreatic stent placed at the end of the procedure. All underwent one successful intraductal RFA session with biliary stent placement and recovered uneventfully. Five (25%) received a pancreatic stent. The rates of residual neoplasia were 15% and 30% at 6 and 12 mo, respectively. Only two patients (10%) were referred for surgery. Eight patients (40%) experienced at least one adverse event between intraductal RFA and 12 mo of follow-up. No major adverse events occurred. Intraductal RFA of residual endobiliary dysplasia after endoscopic papillectomy can be offered as an alternative to surgery with a 70% chance of dysplasia eradication at 12 mo after a single session and a good safety profile[96].

PHOTODYNAMIC THERAPY

PDT is the use of photosensitizing agents that accumulate into the tumor. The agents are activated by laser light. Free oxygen radicals are released and destroy the neoplastic cells[69]. Apoptotic death of cells is another mechanism produced by PDT with an immunomodulatory effect. Hematoporphyrin derivatives, ∂-aminolevulinic acid and meso-tetra (hydroxyphenyl) chlorin are the photosensitizing agents used for CCA treatment[24,97]. Strong phototoxic skin reactions that can persist for weeks are a disadvantage of the use of photosensitive substances such as photofrin (porfimer sodium). The advantage of the ∂-aminolevulinic acid, which is a second generation photosensitizer, is the lack of prolonged photosensitization and laser light exposure.

The endoscopic PDT technique involves intravenous 48-h administration of the photosensitizing agent prior to the laser light illumination. The specific substance is retained in tumor cells and into the skin longer than 48-72 h like in the normal tissues. With a guidewire and a catheter, the light laser fiber is placed across the tumoral stricture (Figure 8). The power density used is 300-400 mW/cm with a power energy of 180-200 J/cm. The irradiation time is 400-600 s[97]. Due to the fact that light laser fiber is stiff, the breakage may occur in up to one third of the procedures, making the procedure a bit more cumbersome and affecting treatment cost[24,44]. The PDT is only performed in some specialized centers.

In addition to facilitating biliary decompression after stenting in patients with locally advanced disease, survival might be improved in patients who undergo PDT[98-107] (Figure 9). The data showed a survival benefit for this approach with favorable early results including longer survival and quality of life[98,105,106] (Table 3). The survival benefit was related to the prolonged relief of obstruction rather than to a reduction of the tumor. Although the factors that are associated with prolonged

Table 3 Photodynamic therapy in patients with cholangiocarcinoma

Ref.	No. patients	Median survival, d/mo	Adjuvant therapy
Ortner *et al*[106]	PDT 20, Control 19	493 d, 98 d	PDT -, Control -
Zoepf *et al*[103]	PDT 16, Control 16	630 d, 210 d	PDT -, Control -
Dumoulin *et al*[100]	PDT 24, Control 23	9.9 mo, 5.6 mo	PDT -, Control -
Kahaleh *et al*[102]	PDT 19, Control 21	8.0 mo, 5.0 mo	PDT, CTX 11; RTX 9; CTX 11, RTX 10
Witzigman *et al*[99]	PDT 68, Control 56	12.0 mo, 6.4 mo	PDT, CTX 6; RTX 2; CTX 5, RTX 1

CTX: Chemotherapy; PDT: Photodynamic therapy; RTX: Radiation therapy.

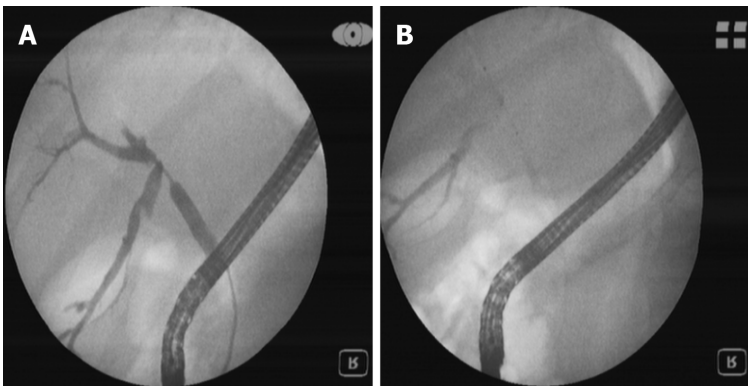

Figure 8 Endoscopic retrograde cholangiopancreatography. Radiologic view. A: Bismuth III cholangiocarcinoma (guidewire is passing through malign stenosis); B: Photodynamic therapy. The laser fiber at the level of stenosis can be seen.

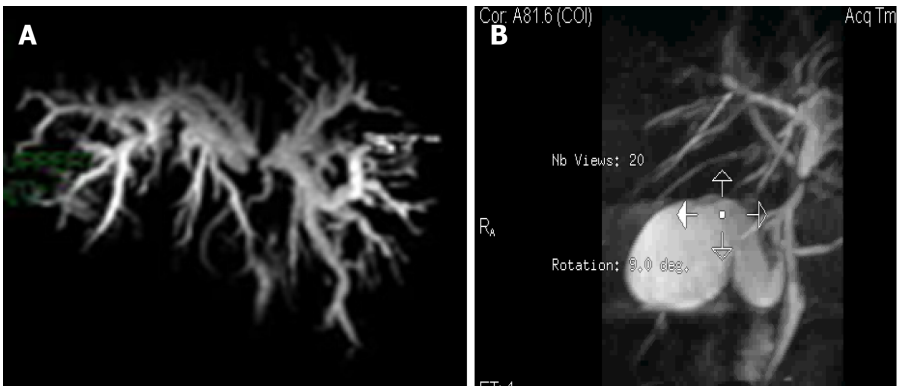

Figure 9 CholangioIRM. A: Before photodynamic therapy: Bismuth III cholangiocarcinoma (large dilatation of intrahepatic ducts can be seen); B: After photodynamic therapy: The stenosis at the level of hilium and intrahepatic dilatation have been reduced.

survival are not completely known, at least some data suggest that the absence of a visible mass on radiographic studies correlates with longer survival after PDT[44,107].

Cholangitis and a liver abscess are the main complications of photodynamic therapy[98-107]. Data suggest that combining photodynamic therapy with systemic combination chemotherapy improved outcomes over PDT alone for patients with nonresectable tumors without increasing toxicity rates, although randomized trials have not been conducted[108-112]. At the moment, PDT is being studied preoperatively as a means of improving the likelihood of achieving a margin-negative resection[113].

In a recent meta-analysis conduct by Lu *et al*[114], overall survival was significantly better in patients who received photodynamic therapy than those who did not. Among the eight trials (642 subjects), five assessed the changes of serum bilirubin levels and/or Karnofsky performance status as other indications for improvement. The incidence of phototoxic reaction was 11.11%. The incidence for other events in photodynamic therapy and the stent-only group was 13.64% and 12.79%, respectively.

A new model of a photosensitizer-embedded self-expanding metal stent (PDT-stent) that provides a photodynamic effect without a systemic injection has been developed. The treatment could be repeated due to the incorporation of the polymeric photosensitizer into the mesh of the stent. The stent maintained its photodynamic power for at least 8 wk. This type of stent after light exposure creates cytotoxic free radical, such as singlet oxygen, in the surrounding tissue and induces destruction of tumoral cells on animal models[115]. Unfortunately, PDT is not widely available and is expensive and uncomfortable for the patient.

BRACHYTHERAPY

The purpose of brachytherapy (BT) is to deliver a high local dose of radiation to the tumoral tissue while sparing healthy tissue around it. It can be adapted for right and left hepatic duct and for common bile duct lesions. It plays a limited but specific role in the curative intent treatment in selected cases of early disease as well as in postoperative small residual tumoral tissue. The indications for BT are as radical or palliative treatment. For radical treatment, it is recommended in small inoperable tumors or in combination with external beam radiation therapy and/or chemotherapy in advanced disease for unresectable tumors. BT may be used as adjuvant treatment after nonradical excision, possibly combined with external beam radiation therapy. The most common indication for BT occurs as palliative in unresectable Klatskin tumors. The purpose is to prevent locoregional disease progression and to facilitate the bile outflow. The major aim is to improve the quality of life and to increase survival. The treatment decision should be personalized[116].

ERCP-directed tumor therapy using iridium-192 ribbons *via* nasobiliary catheters in patients with pCCA as part of a neo-adjuvant treatment protocol that include external beam radiation therapy, radiation-sensitizing chemotherapy and low-dose-rate BT (< 3000 cGy) followed by liver transplant was first described in 2006[117]. High-dose-rate (HDR)-BT using 930-1600 cGy fractionated in 1-4 doses over 1-2 d was introduced in 2009[117]. The benefits of this technique are lack of irradiation of medical staff, lower time span (5-10 min), a better distribution of doses in the tumor and protection of the stomach and duodenum[117]. Using ERCP, an 8.5 Fr or 10 Fr nasobiliary tube is placed into the biliary system with the proximal end of tube at least 2 cm beyond the proximal end of the tumor. In cases of bilateral duct involvement, a second 10 Fr tube is placed. After HDR-BT is completed, the tubes and brachycatheter are removed. Nasobiliary BT catheter displacement, cholangitis, abdominal pain, duodenopathy and gastropathy are possible complications[118,119].

Some studies demonstrated longer survival in patients with CCA due to the BT. Extrahepatic localization of CCA, the absence of metastases, increasing calendar year of treatment and liver transplantation with postoperative radiation therapy were factors significantly associated with improved survival[118,119]. However, another study did not find any benefit regarding the survival in patients treated with PTBD-guided iridium-192, intraluminal BT compared with patients with only PTBD[120]. These results are in accordance with another study that found a correlation only with local tumor control[121].

In a recent study[122], 122 patients with CCA were successfully treated with HDR-BT using the nasobiliary technique. The BT was not completed in three patients because either the catheter migrated between the ERCP and the treatment (two patients) or the HDR after loader was physically unable to extend the source wire into the treatment site (one patient). These three patients benefited from an external beam boost instead of HDR-BT. Intraluminal HDR-BT with a nasobiliary catheter is a minimally invasive method for administering neoadjuvant radiotherapy.

PALLIATIVE AND ADJUVANT CHEMOTHERAPY

The assessment of patients with CCA before starting chemotherapy includes the Eastern Cooperative Oncology Group patient scale used for the evaluation of the patient performance status, disease distribution and accessibility of tumor profiling[123]. The current data support the use of first-line cisplatin and gemcitabine combination regimen chemotherapy. The multicenter phase III ABC-02 study illustrated the superiority of the combination regimen regarding median overall survival (11.7 mo) over the gemcitabine monotherapy (8.1 mo)[124,125].

New combinations and more intensive triple chemotherapy are being explored. The

combinations include: Cisplatin-gemcitabine combined with nab (nanoparticle albumin-bound)-paclitaxel[126]; S1 (tegafur, gimeracil and oteracil)[127]; and FOLFIRI-NOX (5-FU, oxaliplatin and irinotecan; AMEBICA study , NCT02591030). Acelarin is a nucleotide-analogue independent of hENT2 (also known as SLC29A2) cellular transport and is not metabolized by cytidine deaminase, resulting in greater intracellular concentrations. Cisplatin with acelarin was compared with the classic combination regimen of cisplatin and gemcitabine in a phase III study[128].

A recent phase III clinical trial ABC-06[129] randomly assigned 162 patients with advanced biliary cancer (72% with CCA) who obtained symptom control from first-line cisplatin-gemcitabine (81 patients) or second-line chemotherapy with FOLFOX (folinic acid, 5-FU and oxaliplatin) (81 patients). The results showed a benefit from second-line chemotherapy regarding survival at 6 mo (35.5% *vs* 50.6%) and 12 mo (11.4% *vs* 25.9%), but no significant differences regarding overall survival (5.3 mo *vs* 6.2 mo) were observed.

A very difficult to handle and a major issue in the management of patients with CCA is the poor response to pharmacological treatment. A cause could be the poor understanding of the mechanisms of chemoresistance. To identify the so-called "resistome" that includes a set of proteins involved in the lack of response to chemotherapies is required to increase efficacy. Genes involved in mechanisms of chemoresistance are usually expressed by normal cholangiocytes because one of their roles is the protection against potentially harmful compounds present in bile. Their expression during carcinogenesis contributes to intrinsic chemoresistance, and upregulation in response to treatment leads to acquired chemoresistance[130-132].

MOLECULAR TARGETED THERAPY

Recent molecular studies have increased the understanding of the pathogenetic mechanism of CCAs, but to date the clinical data on immune-directed therapies in CCA are limited.

Inhibitors of isocitrate dehydrogenase (IDH) 1, IDH2 and pan-IDH1-IDH2 are currently being tested in patients with intrahepatic CCA. Ivosidenib (IDH1 inhibitor) was tested in 73 patients with IDH1-mutant advanced CCA in a phase I study with no major adverse events reported[133]. A recent preliminary phase III trial showed a benefit for ivosidenib over placebo in terms of progression free-survival. One hundred eighty-five patients with IDH1 mutant CCA were randomly assigned to ivosidenib or placebo. This study highlighted the importance of molecular profiling in CCA[134] .

There are some phase II studies with encouraging preliminary data for fibroblast growth factor receptor inhibitors in patients with CCA. Some fibroblast growth factor receptors inhibitors are currently being evaluated as first-line treatment, for example the FIGHT-302 study (NCT03656536) and the PROOF study (NCT03773302)[135-137].

CONCLUSION

CCAs are heterogeneous and highly aggressive tumors with a poor prognosis despite the progress of the research in this field. Surgical resection is still the only potential curative treatment method. The recent findings on understanding the mechanism of chemoresistance and molecular targeted therapy could bring a new horizon in the approach of these tumors. Currently, endoscopic treatment in patients with CCA and jaundice remain the first choice of biliary duct decompression, either preoperatively or with a palliative purpose. The combination of endoscopic procedures with nonsurgical local methods or additional therapies may increase the quality of life and the rate of survival in patients with locally advanced, unresectable or recurrent disease.

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ORIGINAL ARTICLE

Basic Study

Adult human liver slice cultures: Modelling of liver fibrosis and evaluation of new anti-fibrotic drugs

Daria Kartasheva-Ebertz, Jesintha Gaston, Loriane Lair-Mehiri, Pierre-Philippe Massault, Olivier Scatton, Jean-Christophe Vaillant, Vladimir Alexei Morozov, Stanislas Pol, Sylvie Lagaye

ORCID number: Daria Kartasheva-Ebertz 0000-0001-9509-0253; Jesintha Gaston 0000-0003-4716-7409; Loriane Lair-Mehiri 0000-0003-4428-8272; Pierre-Philippe Massault 0000-0002-4728-3816; Olivier Scatton 0000-0001-8313-3500; Jean-Christophe Vaillant 0000-0003-0563-120X; Vladimir Alexei Morozov 0000-0001-7013-7115; Stanislas Pol 0000-0001-9772-9591; Sylvie Lagaye 0000-0002-9156-3698.

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Daria Kartasheva-Ebertz, Jesintha Gaston, Loriane Lair-Mehiri, Stanislas Pol, Sylvie Lagaye, Institut Pasteur, Immunobiologie des Cellules Dendritiques, INSERM U1223, Paris 75015, France

Daria Kartasheva-Ebertz, Jesintha Gaston, Loriane Lair-Mehiri, BioSPC, Université de Paris, Paris 75005, France

Pierre-Philippe Massault, Service de Chirurgie digestive, Hépato-biliaire et Endocrinienne, AP-HP, Groupe Hospitalier Cochin, Paris 75014, France

Olivier Scatton, Jean-Christophe Vaillant, Service de Chirurgie digestive et Hépato bilio pancréatique, AP-HP, Groupe Hospitalier La Pitié-Salpétrière, Medecine Sorbonne Université, Paris 75013, France

Vladimir Alexei Morozov, Center for HIV and Retrovirology, Department of Infectious Diseases, Robert Koch Institute, Berlin 13353, Germany

Stanislas Pol, Département d'Hépatologie, AP-HP, Groupe Hospitalier Cochin, Université de Paris, Paris 75014, France

Corresponding author: Sylvie Lagaye, DSc, PhD, Senior Scientist, Institut Pasteur, Immunobiologie des Cellules Dendritiques, INSERM U1223, 25-28 rue du Dr Roux, Paris 75015, France. sylvie.lagaye@inserm.fr

Abstract

BACKGROUND

Liver fibrosis can result in end-stage liver failure and death.

AIM

To examine human liver fibrogenesis and anti-fibrotic therapies, we evaluated the three dimensional *ex vivo* liver slice (LS) model.

METHODS

Fibrotic liver samples (F0 to F4 fibrosis stage according to the METAVIR score) were collected from patients after liver resection. Human liver slices (HLS) were cultivated for up to 21 days. Hepatitis C virus (HCV) infection, alcohol (ethanol stimulation) and steatosis (palmitate stimulation) were examined in fibrotic (F2 to

Publique-Hôpitaux de Paris (AP-HP, France).

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F4) liver slices infected (or not) with HCV. F0-F1 HLS were used as controls. At day 0, either ursodeoxycholic acid (choleretic and hepatoprotective properties) and/or α-tocopherol (antioxidant properties) were added to standard of care on HLS and fibrotic liver slices, infected (or not) with HCV. Expression of the biomarkers of fibrosis and the triglyceride production were checked by quantitative reverse transcription polymerase chain reaction and/or enzyme-linked immunosorbent assay.

RESULTS

The cultures were viable *in vitro* for 21 days allowing to study fibrosis inducers and to estimate the effect of anti-fibrotic drugs. Expression of the biomarkers of fibrosis and the progression to steatosis (estimated by triglycerides production) was increased with the addition of HCV and /or ethanol or palmitate. From day 15 of the follow-up studies, a significant decrease of both transforming growth factor β-1 and Procol1A1 expression and triglycerides production was observed when a combined anti-fibrotic treatment was applied on HCV infected F2-F4 LS cultures.

CONCLUSION

These results show that the human three dimensional *ex vivo* model effectively reflects the *in vivo* processes in damaged human liver (viral, alcoholic, nonalcoholic steatohepatitis liver diseases) and provides the proof of concept that the LS examined model permits a rapid evaluation of new anti-fibrotic therapies when used alone or in combination.

Key Words: Human liver fibrosis; Hepatitis C virus; Alcoholic liver disease; Nonalcoholic steatohepatitis; *Ex vivo* model; Drugs

Core Tip: In the developed world, about 45% of deaths are due to fibroproliferative diseases. Liver fibrosis is frequently associated with viral infection (Hepatitis C virus and Hepatitis B virus infection), chronic inflammation and excessive alcohol consumption. Despite the availability of effective antiviral drugs, morbidity, and mortality related to viral hepatitis are still increasing. Moreover, the number of non-viral liver diseases such as nonalcoholic steatohepatitis, and alcoholic liver disease is steadily growing. Our studies provide the proof of concept that the three-dimensional *ex vivo* model of human liver slice culture can be used for the molecular investigation of fibrosis as well as to perform follow-up studies of new anti-fibrotic drugs and therapies for a 21-days period.

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INTRODUCTION

Forty five percent of deaths in the developed countries may be attributed to fibroproliferative diseases[1]. Liver fibrosis is frequently associated with viral infection [Hepatitis C virus (HCV) and Hepatitis B virus (HBV)] infection, chronic inflammation, and excessive alcohol consumption. Despite effective antiviral treatment, morbidity and hepatitis-related mortalities are still increasing. Moreover, the number of non-viral liver diseases such as nonalcoholic steatohepatitis (NASH) and alcoholic liver disease (ALD) is steadily growing[2].

Progression to liver fibrosis is a multistep process, whose development time varies. Fibrosis is initiated by the activation of hepatic stellate cells triggered by several signaling pathways[3]. The activation of stellate cells induces cellular matrix production

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and collagen 1 expression This process is stimulated by transforming growth factor-β1 (TGF-β1), which is a crucial element involved in fibrogenesis[4]. The progression of liver fibrosis frequently results in cirrhosis (liver acini are substituted by regeneration nodules surrounded by fibrosis) and, further on, in the development of hepatocellular carcinoma. Liver fibrosis can persist even with effective treatments. In most cases, the necro-inflammation leading to fibrosis can be effectively treated by treatments with antiviral drugs that target HCV, by nucleoside analogs in patients with HBV, by immune suppression in autoimmune hepatitis, by ethanol weaning and other dietary approaches in ALD and NASH, and iron chelation for hemochromatosis. However, if patients are not treated in a timely manner, and fibrosis progresses to decompensated cirrhosis, the only remaining option is liver transplantation The main obstacles (or delays) to liver transplantation are an insufficient number or a shortage of suitable organs, long waiting lists and high cost of this procedure[5]. Thus, mortality remains high in patients on the waiting list and new anti-fibrotic agents and new clinical strategies to manage patients in the different stages of liver fibrosis are needed.

The liver slices (LS) cultures are appropriate models to study liver fibrosis, because they maintain the complex cellular interactions that occur *in vivo*, which cannot be obtained in co-cultures systems[6]. These cultures can be used to study molecular biological events either in the fibrotic liver tissue or in hepatocellular carcinoma tissue. Although the LS cultures from non-fibrotic and fibrotic rat livers have been used to investigate the early and late phases as well as the resolution of liver fibrosis[7,8], the experiments are limited to 3 days in the rat model[7-9], and to 15 days in the human non-fibrotic LS model[10]. In previous studies, we developed a three dimensional (3D) *ex vivo* model of HCV replication using human LS cultures that were followed for 10 days[11] to evaluate a new antiviral drug[12].

Here, for the first time, human fibrotic LS cultures (stages F2-F4) were successfully maintained and evaluated for 21 days. Using the *ex vivo* LS model for a 21-d period makes it possible to explore molecular fibrogenesis in more detail including the role of important factors such as HCV infection, ethanol (EtOH), or steatosis. Thus, this model can improve the understanding of the three of the main causes of liver injury in clinical practice[2]. In addition, it was demonstrated that LS cultures are efficient instruments to study anti-fibrotic drugs and their combination[13,14].

This study provides the proof of concept that the *ex vivo* model of human LS culture can be used for the molecular evaluation of fibrosis and to perform follow-up studies of new anti-fibrotic drugs and therapies for a 21-days period.

MATERIALS AND METHODS

Patients and human liver tissue specimens

Adult human liver tissue samples were obtained from selected patients with different liver pathologies, as previously described[11,12]. Written informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee. Experimental procedures were carried out in accordance with French laws and Regulations and ethic committees from Pitié-Salpétrière Hospital, Cochin Hospital, and Pasteur Institute (France). The tissue samples from twenty patients were divided into three groups according to their METAVIR score[15]. Liver samples were either non-fibrotic F0-F1, obtained during surgery for colorectal cancer liver metastases or fibrotic ranging from F2 to F4 according to the METAVIR score (Table 1). Significant necrotic inflammation as defined by an activity grade (A) was not always available.

Liver slices preparation, culture and infection

We obtained between 32 to 48 liver slices for each donor sample. On the different days of the kinetic experiments, the results were obtained from the mean of three liver slices from each donor. The liver slices were infected with a same viral stock. The liver slice cultures were inoculated with viral supernatant diluted in fresh medium, at MOI = 0.1 (multiplicity of infection) and incubated overnight at 37 °C. In order to remove free virus, the slices were washed three times with phosphate-buffered saline (PBS) and fresh complete culture medium was added, after which cultures were followed in the absence of additional changes to the media composition or replacement with fresh culture medium. The preparation and culture of the liver slices, HCV RNA transfection, virus production, HCV RNA extraction were performed as previously described[11,12].

Table 1 METAVIR scores and description of clinical liver samples

METAVIR score		Patients (*n*)	Pathology
F0-F1	No fibrosis or mild fibrosis	10	HBV-, HCV-, HIV-seronegative patients who underwent liver resection surgery, mainly for liver metastasis, in the absence of underlying liver disease. A0-F1
F0-F1	No fibrosis or mild fibrosis	1	Prior history: Breast cancer with liver metastases, treated by surgery and radio-chemotherapy. Non-tumoral liver sample: Perisinusoidal and portal fibrosis without septa (F1). No steatosis
		2	Prior history: HCV infection, resected hepato-cholangiocarcinoma. Non tumoral liver samples: A0F0
F2-F3	Moderate to severe fibrosis	2	Cholangiocarcinoma, non-tumor liver samples
		1	Chronic hepatitis B infection, NASH, and two resected hepatocellular carcinoma nodules. Non-tumoral liver sample: Chronic hepatitis with extensive fibrosis A1F3
F4	Cirrhosis	2	HCC, non-tumor liver samples
		1	HCC, treated HCV infection. Non-tumoral liver sample
		1	HCC on untreated HCV infection. Non-tumoral liver sample

A "significant" fibrosis, as defined by a fibrosis grade (F), is greater than 1 by the METAVIR scoring system, with usually a significant necrotic inflammation as defined by an activity grade (A) greater than 1 by the METAVIR scoring system. Fibrosis grade: F0: No fibrosis, F1: Portal fibrosis without septa, F2: Portal fibrosis with few septa, F3: Numerous septa without cirrhosis, F4: Cirrhosis. Activity grade: A0: No activity, A1: Mild activity, A2: Moderate activity, A3: Severe activity. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; HIV: Human immunodeficiency virus; NASH: Nonalcoholic steatohepatitis.

Liver slices viability

Special attention was paid to the condition of clinical liver samples. It is evident that the condition of the liver sections that we obtained was different. Thus, they were carefully selected for *in vitro* studies. In fact, cell viability was estimated by determining the percentage of viable cells upon microscopic examination 10X, using live/dead fixable dead cell stain kit (Molecular Probes, Invitrogen, ThermoFisher, France) and, as the percentage of ATP production determined by enzyme-linked immunosorbent assay (ELISA) assays, while observing the increasing albumin and urea secretion levels throughout the experiments, which indicates that the physiological and biochemical parameters of the liver slices are normal. On day 15, the immunostaining for Ki67, a cellular marker for proliferation confirmed the cell viability. Only slices with viability greater than 80% were used and allowed to obtain all the presented results. The architecture of human LS cultures was accessed by hematoxylin-eosin (HE) staining performed as following: Cryosections were washed with distilled water for 5-10 min and then stained for 8 min with hematoxylin, followed by a washing step with warm water at 30 °C for 10 min. After a short washing step with distilled water, the slices were counter-stained for 6 min with eosin. Washing was followed by dehydration steps in 2 min intervals in 50%, 60%, 70%, 80% and 90% of ethanol.

Experimental set up

The experimental set up was as follows (Figure 1). Non-infected liver slices obtained either from human non-fibrotic (F0-F1) or fibrotic (F2-F3, F4) liver resection and cut in 350 µm-thick slices (approximately 2.7×10^6 cells per slice), were cultivated for up to 21 days either with or without HCV, ethanol (EtOH) (1 mmol/L, 5 mmol/L, 25 mmol/L) or palmitate (500 µmol/L). Liver slices were infected with hepatitis C virus infection from cell culture (HCVcc) supernatant [Con1/C3 (genotype1b)][16] (MOI = 0.1) (INF LS) in presence or not either of EtOH (1 mmol/L, 5 mmol/L, 25 mmol/L) or palmitate (500 µmol/L). The different concentrations of EtOH were added on days 0, 5, 10, 15 during the kinetic studies. Palmitate (500 µmol/L) was added or not to non-infected and infected liver slices on days 0, 5, 10 and 15 of the kinetic studies. As previously described, infectivity (ffu/mL) was measured on days 1, 5, 10, and 21 post-treatment depending on the experiment[11,12]. All experiments were performed in triplicate. All data were presented in relation to the percentage of viable liver slices in culture. Once the model was validated for the presence of "molecular fibrogenesis" defined as a significant increase in fibrosis biomarkers [TGF-β1, Hsp47, α-SMA, Procol1A1, matrix

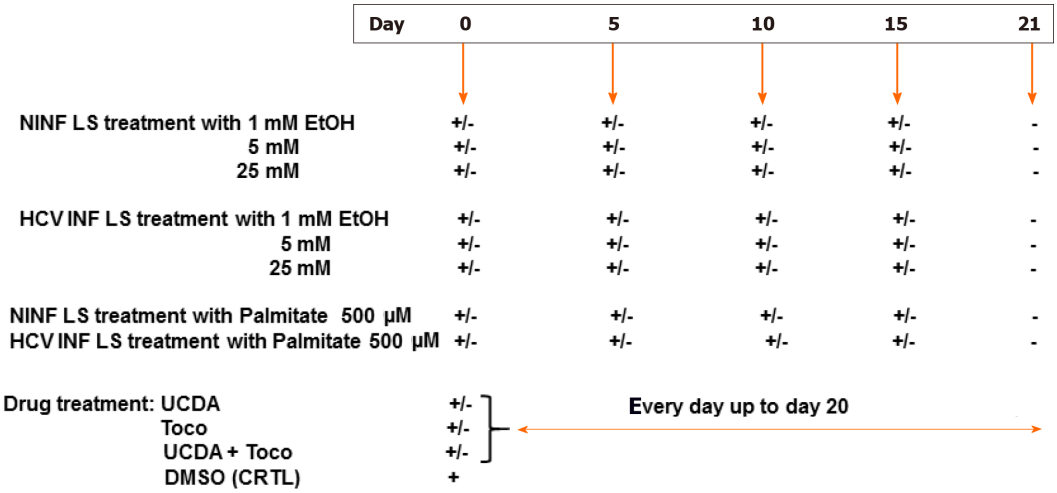


Figure 1 Experimental set up of the different liver slice treatments during the cultures. NINFLS: Non-infected liver slices; EtOH: Ethanol; LS: Liver slices; Toco: Tocopherol; UCDA: Ursodeoxycholic acid; HCV: Hepatitis C virus.

metalloproteinases 2 (MMP-2), MMP-9, and vascular endothelial growth factor (VEGF)], we evaluated the anti-fibrotic properties of two drugs, ursodeoxycholic acid (UCDA) (Sigma-Aldrich, Merck, Germany) and α-Tocopherol (Toco) (Sigma-Aldrich, Merck, Germany). UCDA (240 ng/liver slice) and / or α-Toco (170 ng/liver slice) were added to the culture media from day 0 and every day up to day 20 of the culture. The estimation of the triglyceride content was essential during the different kinetic experiments, since its accumulation in the cytoplasm of hepatocytes indicates cell metabolism disturbances, typical of non-alcoholic fatty liver disease[17].

Quantification of HCV RNA and liver-specific and fibrosis markers genes expression by real-time reverse transcription-quantitative polymerase chain reaction

The liver slices were washed three times in PBS at 4 °C. RNA was extracted from three combined slices using Trizol reagent as described in the protocol (Invitrogen, Cergy Pontoise, France). A strand-specific real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) technique to quantify the intracellular levels of positive and negative-strand HCV RNA was performed during the experiments with the quantification of 28S rRNA used as an internal standard to quantify HCV in total liver RNA as previously described[11], (detection threshold: 25 copies/reaction). Briefly, reverse transcription was performed using an oligo primer and Moloney murine leukemia virus reverse transcriptase (Promega, Charbonnières, France) according to the manufacturer's instructions. Real-time polymerase chain reactions were performed using Light CylclerR (Roche Applied Science, Grenoble, France) and FastStart DNA Master SYBR Green I kit (Roche Applied Science, Grenoble, France) according to the manufacturer's instructions.

The relative expression of each liver-specific transcript (albumin, HNF-1β, HNF-4α transcription factors, cytochrome P450 enzymes, CYP2E1 and CYP3A4) was quantified by qRT-PCR and normalized to 18S RNA transcripts[11,12]. The relative expression level of the transcripts was then determined in relation to the 18S RNA by the (Ct) method[13]. The PCR conditions were as follows: Denaturation for 10 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 30 s. The specificity of the PCR products was checked by a melting curve analysis after amplification. Primer sequences are listed in Table 2.

The expression of fibrosis markers in either non-infected liver slices (used as controls, CRTL) or in HCV-infected (INF) liver slices with or without the presence of EtOH or palmitate were evaluated by RT-qPCR with the SYBR PrimeScript RT-qPCR Kit (TaKaRa Bio Inc., Japan) and performed with the housekeeping gene, GAPDH as an internal control. Real-Time qPCR reaction for fibrosis markers including TGF-β1, heat shock protein 47 (Hsp47), alpha smooth muscle actin (β-SMA), procollagen1 A1 (Procol1A1), and VEGF was performed as follows: Denaturation for 10 min at 95 °C followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min, and elongation at 72 °C for 30 s. Concerning the MMP-2, MMP-9 gene expression, the Real-Time qPCR reaction was performed as follows: Denaturation for 10 min at 95 °C followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C and 68 °C

Table 2 Primers used for real-time reverse transcription-quantitative polymerase chain reaction analysis

Gene	Forward primer sequence	Reverse primer sequence
CYP2E1	AGCACAAACTCTGAGATATGG	ATAGTCACTGTACTTGAACT
CYP3A4	GCCTGGTGCTCCTCTATCTA	ACAGGCTGTTGACCATCATAAAAG
HNF-1β	ACGTCAGAAAGCAACGAGAGATC	CCCAGGCCCATGGCT
HNF-4α	CCTGGAATTTGAGAATGTGCAG	AGGTTGGTGCCTTCTGATGG
Albumin	ATGAGATGCCTGCTGACTTG	GCACGACAGAGTAATCAGGA
18S RNA	CAGAGCGAAAGCATTTGCCAAG	CGGCATCGTTTATGGTCGGAAC
TGF-β1	CCTGGAAAGGGCTCAACAC	CAGTTCTTCTCTGTGGAGCTGA
HSP 47	GCCACCGTGGTGCCGCA	GCCAGGGCCGCCTCCAGGAG
β-SMA	AGGGGGTGATGGGTGGGAA	ATGATGCCATGTTCTATCGG
Procol1A1	CAATCACCTGCGTACAGAACGCC	CGGCAGGGCTCGGGTTTC
MMP-2	CTT CGCCCC AGG CAC TGG TG	CCTCGCTCCCATGGG GTT CGGT
MMP-9	GGT CCCCCCACT GCT GGC CCTTCTACGGCC	GTCCTCAGG GCACTG GAG GAT GTC ATA GCT
VEGF	TACCTCCACCATGCCAAGTG	ATGATTCTGCCCTCCTCCTTC
GAPDH	ACCAGGGCTGCTTTTAACTCT	GGTGCCATGGAATTTGCC

respectively, for 1 min, and elongation at 72 °C for 30 s. Ct (threshold cycle) values were corrected for the Ct values of the housekeeping gene GAPDH. Primer sequences are listed in Table 2.

Albumin enzyme-linked immunosorbent assay: Human liver albumin concentrations were determined by a competitive ELISA as previously described[18,19]. Purified human albumin and peroxidase-conjugated anti-human albumin were obtained from MP Biomedicals Europe (Illkirch, France). To ensure the specificity of the ELISA, human antibodies were incubated for 2 h at 37 °C with 3% BSA in 0.5% Tween-20 in PBS before the sample addition in order to block any cross reaction.

Urea assays: Urea concentrations were determined by colorimetric assay (640-1, Sigma-Aldrich) according to the manufacturer's recommendations and analyzed with BioPhotometer 6131 (Eppendorf, Hamburg, Germany).

Western blotting and antibodies

Western blotting was performed as previously described[11,12], and the antibodies used are described as following. Mouse monoclonal antibodies (mAbs) to HCV core protein (C7-50; dilution 1/10000, Affinity BioReagents, Golden, CO, United States), HCV nonstructural protein 3 (clone1847, dilution: 1/2000, Viro-Stat, Portland, ME, United States) were used to analyze HCV expression, mouse monoclonal antibodies (mAbs) TGF-β1 (ab 190503, dilution: 1/2000, Abcam, United Kingdom), HSP-47 (M16.10A1, dilution: 1/1000, Enzo life sciences, France), Collagen I alpha 1 (NB600-450, dilution: 1/2000, Novus Biologicals, CO, United States), MMP-9 (ab119906, dilution: 1/2000, Abcam, United Kingdom), VEGF (ab69479, dilution: 1/2000, Abcam, United Kingdom), β-actin (A5316, dilution: 1/5000 Sigma-Aldrich, Merck, Germany), and rabbit polyclonal antibodies (rAbs) to MMP-2 (ab92536, dilution: 1/1000, Abcam, United Kingdom), alpha-smooth muscle actin [alpha-SMA (ab 5694, dilution: 1/2000, Abcam, United Kingdom)] allowing fibrosis analysis, were used as primary antibodies. Horseradish peroxidase-conjugated anti-mouse IgG and horseradish peroxidase-conjugated anti-rabbit IgG (Amersham, GeHealthCare Life Sciences, United Kingdom) secondary antibodies, taken 1:50000, were used as secondary antibodies. The reactions were developed using enhanced chemiluminescence detection reagents (ECL Advance kit, Amersham, GeHealthCare Life Sciences, United Kingdom), followed by exposure to X-OMAT film (Amersham, GeHealthCare Life Sciences, United Kingdom).

Histology and immunohistochemistry

Liver sections (7 μm) were stained with Goldner's trichrome (Electron Microscopy Sciences, United States) or picrosirius red (Abcam, United Kingdom), performed

standard protocols for collagen/connective tissue labelling using two slices per human liver sample and two different human liver samples per group. The images were taken with the EVOS XL Core Imaging System (Invitrogen, Thermo Fisher Scientific, France). The average integrated optical density (OD) of collagen deposition was calculated using the image quantification standard software, ImageJ2[20,21] or inform V2.1 (Perkin Elmer, MA, United States) used routinely in the histology (HISTIM) facilities (Cochin Institute, Paris, France). Immunostaining for TGF-β1 (mAbs, ab92486, Abcam, United Kingdom), MMP-9 (mAbs, ab119906, Abcam, United Kingdom), Ki67 (rAbs, ab15580, Abcam, United Kingdom), and alpha-SMA (rAbs, ab5694, Abcam, United Kingdom) was performed after paraffin removal in xylene, rehydratation in EtOH and then distilled water following the manufacturer's instructions. Unmasking of the antigenic sites was performed at 120 °C in 10 mmol/L citrate buffer, pH 6.0. A solution of 3% H_2O_2 was used to eliminate endogenous peroxidases. The sections were washed 3 times for 5 min. in TBS-Triton 0.1% solution. After incubation in a blocking solution (TBS-Triton 0.1%-3% dry milk) for 1 h at room temperature, they were incubated with the primary antibodies. All primary antibodies were diluted at 1/50 in the blocking solution. After incubation for 2 h at room temperature, the sections were washed 3 times and incubated with secondary antibodies. The nuclei were stained with DAPI. All sections were counterstained with hematoxylin for tissue quality control. Control sections incubated with non-immune serum were used as negative controls.

TGF-β1 and Triglyceride quantification

TGF-β1 and triglyceride quantification were performed according to the manufacturer's instructions (TGF-β1 Quantikine ELISA, RD Systems, United States; Triglyceride assays Kit–Quantification, ab65336, Abcam, United Kingdom). For TGF-β1, cellular lysates and culture supernatants were first treated with acid to lower the pH to 2.0, which denatures the latency-associated peptide and allows the detection of active TGF-β1. The supernatant was then brought back to neutral pH before the ELISA assays.

ATP production quantification and LDH assays

To check viability, the percentages of ATP was assessed at each point of the kinetics studies during the liver slices culture and determined by ELISA assays (CellTiter-Glo® 2.0 Assay, Promega, France)[19]. The viability of liver slices and the potential cytotoxicity[20] (cytoTox 96R Non-Radioactive Cytotoxicity Assay, Promega, France) induced by Ethanol, or Palmitate, or drugs treatments was estimated as described previously[11,12], in accordance with the manufacturers' protocols.

Drugs inhibition of fibrosis markers expression and cytotoxicity assays

Human LS were infected or not with the HCVcc Con1/C3 supernatant as previously described[11,12]. On day 0 of the culture, treatment either with (240 ng/Liver slice) UCDA or (170 ng/Liver slice) Toco or both (the recommended standard of care) or 0.5% of dimethyl sulfoxide (Sigma Aldrich, Merck, Germany) as a control, were added to HCV-infected or non-infected LS culture medium every day to day 20. TGF-β1 and Procol1A1 RNA expression were measured at different time points of the kinetic studies. All experiments were performed in triplicate.

Statistical analysis

Liver specimens from 20 individuals were examined. During the kinetic studies, the quantification of gene expression was determined in relation to the percentage of liver slice viability. The results were obtained from the mean of the three liver slices, on the different days of the kinetic studies. Statistical tests were performed using GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, United States). Values are expressed as means ± standard errors of the mean. The data were compared using either the unpaired two-tailed Student's *t*-test or the two-way ANOVA test with multiple comparisons for a given day as compared to the standard LS. A *P* value of 0.05 or less was considered significant.

RESULTS

Maintenance of phenotypic characteristics and viability of three dimensional human non-fibrotic (stages F0-F1) and fibrotic (stages F2-F4) LS cultures for 21 days of cultivation

The viability of human LS cultures during prolonged studies was and is a crucial factor. Liver slices viability (percentage of ATP production) and tissue morphology were assessed daily, until day 21. The architecture of the liver slices was normal (Figure 2A) and human liver slices (HLS) expressed the Ki67 protein, a proliferation marker (Figure 2B). Human LS cultures maintained their differentiation status throughout the entire study period, as previously described (Figure 2A-C)[10,12]. Indeed, LS status was confirmed by analysing various parameters and biomarkers, in particular, albumin content, hepatocyte nuclear factors HNF-1β, HNF-4α, CYP2E1, and CYP3A4 (Figure 2D)[10-12,22-24]. A comparison of the expression of hepatocyte-specific genes in F0-F1 non-infected liver slices and Huh-7.5.1 cells showed increased expression in F0-F1 non-infected liver slices on day 21 compared to that in Huh-7.5.1 cells, either at an exponential growth phase or at the confluence (data not shown). CYP3A4 expression was undetected in Huh-7.5.1 cells whatever the growth stage[25]. Albumin and urea secretion increased throughout, indicating that liver slices had retained normal physiological and biochemical parameters (Figure 2E-F)[11,12]. As previously reported[11], the cell viability and expression of hepatocyte-specific genes were also evaluated post- HCVcc[11]. Results were similar to those in uninfected liver slices, indicating that there was no evident cytopathic effect (Figure 3A-C).

The viability of non-fibrotic (F0-F1) and fibrotic (F2-F4) LS cultures and resistance to EtOH and palmitate treatments were tested during the 21 d follow-up studies by evaluating of the rate of ATP production in the liver slices (Figure 4A, C-E and G)[22], and by quantification of LDH release from liver slices (Figure 4B-F)[23]. On day 21, the F0-F1 and F2-F4 non-infected liver slices had a viability rate of 75% and 50%, respectively (Figure 4A). Following treatment with 25 mmol/L of EtOH, ATP synthesis in F0-F1 infected liver slices was reduced by 55% on day 21 (Figure 4D), and tissue viability decreased by nearly 25% compared to untreated F0-F1 non-infected/infected liver slice cultures (Figure 4C and D). However, the addition of EtOH (25 mmol/L) did not change LDH release in F0-F1 and F4 non-infected LS cultures (Figure 4F). Treatment with palmitate (500 μmol/L) did not reduce significantly the viability rate of F0-F1 non-infected and infected LS cultures, compared to untreated and non-infected LS cultures (55% and 65%, respectively) (Figure 4G). There was no significant difference in LDH release from F0-F1, F2-F3, and F4 non-infected and infected LS cultures after treatment with the combination of UCDA and alfa-Toco (Figure 4H and I). Results of ATP production in F0-F1, F2-F3, and F4 non-infected and infected LS cultures after treatment with the combination of UCDA and alfa-Toco were significantly positive (Figure 4J-K) with increased ATP production in Fibrotic treated liver slices. These results, showing no significant changes in viability (with increasing levels of albumin, urea secretion as well as ATP production throughout the experiments) or morphology (Ki67 marker expression), confirm that the non-fibrotic (F0-F1) and fibrotic (F2-F4) LS cultures can survive for 21 days, and that the 3D LS cultures tolerated the different treatments (Figure 4H-K). Thus, LS cultures from selected donors can be used in extended research.

Evaluation of the expression of fibrogenesis liver biomarkers in 3D LS cultures from non-fibrotic (F0-F1) and fibrotic (F2-F4) livers

Activation or down-regulation of certain biomarkers reflects the process of the transition of the non-fibrotic liver to the fibrotic liver designated as the molecular fibrogenesis. We measured the expression of seven fibrosis biomarkers (TGF-β1, Hsp47, α-SMA, Procol1A1, MMP-2, MMP-9, and VEGF)[1,26] by RT-qPCR to analyse both non-fibrotic (F0-F1) and fibrotic (F2-F3, F4) stages of the liver in human LS cultures.

Induction of fibrogenesis by three exogenous factors: HCV, EtOH, and fatty acids (palmitate) in non-fibrotic (F0-F1) and fibrotic (F2-F4) LS cultures

HCV efficiently replication in LS cultures: A model of the viral liver disease: Robust replication of HCVcc and production of infectious viral particles were detected up to day 21 in human F0-F1 LS (Figure 5). Intracellular replication of the viral genome was assessed by a strand-specific RT-qPCR, as previously described[11]. The HCV RNA negative strand, proof of HCV genome replication, could be detected as early day 1

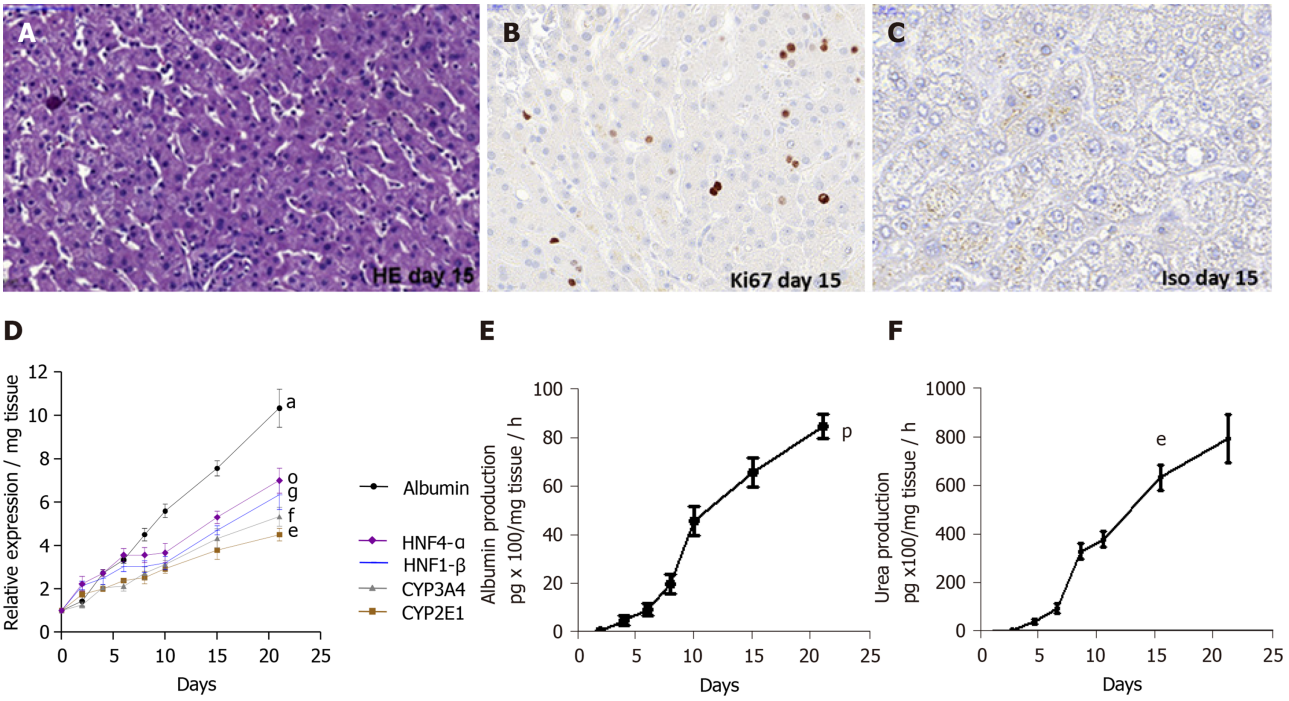


Figure 2 Maintenance of phenotypic characteristics of human non-fibrotic (F0-F1), and fibrotic (F2-F3, F4) liver slices during the culture, demonstrated by histochemistry, real-time reverse transcription-quantitative polymerase chain reaction and biochemical assays. A: Light microscopy of human liver tissue 7 μm-thick section stained with hematoxylin and eosin showing non-fibrotic (F2-F3) liver lobular architecture on day 15, magnification × 20. Scale bars 100 μm; B: Representative human liver tissue 7 μm-thick sections from fibrotic (F2-F3) liver patient showing immunostaining for Ki67, a proliferation marker, on day 15, magnification × 40, Scale bars, 20 μm; C: Representative human liver tissue 7 μm-thick sections from fibrotic (F2-F3) liver patient showing immunostaining with isotype as negative control, on day 15, magnification × 40, Scale bars, 20 μm; D: Hepatocyte-specific gene mRNA expression (relative expression/mg tissue) during the 21 days follow up studies. Maintenance of hepatocyte-specific gene expression patterns in human non-fibrotic (F0-F1) non-infected liver slices during culture. The real-time reverse transcription-quantitative polymerase chain reaction analyses were performed from five independent human non-fibrotic (F0-F1) livers using slices in triplicate from each liver. All liver-specific gene expression values were normalized to 18S RNA as an internal standard and expressed relative to the zero-time point. Values are expressed as mean ± standard errors. The results were compared using the two-paired Student's *t*-test: Albumin: $^{a}P < 0.0001$; CYP2E1: $^{e}P < 0.001$; CYP3A4: $^{f}P < 0.0003$; HNF1-β: $^{g}P < 0.01$; HNF4-α: $^{o}P < 0.008$; E and F: Biochemical functional assays; E: Albumin production (pg x 100/mg tissue/hour) during the 21 days follow up studies; and F: Urea production (pg/mg tissue/hour) during the 21 days follow up studies. Studies were done in triplicate and repeated twice for each liver sample. Values are expressed as means ± standard errors ($n = 5$). The results were compared using the two-paired Student's *t*-test: albumin production ($^{p}P < 0.02$), urea production ($^{e}P < 0.001$).

post-infection, and the intracellular levels of both negative and positive strands increased significantly during LS culture. These results confirmed active viral replication in LS cultures (Figure 5A). The HCV expression level was significantly increased in the LS culture on day 5 post-infection (Figure 5A). HCV protein expression was confirmed by Western blotting. Detection of core and nonstructural protein 3 proteins confirmed effective intracellular processing of the viral protein precursor[11] (Figure 5B and C).

The virus titer was estimated in LS culture supernatants using a classic titration assay on Huh-7.5.1 cells to determine whether progeny virions released from the infected LS could replicate[11]. Infectivity increased during the culture and reached a peak of up to 1.7×10^5 ffu/mL respectively, by day 21 post-infection (Figure 5D). To further confirm that the new progeny virus produced by the human LS called the primary-culture-derived virus was indeed infectious, naive human LS were infected *de novo* with primary-culture-derived virus Con1/C3 at MOI = 0.1. A *de novo* productive infection of LS was obtained with higher infectivity titers on day 21, genotype1b (180000 ffu/mL) (Figure 5E). Thus, HCV RNA replication, the expression of viral proteins, and the production of highly infectious particles were demonstrated.

HCVcc infection of non-fibrotic (F0-F1) LS activated the expression of the main pro-fibrogenic markers. During follow-up studies, in non-fibrotic (F0-F1) LS cultures (Figure 6), RNA, and protein expression of TGF-β1 (Figure 6A-C), α-SMA, Hsp47, Procol1A1, (Figure 6D-F) had increased significantly in non-infected and infected LS on day 21. A marked 2.6 to 3.6 fold increase of α-SMA, Hsp47, Procol1A1 RNA expression was observed in non-fibrotic (F0-F1) HCV infected LS cultures, compared to non-infected LS cultures on day 21. MMP-2 RNA expression was also significantly increased after HCV infection in non-fibrotic F0F1 LS, (Figure 6G). On the contrary,

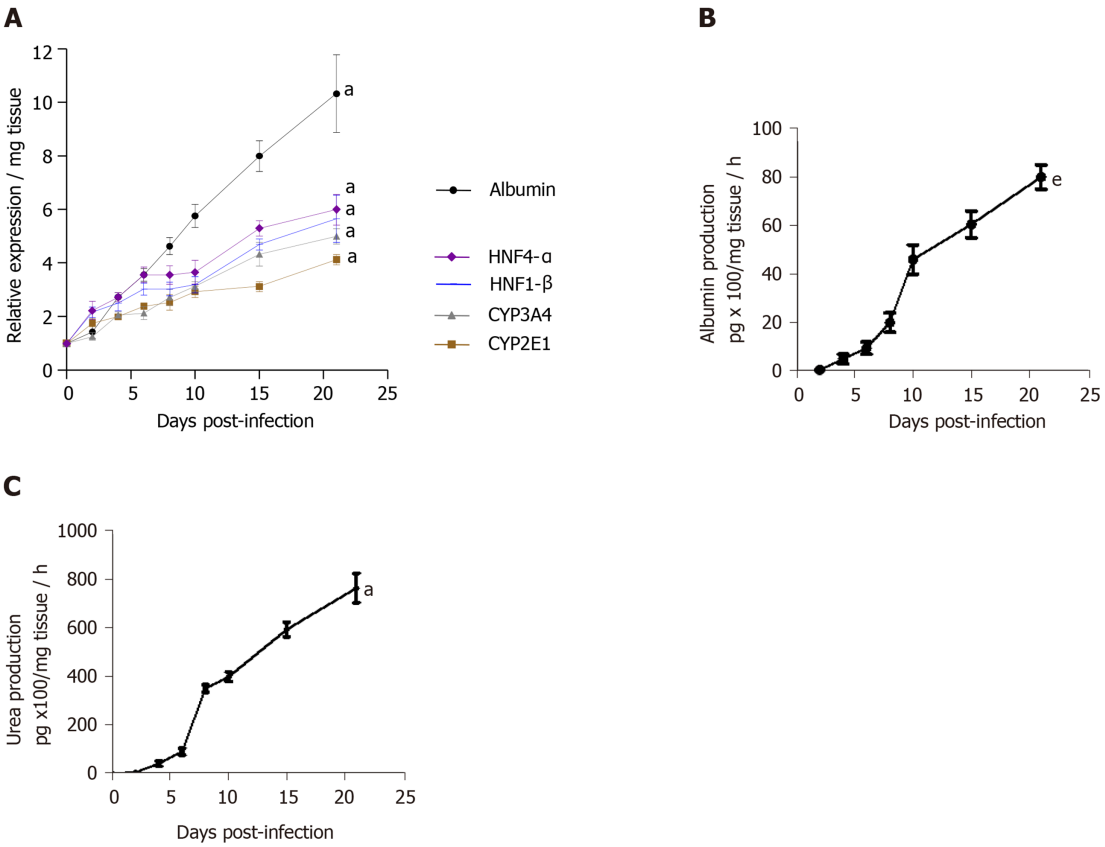
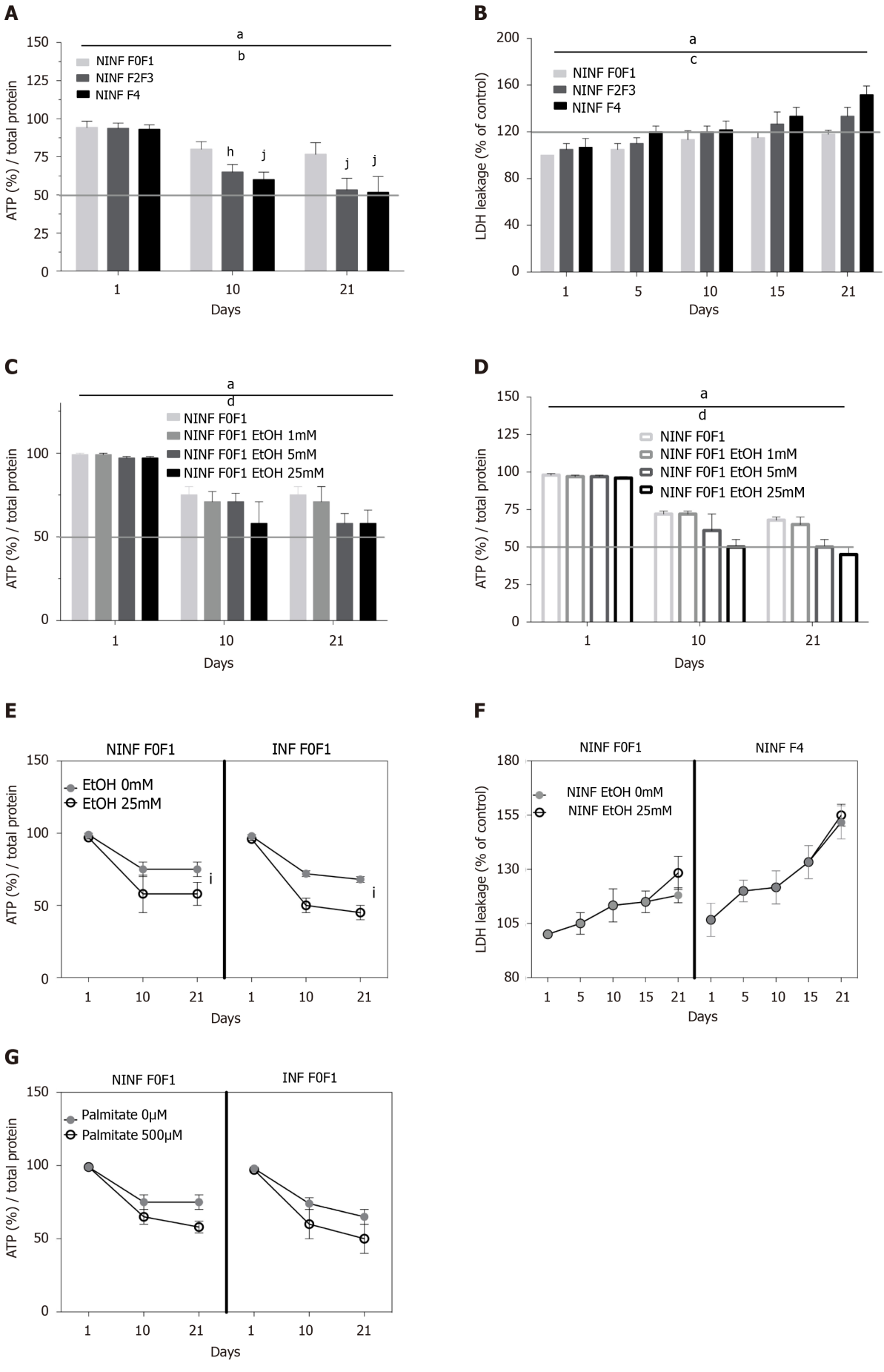


Figure 3 Maintenance of phenotypic characteristics of human non-fibrotic (F0-F1) hepatitis C virus-infected liver slices during the culture, demonstrated by real-time reverse transcription-quantitative polymerase chain reaction and biochemical assays. A: Hepatocyte-specific gene mRNA expression (relative expression/mg tissue) during the 21 days follow up studies. Maintenance of hepatocyte-specific gene expression patterns in human non-fibrotic (F0-F1) hepatitis C virus (HCV) infected liver slices during the culture. The real-time reverse transcription-quantitative polymerase chain reaction analyses were performed from five independent human non-fibrotic (F0-F1) liver samples, using HCV- infected slices in triplicate from each liver. Liver slices were infected with HCVcc, on day 0, at MOI = 0.1. All liver–specific gene expression values were normalized to 18S RNA as an internal standard and expressed in relation to the zero-time point. Values are expressed as mean ± standard errors. The results were compared using the two-paired Student's *t*-test: Albumin, $^{a}P < 0.0001$; CYP2E1: $^{a}P < 0.0001$; CYP3A4: $^{a}P < 0.0001$; HNF1-β: $^{a}P < 0.0001$; HNF4-α: $^{a}P < 0.0001$); B and C: Biochemical functional assays: B: Albumin production (pg x 100/mg tissue/ hour) during the 21d- follow up studies (days). C: Urea production (pg/mg tissue/hour during the 21d- follow up studies (days) by human F0-F1 cultured HCV-infected liver slices ($n = 5$). The assays were performed as previously described[11,12]. Studies were performed in triplicate and repeated twice for each liver sample. Values are expressed as means ± standard errors ($n = 5$). The results were compared using the two-paired Student's *t*-test: Albumin production: $^{e}P < 0.001$; urea production: $^{a}P < 0.0001$.

there was no significant difference in MMP-9 RNA expression between F0-F1 non-infected and infected liver slices (Figure 6H). VEGF RNA expression increased irregularly up to day 21 and seemed to be influenced by HCV infection until day 5 compared to non-infected LS (Figure 6I). The triglyceride production increased in both F0-F1 non-infected and infected LS cultures (Figure 6J) with no significant difference between them.

Expression of fibrosis biomarkers was higher in fibrotic LS culture (stages F2-F3 and F4), than in non-fibrotic LS cultures, with a significant 4 to 8 fold increase compared to controls (day 1) (Figure 7). This mainly concerned TGF-β1 (Figure 7A), Procol1A1 (Figure 7C), α-SMA (Figure 7D), Hsp47 (Figure 7E) as well as an increased triglyceride production in fibrotic LS (approximately 3.2 fold) (Figure 7B). After day 10, RNA expression increased with the progression of fibrosis. MMP-2 RNA expression (Figure 7F), as well as MMP-9 and VEGF expression (Figure 7G and H), did not differ between fibrosis stages F2-F3 and F4. It is interesting to note that HCV infection significantly increased TGF-β1, Hsp47, α-SMA, Procl1A1, MMP-2, MMP-9, VEGF expression as well as triglyceride production in fibrotic (F2-F3, F4) infected LS cultures. A significant 2 to 4 fold increase in fibrosis biomarkers was observed on day 21 in F2-F3 and F4 HCV infected LS compared to F2-F3, F4 non-infected LS. Thus, the TGF-β1 (Figure 6A-C), α-SMA, Hsp47, Procol1A1, MMP-2, MMP-9, VEGF expression increased in non-infected and infected LS cultures with a greater increase in infected LS cultures than in controls. On day 21, a significant 2 to 13 fold increase in fibrosis biomarkers was observed in F2-F3, F4 infected LS cultures compared to F2-F3, F4 non-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NINF F2F3
NINF F4
Days
B
LDH leakage (% of control)
NINF F0F1
NINF F2F3
NINF F4
Days
C
ATP (%) / total protein
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NINF F0F1 EtOH 1mM
NINF F0F1 EtOH 5mM
NINF F0F1 EtOH 25mM
Days
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ATP (%) / total protein
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Days
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NINF F0F1
INF F0F1
EtOH 0mM
EtOH 25mM
ATP (%) / total protein
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F
NINF F0F1
NINF F4
NINF EtOH 0mM
NINF EtOH 25mM
LDH leakage (% of control)
Days
G
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Palmitate 0μM
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ATP (%) / total protein
Days

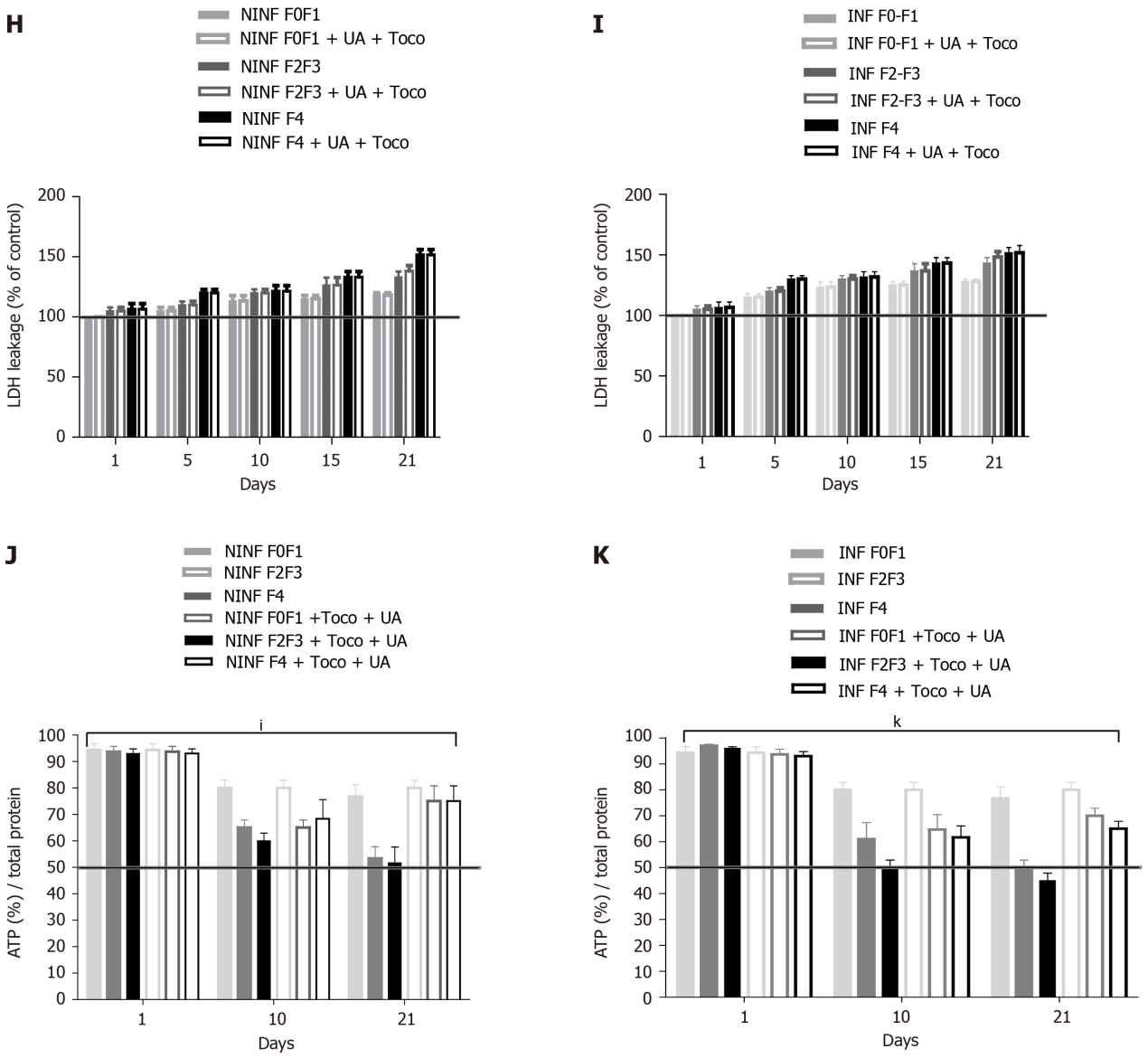


Figure 4 Viability of human non-fibrotic (F0-F1), and fibrotic (F2-F3, F4) non-infected or hepatitis C virus-infected liver slices during the different kinetic studies, with no treatment cytotoxicity as shown by ATP and LDH dosages. A: Percentage of ATP synthesis/total protein in non-infected (NINF) liver slice (LS) with F0-F1 to F4 stage fibrosis during the 21 days-follow up kinetics; B: The percentage of LDH release/control in NINF LS with a F0-F1 to F4 stage fibrosis during the 21d-follow up kinetics (d: days); C and D: The percentage of ATP synthesis /total protein in F0-F1 NINF and hepatitis C virus (HCV)-infected (INF) LS treated with 1 mmol/L, 5 mmol/L and 25 mmol/L of EtOH during the 21d-follow up kinetics; E: The percentage of ATP synthesis / total protein in the presence of 25 mmol/L of EtOH on F0-F1 NINF and INF LS during the 21d-follow up kinetics; F: LDH release (% of control) in F0-F1 non-infected LS cultures treated or non-treated with 25 mmol/L of EtOH compared to F4 non-infected treated or non-treated with 25 mmol/L of EtOH during the 21d-follow up kinetics. Values are expressed as means ± standard errors (SEMs), (n = 5). $^{a}P < 0.0001$ time factor; $^{b}P < 0.01$ fibrosis stage; $^{c}P < 0.05$ fibrosis stage; $^{d}P < 0.05$ alcohol factor; $^{i}P < 0.01$ subject *vs* control (non-treated) (two-way ANOVA test).There is no significant toxic effect of EtOH (25 mmol/L) on F0-F1 NINF and INF LS and F2-F3, F4 NINF LS; G: The percentage of ATP synthesis/total protein during the 21-follow up kinetics showing the viability of F0-F1 NINF or INF LS cultures with or without the presence of palmitate (500 µmol/L); H and I: Absence of drug cytotoxicity (LDH release, (% of control)) on the viability of human F0-F4 LS NINF or infected (INF) by HCVcc Con1/C3 during the treatment with either UCDA (UA) or Toco or both for 21 days. It is important to note that under 150%, there is no cytotoxic effect of the drugs on LS viability. Values are expressed as means ± SEMs, (n = 5); J and K: The percentage of ATP synthesis / total protein during the 21 days follow up kinetics, in F0-F1 to F4 NINF or infected (INF) LS with combined treatment [Toco + UCDA (UA)]. Values are expressed as means ± SEMs, (n = 5); Levels of significance are as follows between: Subject *vs* control, $^{k}P < 0.0001$; $^{i}P < 0.001$; $^{i}P < 0.01$; $^{h}P < 0.05$) (two-way ANOVA test).

infected LS cultures. Triglyceride production increased in both non-infected and infected LS cultures, independent from the stage of fibrosis. After 21 d of the culture, the amount of triglyceride in the supernatant of F2-F3 and F4 LS cultures increased by 1.36 and 2.7 folds, respectively (Figure 7B). Increased expression of the TGF-β1, α-SMA, Procol1A1, MMP-2, MMP-9, and VEGF in F2-F3 LS cultures throughout the 21-d of follow-up was confirmed by Western blotting (Figure 7I and J). On day 10, immunohistochemistry showed that TGF-β1, α-SMA and MMP-9 expression (Figure 7K) was increased by about 20% in F2-F3 LS compared to day 0.

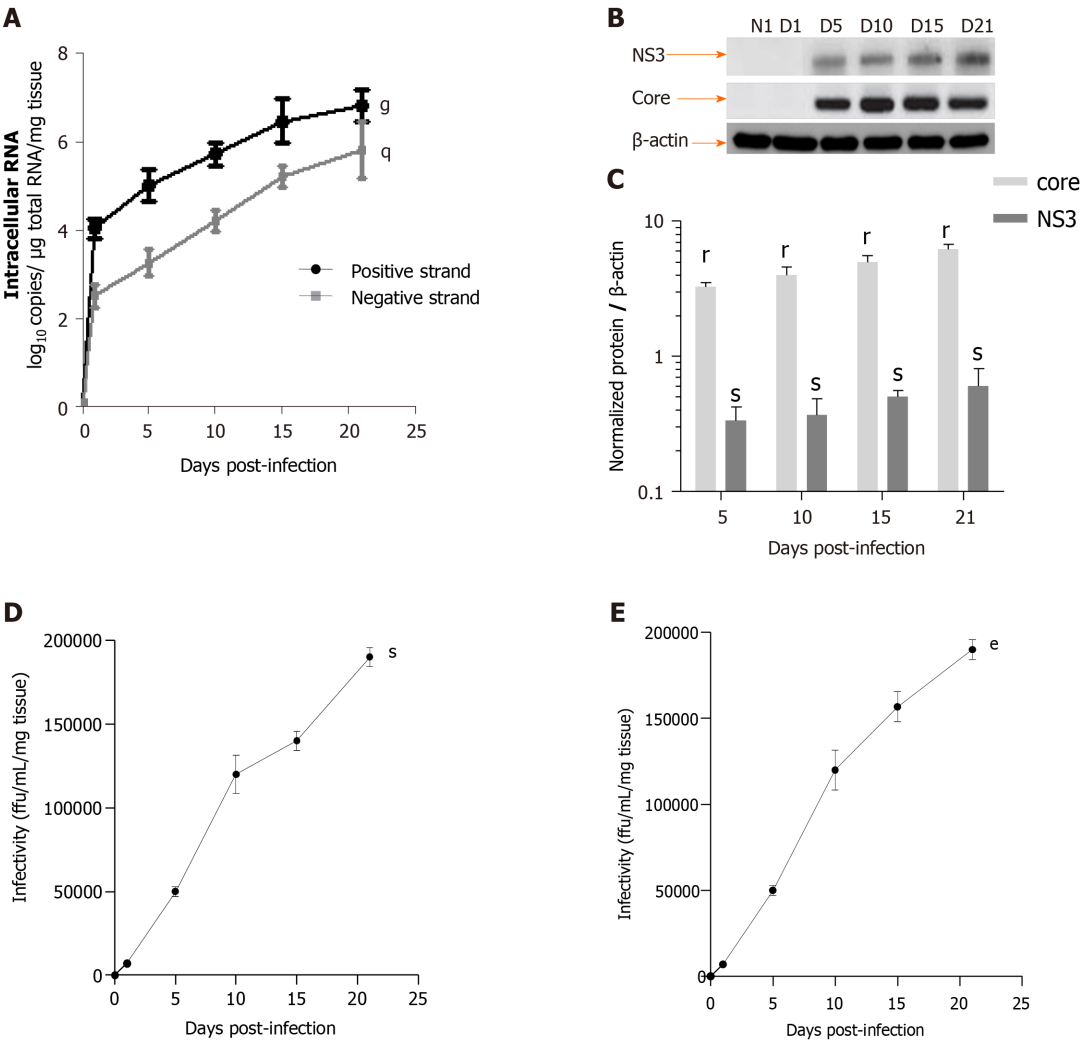
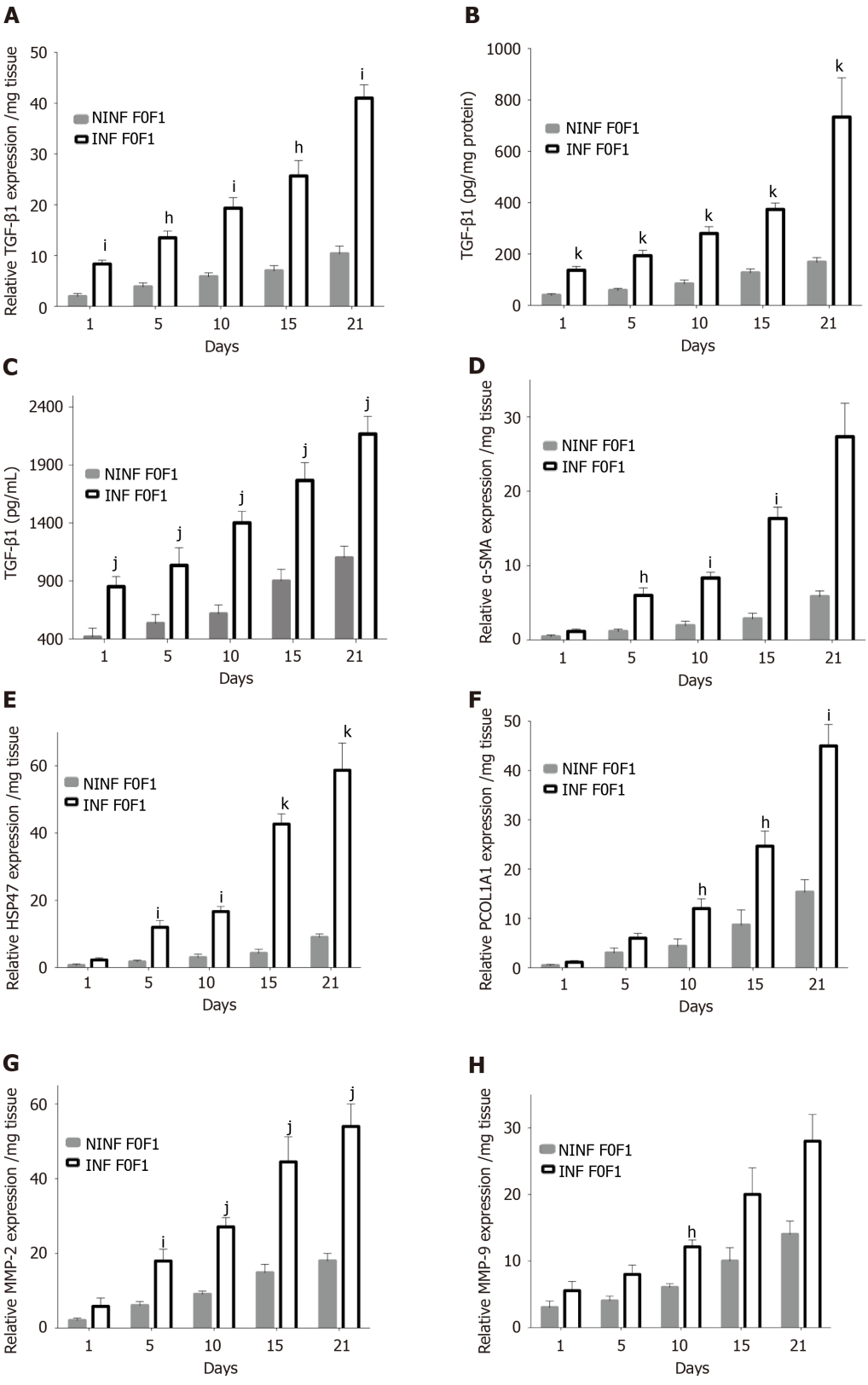


Figure 5 Efficient replication of hepatitis C virus RNA, and hepatitis C virus core and NS3 proteins expression in human F0-F1 liver slice culture as shown by real-time reverse transcription-quantitative polymerase chain reaction and western blotting analysis. A: Quantification of intracellular levels of positive- and negative-strand hepatitis C virus (HCV) RNA (log10 copies/µg total RNA/mg tissue) in primary human F0-F1 HCVcc Con1/C3 -infected liver slice (LS) by specific- strand real-time reverse transcription-quantitative polymerase chain reaction on day 5, day 10, day 15 and day 21 post-infection. Values are expressed as mean ± SEMs. All results were compared using the two-paired Student *t*-test, time factor: Positive strand: $^{g}P < 0.01$; negative strand: $^{q}P < 0.04$, ($n = 3$). Detection of the negative strand of HCV RNA evidences active replication as well as an increase over time of both positive and negative strands of HCV RNA; B: Western blotting analysis of human F0-F1 HCVcc Con-1/C3 -infected LS lysates with mAbs against HCV NS3 or core proteins on day 5, day 10, day 15, and day 21, post-infection (MOI = 0.1) was performed and analyzed ($n = 3$). Lysates of naïve human F0-F1 LS lysates were run in parallel to serve as a negative controls (NI). β-actin was used as a loading control; C: Normalization of Core and NS3 protein expression compared to b-actin expression (Normalized protein / β-actin) during the 21 days follow-up kinetics using the image quantification standard software, ImageJ2[21]. The position of molecular-weight markers is indicated in kDa. Values are expressed as means ± SEMs ($n = 3$): Core $^{r}P < 0.002$; NS3 $^{s}P < 0.02$ (two-paired Student *t*-test); D: Production of HCV infectious particles (genotype 1b) in primary adult human F0-F1 LS: Infectivity titers [*i.e.*, infectivity (ffu/mL/mg tissue)] of culture supernatants from human F0-F1 LS infected by the Con1/C3 virus during the 21 days follow up kinetics. The curve represents the average of three independent infections from 3 different donors. Each kinetic study was performed in triplicate. Values are expressed as means ± SEMs. Results were compared using the two-paired Student *t*-test: $^{s}P < 0.02$; and E: Infectivity titers [*i.e.*, infectivity (ffu/mL/mg tissue)] of culture supernatants of naive F0-F1 LS infected with supernatants from human F0- F1 HCV-infected LS culture (HCVpc) during the 21 days follow up kinetics. The infection of naive F0-F1 LS with supernatants from human F0-F1 HCV-infected LS culture (HCVpc) clearly indicates the infectivity of extracellular viral particles, which are produced by HVCcc Con1/ C3 (genotype 1b) infection. Values are expressed as means ± SEMs ($n = 3$). Levels of significance: $^{e}P < 0.001$ (two-paired Student *t*-test).

Exposition of LS cultures to ethanol: A model of the alcoholic liver disease: The effect of EtOH exposure on LS cultures was estimated using non-fibrotic (F0-F1) HCV infected or non-infected LS cultures (Figure 8A-F and Figure 9) and fibrotic (F2-F4) HCV infected or non-infected LS cultures (Figure 8G-L) and Figure 10A-L). One mmol/L, 5 mmol/L or 25 mmol/L of EtOH was added to F0-F1 HCV infected, or non-infected LS cultures (Figure 8A-F). Only the highest concentration of EtOH was studied in fibrotic (F2-F3 and F4) non-infected or HCV-infected LS cultures, (Figure 8G-L and Figure 10A-L) respectively. During the follow-up studies (Figure 8A-F), EtOH enhanced the RNA expression of fibrosis markers in a dose-dependent manner in F0-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NINF F0F1
INF F0F1
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TGF-β1 (pg/mg protein)
C
TGF-β1 (pg/mL)
D
Relative α-SMA expression /mg tissue
E
Relative HSP47 expression /mg tissue
F
Relative PCOL1A1 expression /mg tissue
G
Relative MMP-2 expression /mg tissue
H
Relative MMP-9 expression /mg tissue

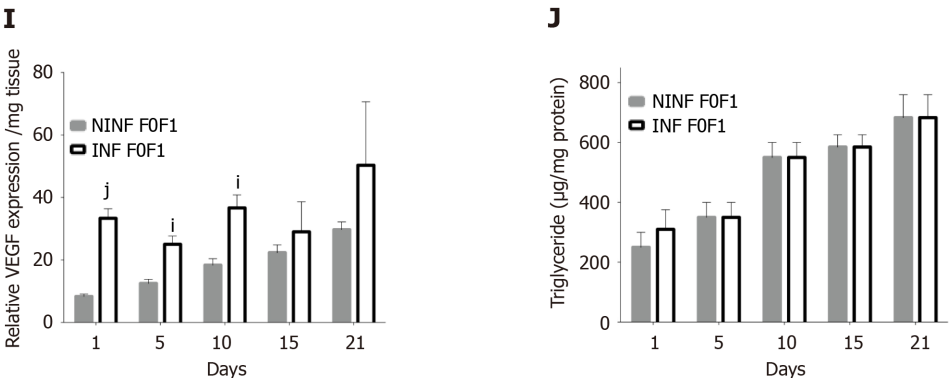
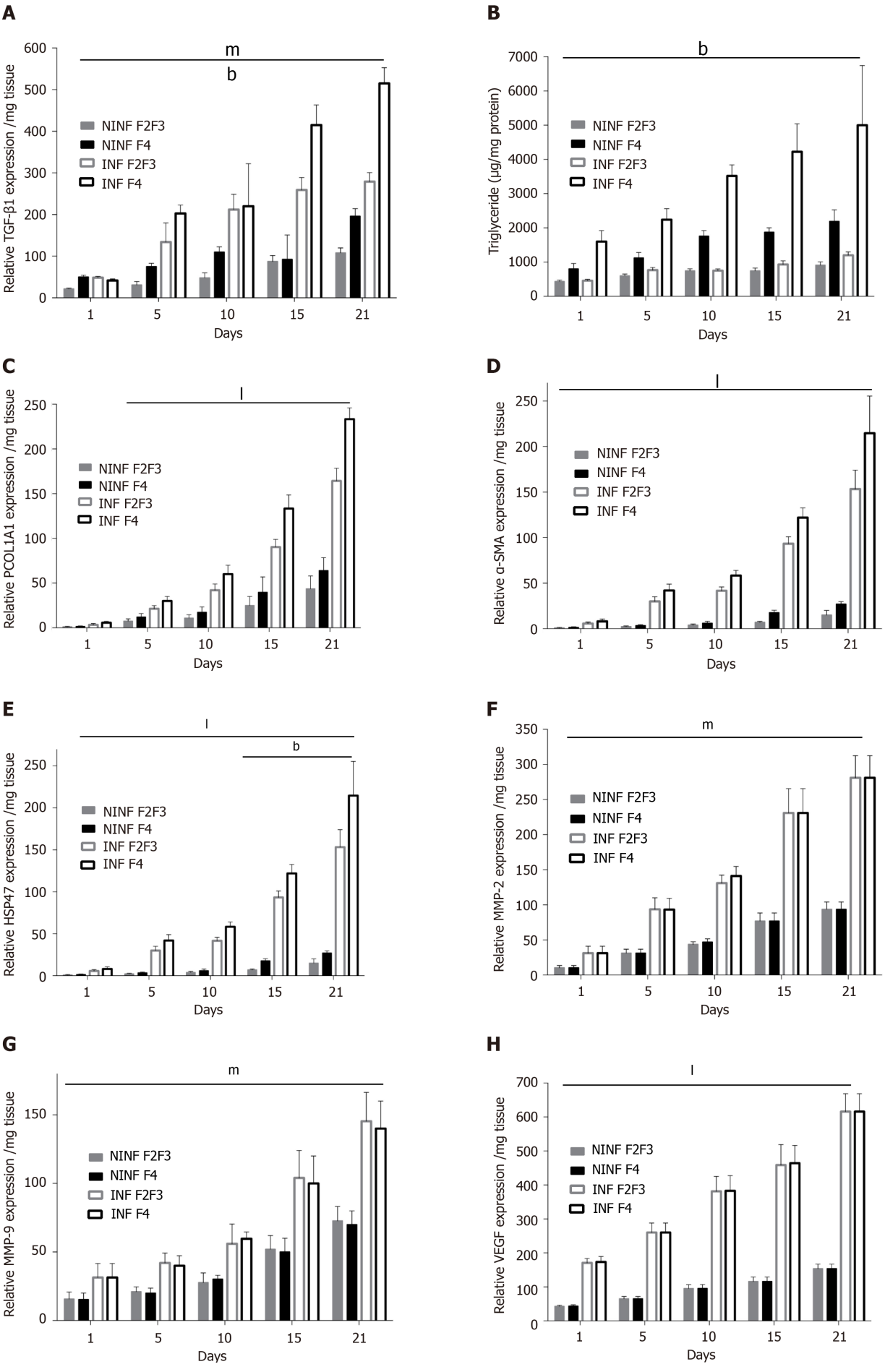


Figure 6 Real-time reverse transcription-quantitative polymerase chain reaction analysis evidencing the significant increase of fibrosis markers expression at the transcriptional level in human F0-F1 non-infected or hepatitis C virus infected liver slice during the kinetics. A: TGF-β1 expression at mRNA level (relative RNA expression / mg tissue) during 21 days follow up kinetics; B: TGF-β1 expression at intracellular protein level (pg/mg protein) during 21 days follow up kinetics; C: TGF-β1 expression at extracellular secretion level (pg/mL) during 21 days follow up kinetics; D-F: mRNA expression (relative RNA expression / mg tissue) of (D) α-SMA, HSP47 (E) and ProCOL1A1 (F) during 21 days follow up kinetics; G and H: MMP-2 and MMP-9 mRNA expression (relative RNA expression / mg tissue) during 21 days follow up kinetics; I: VEGF mRNA expression (relative RNA expression/mg tissue) during 21 days follow up kinetics; J: Triglyceride production (µg/mg protein) raised during the during the 21 days follow up kinetics. All data are presented considering the percentage of viable liver slices in culture. Data are expressed as means ± SD (n = 5), subject vs control, $^{h}P < 0.05$; $^{i}P < 0.01$; $^{j}P < 0.001$; $^{k}P < 0.0001$, (two-way ANOVA test).

F1 LS cultures. Increased expression of TGF-β1, Procol1A1 RNA was further detected in F0-F1 infected LS (Figure 8B, D and F, Figure 9B, Figure 10B), compared to non-infected LS (Figure 8A, C and E, Figure 9A, Figure 10A). Similar results were found in fibrotic F2-F4 LS (Figure 8G-L, Figure 10A-L). Interestingly, there was no significant increase in Procol1A1 or α-SMA RNA expression in F0-F1 non-infected LS except on day 21 when 25 mmol/L of EtOH was added to the culture (Figure 8E and Figure 9C, respectively). However, a significant dose-dependent increase of the Procol1A1 and α-SMA RNA expression occurred whatever the dose of EtOH added to F0-F1 infected LS cultures (Figure 8F, Figure 9D). There was a dose-dependent increase in the RNA expression of the other fibrosis markers such as α-SMA (Figure 9C and D; Figure 10E and F), and HSP47 (Figure 9E and F, Figure 10C and D) with the addition of EtOH in F0-F1 to F4 infected LS which was less marked in F0-F1 to F4 non-infected LS. Analysis of F0-F1 to F4 HCV non-infected or infected LS showed a significant dose-dependent increase in MMP-2, MMP-9, and VEGF expressions in response to EtOH (Figure 10G-L). Masson's trichrome staining showed a significant increase in collagen fibers (%) between day 1 (1.242% of collagen) and day 6 (2.076% of collagen) in F0-F1 HCV infected LS treated with 5 mmol/L of EtOH (Figure 8M) but not in F0-F1 non-infected LS with the same treatment (Figure 8N). Picro Sirius red staining confirmed the significant increase in collagen fibers (%) between day 1 (0.55% of collagen) and day 6 (1.53% of collagen) in F0-F1 HCV infected LS treated with 5 mmol/L of EtOH compared to non-treated LS (data not shown).

Exposition of LS cultures to palmitate: a model of NASH. To imitate NASH, non-fibrotic (F0-F1) LS cultures infected (or not infected) with HCV were exposed to 500 µmol/L of palmitate (Figure 11). More marked triglyceride synthesis was noted in F0-F1 palmitate treated HCV-infected LS cultures, than in F0-F1 untreated non-infected LS cultures (Figure 11A). The F0-F1 infected LS cultures treated with palmitate demonstrated more marked expression of the fibrotic markers such as TGF-β1 (Figure 11B), intracellular expression of TGF-β1 (Figure 11C), and secretion of the extracellular TGF-β1 (Figure 11D). A similar increase was observed with Procol1A1, α-SMA, and HSP47 (Figure 11E-G) on day 21. The expression of markers (RNA) involved in liver fibrolysis, (MMP-2, -9), and VEGF increased significantly in both F0-F1 non-infected LS cultures treated or not with palmitate (Figure 12A). But, the treatment of F0-F1 non-infected LS cultures with palmitate showed a greater significant increase of the expression of MMP-2, -9, and VEGF compared to those of F0-F1 untreated non-infected LS cultures. The treatment of the F0-F1 infected LS cultures with palmitate, increased significantly VEGF, MMP-2, and MMP-9 from day 10, 15, and day 21 respectively (Figure 12B). Fibrotic marker expression increased both in F0-F1 LS cultures HCV infected or non-infected treated with palmitate but with a greater increase in F0-F1 HCV infected LS treated with palmitate.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Triglyceride (μg/mg protein)
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Relative PCOL1A1 expression /mg tissue
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Relative HSP47 expression /mg tissue
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Relative MMP-2 expression /mg tissue
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Relative VEGF expression /mg tissue

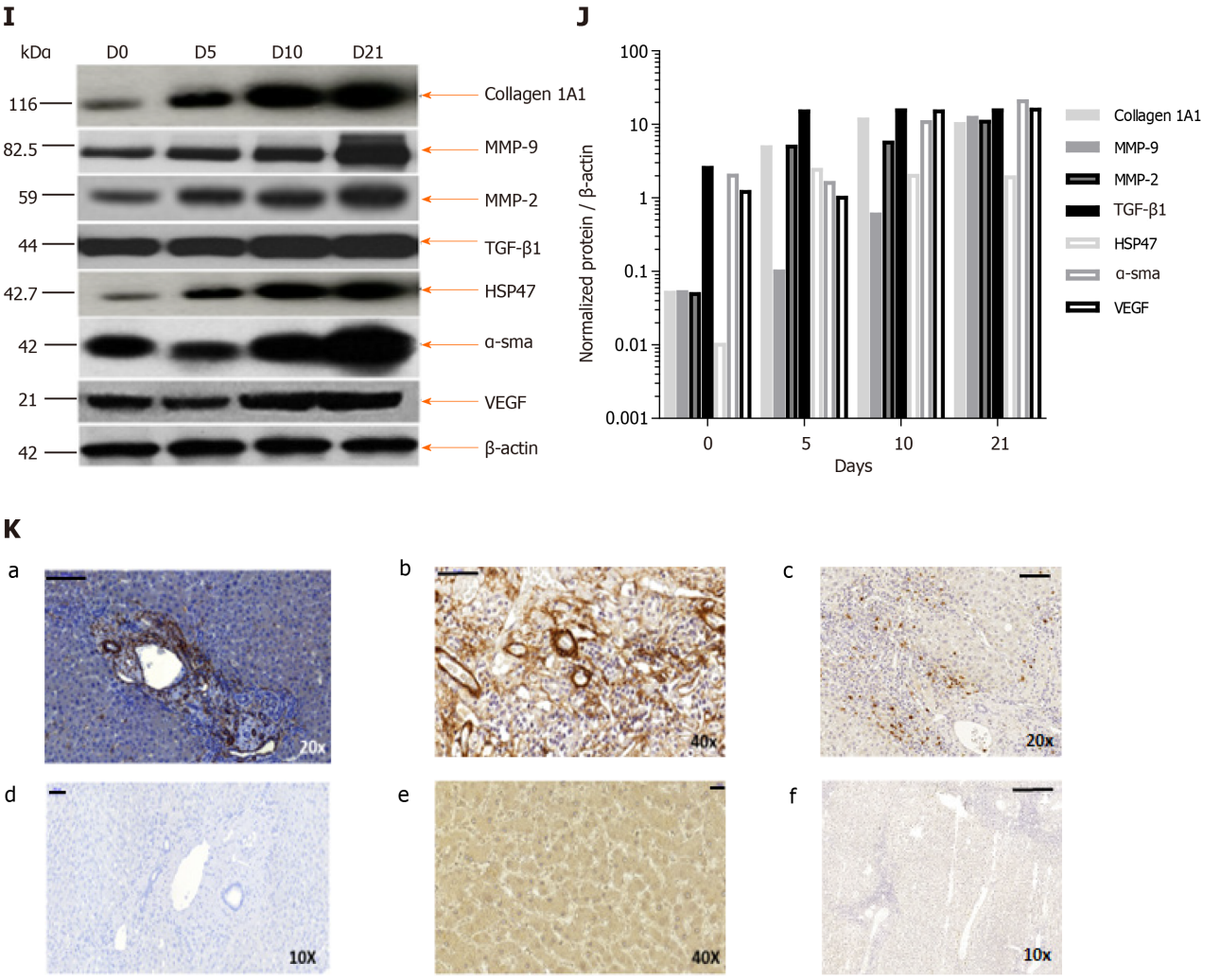
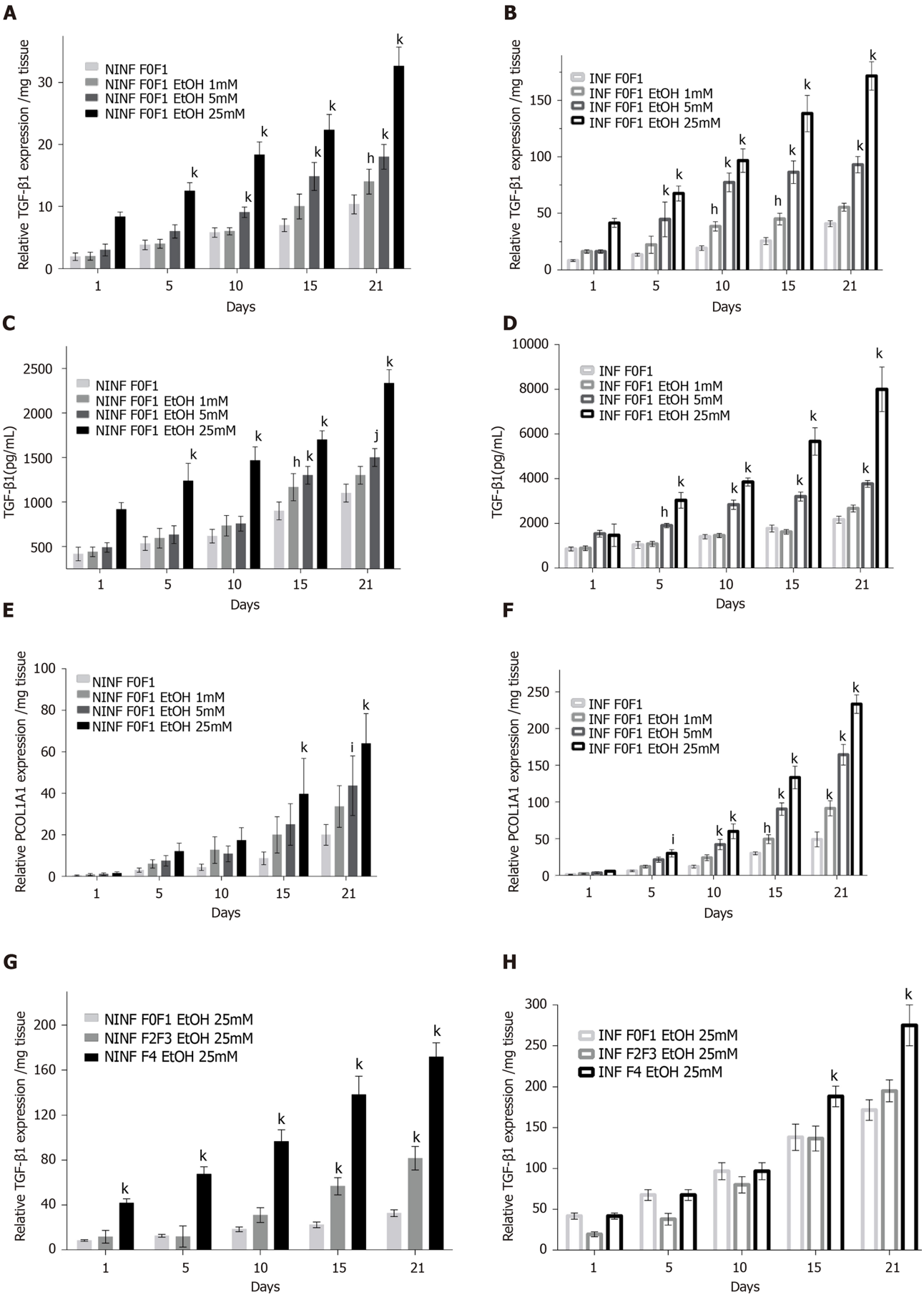


Figure 7 Real-time reverse transcription-quantitative polymerase chain reaction analyses of RNA expression of liver fibrosis markers (TGF-β1, Procol1A1, α-SMA, HSP47, MMP-2, MMP-9, VEGF), and triglyceride production in non-infected or hepatitis C virus infected liver slice cultures from fibrotic liver (F2-F3, F4) showing a significant increase during the kinetics. A: TGF-β1 mRNA Expression (relative RNA expression/mg tissue) during 21 days follow up kinetics; B: Triglyceride production (μg/mg protein); C–E: Procol1A1, α-SMA, HSP47 mRNA expression (relative RNA expression / mg tissue) during 21 days follow up kinetics; F- H: MMP-2, MMP-9 and VEGF mRNA expression (relative RNA expression/mg tissue) during 21 days follow up kinetics. Data are expressed as mean± SD (F2-F3 liver samples, $n = 2$, F4 liver samples, $n = 2$). $^{b}P < 0.01$ fibrosis stage factor; $^{m}P < 0.001$ Infection factor; $^{I}P < 0.0001$ infection factor; (two-way ANOVA test); I: TGF-β1, HSP-47, Collagen I alpha 1, MMP-9, MMP-2, a-SMA, VEGF proteins expression in F2-F3 liver slice performed in western blotting and normalized. Positions of molecular-weight markers are indicated in kDa; J: Normalization of the proteins expression compared to β-actin expression (Normalized protein/b-actin) during the 21 days follow-up kinetics using the image quantification standard software, ImageJ2; and K: Representative human liver tissue 7 μm-thick sections from F2-F3 liver patient showing immunohistochemistry staining for fibrosis markers, TGF-β1 (a), α-SMA (b), MMP-9 (c) on day 10, magnification 20×, Scale bars, 100 μm; 40×, Scale bars 50 μm; 10×, Scale bars 100 μm, respectively. (d-f) isotypes controls staining, magnification 10×, Scale bars, 100 μm; 40×, Scale bars 20 μm; 10×, Scale bars 200 μm, respectively.

LS treatment with a combination of the "hepatoprotective" UCDA and anti-fibrotic α-Toco drugs significantly reducing the expression of the main fibrosis markers TGF-β1, Procollagen1A1, and triglyceride production

To validate the LS culture as a model for drug screening, the "hepatoprotective" (UCDA) and "anti-fibrotic (Toco) drugs were tested on non-fibrotic (F0-F1), or fibrotic (F2-F3, F4) LS cultures, infected or non-infected with HCV. UCDA and Toco were dosed according to the standard of care in humans (Figure 13). On day 0, LS cultures were infected with HCVcc Con1/C3 (MOI = 0.1) and treated either with daily doses of UCDA and /or with Toco for up to day 21.

During the 21-days long follow-up studies of F0-F1, F2-F3, and F4, LS cultures, a significant, 25% to 50%, reduction in TGF-β1 RNA expression was only identified in F4 LS cultures treated with Toco, from day 5 and day 10 of the culture in non-infected and HCV infected LS cultures, respectively (Figure 13A and B). Treatment with UCDA did not induce a significant reduction in TGF-β1 RNA expression in any non-infected F0-F4 LS cultures (Figure 13C). Interestingly, from day 15, at least a two-fold reduction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Days
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Relative TGF-β1 expression /mg tissue
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INF F0F1 EtOH 1mM
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INF F0F1 EtOH 25mM
Days
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NINF F0F1 EtOH 1mM
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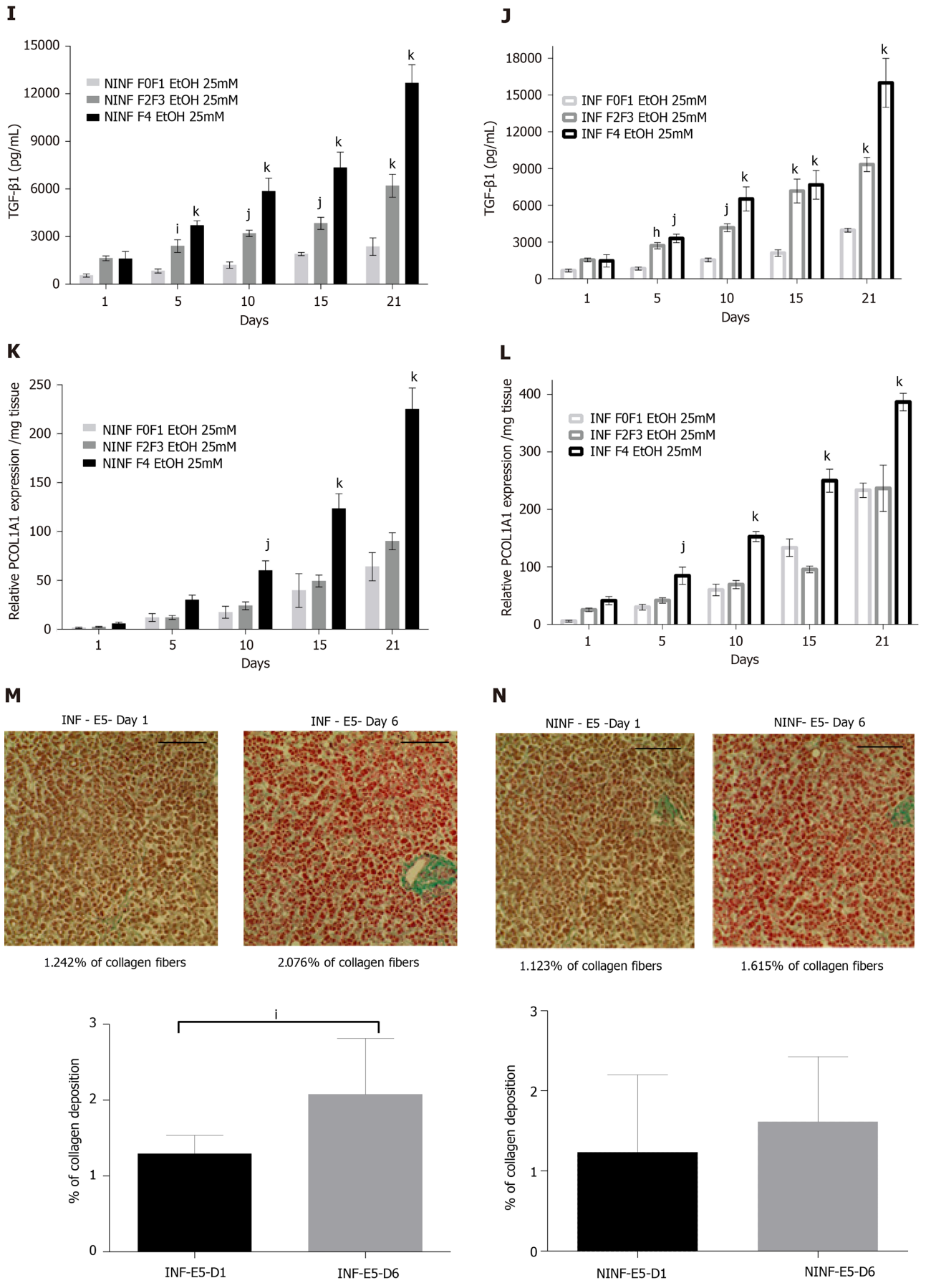


Figure 8 Significant increased expression of TGF-β1 and Procol1A1 mRNA with ethanol (1 mmol/L, 5 mmol/L, 25 mmol/L) treatment of non-infected or hepatitis C virus INF liver slice cultures from non-fibrotic (F0-F1) and ethanol (25 mmol/L) treatment of non-infected or

hepatitis C virus infected liver slice cultures from fibrotic (F2-F3, F4) liver samples as shown by real-time reverse transcription-quantitative polymerase chain reaction and ELISA and histochemistry. A and B: TGF-β1 mRNA expression (relative RNA expression / mg tissue) during 21 days follow up kinetics with ethanol (EtOH) (1 mmol/L, 5 mmol/L, 25 mmol/L) treatment in non-infected (NINF) or hepatitis C virus (HCV) INF liver slice (LS) cultures from non-fibrotic (F0-F1); C and D: Extracellular TGF-β1 protein expression (pg/mL) during 21 days follow up kinetics, with EtOH (1 mmol/L, 5 mmol/L, 25 mmol/L) treatment of NINF or HCV INF LS cultures from non-fibrotic (F0-F1); E and F: Procol1A1 mRNA expression (relative RNA expression/mg tissue) during 21 days follow up kinetic, with EtOH (1 mmol/L, 5 mmol/L, 25 mmol/L) treatment of NINF or HCV INF LS cultures from non-fibrotic (F0-F1); G and H: TGF-β1 mRNA expression (relative RNA expression/mg tissue) during 21 days follow up kinetics, in fibrotic (F2-F3, F4) NINF and HCV INF LS treated with 25 mmol/L of EtOH compared to F0F1 NINF or HCV INF LS cultures in presence of the 25 mmol/L EtOH; I and J: Extracellular TGF-β1 protein expression (pg/mL) during 21 days follow up kinetics, in fibrotic (F2-F3, F4) NINF and HCV INF LS after treatment with 25 mmol/L of EtOH compared to F0F1 NINF or HCV INF LS cultures in presence of 25 mmol/L EtOH; K and L: Relative Procol1A1 mRNA expression (relative RNA expression/mg tissue) during 21 days follow up kinetics, in fibrotic (F2-F3, F4) NINF and HCV INF LS treated with 25 mmol/L of EtOH compared to F0F1 NINF or HCV INF LS cultures in presence of 25 mmol/L EtOH. Data are expressed as means ± SEM (F0-F1, n = 5; F2-F3, n = 2; F4, n = 2). $^{k}P < 0.0001$ subject *vs* control (non-treated); $^{i}P < 0.001$ subject vs control (non-treated); $^{i}P < 0.01$ subject *vs* control (non-treated), $^{h}P < 0.05$ subject vs control (non-treated) (two-way ANOVA test); M: Significant increase of collagen deposition (% of collagen deposition) in F0-F1 HCV INF LS treated with 5 mmol/L of EtOH (E5) on day 6 (D6) compared to day 1 (D1); N: No significant change of collagen deposition (% of collagen deposition) in F0-F1 non-infected (NINF) LS treated with 5 mmol/L of EtOH (E5) on day 6 (D6) compared to day 1 (D1). Data are expressed as means ± SEM (n = 8). $^{i}P < 0.01$ subject *vs* control (non-treated), (unpaired two-tailed Student's *t*-test). Magnification 20X, Scale bars 100 μm.

in TGF-β1 RNA expression, at least, F2-F3, and F4 infected LS cultures was observed (Figure 13D). There was no change in TGF-β1 RNA expression in non-infected LS treated with both UCDA and Toco, whatever the stage of disease (Figure 13E). TGF-β1 RNA expression in F2-F3 and F4 infected LS cultures on days 5 and 15 was reduced by nearly two fold. On day 21, TGF-β1 RNA expression in F4 LS cultures were reduced 2.5 fold (Figure 13F). During the 21-days follow-up studies of infected and non-infected F0-F1, F2-F3, and F4 LS cultures treated with both UCDA and Toco, procollagen1A1 expression was significantly reduced in non-infected and infected F0F1- F4 LS cultures compared to untreated cultures from day 15 (Figure 13G and H). In particular, the significant reduction of procollagen1A1 RNA expression (around two-fold) in treated F2-F3 infected LS cultures was observed from day 10 and from day 15 for treated F4 infected LS cultures. Triglyceride production in HCV non-infected and infected LS from F0-F1, F2-F3 and F4 LS cultures was significantly reduced by the combination treatment from day 10 in F4 HCV non-infected LS cultures (Figure 13I) and from day 1 in F4 HCV infected LS cultures (Figure 13J).

DISCUSSION

For the first time, different stages of human liver fibrogenesis were investigated *ex vivo* and for a relatively long period. Indeed, liver tissue slices remained viable for at least 21 days, as shown by the secretion of albumin and urea, the percentage of ATP production and LDH release observed during the kinetic experiment. However, the secretion of albumin and urea was lower than that in micropatterned hepatocyte co-cultures models[27]. Both fibrotic (stages F2-F4) and non-fibrotic (stages F0-F1) liver samples remained viable *ex vivo* for this period. Twenty one-day follow-up studies of LS cultures significantly improved the investigation of fibrogenesis in general, and fibrotic biomarkers, in particular. We obtained RT-qPCR analyses of the biomarkers (TGF-β1, procol1A1, MMP-2, MMP-9, α-SMA, HSP47, and VEGF) involved in molecular fibrogenesis, and estimation of anti-fibrotic drugs potency, in both non-fibrotic (F0-F1) and fibrotic livers samples (F2-F3, F4). Additional evaluation of fibrotic biomarkers performed by ELISA, histology, and by Western blotting supported RT-qPCR data. With this *ex vivo* model, sustaining hepatocyte-specific gene expression for 21 days, we induced molecular fibrogenesis using HCV, EtOH, or palmitate, thus mimicking human viral, alcoholic, and NASH liver diseases.

The most important property of this LS model is cell viability for a relatively long period of time. The expression of diverse biomarkers of fibrosis was analyzed in the presence of HCV, EtOH or palmitate using non-fibrotic F0-F1 LS cultures. The markers of fibrogenesis and triglyceride production were found to be increased in both non-infected and infected LS cultures. The addition of either EtOH or palmitate significantly increased the expression of fibrotic biomarkers. Moreover, this increase was found to be greater in HCV infected than in non-infected LS with increased triglyceride production higher in infected LS. HCV infection seemed to enhance fibrosis marker expression in the presence of ethanol or palmitate.

It is important to mention, that TGF-β1 expression, the principal marker of fibrogenesis, was higher in non-fibrotic (F0-F1) LS cultures cultivated in the presence

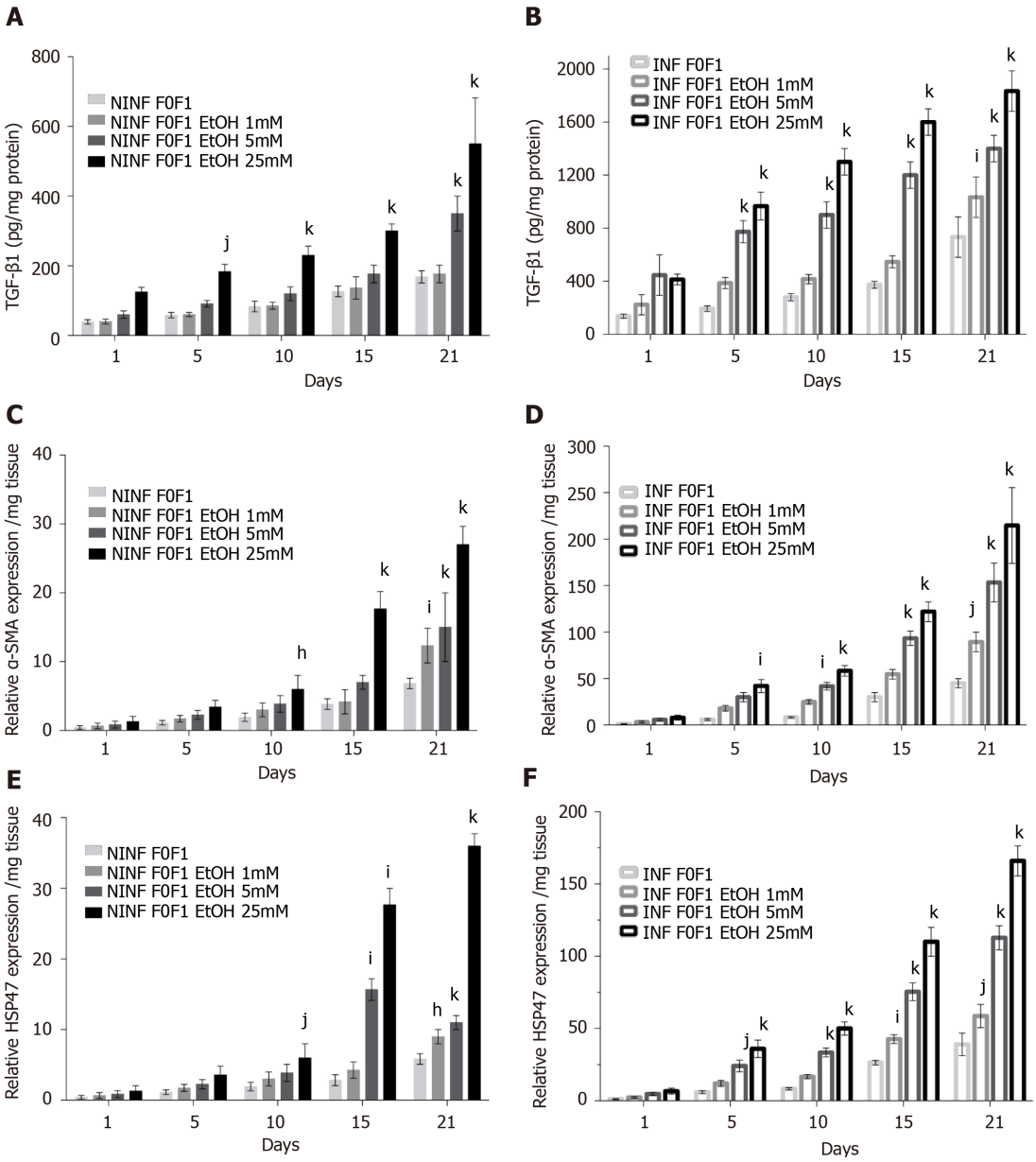
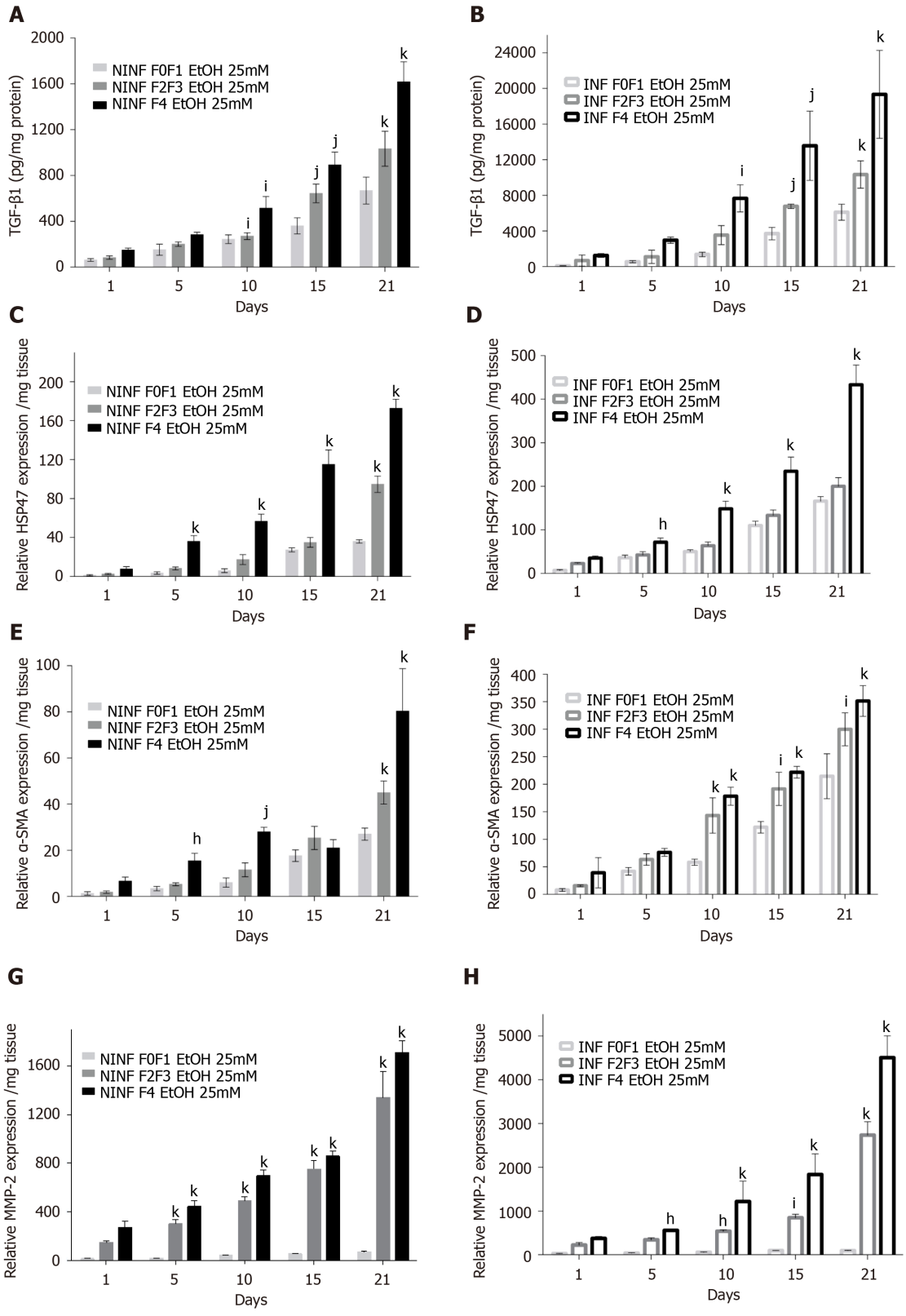


Figure 9 Significantly increase in TGF-β1 protein and RNA expression of α-SMA, and HSP47 in non-infected or hepatitis C virus-infected non-fibrotic (F0-F1) liver slice cultures treated with 1 mmol/L, 5 mmol/L and 25 mmol/L of ethanol was shown by enzyme-linked immunosorbent assay and real-time reverse transcription-quantitative polymerase chain reaction analyses. A and B: TGF-β1 intracellular protein expression (pg/mg protein) during 21 days follow up kinetics, in non-infected (A) and hepatitis C virus (HCV)-infected (B) F0-F1 liver slice (LS) cultures, treated with 1 mmol/L, 5 mmol/L, 25 mmol/L of ethanol (EtOH); C and D: Relative α-SMA RNA expression level (relative RNA expression/mg tissue) during 21 days follow up kinetics, in non-infected (C) and HCV-infected (D) F0-F1 LS cultures treated with 1 mmol/L, 5 mmol/L, 25 mmol/L of EtOH; E and F: Relative HSP47 RNA expression level expression (relative RNA expression / mg tissue) during 21 days follow up kinetics, in non-infected (E) and HCV-infected (F) F0-F1 LS cultures treated with 1 mmol/L, 5 mmol/L, 25 mmol/L of EtOH. All presented data take into account the viability of the liver slice cultures. Values are expressed as means ± SEMs (n = 5); Levels of significance: $^{k}P < 0.0001$ subject *vs* control (non-treated); $^{j}P < 0.001$ subject *vs* control (non-treated); $^{i}P < 0.01$ subject *vs* control (non-treated), $^{h}P < 0.05$ subject *vs* control (non-treated) (two-way ANOVA test).

of HCV and /or EtOH, or palmitate treatment. This effect was greater in fibrotic (F2-F4) LS cultures. Moreover, when fibrotic LS cultures were exposed to EtOH, a significant increase of α-SMA, HSP47, procol1A1 expression as well as the other markers involved in liver fibrolysis such as (MMP-2, -9) and VEGF was identified in both non-infected and infected liver slices.

The increased expression of fibrogenesis biomarkers was throughout the twenty-one days follow-up studies. RT-qPCR showed that the effect was more marked in LS cultures obtained from livers with advanced stages of fibrosis. This was confirmed for the following biomarkers: TGF-β1, α-SMA, HSP47, Procol1A1, MMP-2, -9, and VEGF. These results were further confirmed by Western blot analyses for TGF-β1, α-SMA, Col1A1, HSP47, MMP-2, -9, and VEGF. Thus, analyses of LS cultures revealed, that the


A
TGF-β1 (pg/mg protein)
NINF F0F1 EtOH 25mM
NINF F2F3 EtOH 25mM
NINF F4 EtOH 25mM
Days
B
TGF-β1 (pg/mg protein)
INF F0F1 EtOH 25mM
INF F2F3 EtOH 25mM
INF F4 EtOH 25mM
Days
C
Relative HSP47 expression /mg tissue
NINF F0F1 EtOH 25mM
NINF F2F3 EtOH 25mM
NINF F4 EtOH 25mM
Days
D
Relative HSP47 expression /mg tissue
INF F0F1 EtOH 25mM
INF F2F3 EtOH 25mM
INF F4 EtOH 25mM
Days
E
Relative α-SMA expression /mg tissue
NINF F0F1 EtOH 25mM
NINF F2F3 EtOH 25mM
NINF F4 EtOH 25mM
Days
F
Relative α-SMA expression /mg tissue
INF F0F1 EtOH 25mM
INF F2F3 EtOH 25mM
INF F4 EtOH 25mM
Days
G
Relative MMP-2 expression /mg tissue
NINF F0F1 EtOH 25mM
NINF F2F3 EtOH 25mM
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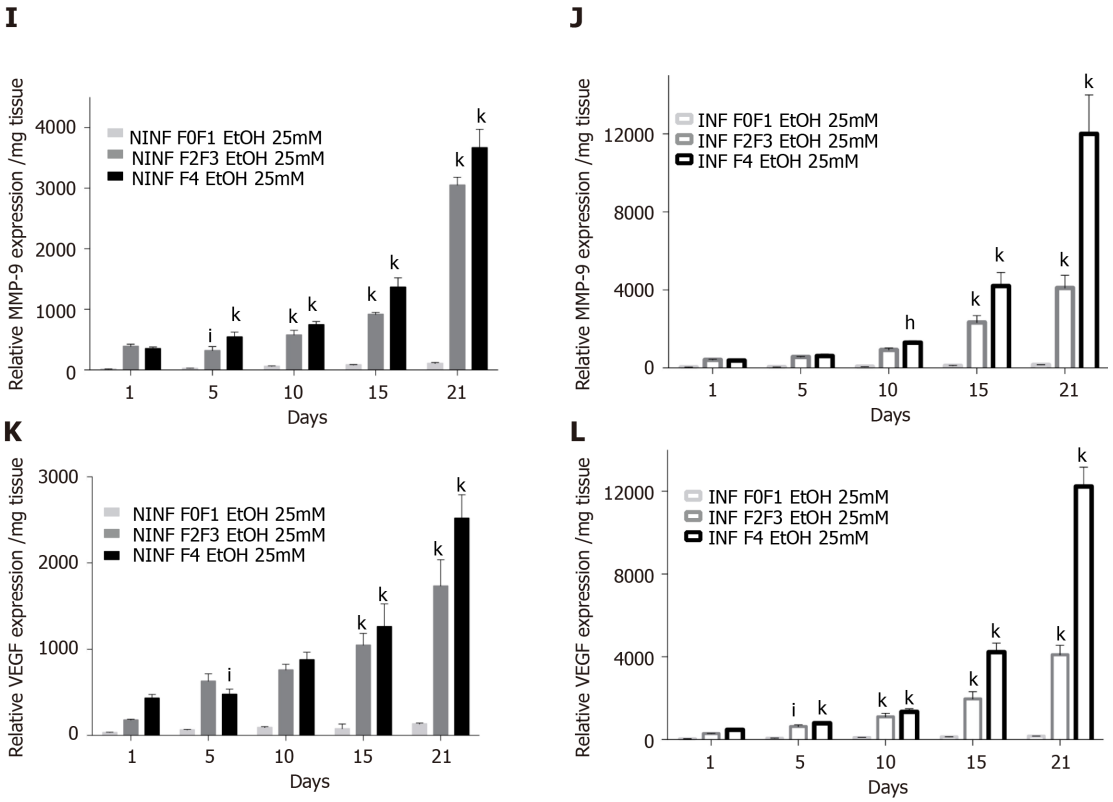

Figure 10 By real-time reverse transcription-quantitative polymerase chain reaction and enzyme-linked immunosorbent assay, significantly increase of TGF-β1 protein and RNA expression of fibrosis biomarkers HSP47, α-SMA, MMP- 2, MMP-9, VEGF increased in non-infected or hepatitis C virus-infected liver slice cultures from stages F0-F1 to stage F4 treated with 25 mmol/L of ethanol. A and B: TGF-β1 intracellular protein expression (pg/mg protein) during the 21 days follow up kinetics, in F0-F1 to F4 non-infected (A) and hepatitis C virus (HCV)-infected (B) liver slice (LS), treated with 25 mmol/L of ethanol (EtOH); C and D: Relative HSP47 RNA expression (relative RNA expression/mg tissue) during the 21 days follow up kinetics, in F0-F1 to F4 non-infected (C) and HCV infected (D) LS treated with 25 mmol/L of EtOH; E and F: Relative α-SMA RNA expression (relative RNA expression / mg tissue) during the 21 days follow up kinetics, in F0-F1 to F4 non-infected (E) and HCV-infected (F) LS treated with 25 mmol/L of EtOH; G and H: Relative MMP-2 RNA expression (relative RNA expression / mg tissue) during the 21 days follow up kinetics, in F0-F1 to F4 non-infected (G) and HCV-infected (H) LS cultures treated with 25 mmol/L of EtOH; I and J: Relative MMP-9 RNA expression (relative RNA expression / mg tissue) during the 21 days follow up kinetics, in F0-F1 to F4 non-infected (I) and HCV-infected (J) LS treated with 25 mmol/L of EtOH; K and L: Relative VEGF RNA expression (relative RNA expression/mg tissue) during the 21 days follow up kinetics, in F0-F1 to F4 non-infected (K) and HCV-infected (L) LS cultures treated with 25 mmol/L of EtOH. Values are expressed as mean ± SEMs (F0-F1: $n = 5$; F2-F3, $n = 2$; F4, $n = 2$). $^{k}P < 0.0001$ subject *vs* control (F0-F1); $^{i}P < 0.001$ subject *vs* control (F0-F1); $^{i}P < 0.01$ subject *vs* control (F0-F1), $^{h}P < 0.05$ subject *vs* control (F0-F1) (two-way ANOVA test).

progression of fibrosis is associated with an increase in the expression of certain biomarkers, in particular, α-SMA expression, and resembles a snowball effect, as shown by histochemistry results with a significant increase of collagen production in F0-F1 EtOH treated HCV infected LS on day 6 compared to day 1. As might be expected, a more marked fibrogenesis reaction was observed in fibrotic (F2-F3, F4) LS cultures, than that in non-fibrotic (F0-F1) LS cultures.

Thus, the LS model well responded to fibrotic inducers and, then, released a set of biomarkers that are usually detected during clinical studies in patients with fibrosis. In particular, this included TGF-β, α-SMA, Procollagen1A1, MMP-2, MMP-9, VEGF, the markers of liver fibrogenesis, whatever the origin of fibrosis. This study also showed the synergistic effect of liver comorbidities (virus, alcohol, and fat) on fibrogenesis and its consequences[28,29]. Finally, the efficacy of hepatoprotective[28] or anti-fibrotic drugs[29] was suggested in the LS cultures model. Recently, Wu *et al*[10] demonstrated that Human liver slices collected from resected livers could be maintained in *ex vivo* culture over a two-week period.

Several anti-fibrotic drugs are now in development[27-29], following validation in animal models[30], in particular, target inhibitors for the treatment of NASH-related fibrosis. This includes NGM282, an FGF19 analog that reduces steatosis, biliary acids injury, and lipotoxicity *via* 2 receptors, the MGL-3196, a THR-β1 agonist that decreases LDL-cholesterol, triglyceride and fatty liver, thus lipotoxicity[30,31]. Randomized controlled trials are known to take time and the results may be disappointing despite the encouraging results of the recent REGENERATE trial, in obeticholic acid[32-36]. For example, Cenicriviroc, a dual CCR2/CCR5 antagonist with positive results in mice,

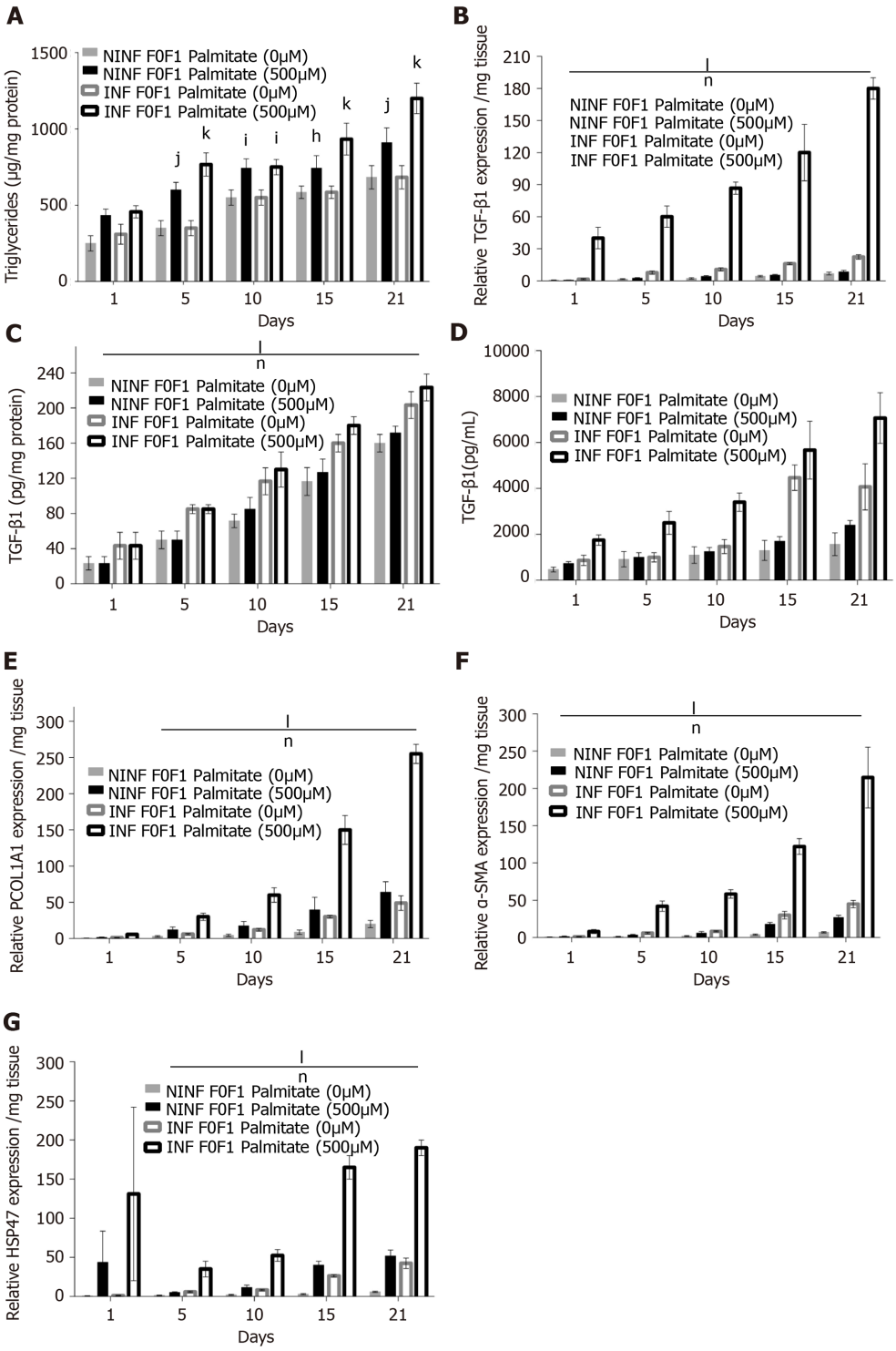

Figure 11 Significant increase of intracellular triglyceride production and RNA expression of fibrosis liver markers in non-fibrotic (F0-F1) hepatitis C virus INF liver slice cultures treated with palmitate (500 µmol/L) compared to non-infected and non-treated liver slice showed by enzyme-linked immunosorbent assay and real-time reverse transcription-quantitative polymerase chain reaction analyses, respectively. A: Triglyceride production (µg/mg protein) during the 21 days follow up kinetics: Non-significant production in hepatitis C virus (HCV) INF liver slice (LS) compared to non-infected (NINF) LS: (ns NINF *vs* INF); significant increase in HCV INF LS treated with palmitate compared to NINF; B: Significant increase of TGF-β1 mRNA expression (Relative RNA expression /mg tissue) during the 21 days follow up kinetics, in HCV INF LS compared to NINF LS and in HCV INF LS treated with palmitate compared to NINF; C and D: (C) Intracellular (pg/mg protein) and (D) extracellular (pg/mL) TGF-β1 protein production during the 21 days follow up kinetics, measured by enzyme-linked immunosorbent assay assays, in F0-F1 NINF and HCV INFLS cultures treated or non-treated with palmitate; Significant increase in HCV INF LS compared to NINF LS; Significant increase in HCV INF LS treated with palmitate compared to NINF; E-G: Intracellular mRNA expression (Relative RNA expression /mg tissue) of the Procol1A1 (E), α-SMA (F), HSP47 (G) during the 21 days follow up kinetics: Significant increase in HCV INF LS compared to NINF LS; Significant increase in HCV INF LS treated with palmitate compared to NINF LS. Data are expressed as mean± SEM (F0-F1, n = 5); ^{l}P < 0.0001 infection factor; ^{m}P < 0.001 infection factor; ^{n}P < 0.0001 palmitate factor (two-way ANOVA test).

did not result in any significant reduction in NASH-related fibrosis after 2 years of studies[37], and Selonsertib, an ASK1 inhibitor with putative anti-fibrotic properties, was recently withdrawn in the Stellar-3 and Stellar-4 Randomized controlled trials (Gilead, Press release, April 2019).

Although randomized clinical trials are the best way to prove the drug efficacy of a drug, there are several important limitations to this approach including the need for serial liver biopsies, suboptimal dosage schedules, or placebo double-blinded controls with a single drug. All of this can require about three years. With existing LS models, anti-fibrotic drug testing can be performed for 2-3 weeks (wk). Testing is possible for single drugs, drug combinations with similar or different agents, dose effects, stability in the liver, *etc*…

In the present study, we used our 3D LS *ex vivo* models to investigate the anti-fibrotic properties of two drugs, being tested in clinical trials. Ursodeoxycholic acid is indicated in the treatment of primary biliary cirrhosis and dissolve radiolucent gallstones in patients with a functioning gallbladder. Alpha-Tocopherol (Toco, vitamin E) is tested currently in patients with high cholesterol and NASH. A meta-analysis in a sub-group analysis of random clinical trials has shown that alpha-tocopherol has an anti-fibrotic effect compared to UCDA[36,38]. These drugs were tested alone and in a combination with the LS model. The combined treatment is not tested during the first phase of the clinical trials. The half-life of UCDA is 3.5 to 5.8 days and that of Toco is 44.5 hours. Patients must be treated daily with UCDA for 2 to 3 months and for 96 wk with Toco to obtain some clinical effects. In the LS model, Toco treatment only reduced the TGF-β1 expression in non-infected and infected LS with stage F4 after day 10. After day 15, UCDA reduced TGF-β1 expression in stage F2 to F4 infected LS. It is interesting to note that with a combination of both drugs, TGF-β1 and Procol1A1 expression was reduced significantly in LS. The level of TGF-β1 decreased nearly 2 fold in F2-F3 infected LS on day 15 and 2.5 fold on day 21, in F4 infected LS cultures. A significant reduction in procol1A1 RNA expression was found with the combination treatment in F2-F3, and in F4 infected and non-infected LS cultures with a two-fold decrease on days 15 and 21. Obviously, to confirm the results, the other dosages and proportions of drugs (in combination) should be tested. In fact, this model showed a clear decrease in the main hepatic fibrogenesis biomarker TGF-β1, in the presence of a combination of anti-fibrotic drugs (UCDA and Toco) in F2-F4 infected LS cultures with a significant decrease in both triglyceride production and Procol1A1 expression. Procol1A1 expression was significantly reduced in F2-F3, and F4 infected or non-infected LS cultures during combined treatment (UCDA and Toco). Thus, these data provide a proof of concept that this proposed 3D *ex vivo* model effectively allows a rapid evaluation of new anti-fibrotic drugs.

CONCLUSION

In summary, the 3D *ex vivo* LS model provides hepatocyte-specific gene expression for 21 days, and effectively reproduces liver fibrogenesis related to HCV infection, EtOH, or lipids exposure, thus, mimicking human viral, alcoholic, and NASH liver diseases. Our study is the proof of concept that this relatively easy model can be used to study human liver fibrogenesis of different origins and evaluate the potency of new anti-fibrotic therapies that are currently under development. In particular, this system might estimate unpredictable side effects when testing certain drug combinations.

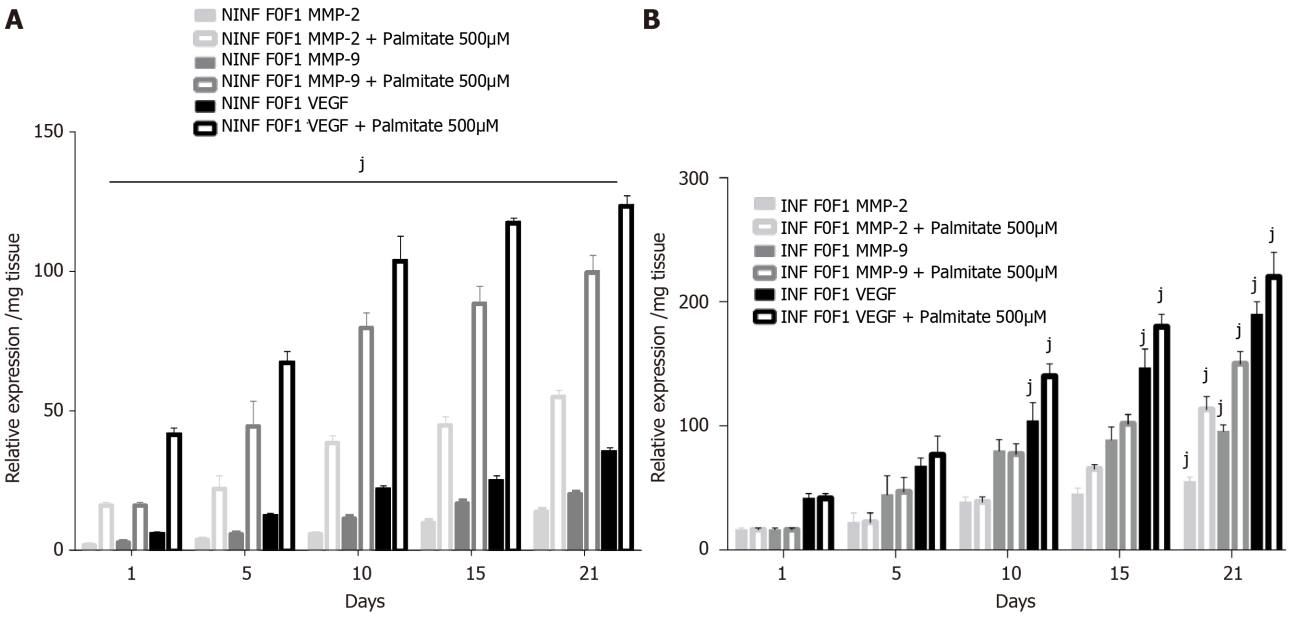

Figure 12 Significant increase of matrix metalloproteinases -2, -9, and vascular endothelial growth factor RNA expression after treatment of F0-F1 non-infected and infected liver slice cultures with palmitate (500 μmol/L). Biomarker expression estimated by real-time reverse transcription-quantitative polymerase chain reaction. A: Matrix metalloproteinases (MMP)- 2, MMP-9 and vascular endothelial growth factor mRNA expression (relative expression /mg tissue) during the 21 days follow up kinetics, in F0-F1 non-infected liver slice (LS) cultures treated without or with palmitate (500 μmol/L); B: MMP- 2, MMP-9 and vascular endothelial growth factor mRNA expression (relative expression /mg tissue) during the 21 days follow up kinetics, in F0-F1 infected LS cultures treated without or with palmitate (500 μmol/L). Real-time reverse transcription-quantitative polymerase chain reaction experiments were performed with five independent human F0-F1 liver samples (n = 5). LS were obtained in triplicate for each liver sample, at each time point in the kinetic studies. Values are expressed as means ± standard errors (n = 5). Levels of significance were as follows: $^{j}P < 0.001$ subject *vs* control (non-treated palmitate), (two-way ANOVA test).

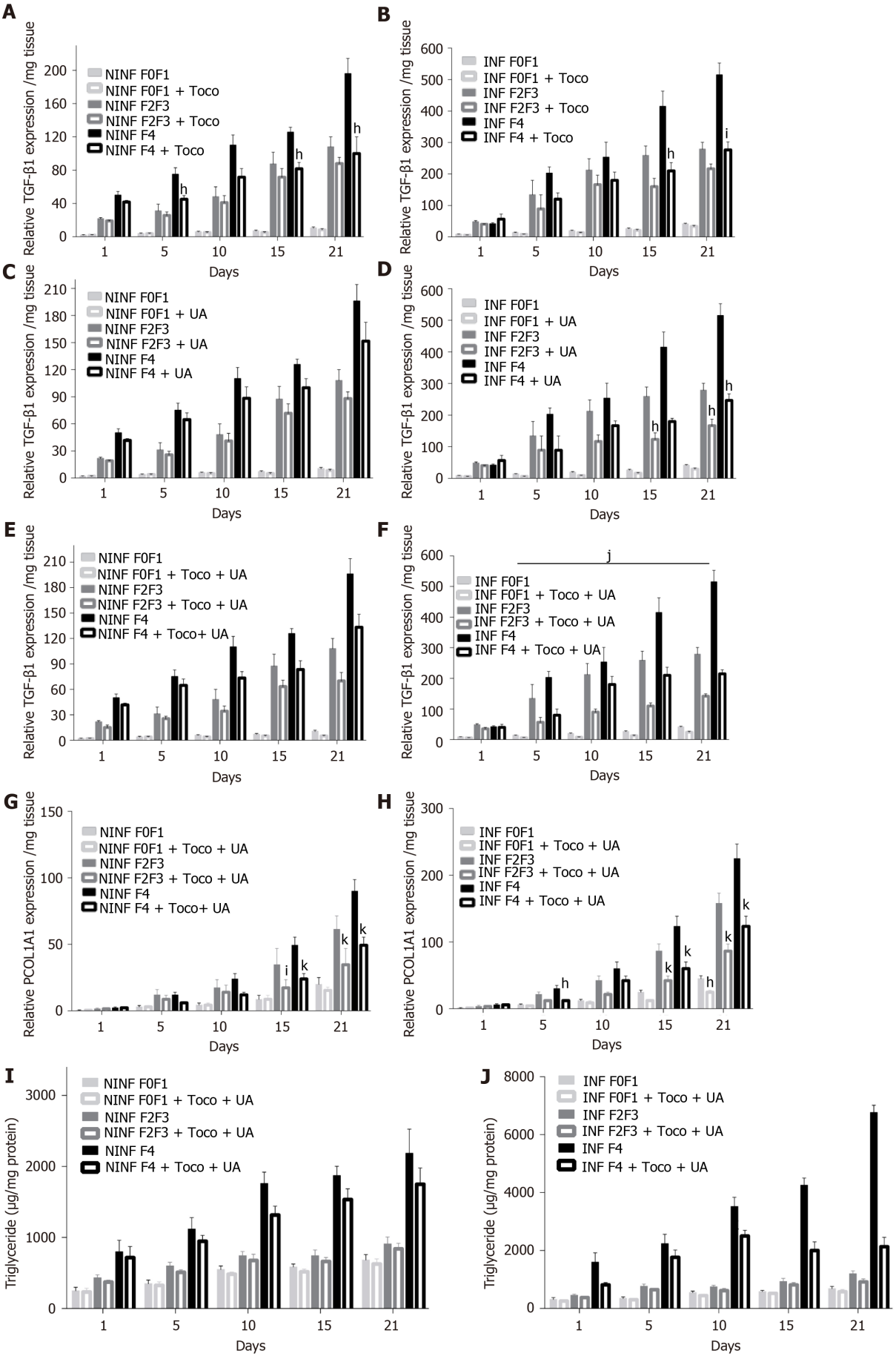

Figure 13 During treatment with alpha-Tocopherol and ursodeoxycholic acid in combination, significant inhibition of the TGF-β1 mRNA

expression of fibrotic (F2-F3, F4) hepatitis C virus INF liver slice cultures from day 5 and significant reduction of Procol1A1 mRNA expression and the triglyceride production in F0 to F4 non-infected and hepatitis C virus INF liver slice cultures during the follow-up kinetics, as evidenced the real-time reverse transcription-quantitative polymerase chain reaction analysis and enzyme-linked immunosorbent assays, respectively. A and B: TGF-β1 mRNA expression (relative TGF-β1 expression /mg tissue) during the 21 days follow up kinetics, in α-Tocopherol (Toco) treated liver slice (LS); C and D: TGF-β1 mRNA expression (relative TGF-β1 expression /mg tissue) during the 21 days follow up kinetics, in ursodeoxycholic acid (UCDA) treated LS; E and F: TGF-β1 mRNA expression (relative TGF-β1 expression /mg tissue) during the 21 days follow up kinetics, in LS during the combined treatment, Toco + UCDA. Data are expressed as means ± SEM (F2-F3 liver samples, n = 2; F4 liver samples, n = 2). $^{k}P < 0.0001$ subject *vs* control (non-treated); $^{j}P < 0.001$ subject *vs* control (non-treated); $^{i}P < 0.01$ subject *vs* control (non-treated), $^{h}P < 0.05$ subject *vs* control (non- treated) (two-way ANOVA test); G and H: Procol1A1 mRNA expression (relative Procol1A1 expression /mg tissue) during the 21 days follow up kinetics, in LS during the combined treatment, Toco + UCDA. Data are expressed as means ± SEM (F0-F1 liver samples, n = 10; F2-F3 liver samples, n = 2; F4 liver samples, n = 2). $^{k}P < 0.0001$ subject *vs* control (non-treated); $^{j}P < 0.001$ subject *vs* control (non-treated); $^{i}P < 0.01$ subject *vs* control (non-treated), $^{h}P < 0.05$ subject *vs* control (non-treated); (two-way ANOVA test); I and J: Triglyceride production (μg/mg protein) during the 21 days follow-up kinetics, in NINF and hepatitis C virus INF LS from F0-F1 to F4 LS cultures significantly reduced by the combined treatment [Toco + UCDA (UA)], more particularly from day 15 in F4 hepatitis C virus infected LS cultures. Data are expressed as means ± SEM (F0-F1, n = 5, F2-F3 liver samples, n = 2; F4 liver samples, n = 2). $^{k}P < 0.0001$ subject *vs* control (non- treated); $^{j}P < 0.001$ subject *vs* control (non-treated); $^{i}P < 0.01$ subject *vs* control (non-treated), $^{h}P < 0.05$ subject *vs* control (non-treated) (two-way ANOVA test).

ARTICLE HIGHLIGHTS

Research background

Liver fibrosis is frequently associated with viral infection [Hepatitis C virus (HCV) and Hepatitis B virus] infection, chronic inflammation, and excessive alcohol consumption. Despite effective antiviral treatment, morbidity and hepatitis-related mortalities are still increasing. Moreover, the number of non-viral liver diseases such as nonalcoholic steatohepatitis and alcoholic liver disease is steadily growing.

Research motivation

In previous studies, we developed a three dimensional (3D) *ex vivo* model of HCV replication using human liver slice cultures that were followed for 10 days to evaluate a new antiviral drug.

Research objectives

We aimed to establish a 3D *ex vivo* liver slice model viable *in vitro* for 21 days allowing us to examine human liver fibrogenesis by fibrosis inducers and anti-fibrotic therapies.

Research methods

The adult human liver tissue samples from twenty patients were collected after liver resection, and divided into three groups according to their METAVIR score (F): Non-fibrotic F0-F1, obtained during surgery for colorectal cancer liver metastases or fibrotic ranging from F2 to F4. HCV infection, alcohol (ethanol stimulation), and steatosis (palmitate stimulation) were examined in non-fibrotic F0-F1 human liver slices (HLS) compared to fibrotic (F2 to F4) liver slices (FLS) infected (or not) with HCV [Con1/C3 (genotype1b)] (INF). HLS of 350 μm (2.7×10^{6} cells per slice) were cultivated for up to 21 days. At day 0, either ursodeoxycholic acid (only choleretic and hepatoprotective properties) and/or α-tocopherol (Toco, anti-oxidant properties which could reduce fibrosis progression) were added to standard of care concentrations on HLS and FLS. The following fibrosis markers expression were assayed in HLS, in FLS and in INF FLS, [tumor growth factor-beta (TGF-β1), Hsp47, Alpha smooth muscle actin, Procol1A1, Matrix metalloproteinases 2, 9 (MMP-2, 9), Vascular endothelial growth factor] and checked by real-time reverse transcription-quantitative polymerase chain reaction and the triglyceride production by enzyme-linked immunosorbent assay assays.

Research results

Here, for the first time, human LS cultures (stages F0-F4) were successfully maintained and evaluated for 21 days allowing to explore molecular fibrogenesis in more detail including the role of important factors such as HCV infection, ethanol (EtOH), or steatosis, three of the main causes of liver injury in clinical practice. In addition, it was demonstrated that LS cultures are efficient instruments to study anti-fibrotic drugs and their combination. We obtained real-time reverse transcription-quantitative polymerase chain reaction analyses of the biomarkers (TGF-β1, procol1A1, MMP-2, MMP-9, Alpha smooth muscle actin, HSP47, and Vascular Endothelial Growth Factor) involved in molecular fibrogenesis, and estimation of anti-fibrotic drugs potency, in

both non-fibrotic (F0-F1) and fibrotic livers samples (F2-F3, F4). Expression of the fibrosis biomarkers and the progression to steatosis (estimated by triglyceride production) increased with the addition of HCV and /or EtOH or palmitate. We observed a significant decrease in both of the expression of TGF-β1, and procollagen1A1 as well as in the production of triglycerides observed in a combined anti-fibrotic treatment applied to the F2-F4 LS cultures infected with HCV.

Research conclusions

The 3D *ex vivo* LS model provides hepatocyte-specific gene expression for 21 days, and effectively reproduces liver fibrogenesis related to HCV infection, EtOH, or lipids exposure, thus, mimicking human viral, alcoholic, and nonalcoholic steatohepatitis liver diseases. Our study is the proof of concept that this relatively easy model can be used to study human liver fibrogenesis of different origins and evaluate the potency of new anti-fibrotic therapies that are currently under development. In particular, this system might estimate unpredictable side effects when testing certain drug combinations.

Research perspectives

Using the *ex vivo* model of human liver slice culture, the perspectives would be to evaluate the potency of new anti-fibrotic therapies alone or in combination and to study the immune components of liver disease.

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ORIGINAL ARTICLE

Clinical and Translational Research

Production and activity of matrix metalloproteinases during liver fibrosis progression of chronic hepatitis C patients

Moises Martinez-Castillo, Abigail Hernandez-Barragan, Ivonne Flores-Vasconcelos, Marina Galicia-Moreno, Dorothy Rosique-Oramas, Jose Luis Perez-Hernandez, Fatima Higuera-De la Tijera, Eduardo E Montalvo-Jave, Aldo Torre-Delgadillo, Paula Cordero-Perez, Linda Muñoz-Espinosa, David Kershenobich, Gabriela Gutierrez-Reyes

ORCID number: Moises Martinez-Castillo 0000-0003-1735-502X; Abigail Hernandez-Barragan 0000-0003-4725-0536; Ivonne Flores-Vazconcelos 0000-0002-2906-3983; Marina Galicia-Moreno 0000-0002-7435-1861; Dorothy Rosique-Oramas 0000-0002-6255-1516; Jose Luis Perez-Hernandez 0000-0003-4367-8463; Fatima Higuera-De la Tijera 0000-0003-1862-7541; Eduardo E Montalvo-Jave 0000-0003-4616-1275; Aldo Torre-Delgadillo 0000-0002-9299-3075; Paula Cordero-Perez 0000-0001-7747-8895; Linda Muñoz-Espinosa 0000-0002-0455-9325; David Kershenobich 0000-0001-6178-9170; Gabriela Gutierrez-Reyes 0000-0003-1961-9885.

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Moises Martinez-Castillo, Abigail Hernandez-Barragan, Ivonne Flores-Vasconcelos, Dorothy Rosique-Oramas, Gabriela Gutierrez-Reyes, Liver, Pancreas and Motility Laboratory, Unit of Experimental Medicine, School of Medicine, General Hospital of Mexico, Universidad Nacional Autonoma de Mexico, Mexico City 06726, Mexico

Marina Galicia-Moreno, Department of Molecular Biology and Genomics, Institute of Molecular Biology in Medicine and Gene Therapy, Health Science University Center, University of Guadalajara, Guadalajara 06726, Mexico

Jose Luis Perez-Hernandez, Fatima Higuera-De la Tijera, Department of Gastroenterology, General Hospital of Mexico "Dr. Eduardo Liceaga," Mexico City 06726, Mexico

Eduardo E Montalvo-Jave, Department of General Surgery, General Hospital of Mexico "Dr. Eduardo Liceaga," School of Medicine, Universidad Nacional Autonoma de Mexico, Mexico City 06726, Mexico

Aldo Torre-Delgadillo, David Kershenobich, Hepatology and Liver Transplant, National Institute of Medical Sciences and Nutrition "Salvador Zubirán," Mexico City 06726, Mexico

Paula Cordero-Perez, Linda Muñoz-Espinosa, University Hospital "Dr. José Eleuterio González," Autonomous University of Nuevo Leon, Monterrey 06726, Mexico

Corresponding author: Gabriela Gutierrez-Reyes, PhD, Academic Researcher, Professor, Research Scientist, Liver, Pancreas and Motility Laboratory, Unit of Experimental Medicine, School of Medicine, General Hospital of Mexico, Universidad Nacional Autonoma de Mexico, Dr. Balmis 148 Col Doctores Cuauhtemoc, Mexico City 06726, Mexico.
gabgurey@yahoo.com.mx

Abstract

BACKGROUND

Matrix metalloproteinases (MMPs) participate in the degradation of extracellular matrix compounds, maintaining the homeostasis between fibrogenesis and fibrolytic processes in the liver. However, there are few studies on the regulation of liver MMPs in fibrosis progression in humans.

M contributed to the matrix metalloproteinases assessment; Martinez-Castillo M and Gutierrez-Reyes G contributed to the manuscript writing; Gutierrez-Reyes G contributed to the critical revision of the manuscript; All authors read and approved the final manuscript.

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Institutional review board statement: The study was previously approved by the institutional ethics committees of the Hospital General de México (HG/DI/16/107/03/082) and the Universidad Nacional Autónoma de México (FMD/DI/15/2015), guaranteeing its performance in accordance with the ethical principles described in the 1975 Declaration of Helsinki.

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AIM

To assess the production activity and regulation of matrix metalloproteinases in liver fibrosis stages in chronic hepatitis C (CHC).

METHODS

A prospective, cross-sectional, multicenter study was conducted. CHC patients were categorized in fibrosis grades through FibroTest® and/or FibroScan®. Serum MMP-2, -7, and -9 were determined by western blot and multiplex suspension array assays. Differences were validated by the Kruskal-Wallis and Mann-Whitney U tests. The Spearman correlation coefficient and area under the receiver operating characteristic curve were calculated. Collagenolytic and gelatinase activity was determined through the Azocoll substrate and zymogram test, whereas tissue inhibitor of metalloproteinase-1 production was determined by dot blot assays.

RESULTS

Serum concentrations of the MMPs evaluated were higher in CHC patients than in healthy subjects. MMP-7 distinguished early and advanced stages, with a correlation of 0.32 ($P < 0.001$), and the area under the receiver operating characteristic displayed moderate sensitivity and specificity for MMP-7 in F4 (area under the receiver operating characteristic, 0.705; 95% confidence interval: 0.605-0.805; $P < 0.001$). Collagenolytic activity was detected at F0 and F1, whereas gelatinase activity was not detected at any fibrosis stage. Tissue inhibitor of metalloproteinase-1 determination showed upregulation in F0 and F1 but downregulation in F2 ($P < 0.001$).

CONCLUSION

High concentrations of inactive MMPs were present in the serum of CHC patients, reflecting the impossibility to restrain liver fibrosis progression. MMPs could be good diagnostic candidates and therapeutic targets for improving novel strategies to reverse liver fibrosis in CHC.

Key Words: Extracellular matrix; Matrix metalloproteinases; Liver fibrosis; Chronic hepatitis C; Fibrogenesis; Fibrolysis

Core Tip: The relevance of this prospective study was to evaluate the role of matrix metalloproteinases in the pathophysiology of liver fibrosis in chronic hepatitis C patients. Matrix metalloproteinases could be used as possible therapeutic targets and as a monitoring tool in treatment-experienced patients that continue to present with liver fibrosis and develop cirrhosis and/or hepatocellular carcinoma.

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INTRODUCTION

Liver fibrosis is a convergence of repair mechanisms for chronic cellular damage, which can be induced by several etiologies, including the hepatitis B and C viruses, alcoholic liver disease (ALD) and nonalcoholic fatty liver disease, among others[1]. The intricate mechanism of the tissue repair response of extracellular matrix (ECM) compounds has been associated with the balance between fibrogenesis and fibrolysis[2]. In a normal liver, ECM proteins, fibronectin, laminin, proteoglycans and

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collagen types I, III, IV and V comprise approximately 0.5% of the wet weight[2,3]. The unregulated accumulation of ECM can result in chronic liver damage, promoting cirrhosis and hepatocellular carcinoma (HCC), with the subsequent death of patients. The uncontrolled deposition of collagen in the liver parenchyma involves the unceasing activation of hepatic stellate cells (HSCs), which represent approximately 5% to 10% of resident liver cells[4].

Some of the representative features of activated or transdifferentiated HSCs to myofibroblast-like cells are the loss of vitamin A storage, proliferation, inflammation, chemotaxis and ECM production[5,6]. Transforming growth factor beta (TGF-β) is the most potent fibrogenic cytokine and promotes smad-3 protein activation, stimulating the active transcription of collagen type I and III[7]. TGF-β can also activate the MAPK/p38/c-JNK pathway[8], inducing a continuous proinflammatory milieu. Other proliferative HSC inductors have been well described, such as PDGF, CTGF and VEGF[1].

The control of collagen and other ECM elements is known to be regulated by the family of matrix metalloproteinases (MMPs)[9]. The MMPs have been classified into broad groups related to their activity: Collagenases, gelatinases, membrane-type MMPs, stromelysins and matrilysins[10]. MMPs also play important roles in the degradation and activation of immune mediators (*e.g.*, cytokines and antimicrobial peptides)[2]; those biologic regulators are usually released as zymogens that require additional processing in the extracellular space by self-activation, the indirect action of plasminogen and the assistance of transmembrane MMP activity. Thus, some reports state that MMPs may display dual roles in liver fibrosis, depending on the timing of action. Proteolytic activity is mainly controlled by reversible tissue inhibitors of metalloproteinases (TIMPs, 1-4)[11,12]. In fact, activated HSCs have been reported to upregulate TIMP-1, enabling the accumulation of ECM proteins in the extracellular space[13,14].

Approximately 20 years ago, MMP-2 was described to be overexpressed in the liver parenchyma of human fibrotic and cirrhotic patients[15], and a direct association with collagen I expression was also reported in an animal model[14]. In addition, MMP-2 participated in the activation of TGF-β and the modulation of IL-1β, TNF-α and MCP-3 by proteolytic cleavage[16]. In 2000, Lichtinghagen *et al*[17] reported that peripheral blood cells revealed a correlation between the MMP-2/TIMP-1 ratio and the histologic grade of fibrosis in patients with chronic active cirrhosis due to hepatitis C. The authors concluded that said ratio could be used as a progression marker in patients with chronic liver disease[17]. MMP-2 (gelatinases A) and MMP-9 (gelatinases B) were recently suggested as serum biomarkers of ALD severity in a region in Poland[18]. The chronologic expression of MMP-2 and MMP-9 in hepatic fibrosis was proposed using different animal models of fibrosis. Those MMPs were relatively overexpressed and TIMP-1 was downregulated after the fibrosis inductor was eliminated[14].

A recent multi-analysis of serum proteins demonstrated that MMP-7 was directly associated with fibrosis. The authors suggested that MMP-7 could be a valuable indicator of advanced fibrosis and might play a role in liver fibrogenesis, due to its role as a matrix remodeling factor[19]. However, there is little information about MMP-7 and liver fibrosis progression in the liver as well as at the serum level.

The correct determination of liver fibrosis stages is imperative for making the diagnosis and implementing therapeutic decisions. At present, there is no evidence of the production and activity of MMP-2, MMP-7 or MMP-9 or their correlation with fibrosis progression in serum samples from patients. In the present work, we evaluated the serum concentration and proteolytic capacity of MMP-2, -7 and -9 in chronic hepatitis C (CHC) patients according to fibrosis progression.

MATERIALS AND METHODS

Patient selection

A prospective, cross-sectional, observational study was conducted. Patients were carefully selected from the Hospital General de México, "Dr. Eduardo Liceaga," the Universidad Autónoma de Nuevo Leon and the Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán." The patients included in the study were diagnosed with CHC (n = 119) and were treatment naïve. Fibrosis degrees were classified according to international guidelines by the FibroTest® and/or FibroScan® methods (F0, F1, F2, F3 or F4). The fibrosis stages of patients classified by FibroScan® and FibroTest® were grouped into similar intermediate classifications (F0-F1, F1-F2, F2-F3 and F3-F4). Patients whose tests were concordant or in close stages were included,

whereas patients whose results were discrepant were discarded. Patients with clinical evidence of risk alcohol consumption (AUDIT > 8) and/or systemic infections (*e.g.*, bacteria, flu, autoimmune diseases, *etc.*) and comorbidities (*e.g.*, diabetes and hypertension) were excluded. The control group consisted of blood bank donors from the Hospital General de México with negative serology for HIV and hepatitis A, B and C viruses and classified as non-risk drinkers (AUDIT < 8) (n = 119).

Clinical and biochemical values

The anthropometric variables collected for both sexes were age, height, weight and body mass index (kg/m^2; weight/height^2), and the biochemical parameters were hemoglobin, hematocrit, leukocyte count, platelets, total bilirubin, direct bilirubin, aspartate aminotransferase, alanine aminotransferase and gamma glutamyl transpeptidase.

Sample collection

A total of 30 mL of blood was drawn from all participants for the samples; 20 mL were used for the biochemical tests and 10 mL to obtain serum. The samples were centrifuged at 3500 rpm for 10 min, and the serum was recovered and stored at -80 °C until its use for evaluating MMP concentration and regulation.

Serum MMP-2, -7, and -9 determination by western blot

Serum samples were randomly collected from CHC patients and healthy individuals and incubated at 65 °C for 7 min. Total protein concentration was evaluated by the Bradford method[20]. The sample buffer (Laemmli 2X) and 10% β-mercaptoethanol (Bio-Rad) were then added and the final protein concentration was adjusted to 10 μg/μL for all the samples evaluated.

The proteins were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Electrophoresis was performed at 100 V for 1 h, and the proteins were then transferred to PVDF membranes (Perkin-Elmer) at 400 mA for 60 min. The membranes were blocked with 7% skim milk dissolved in PBS and incubated for 2 h. Solid phase detection was carried out overnight at 4 °C in agitation, utilizing MMP-2, MMP-7, and MMP-9 mouse polyclonal antibodies (1:1000) (Santa Cruz Biotechnology). The membranes were washed three times with 0.05% Tween 20 in PBS and incubated with secondary goat-anti-mouse-IgG peroxidase-conjugated antibodies (1:2500) (Santa Cruz Biotechnology) for 1 h at 37 °C. The membranes were then washed with 0.05% Tween 20 in PBS and exposed to a luminol kit reagent (Santa Cruz Biotechnology) using Kodak film. Densitometry analysis was performed using the ImageJ program (http://rsb.info.nih.gov/nih-image), and the results were expressed as relative optical density.

MMP-2, -7 and -9 serum concentration

MMP concentration was evaluated by multiplex suspension array technology (Millipore®). Nontreated serum samples from patients and controls (25 μL) were evaluated using the HMMP2MAG-55K kit, which allowed the simultaneous determination of MMP-2, -7 and -9 concentrations with no cross-reactivity and minimal intra- and interassay error (% CV < 10) (Merck, Millipore ®, United States). The data were acquired utilizing Luminex200 MAGPIX® Systems equipment, following the supplier's specifications (series number 10294005; Merck, Millipore, United States). The data were validated with internal standards and controls, and minimum and maximum detection values for each protein were obtained using Luminex XPONENT software.

Azocoll quantitative assays

To determinate the collagenolytic activity of MMPs in serum, we used the chromogenic substrate, Azocoll. Two milligrams of Azocoll were incubated with 10 μg/μL of total serum protein and adjusted to 500 μL by the addition of an activation buffer at pH 9.0 (100 mmol/L glycine, 2 mmol/L $CaCl_2$) (J.T. Baker, PA, United States). The serums were incubated overnight with shaking at 37 °C in duplicate. The reaction was stopped with 10% trichloroacetic acid (500 μL) (Sigma-Aldrich, MO, United States), and the samples were centrifuged at 4600 × g for 15 min. The supernatants were collected, and their absorbances were evaluate at 520 nm. A total of 2.5 μg/mL (4.5 U/mL) of collagenase from *Clostridium histolyticum* was used for the positive control. Protease activity was reported as units per milligram; that unit of measure is equivalent to the amount of substrate degraded in 1 min per milliliter[21].

Detection of MMP activity in gelatin-zymogram

MMP activity was analyzed in serum from CHC patients and control subjects by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, copolymerized with 0.1% porcine skin gelatin (type A) (Sigma-Aldrich) or collagen (Collagen Standard from SIRCOL kit assays; Biocolor, United Kingdom) as the substrate. Concentrations of 10 μg of protein were loaded per well. Electrophoresis was performed at 100 V at 4 ^{o}C for 3 h. A total of 5 μg/mL of broad-spectrum collagenase from *Clostridium histolyticum* was used for the positive control (Collagenase P; Roche, United States). After electrophoresis, the gels were washed twice with a 2.5% Triton X-100 (Sigma-Aldrich) solution for 15 min with shaking. For MMP activation, the gels were incubated overnight with buffer solution at pH 9.0 (100 mmol/L glycine and 2 mmol/L $CaCl_2$ with or without 2 mmol/L dithiothreitol). Finally, the gels were stained with 0.5% (w/v) Coomassie brilliant blue R-250 for 30 min. Protease activities were observed after the gels were decolored with methanol-acetic acid-water (%) (50:10:40) until clear bands on a blue background were obtained.

TIMP-1 determination in fibrosis grades by dot blot

To determine TIMP-1 in the different grades of liver fibrosis, we performed dot blot assays. All samples (5 μg/mL) were placed by drops onto PVDF membranes (0.22 μm), and bound protein was determined with Ponceau S solution (Sigma-Aldrich). Dot blots were determined using an anti-TIMP-1 polyclonal antibody (1:500) overnight, followed by incubation with secondary anti-goat antibody (1:2500; Invitrogen, MA, United States). The blots were exposed to a luminol kit reagent (Santa Cruz Biotechnology, TX, United States) using Kodak photographic film. The densitometry analysis was evaluated with the ImageJ program (http://rsb.info.nih.gov/nih-image), and the data were expressed as relative optical density.

Statistical analysis

The continuous variables were described as mean ± standard error of the mean and the qualitative variables as absolute and relative frequencies (%). The qualitative variables were analyzed using the chi-square test, and the continuous parameters were analyzed using the Mann-Whitney U test. Relative optical density data and MMP and TIMP-1 activity from the western blot and Azocoll assays were plotted using GraphPad Prism Software V6 (CA, United States). The *P* values were calculated using two-way ANOVA and Bonferroni's Multiple Comparisons Test. The Pearson correlation was calculated, and the area under the receiver operating characteristic curve for MMP-7 was determined in all the fibrosis stages to determine its relevance as a biomarker. Differences were considered statistically significant when the *P* value was less than 0.05. The statistical analysis was performed using the IBM SPSS Statistics for Windows, Version 22 program (IBM Corp, NY, United States).

RESULTS

Demographic and biometric analyses

A total of 119 patients with CHC were included. Unexpectedly, the CHC group mainly consisted of women. The demographics and biochemical information were contrasted with 119 healthy subjects that were predominantly male (Table 1). Only body mass index did not display differences in the biometric analysis. The data are summarized in Table 1.

Overproduction of MMP-2, -7 and -9 in the serum of CHC patients

To evaluate the presence of MMPs in serum from patients and healthy individuals, we first determined the presence of each of the MMPs by western blot assays. The results showed the presence of bands with molecular weights of 75, 29 and 50 kDa, using specific antibodies against MMP-2, MMP-7, and MMP-9, respectively (Figure 1A). The densitometric analysis of all the MMPs evaluated showed an evident increase in the CHC patients (Figure 1B).

After observing those differences, we evaluated the specific concentration of each MMP by multiplex suspension array technology. Interestingly, the multiplexed determination of MMPs in the CHC patients (n = 119) and controls (n = 119) showed that MMP-2 had higher concentration values compared with MMP-7 and MMP-9 (Figure 2). The results correlated with the western blot assays.

Table 1 Demographic and clinical data of chronic hepatitis C patients compared with healthy individuals

	CHC, *n* = 119	CT, *n* = 119	*P* value
Sex, *n* (%)			< 0.001
Men	53 (45)	75 (63)	
Women	66 (55)	44 (37)	
Age in yr	54 ± 13	37 ± 10	< 0.001
BMI in kg/m^2	27 ± 1	28 ± 1	0.340
Hb in g/dL	14 ± 2	16 ± 1	< 0.001
Leu as 10^3/μL	5.1 ± 1.0	7.4 ± 0.2	< 0.001
Platelets as × 10^3	177 ± 78	272 ± 62	< 0.001
Total bilirubin in mg/dL	2.52 ± 1.70	0.75 ± 0.29	0.001
Direct bilirubin in mg/dL	0.18 ± 0.15	0.07 ± 0.06	0.001
AST in UI/L	60 ± 5	30 ± 11	< 0.001
ALT in UI/L	63 ± 5	26 ± 18	< 0.001
GGT in UI/L	92.50 ± 10.67	30.55 ± 2.43	< 0.001

Data were expressed as the mean ± standard deviation. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; CHC: Chronic hepatitis C; CT: Control; GGT: Gamma glutamyl transpeptidase; Hb: Hemoglobin; Leu: Leukocytes.

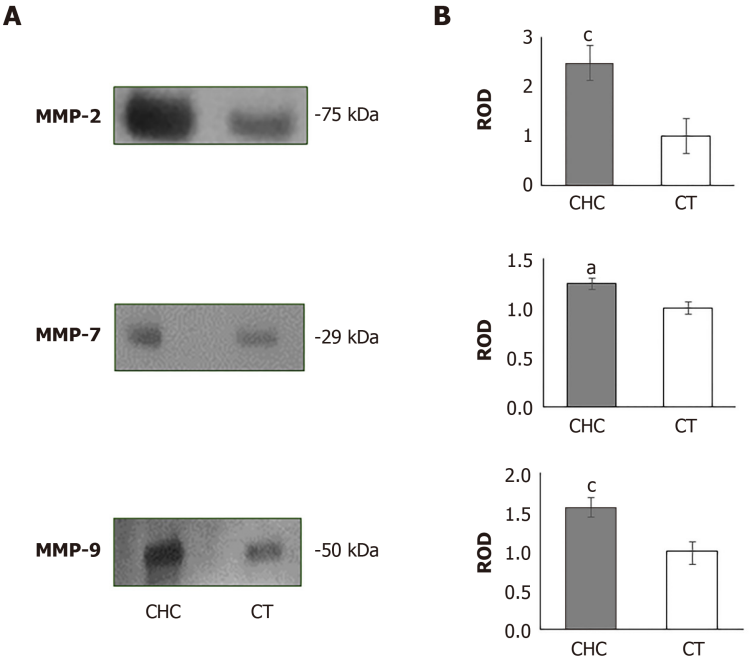


Figure 1 Matrix metalloproteinase-2, -7 and -9 determination in serum. A: Detection of bands at 75, 29 and 50 kDa in chronic hepatitis C (CHC) patients and healthy individuals corresponding to matrix metalloproteinase (MMP)-2, MMP-7 and MMP-9, respectively; B: Densitometric analysis of each of the MMPs in the patients (gray bars) and the control (CT) group (white bars) expressed as relative optical density (ROD). Relative optical density analysis was performed using ImageJ software. Bars display the mean ± standard error of the mean of five independent assays of random samples from CHC samples and CT subjects. $^aP < 0.05$; $^cP < 0.001$.

Differential production of MMPs in fibrosis stages

After determining MMP overproduction, the CHC patients were categorized into fibrosis stages (F0, F1, F2, F3 and F4) (Supplementary Table 1). Mean patient age was between 55 and 60 years, and body mass index was higher in F4 (Table 2). The comparative results of serum MMP-2 concentrations suggested decreases in stages F0, F1, and F2, but no differences were observed in any of the fibrosis grades (Figure 3A).

Table 2 Demographic and biochemical data according to fibrosis stages

	F0 (36)	F1 (11)	F2 (14)	F3 (20)	F4 (38)	Differences
Sex, *n* (%)						
Men	10 (30)	6 (60)	7 (50)	9 (45)	13 (37)	N/A
Women	26 (70)	5 (40)	7 (50)	11 (55)	25 (63)	N/A
Age in yr	50 ± 12	39 ± 7	55 ± 13	59 ± 10	55 ± 13	F0-F1[a], F0-F3[a], F1-F2[b], F1-F3[b], F1-F4[b]
BMI in kg/m^2	25 ± 4	26 ± 4	26 ± 3	27 ± 3	27 ± 5	F0-F4[a]
Hb in g/dL	15.0 ± 1.3	14.0 ± 2.0	14.0 ± 2.0	14.0 ± 2.0	14.0 ± 2.0	N/S
Leu in g/dL	5.6 ± 1.3	6.5 ± 0.8	4.2 ± 1.4	4.7 ± 1.2	4.3 ± 2.2	F0-F4[b]
Platelets as × 10^3	231 ± 56	233 ± 20	193 ± 74	163 ± 54	92 ± 39	F0-F3[b]
						F0-F4[b]
						F1-F4[b]
						F2-F4[b]
						F3-F4[b]
Total bilirubin in mg/dL	0.72 ± 0.31	0.44 ± 0.22	0.73 ± 0.28	1.75 ± 0.85	2.39 ± 2.7	F0-F1[b]
						F1-F4[b]
						F2-F4[a]
						F3-F4[a]
Direct bilirubin in mg/dL	0.18 ± 0.10	0.07 ± 0.05	0.12 ± 0.10	0.23 ± 0.15	0.27 ± 0.3	F0-F1[a]
AST in UI/L	45 ± 7	38 ± 12	49 ± 11	74 ± 10	86 ± 16	F0-F3[b]
						F0-F4[b]
						F1-F3[a]
						F1-F4[a]
ALT in UI/L	56 ± 11	56 ± 8	57 ± 10	69 ± 10	80 ± 13	F0-F4[b]
GGT in UI/L	70.49 ± 15.65	51.30 ± 19.43	52.62 ± 14.10	91.58 ± 18.74	144.00 ± 25.29	F0-F4[a]
						F1-F3[a]
						F1-F4[b]
						F2-F4[a]

[a]$P < 0.05$.

[b]$P < 0.01$. Data are expressed as the mean ± standard deviation. Fibrosis stages: F0, F1, F2, F3 and F4. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index, GGT: Gamma glutamyl transpeptidase; Hb: Hemoglobin; Leu: Leukocytes; N/A: Not applicable; N/S: Not significant.

In contrast, MMP-7 displayed a continuous increase according to fibrosis stage progression. Statistical differences were found in F0 *vs* F1, F0 *vs* F3, F0 *vs* F4, F1 *vs* F3, F1 *vs* F4, F2 *vs* F3 and F2 *vs* F4 (Figure 3B). Finally, the MMP-9 analysis showed no tendency or difference between each fibrosis stage.

MMPs as indicators of fibrosis in CHC patients

After determining that MMP-7 showed differences between fibrosis stages, we performed the Spearman correlation, and MMP-7 displayed moderate correlation (r = 0.32, $P < 0.001$) with CHC. We then determined the area under the receiver operating characteristic curve to evaluate MMP-7 as a candidate marker for fibrosis. Receiver operating characteristic (ROC) values > 0.7 were considered acceptable, whereas values below that point were discarded. The results showed that MMP-7 was not effective for distinguishing F0, F1, F2 or F3 (Figure 4A). However, the ROC values were acceptable in F4, the advanced fibrosis stage (Figure 4A and 4B). The use of MMP-7 as a complementary protein could improve the specificity and sensitivity of

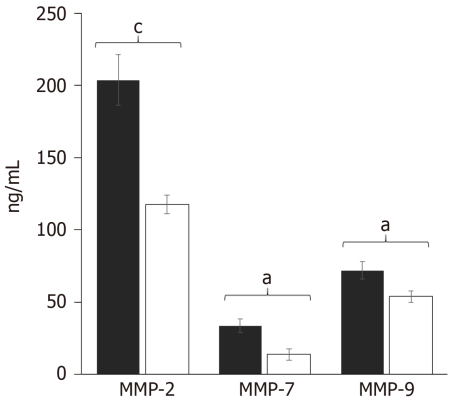


Figure 2 Matrix metalloproteinase concentrations in serum of chronic hepatitis C patients and healthy individuals. The serum concentrations of matrix metalloproteinase (MMP)-2, -7 and-9 were simultaneously determined in chronic hepatitis C patients (black bars) and healthy subjects (white bars) by multiplex suspension array technology, and the values were expressed in ng/mL. Statistical differences were obtained through the Mann-Whitney U test. Data are expressed as mean ± standard error of the mean. $^{a}P < 0.05$; $^{c}P < 0.001$.

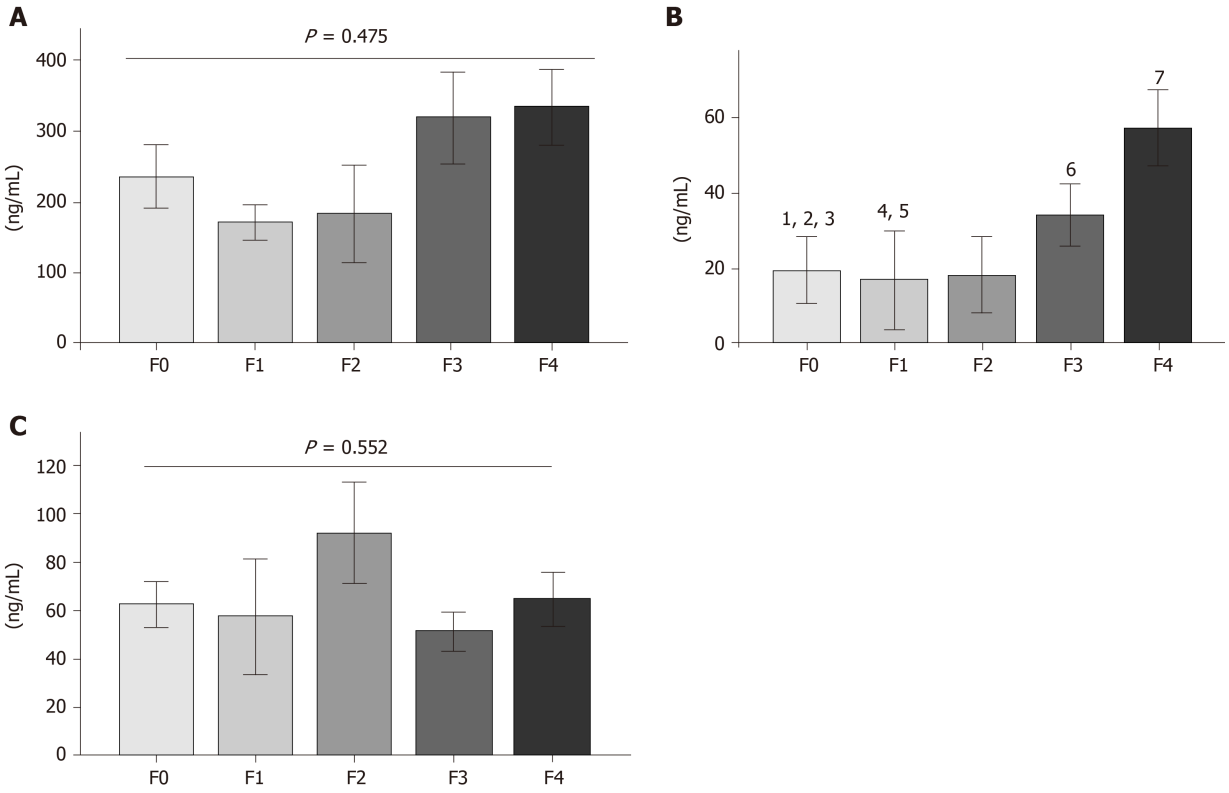


Figure 3 Matrix metalloproteinase productions according to fibrosis stage. Serum concentrations of A: Matrix metalloproteinase (MMP)-2; B: MMP-7 and C: MMP-9 of chronic hepatitis C patients classified according to fibrosis grades F0, F1, F2, F3 and F4. Statistical differences of MMP-7 were observed in 1 = F0 *vs* F1^{a}; 2 = F0 *vs* F3^{b}; 3 = F0 *vs* F4^{b}; 4 = F1 *vs* F3^{b}; 5 = F1 *vs* F4^{b}; 6 = F2 *vs* F3^{a}, 7 = F3 *vs* F4^{b}. Data are expressed as mean ± standard error of the mean. $^{a}P < 0.05$; $^{b}P < 0.01$.

the available methods for determining that fibrosis stage.

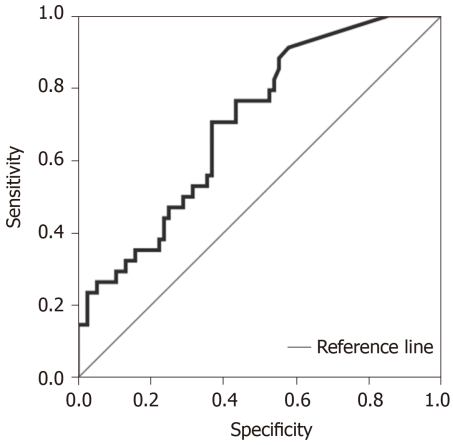
Collagenolytic and gelatinase activity of MMPs in CHC

Zymograms and Azocoll assays were performed to evaluate whether serum MMPs had proteolytic activity. The quantitative analysis of the degradation of Azocoll demonstrated that activity in the CHC patients (n = 119) was slightly higher than in the controls (n = 119) (Figure 5A). The evaluation of activity in relation to fibrosis grades showed that F0 and F1 had collagenolytic activity and that F2, F3, and F4 values were lower than those of F1 (Figure 5B). Activity was adjusted to the positive collagenase activity of *Clostridium histolyticum* reaching absorbance at 4.5 U/mL. The

A

	ROC	*P*	Lower-upper limit 95%CI
F0	0.378	0.044	0.264-0.493
F1	0.296	0.033	0.078-0.504
F2	0.338	0.058	0.190-0.486
F3	0.605	0.144	0.487-0.722

B



F4. ROC: 0.705; *P* = 0.001, 0.605-0.805

Figure 4 Area under the receiver operating characteristic analysis of matrix metalloproteinase-7 in the fibrosis stages. A: Area under the receiver operating characteristic (ROC) values and lower and upper limits of matrix metalloproteinase (MMP)-7 in F0, F1, F2 and F3 were calculated; B: Area under the ROC curve of MMP-7 in F4; area under the ROC value, statistical significance and lower-upper limits are indicated. CI: Confidence interval.

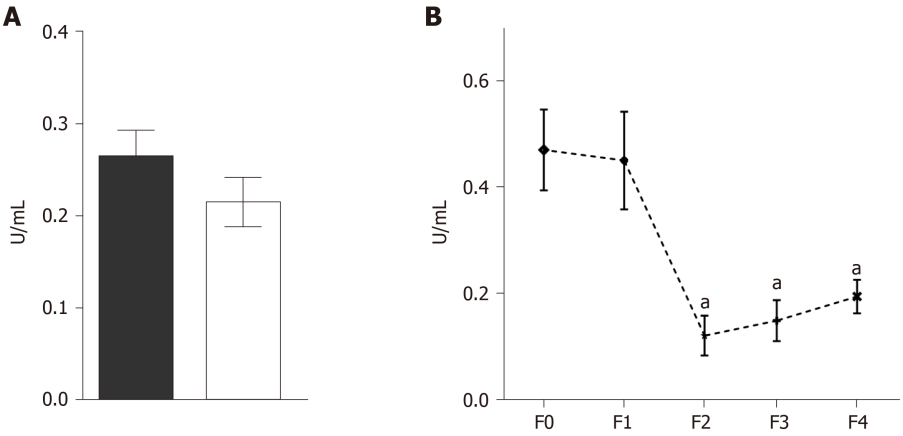


Figure 5 Collagenolytic matrix metalloproteinase activities in serum. A: Representative degradation of the Azocoll substrate reflecting collagenolytic activity in the chronic hepatitis C (CHC) patients and controls (CTs); B: Representative enzymatic activity of the matrix metalloproteinases in F0, F1, F2, F3 and F4. Matrix metalloproteinase activation was carried out with a pH 9.0 buffer at 37 °C for 2 h. Absorbance was obtained through spectrophotometry at 520 nm, and the values were adjusted to the maximal activity reached by *Clostridium histolyticum* collagenase and expressed as U/mL. Bars are expressed as mean ± standard error of the mean. [a]*P* < 0.05.

zymography assays did not detect any apparent activity of gelatinase or collagenase in either the patients or the controls (Figure 6). In contrast, the positive controls showed activity in the range of 150 to 250 kDa and activity close to 62 kDa. Similar results were observed in collagen-zymogram (data not shown). Regarding the zymography assays of fibrosis stages in the CHC patients, no enzymatic activity was shown in the sample evaluated (Figure 6B).

Differential TIMP-1 production in different fibrosis stages

To explore TIMP-1 regulation in the serum from patients with different fibrosis grades, we performed dot blot assays. At the early stages of fibrosis (F0 and F1), the patients had high levels of TIMP-1. However, at F2, TIMP-1 diminished sharply, but the levels were recovered in F3 and F4 (Figure 7B). The data were compared with the control levels. The PVDF membrane was stained with Ponceau S solution as the protein control load (Figure 7A). Statistical differences were determined through the densitometric analysis of TIMP-1 in the different fibrosis grades and the control subjects (Figure 7C).

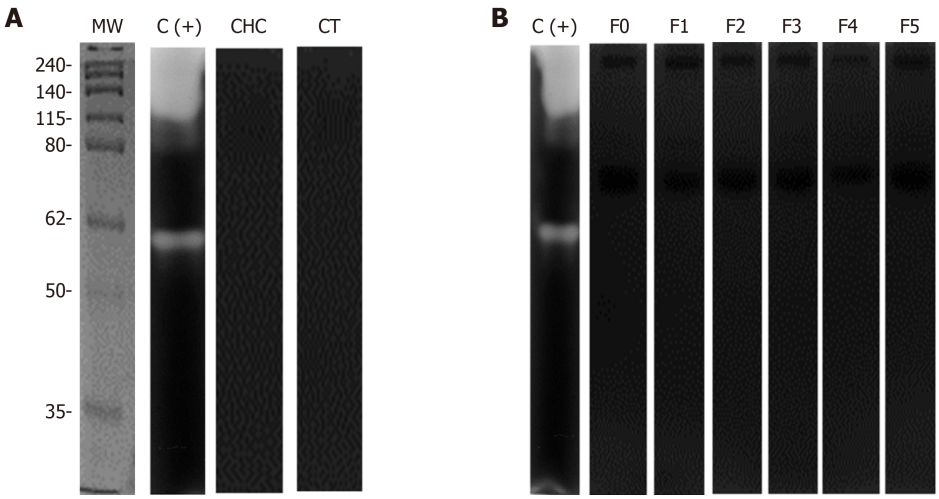


Figure 6 Evaluation of gelatinase activity by zymography. A: Representative zymography assays in 10% polyacrylamide gel electrophoresis copolymerized with 0.1 % (w/v) gelatin (molecular weight pattern). A total of 10 μg/mL of serum from chronic hepatitis C (CHC) patients and healthy individuals (n = 20) was loaded. Zymograms were activated overnight at pH 9.0 at 37 °C; B: Determination of gelatinases in different fibrosis stages (F0, F1, F2, F3 and F4) in the serum of CHC patients. Collagenase from *Clostridium histolyticum* (5 μg/mL) [C (+)] was used as the experimental control (CT). Five samples of each fibrosis stage and CT were evaluated in triplicate. MW: Molecular weight.

DISCUSSION

Up to the year 2010, approximately two million deaths worldwide (an estimated 4% of total deaths) were associated with liver diseases that included acute hepatitis, cirrhosis and liver cancer[22]. Novel antiviral treatments and changes in lifestyle have improved survival and quality of life, albeit treatment is not always successful in cases of chronic hepatitis C virus (HCV) infection. In addition, achieving alcohol abstinence and adherence to diet is no easy task[23-25]. Importantly, some patients that have been treated, and in whom viral infection is clinically eliminated continue to present with liver damage and develop cirrhosis and/or HCC[26]. Fibrosis is a common feature in the wound-healing response for most damage inductors and can be considered the key to adequate or inadequate liver parenchyma function. HSCs activated by TGF-β are thought to be the major source of collagens and TIMPs, but inactivation of that cell line is not enough to restore normal liver function. The degradation of excessive ECM is also necessary[14,27]. Collagens are degraded by MMPs, which are secreted by Kupffer cells and HSCs as proenzymes, and their activation occurs in the extracellular space. In the present work, we evaluated the serum concentration and proteolytic capacity of MMP-2, -7 and -9, in CHC patients according to fibrosis progression.

The demographic data revealed that the CHC population was older than the control group, correlating with progression and evolution time of hepatitis C, which is usually diagnosed in advanced-age patients. In contrast, the control individuals were young, which is the common age range of blood donors (35 to 45 years)[28]. Furthermore, both the CHC and control subjects presented with the obesity criteria with no apparent impact on biochemical values, including platelets and liver enzymes (aspartate aminotransferase, alanine aminotransferase and gamma glutamyl transpeptidase), which showed evident clinical alterations in CHC. Moreover, young patients were mainly identified as F1, which possibly was related to the fact that some of the study subjects then became blood donors (whose common age is from 35 to 45 years). Through the viral panel, these donors were found to be positive for HCV. Advanced stages of fibrosis were found in patients whose age ranged from 55 to 60 years.

Despite the fact that MMP-1, MMP-2, MMP-9 and MMP-13 are the most common MMPs related to liver fibrosis regulation[29], in 2015 MMP-7 was observed to be associated with liver fibrosis in biliary atresia[30]. In our study, the CHC patients had higher concentrations of MMP-7 compared with the healthy individuals, which correlated with the report of upregulation of MMP-7 in cirrhosis[19]. We provided evidence that absolute values of MMP-7 were able to distinguish mild, moderate and advanced fibrosis stages.

After evaluating the regulation of MMP-7 through fibrosis progression and observing the differences according to fibrosis stages, we performed ROC analyses in each of the stages of fibrosis (data not shown). MMP-7 showed acceptable ROC values for distinguishing F4 from the other stages of fibrosis. In previous reports, multiple

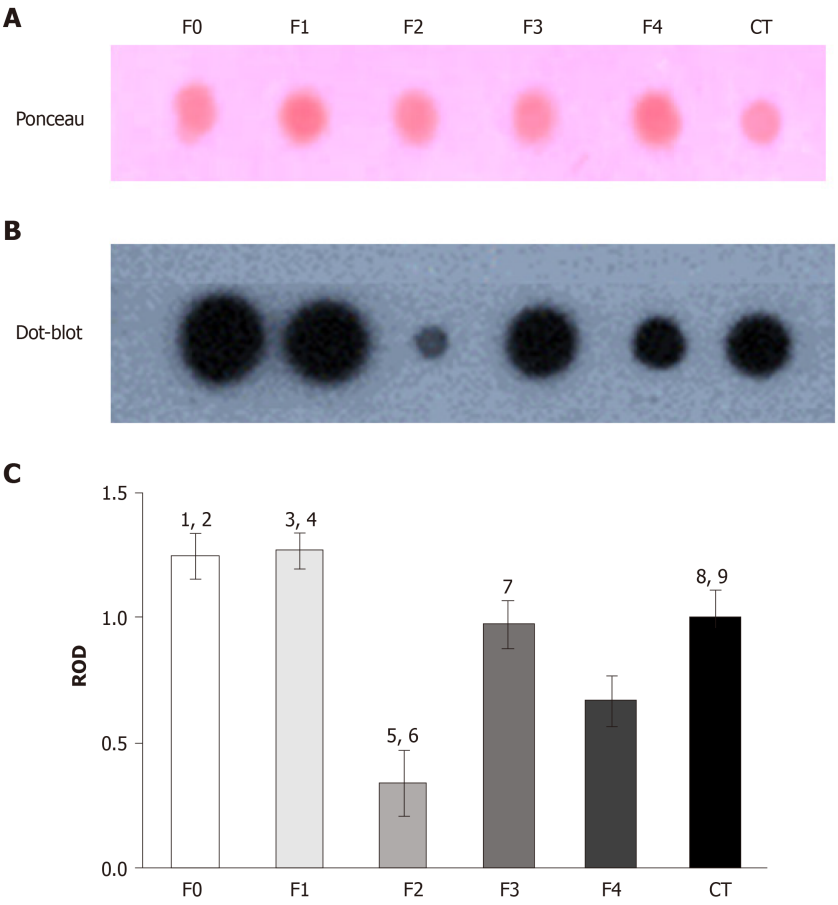


Figure 7 Tissue inhibitor of metalloproteinase -1 evaluations in the fibrosis grades. A: Representative serum protein load (5 μg/μL) onto the PVDF membrane stained with the Ponceau S solution; B: Representative dot blot assay of tissue inhibitor of metalloproteinase-1 in the different fibrosis stages (F0-F4) compared with healthy subjects; C: Densitometric analysis of each fibrosis stage and the controls (CTs; black bars) was expressed as relative optical density (ROD). Random serum samples (n = 5) of each fibrosis stage were evaluated in triplicate. ROD analysis was performed using ImageJ software, and bars display the mean ± standard error of the mean. Group comparisons: 1 = F0 *vs* F2[c]; 2 = F0 *vs* F4[c]; 3 = F1 *vs* F2[c]; 4 = F1 *vs* F4[c]; 5 = F2 *vs* F3[c]; 6 = F2 *vs* F4[c]; 7 = F3 *vs* F4[c]; 8 = CT *vs* F2[c]; 9 = CT *vs* F4[c]; [c]P < 0.001 in all the groups compared.

analyses and multivariate logistic regression modeling of MMP-7 with hyaluronic acid, MMP-1, α-fetoprotein and APRI enhanced diagnostic accuracy to 0.938 in advanced fibrosis[19]. Those types of analyses could possibly improve area under the receiver operating characteristic values in the other fibrosis stages. MMP-7 and other liver proteins in serum (*e.g*, TIMP-1 and IGFBP-7, among others)[19] can potentially improve the available diagnostic methods by enabling the precise discrimination of mild, moderate and advanced fibrosis stages. Those results are promising, and MMP-7 has been considered a predictive biomarker in other fibrogenesis pathologies, such as kidney fibrosis and idiopathic pulmonary fibrosis[31,32]. Thus, the correct clinical evaluation is crucial before using serum markers as diagnostic tools. On the other hand, both MMP-2 and MMP-9 have been proposed as serum biomarkers in ALD, and their concentrations have increased according to Child-Pugh score progression[18]. However, their activity and regulation in fibrosis stages has not been previously evaluated in detail in CHC patients.

MMP-1 is known to degrade collagen I and III, which are typical indicators of liver fibrosis[27,33]. However, in human liver biopsy samples, active MMP-2 expression in the liver parenchyma has been observed[15]. In 2011, MMP-2 was reported to suppress collagen I expression in a murine toxin-induced liver fibrosis model[34]. In the present study, we identified higher concentrations of MMP-2 in the serum of CHC patients compared with healthy subjects. Similarly, HCV-infected patients were reported to have higher circulating levels of MMP-2 than healthy donors[35,36].

Additionally, TIMP-1 levels have been shown to be significantly higher in HCV *vs* healthy donors, suggesting the presence of the inactive form of MMP-2[17]. Interestingly, we found no proteolytic activity of that gelatinase type A in our results. Our findings correlate with the histopathologic events of human liver biopsies of patients with chronic hepatitis and cirrhosis of the liver, in which *in situ* hybridization

showed a strong label of inactive MMP-2 in HSCs located in the lobules and periportal areas and in fibroblasts in the fibrous septa[15]. Our dot blot results showed that TIMP-1 had a pattern like that of MMP-2 production, demonstrating an apparent expression in mild fibrosis (F0-F1) and an important reduction in F2 suggesting that TIMP-1 acts on serum MMP-2 regulation in patients. Perhaps MMP-2 downregulation requires less regulation by its inhibitor. In addition, the analysis of fibrosis grades did not display differences in MMP-2 at any stage evaluated. A recent meta-analysis suggests that lower serum levels of MMP-2 can be found in F2 and F3. The production of MMP-2 has been reported in HSCs, monocytes, lymphocytes, dendritic cells and fibroblasts[37]. However, secretion into serum could be due to alterations in the cellular mechanism caused by HCV (*e.g.*, methylation and acetylation)[38]. Those results support the evidence that inactive MMP-2 is overproduced in CHC, but its role in fibrosis progression is uncertain.

We also found that the behavior of gelatinase B, or MMP-9, was like that of MMP-2. Our results described high levels of MMP-9 in the CHC patients, and the zymography analysis showed no gelatinase activity in the general substrate under physiologic conditions in any of the fibrosis stages evaluated (pH 7.0 and 37 °C). MMP-9 is mainly produced by Kupffer cells, but it can also be produced by lymphocytes and endothelial cells. The inactive presence of MMP-9 in the serum of patients with CHC in our study could be explained by the inactive form of MMP-2, which is its natural activator[39]. Furthermore, denaturalized collagen (Azocoll) was used as the specific substrate for MMP-7[40] showing activity in F0 and F1. However, the collagenolytic activity was drastically reduced after F2, as occurs with TIMP-1 at those stages. TIMP-1 can act as an inhibitor of MMP-7[41], but our results showed strong collagenase activity and higher levels of TIMP-1 at the same stages of fibrosis, suggesting that TIMP-1 could be involved in the partial regulation of MMP-7[38]. Similarly, TIMP-1 has also been reported to directly inhibit MMP-9. In fact, MMP-9 has been suggested as a therapeutic target for fibrosis resolution because it is related to the transdifferentiation process of HSCs and apoptosis of that cell line[29]. A longitudinal study reported an approximate 40% reduction of MMP-9 levels in patients treated with dual and triple antiviral therapies but no changes in MMP-2, TIMP-1, or TIMP-2, which the authors suggested was related to the reduction of liver inflammation[35].

Taken together, our results strongly suggest that MMPs and their activity, when determined in serum, could be complementary indicators in the diagnosis of inflammation and fibrosis, especially MMP-7 in advanced stages. The inactive stage of MMPs could be due to alterations in synthesis and production (acetylation and deacetylation, translation or post-transduction modification) caused by the HCV[38]. The identification of novel strategies or therapeutic targets to induce the fibrolytic function of MMPs could be crucial for improving the recovery from liver damage, preventing patients from progressing to HCC, even after receiving direct-acting antiviral treatment. It is also important to be familiar with the fibrolytic process in other liver diseases (nonalcoholic fatty liver disease and ALD) to understand and distinguish molecular and cellular events so that strategies can be implemented to reduce the exacerbated production of ECM and the consequent development of cirrhosis. In short, our results strongly suggest that serum MMP-7 could be used as a complementary indicator in the diagnosis of advanced fibrosis in CHC. Collagenolytic activity occurred mainly at early fibrosis stages (F0 and F1), but gelatinase activity was not detected at any fibrosis stage. Our study provides novel evidence of the increasing production and downregulation of serum MMP activity in CHC patients during the fibrolytic process and CHC progression.

CONCLUSION

Serum concentrations of MMPs were upregulated in patients with CHC, but their collagenolytic activity was limited to early fibrosis stages, whereas gelatinase functions were inactive during fibrosis progression despite their higher circulating concentrations.

The concentration and activity of MMPs, especially MMP-7, could be complementary indicators in the diagnosis of advanced fibrosis. It is possible that the HCV modulates the cellular and molecular mechanisms of MMP production affecting their correct fibrolytic functions and potentially resulting in progression to HCC. Further studies are needed to determine the exact mechanisms by which the HCV maintains MMPs inactive.

ARTICLE HIGHLIGHTS

Research background

Matrix metalloproteinases (MMPs) maintain the homeostasis between fibrogenesis and fibrolytic processes in the liver. Few studies on the production and activity of liver MMPs and fibrosis progression have been performed in humans.

Research motivation

The correct determination of liver fibrosis stages is imperative for making the diagnosis and implementing therapeutic decisions. At present, there is no evidence of the production and activity of MMP-2, MMP-7 or MMP-9 or their correlation with fibrosis progression in serum samples from patients with liver diseases.

Research objectives

In the present prospective, cross-sectional, multicenter study, we assessed the production, activity and regulation of matrix metalloproteinases in liver fibrosis stages in chronic hepatitis C (CHC).

Research methods

We selected CHC patients from the Hospital General de México, "Dr. Eduardo Liceaga," the Universidad Autónoma de Nuevo Leon and the Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán." Patients were categorized in fibrosis grades through FibroTest® and/or FibroScan® (F0, F1, F2, F3 or F4). Serum concentrations of MMP-2, -7 and -9 were determined. Differences were validated by the Kruskal-Wallis and Mann-Whitney U tests. Area under the receiver operating characteristic curve was calculated in fibrosis degrees. Proteolytic activity was validated by chromogenic and enzymatic assays and serum concentration, and the regulation of tissue inhibitor of metalloproteinases-1 was tested in fibrosis progression.

Research results

We compared 119 CHC patients with 119 healthy subjects. MMP-2, -7 and -9 concentrations were higher in the patients with CHC than in the control subjects. No differences between the serum concentrations of MMP-2 and MMP-9 were found, but MMP-7 showed differential regulation in accordance with fibrosis stages as well as an acceptable receiver operating characteristic (0.705), in advanced fibrosis (F4). Collagenolytic MMP activity was maintained in F0 and F1 but decreased significantly in F2, F3 and F4. Gelatin activity was not observed in any stage of fibrosis. The concentration of tissue inhibitor of metalloproteinases-1 was lower in F2 and F4 compared with F0, F1 and healthy subjects. Inactive MMPs were found in the serum of the CHC patients.

Research conclusions

Elevated concentrations of inactive MMPs were present in the serum of CHC patients, reflecting the impossibility to restrain liver fibrosis progression. MMPs could be used in the diagnosis of liver fibrosis and the treatment for its reversal in CHC.

Research perspectives

Given that MMP-2, -7 and -9 have not been simultaneously evaluated in the serum from liver fibrosis patients, MMPs could be used to improve the currently available diagnostic methods and as therapeutic targets. They could also be used as a monitoring tool in treatment-experienced patients that continue to present with liver fibrosis and develop cirrhosis and/or hepatocellular carcinoma.

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EVIDENCE-BASED MEDICINE

Awareness of non-alcoholic steatohepatitis and treatment guidelines: What are physicians telling us?

David Hermanus Wessels, Zeil Rosenberg

ORCID number: David Hermanus Wessels 0000-0002-3811-288X; Zeil Rosenberg 0000-0003-1893-7689.

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David Hermanus Wessels, Medical Office, A.E.S., Chorley PR7 1NY, Lancashire, United Kingdom

Zeil Rosenberg, Chief Medical Office, Accelerated Enrollment Solutions, Horsham, PA 19044, United States

Corresponding author: David Hermanus Wessels, MBChB, Chief Doctor, Medical Office, A.E.S., Sandringham House Ackhurst Park Foxhole Road, Chorley PR7 1NY, Lancashire, United Kingdom. dawie.wessels@globalaes.com

Abstract

BACKGROUND

There is an acute need to raise awareness of non-alcoholic fatty liver disease/non-alcoholic steatohepatitis (NAFLD/NASH) among primary care physicians, endocrinologists and diabetologists to improve patient identification and address the current difficulties in NASH clinical trial enrollment. We examined the extent of knowledge and practice regarding NASH diagnosis and management guidelines. A randomized online convenience survey of 12869 physicians drawn from a national physician database of primary care physicians (PCPs), and gastroenterology and endocrinology specialists were queried *via* online survey. Our results, based on a cohort of 185 respondents, showed gaps in knowledge and practice between these three groups of practitioners, with primary care providers having the lowest adherence to published guidelines for diagnosis of NASH. Without clear knowledge and patient identification at the point of presentation - which is often in primary care or with specialties other than hepatology–many patients with NAFLD and NASH will remain undiagnosed and untreated, and clinical studies will continue to struggle with patient recruitment, hindering clinical development and optimal patient care.

AIM

To determine knowledge base concerning NASH diagnosis amongst gastroenterologists, endocrinologists and primary care physicians to improve referrals into clinical trials.

METHODS

A randomized online convenience survey of 12869 physicians drawn from a national physician database of PCPs, and gastroenterology and endocrinology specialists was conducted yielding a sample of 185 respondents.

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RESULTS

The survey revealed that many physicians are either unaware of testing options other than biopsy, or do not use them in practice. Only 46% of endocrinologists and 42% of primary care physicians indicated they would refer a patient for specialist workup if they suspected NASH. Risk (25%) and inconvenience to patients (18%) are given as reasons for not referring those with suspected NASH for biopsy. For standard diagnostic algorithms such as Fibrosis-4 score, 18% of PCPs, 30% of endocrinologists and 65% gastroenterologists reported using these tests in clinical practice.

CONCLUSION

Substantial gaps in knowledge of the differences between NAFLD and NASH exist between these physician groups, with knowledge being particularly low among primary care doctors and endocrinologists. The use of a simple non-invasive screening algorithm may help to identify the right patients for clinical trials, which in turn will be vital to the development of effective and well-tolerated treatments for this increasingly ubiquitous condition.

Key Words: Non-alcoholic steatohepatitis; Non-alcoholic fatty liver disease; Enrollment; Screening; Diagnostics; Guidelines

Core Tip: Primary care physician knowledge of non-alcoholic steatohepatitis (NASH) diagnostics guidelines is key for appropriate patient management. We conducted a national online survey of physicians regarding their awareness of NASH guidelines. Endocrinologists and primary care physicians were significantly less likely than gastroenterologists to understand the differences between NASH and non-alcoholic fatty liver disease, as well as undertake diagnostic testing and necessary referrals for NASH. Only 18% of primary care physicians and 30% of endocrinologists were familiar with common indices such as the Fibrosis-4 score. Better education of primary care physicians about NASH could also serve as one way to identify candidates for important NASH clinical trials.

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INTRODUCTION

Prevalence and challenges

Non-alcoholic fatty liver disease (NAFLD), defined as the presence of ≥ 5% steatosis in the absence of secondary causes of fat accumulation in the liver, is the most prevalent chronic liver disease worldwide, and is thought to affect about 25% of the adult population globally[1,2]. There is some variation regionally, from 13% in Africa to more than 30% in South America and the Middle East[3]. An increasing prevalence is being seen in the developed world; NAFLD is closely associated with metabolic syndrome with the conditions being found concurrently in a substantial proportion of patients. Indeed, both the NAFLD phenotype as well as its progression to more serious disease may be viewed as an outgrowth of metabolic alterations in the context of a genetic predisposition associated with higher energy intake[4]. Up to two-thirds of patients with type 2 diabetes, and more than 90% of patients undergoing bariatric (weight loss) surgery to treat obesity present with NAFLD. Similarly, approximately a third of patients with hypertension and half of patients with dyslipidemia show evidence of the condition[5]. In the United States, there also has been an increase in the prevalence of NAFLD in children, with estimated rates up to 17%. The condition is more common in boys and a higher prevalence is seen in Hispanic children compared with white,

Asian or African-American children[6].

The natural history of NAFLD is such that the majority of patients will eventually succumb to closed volume-related mortality. However, it is estimated that up to 20% of patients with NAFLD will, during the clinical course of their disease, progress to non-alcoholic steatohepatitis (NASH), which is associated with liver inflammation and hepatocyte injury[7]. NASH also is associated with significant liver-related outcomes including fibrosis, cirrhosis, hepatocellular carcinoma, liver failure and liver death in 15%-25% of patients[8-11]. The prevalence of NASH is difficult to determine, as an unambiguous diagnosis requires a liver biopsy. In 2016 Younossi *et al*[12] reported rates of NASH among patients with NAFLD ranging from almost 7% for those without an indication for biopsy to 59% in biopsied patients[12]. Similarly, the rates of further progression of NASH are unclear, but it is thought that 10%-20% of patients will develop higher-grade fibrosis and < 5% will progress to cirrhosis[10]. NAFLD is also the most rapidly increasing indication for liver transplant[11]. The substantial prevalence of NAFLD, with an estimated 65 million patients in the United States. And 52 million in Europe (Germany, France, Italy and United Kingdom), is associated with a significant economic burden from direct medical costs estimated at $103 billion and $37 billion, respectively. The burden is significantly higher when indirect and societal costs are included[12].

In spite of its ubiquity, knowledge of NAFLD and NASH is suboptimal in clinical practice. Patients frequently present late in the NAFLD spectrum, as the condition is often silent and asymptomatic. Thus, NASH diagnosis and referral remain low. Although many potential treatment options are in clinical development, it follows that recruitment for clinical trials is extremely challenging. In April of this year, 35 clinical trials of products to treat NASH at Phase II or III were listed as recruiting globally, and requiring at least 13000 patients. However, enrollment rates are typically less than one patient *per* clinical research site *per* month, with less than 25% of recent trials achieving > 0.5 patients *per* site *per* month. Clearly, this dearth of patient enrollment will severely hamper the development and approval of new treatment options.

Knowledge of diagnostics guidelines

Several guidelines for the diagnosis and management of NAFLD and NASH have been published, including by EASL[13] and National Institute for Health and Care Excellence[14]. These guidelines have been reviewed and compared elsewhere[15]. Updated guidelines were published in 2018[1] and a clinical guidelines synopsis followed some months later[2]. However, anecdotal evidence suggests knowledge of the guidelines is poor outside of specialist physicians, and that guidelines are not being followed to the same extent that is seen in other chronic disease settings such as diabetes. The impact of this is far-reaching. Without clear knowledge and patient identification at the point of presentation – which is often in primary care or with specialties other than hepatology – many patients with NAFLD and NASH will remain undiagnosed and untreated, and clinical studies will continue to struggle with patient recruitment, hindering clinical development and optimal patient care.

MATERIALS AND METHODS

To investigate this further, a recent survey carried out by Accelerated Enrollment Solutions (AES) examined the extent of knowledge and practice regarding NASH diagnosis and management guidelines. A randomized online convenience survey of 12869 physicians drawn from a national physician database of primary care physicians (PCPs), and gastroenterology and endocrinology specialists was undertaken, yielding a cohort of 185 (response rate of 1.13%) primary care physicians and medical specialists across a number of disciplines in the United States (Table 1). Respondent physicians in the survey came from 34 states and were generally representative of the population as a whole. When asked how many years the respondents were in practice, 0.5% were in practice 0-5 years, 13.5% for 6-10 years, 38.4% for 11-20 years, 28.1% for 21-30 years and 19.5% for greater than 30 years.

The survey aimed to shed light on medical specialists' and primary care physicians' knowledge and practice regarding NASH diagnosis and management guidelines, and also to identify whether any recommendations could be made to improve adherence to guidelines in clinical practice. To determine "best practice" baseline for comparison purposes, and to identify practices of greatest importance for clinicians, we utilized practice guidelines developed in 2018 by the American Association for the Study of Liver Diseases. Results are presented here for the three largest groups – gastroenter-

Table 1 Number and proportion of participants by specialty

Specialty	*n* (%)
Gastroenterology	64 (35)
Endocrinology	60 (32)
Primary care	
Family practice	39 (21)
Internal medicine	6 (3)
General practice	2 (1)
Other	14 (8)

ologists, endocrinologists and primary care physicians. Statistical work was done in Statistical Analysis Software, with significance determined by chi-squared tests.

RESULTS

Appreciation of disease pathology and progression is important in the identification of at-risk and existing patients. However, the survey revealed substantial gaps in knowledge of the differences between NAFLD and NASH in these physician groups, with knowledge being particularly low with primary care doctors and endocrinologists (Figure 1).

Gastroenterologists were generally well informed, and therefore, it was not surprising that physicians in this group were most likely to undertake diagnostic tests firsthand (blood tests, imaging or liver biopsy) and least likely to refer the patient to another specialist (Figure 2). However, the likelihood of referral was relatively low for other groups, with only 46% of endocrinologists and 42% of primary care physicians indicating they would refer a patient for specialist workup if they suspected NASH. The lack of referral is worrying, considering the low levels of confidence in differentiating NAFLD and NASH, as well as suboptimal disease awareness among these specialties, particularly given the risk that many patients may remain undiagnosed.

Although liver biopsy is the gold standard for diagnosis of NAFLD and NASH, its invasive nature means it is rarely used outside specialist care. Risk (25%) and inconvenience to patients (18%) are given as reasons for not referring those with suspected NASH for biopsy. However, most frequently, physicians in all disciplines fail to recommend biopsy because they believe the outcome will not affect any subsequent treatment plan (34%). With the current lack of treatment availability, this is true, but with many products in the development pipeline, unambiguous identification of NASH patients is fundamental for the clinical trials that ultimately will lead to approval of new, effective and well-tolerated treatments.

Guidelines recommend that patients who are at increased risk of having steatohepatitis and/or advanced fibrosis should routinely be referred for further investigation by biopsy. Many of these patients will be those with concurrent metabolic syndrome–*i.e.*, those presenting at primary care or in endocrinology clinics. The low referral rate for biopsy suggests either a deeper lack of willingness to recommend this procedure on the part of the physician, or a suboptimal knowledge of the guidelines for diagnosis and management of NAFLD and NASH.

The survey also revealed that many physicians are either unaware of testing options other than biopsy, or do not use them in practice. NAFLD is generally recognized through abnormal liver chemistries–most commonly patients have a mildly elevated aspartate transaminase (AST) and/or alanine transaminase (ALT), with an AST:ALT ratio < 1, which in later stages may reverse. Thus, AST:ALT > 1[16,17], although a normal or near normal ALT level, does not preclude NASH.

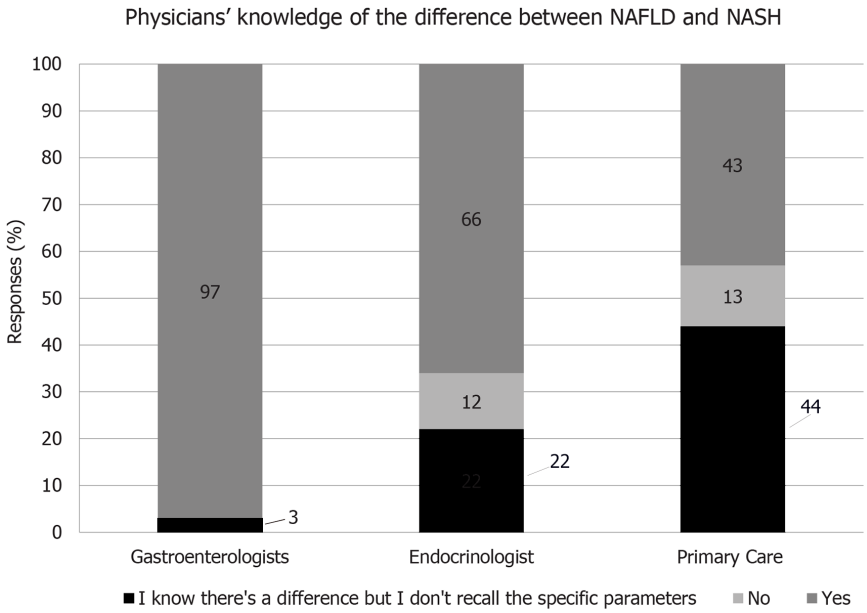

Figure 1 Knowledge of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis among physicians. NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

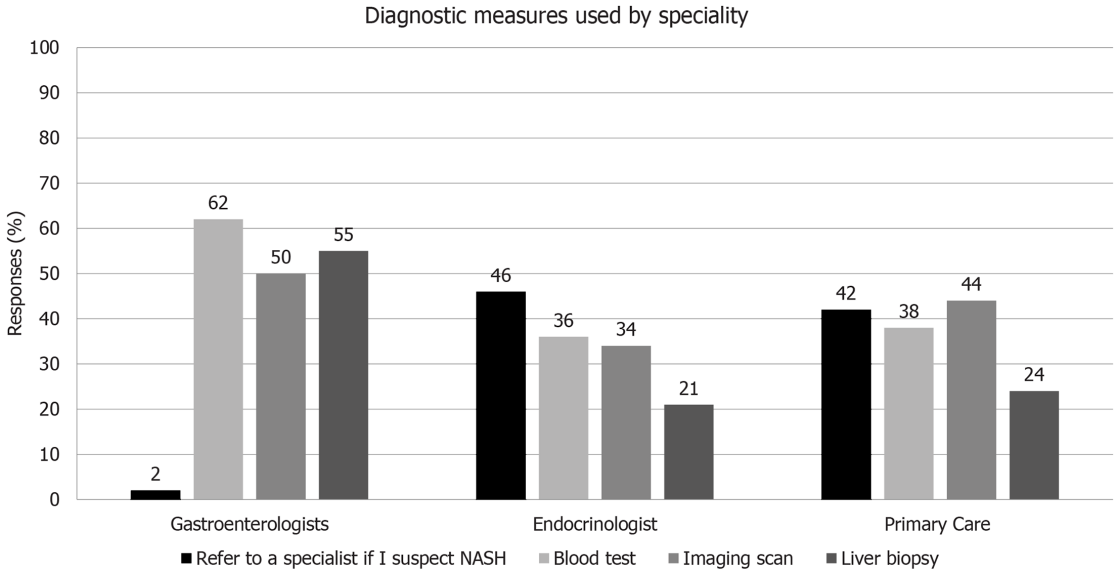

Figure 2 Diagnosis measures for non-alcoholic fatty liver disease/non-alcoholic steatohepatitis by physician specialty. NASH: Non-alcoholic steatohepatitis.

DISCUSSION

How to identify the NASH patient?

The fibrosis-4 (FIB-4) index is a biomarker test that uses outcomes from standard and easily available blood serum tests to generate a score that is correlated with the degree of liver damage in people with a variety of liver diseases. A score can be derived from age, AST and ALT, and platelet counts, and can be used as an indicator of NASH. However, only 36% of PCPs had knowledge of either this or the NAFLD Fibrosis Score (NFS), a non-invasive scoring system that takes into consideration age, hyperglycemia, body mass index, platelet count, albumin and AST/ALT ratio, as diagnostic determinants of NAFLD. In endocrinologists and gastroenterologists, these tests were familiar to 58% and 82%, respectively. However, only 18% of PCPs, 30% of endocrinologists and 65% gastroenterologists reported using these tests in clinical practice. There were significant differences between physician groups ($P < 0.0001$) in both of these cases. Given that many physicians do not opt for liver biopsy, these non-

invasive tests could be crucial to more widespread patient identification. Both of these tests are recommended in the 2018 guidelines[1] as clinically useful tools and decision aids that should be used to differentiate patients at higher risk of advanced fibrosis or cirrhosis. Thus, the lack of awareness in this area is of real concern.

Imaging studies are also an important part of the workup for NAFLD, and while imaging is used by 98% of gastroenterologists, only 83% and 82% of endocrinologists and PCPs, respectively, use the technique. Again, a significant difference ($P < 0.0004$) was seen between physician groups. Outside of liver biopsy in the gastroenterology cohort, vibration-controlled transient elastography [VCTE (FibroScan®)] and computed tomography-guided ultrasound were the techniques most commonly employed (Figure 3).

While the survey sample was small, some clear trends emerged. Although the prevalence of NAFLD and NASH is high in the general population, there are no widely accepted screening processes, even in high-risk patients[7]. As well as exacerbating under-diagnosis and under-treatment, the absence of a standardized screening system contributes to the inadequacy of the numbers of patients available for clinical trials. Currently, trials in NAFLD and NASH tend to recruit and enroll patients who already have, or are very likely to have, a diagnosis. Therefore, to optimize enrollment, an improved process for patient identification would be of great value. This need not involve invasive procedures; rather, the focus should be on identifying those individuals who are most likely to meet clinical trial eligibility criteria. This key subset of patients then can be referred for biopsy to obtain a definitive histological diagnosis. As up to 25% of patients with NAFLD are expected to show evidence of NASH on biopsy, such a screening algorithm is perhaps the most efficient and cost-effective way to identify appropriate patients. It is estimated this process would allow the screening of up to 120000 patients annually, which would greatly aid drug developers and researchers in populating clinical studies in the coming years (Figure 4).

CONCLUSION

Guidelines exist for the diagnosis of NAFLD and NASH. However, disease awareness is low, and therefore, patients are not coming through the referral pathway into the clinical studies required to push forward the development of new treatment options. This will be essential given the rise in the prevalence of NASH and the lack of approved treatment options. The clear association between NAFLD/NASH and metabolic disorders is well known, and reflected in guidance statements. Although routine screening is not recommended, the guidelines indicate physicians should have a high index of suspicion when dealing with patients presenting with these conditions. Furthermore, physicians are advised to use clinical decision aids such as NFS, FIB-4 or VCTE to identify patients who are at risk, and who would benefit from a further referral or more conclusive diagnostic testing[1]. The results from this survey suggest these recommendations are not being implemented in clinical practice, with many physicians having a poor understanding of the stages of disease and the available diagnostic techniques.

The majority of patients with NASH will present at primary care, or specialties other than hepatology. For example, endocrinologists or diabetologists are likely to see a substantial number of high-risk patients. Although it is important to raise awareness across all specialties, there is an acute need to raise awareness and improve the knowledge of NAFLD/NASH among primary care physicians, endocrinologists and diabetologists to improve patient identification and address the current difficulties in NASH clinical trial enrollment. The use of a simple non-invasive screening algorithm may help to identify the right patients for clinical trials, which in turn will be vital to the development of effective and well-tolerated treatments for this increasingly ubiquitous condition.

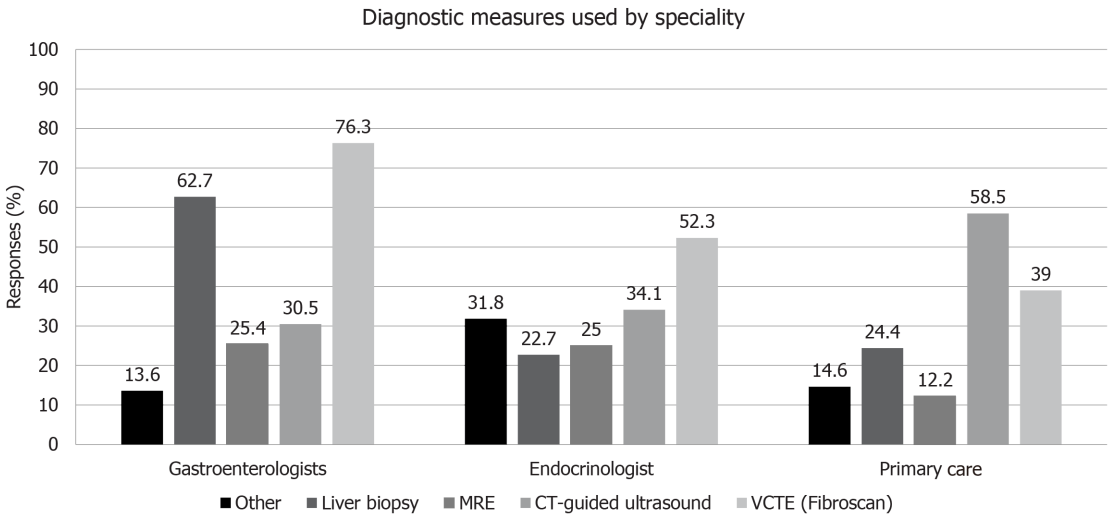

Figure 3 Diagnostic techniques used in non-alcoholic steatohepatitis physicians of different specialties. MRE: Magnetic resonance elastography; VCTE: Vibration-controlled transient elastography; CT: Computed tomography.

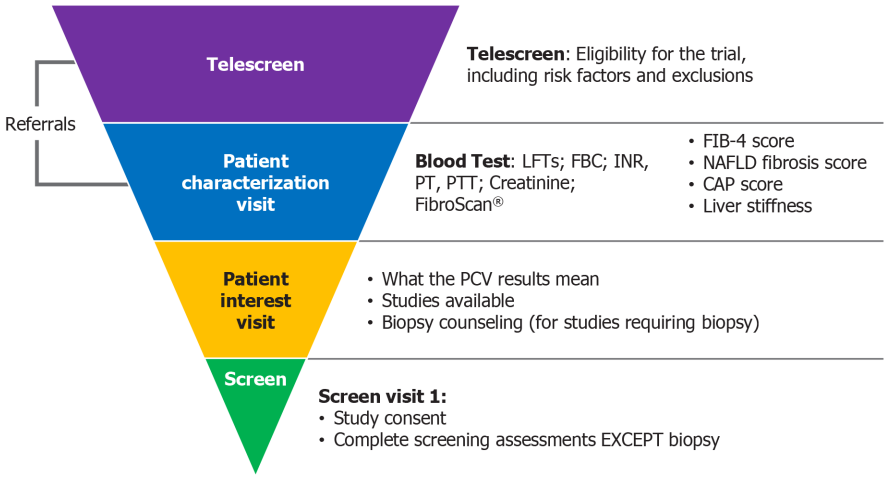

Figure 4 The non-alcoholic steatohepatitis patient recruitment screening pathway. LFT: Liver function test; FBC: Full blood count; INR: International normalized ratio; PT: Prothrombin time; PTT: Partial thromboplastin time; CAP: Controlled attenuation parameter; PCV: Porcine circovirus; NAFLD: Non-alcoholic fatty liver disease; FIB: Fibrosis.

ARTICLE HIGHLIGHTS

Research background

Medical specialist and primary care physician knowledge of non-alcoholic steatohepatitis (NASH) treatments, especially those contained in international guidelines, is important to standardize for the benefit of patient care.

Research motivation

We sought to document to what degree knowledge of NASH diagnostics, as recommended in United States guidelines, varied among United States specialists and primary care providers.

Research objectives

We sought to document to what degree knowledge of NASH diagnostics, as recommended in United States guidelines, varied among United States specialists and primary care providers.

Research methods

We utilized a randomized, online national convenience survey sample of gastroenter-

ologists, endocrinologists, and primary care physicians to inquire about their knowledge and practice regarding NASH.

Research results

While gastroenterologists were relatively well informed, endocrinologists and primary care physicians were less likely to understand the differences between NASH and non-alcoholic fatty liver disease (NAFLD), as well as undertake diagnostic testing and necessary referrals for NASH. Only 18% of primary care physicians and 30% of gastroenterologists were familiar with common indices such as the Fibrosis-4 score by which suspect NASH patients might be identified. Only 46% of endocrinologists and 42% of primary care physicians would refer a patient with a NASH profile for a NASH work-up by a specialist. Risk (25%) and inconvenience to patients (18%) were given as reasons for not referring those with suspected NASH for biopsy.

Research conclusions

Suboptimal knowledge of NASH and NAFLD by primary care physicians and by endocrinologists, both groups to which many NASH patients would be likely to present, may impair the definitive diagnosis of NASH and actions to minimize its effects. Reversing this knowledge gap can help in identification of additional and appropriate patients for enrollment into important NASH clinical trials.

Research perspectives

It is important to raise awareness of NASH among physicians of all kinds. Improved patient identification can not only improve care for the individual patient, but is also necessary to assure sufficient participation of confirmed NASH patients into randomized, placebo-controlled clinical trials for new treatment modalities.

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META-ANALYSIS

Occult hepatitis C virus infection in the Middle East and Eastern Mediterranean countries: A systematic review and meta-analysis

Mohammad Reza Hedayati-Moghaddam, Hossein Soltanian, Sanaz Ahmadi-Ghezeldasht

ORCID number: Mohammad Reza Hedayati-Moghaddam 0000-0001-9133-5006; Hossein Soltanian 0000-0001-6774-7027; Sanaz Ahmadi-Ghezeldasht 0000-0002-0975-6133.

Author contributions: Hedayati-Moghaddam MR conception and design of the study, analysis and interpretation of data, drafting the paper, revising the article, and final approval; Soltanian H acquisition of data, and final approval; Ahmadi-Ghezeldasht S acquisition of data, drafting the paper, and final approval.

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Mohammad Reza Hedayati-Moghaddam, Hossein Soltanian, Sanaz Ahmadi-Ghezeldasht, Blood Borne Infections Research Center, Academic Center for Education, Culture and Research (ACECR), Razavi Khorasan Branch, Mashhad 91779-49367, Iran

Corresponding author: Mohammad Reza Hedayati-Moghaddam, MD, Associate Professor, Blood Borne Infections Research Center, Academic Center for Education, Culture and Research (ACECR), Razavi Khorasan Branch, Ferdowsi University campus, Azadi sq, Mashhad 91779-49367, Iran. drhedayati@acecr.ac.ir

Abstract

BACKGROUND

The presence of hepatitis C virus (HCV) RNA in liver tissue or peripheral blood mononuclear cells with no identified virus genome in the serum has been reported worldwide among patients with either normal or elevated serum liver enzymes. The characterization of occult HCV infection (OCI) epidemiology in the Middle East and Eastern Mediterranean (M and E) countries, a region with the highest incidence and prevalence rates of HCV infection in the world, would be effective for more appropriate control of the infection.

AIM

To estimate the pooled prevalence of OCI in M and E countries using a systematic review and meta-analysis.

METHODS

A systematic literature search was performed using international, regional and local electronic databases. Some conference proceedings and references from bibliographies were also reviewed manually. The search was carried out during May and June 2020. Original observational surveys were considered if they assessed the prevalence of OCI among the population of M and E countries by examination of HCV nucleic acid in peripheral blood mononuclear cells in at least 30 cases selected by random or non-random sampling methods. The meta-analysis was performed using Comprehensive Meta-analysis software based on heterogeneity assessed by Cochran's Q test and I-square statistics. Data were considered statistically significant at a P value < 0.05.

RESULTS

A total of 116 non-duplicated citations were found in electronic sources and grey literature. A total of 51 non-overlapping original surveys were appraised, of which 37 met the inclusion criteria and were included in the analysis. Data were

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available from 5 of 26 countries including Egypt, Iran, Pakistan, Saudi Arabia, and Turkey. The overall prevalence rate of OCI was estimated at 10.04% (95%CI: 7.66%-13.05%). The lowest OCI rate was observed among healthy subjects (4.79%, 95%CI: 2.86%-7.93%). The higher rates were estimated for patients suffering from chronic liver diseases (12.04%, 95%CI: 5.87%-23.10%), and multi-transfused patients (8.71%, 95%CI: 6.05%-12.39%). Subgroup analysis indicated that the OCI rates were probably not associated with the studied subpopulations, country, year of study, the detection method of HCV RNA, sample size, patients' HCV serostatus, and sex (all $P > 0.05$). Meta-regression analyses showed no significant time trends in OCI rates among different groups.

CONCLUSION

This review estimated high rates of OCI prevalence in M and E countries, especially among multi-transfused patients as well as patients with chronic liver diseases.

Key Words: Occult hepatitis C; Prevalence; Review; Meta-analysis; Middle East; Eastern Mediterranean region

Core Tip: No comprehensive reported data are available in the literature regarding the estimated prevalence rate of occult hepatitis C virus (HCV) infection in the Middle East and Eastern Mediterranean countries. This is the first systematic review and meta-analysis to calculate occult HCV infection rate in this region. We estimated the overall rate as well as the rates among both healthy and high-risk populations such as those infected with human immunodeficiency virus, patients with end-stage renal diseases, cryptogenic liver diseases, cleared or treated HCV infection, lymphoproliferative disorders, and multi-transfused patients.

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INTRODUCTION

The World Health Organization set the global health sector strategy on viral hepatitis in 2015 and established some service coverage targets, including the diagnosis of 90% of persons with chronic hepatitis C and treatment of 80% of the diagnosed cases to eliminate hepatitis C as a public health concern by 2030[1]. Occult hepatitis C virus (HCV) infection (OCI) was introduced as a new and challenging form of this infection in 2004[2]. OCI is characterized by the presence of HCV RNA in the liver samples of patients who were seronegative for the viral RNA[2]. Although liver biopsy is the most accurate way to diagnose OCI cases[3], a reliable and non-invasive alternative method is the examination of the peripheral blood mononuclear cells (PBMCs) for the presence of HCV genome[4,5].

Occult hepatitis C has been proposed to occur in two different clinical conditions. The first category has been described in people reactive to HCV antibodies (anti-HCV) but with normal serum levels of liver enzymes. The majority of these patients are those with HCV infection treated with antiviral drugs or cleared spontaneously. In the second type of OCI, called serologically silent, cryptogenic, or secondary OCI, both anti-HCV and serum HCV-RNA are consistently negative but an increase in liver enzymes is observed[6]. Cryptogenic OCI is found mostly in patients with cryptogenic liver disease; however, the incidence of this type of OCI was also reported among blood donors[7].

Occult hepatitis C might be a long-standing infection[8]. OCI appears to be milder than classic chronic HCV infection; however, it is likely related to the development of

liver cirrhosis or even hepatic cancer[3,9,10]. Additionally, patients with OCI may benefit from antiviral therapies[11]. OCI is a common condition worldwide and all HCV genotypes can be involved in this form of infection[11]. This infection has been described in high-risk populations, such as patients with chronic liver disease, dialysis patients, those infected with HBV or HIV, the family members of patients with HCV infection, and even apparently healthy populations[3].

The Middle East and Eastern Mediterranean (M and E) region has been reported to have the highest rates of HCV infection in the world, with an incidence of 62.5 per 100000 person-years and prevalence of 2.3% among the general population (GP). In 2015, it was estimated that approximately one-fourth of 1.75 million newly HCV-infected persons and one-fifth of 71 million chronically infected individuals in the world resided in M and E countries[12]. The median of the anti-HCV seropositivity rate in the GP of this region ranged broadly from 0.3% in Iran[13] to 13.0% in Egypt[10]. In addition, the rate of HCV viremia among anti-HCV positive individuals in M and E countries varies widely from 9% to 100% with a median of 68.8%; the overall pooled rate was averagely estimated as 67.6% (95%CI: 64.9 ± 70.3%)[14].

The prevalence rate of OCI ranged from zero to 60% among the different studied populations in various M and E countries[15-17]. To our knowledge, no review has yet been performed to provide a pooled estimate for the OCI prevalence rate in this region. In the current systematic review and meta-analysis, we aimed to determine OCI epidemiology among both healthy and risk populations in this region by (1) providing pooled mean estimates for the OCI rate through systematically reviewing and analyzing existing data in various subpopulations; (2) assessing the possible factors contributing to between-study heterogeneity; and (3) evaluating the change in OCI prevalence in different studied populations over time. The results of the present review would help professionals to make appropriate decisions for the detection and management of OCI, particularly in at-risk patients.

MATERIALS AND METHODS

Literature search

We performed this review and meta-analysis following the PRISMA 2009 statement[18]. The main object was the presence of HCV RNA in PBMCs detected by a reverse transcription-PCR (RT-PCR) technique in the blood samples of healthy individuals or different patient categories from M and E countries. The search strategy included "Occult Hepatitis C" or "Occult HCV" along with "Middle East", "Eastern Mediterranean", or the names of M and E countries. In this study, the Middle East and Eastern Mediterranean region consisted of 26 countries: Afghanistan, Algeria, Bahrain, Cyprus, Djibouti, Egypt, Iran, Iraq, Israel, Jordan, Kuwait, Lebanon, Libya, Morocco, Oman, Pakistan, Palestine, Qatar, Saudi Arabia, Somalia, Sudan, Syria, Tunisia, Turkey, United Arab Emirates, and Yemen. The considered terms were searched in the title, abstract, and keywords using Web of Science and SCOPUS and in the text using PubMed, ScienceDirect, and ProQuest databases. Likewise, some regional and local databases were searched as "Occult Hepatitis C" or "Occult HCV" to find the articles published in the English language. These databases included the Index Medicus for the Eastern Mediterranean Region, Scientific Information Database, Iranian Database of publication (Magiran), and Iranian Databank of Medical Literature. In addition, some appropriate available abstract booklets and conference proceedings were manually reviewed. The search was performed from 21 May to 08 June 2020 and then expanded by manual cross-checking all references found from bibliographies of retrieved citations.

Study selection and data extraction

The two authors screened the titles and abstracts of the documents identified in the electronic and grey literature. Duplicate and overlapping surveys (the same studied population, methods, and findings) were excluded. Regarding articles, which reported the OCI prevalence in the region, various methodological aspects of the studies were assessed using a 10-items checklist, specifically developed to evaluate both internal and external validity of the prevalence studies[19]. These aspects encompassed the representativeness of the target population, sampling methods, sample size, data collection methods and instruments, response rate, and statistical analysis. The main inclusion criteria were detection of the HCV genome in PBMCs of at least 30 healthy or high-risk subjects selected by probable or non-probable sampling methods in an original observational survey. Review articles, case reports, editorials, or letters were

removed. Surveys that evaluated OCI in hepatocytes or other samples except PBMCs were not included. In addition, studies were not entered into the meta-analysis if they did not use an acceptable case definition and/or not apply an appropriate numerator and denominator for calculating the event rates.

The full texts, tables, and figures of all relevant articles were reviewed for data extraction by the two authors. The following variables were listed for each study: First author, year of publication, years of data collection, study type, study location, studied population, sampling method, the number of cases with anti-HCV and HCV RNA seropositivity, methods used to assess HCV genome, and the number and demographic features of cases with detectable HCV RNA in their PBMCs samples. Since the surveys in this field were restricted, no specific exclusion criteria were set for the studied population, year of data collection, and patients' age and sex.

Statistical analysis

The meta-analysis was performed using Comprehensive Meta-analysis software 2.2.064 (Biostat, Englewood, NJ, United States). With inverse variance weighting, a random-effect model was applied using the DerSimonian and Laird method if heterogeneity between studies was observed based on Cochran's Q test ($P < 0.05$) and I-square statistics (I^2, values of > 50%). Forest plots were applied to demonstrate the point prevalence rates and the 95% confidence intervals (CIs). Subgroup and meta-regression analyses were implemented to identify the possible factors related to heterogeneity between surveys. HCV serostatus was classified as seronegative (negative results for both anti-HCV and serum HCV RNA) and seropositive (tested positive for anti-HCV but negative for serum HCV genome). All statistical data were considered significant at a P value < 0.05.

RESULTS

Study selection

Among 151 citations retrieved from electronic sources, 107 non-duplicated items were selected to review the titles and abstracts (Figure 1). Fifty-two surveys discussed the prevalence of OCI in M and E countries[15-17,20-68], of which 5 review articles and 2 letters were excluded[62-68]. In addition, 4 pertinent documents were identified following a review of abstracts[69-72], and 5 documents were found by a manual screening of bibliographies[73-77]. After removing 3 overlapping surveys[71,72,75], 51 original articles were chosen for a thorough review of the full-text[15-17,20-61,69,70,73,74,76,77]. Most of the surveys had used a non-probable method to select studied samples and none of them had discussed non-response bias. Fourteen articles were not included owing to methodological difficulties, small sample size, and/or analysis of liver tissue or centrifuged serum[20,24,28,30,32,34,37,40,52,56,61,70,73,77].

Finally, 37 non-duplicate and non-overlapping articles met the inclusion criteria and were included in the analysis[15-17,21-23,25-27,29,31,33,35,36,38,39,41-51,53-55,57,58,59,60,69,74,76]. All studies were cross-sectional investigations and almost all of them were based on consecutive samples selected by a non-random convenience sampling method. The mean sample size was 141 (range: 30-1280); less than 100 cases in 23 studies, 100-200 cases in 11 surveys, and more than 200 cases in three studies.

The overall prevalence of occult hepatitis C in M and E countries

Of the 26 included countries, data were available only from five countries. Egypt (n = 17) and Iran (n = 17) were the countries with the largest number of studies reporting OCI prevalence but Pakistan, Saudi Arabia, and Turkey contributed to only one data point. These five countries had surveyed OCI prevalence among a total of 5200 individuals between 2009 and 2019 (Table 1). The studied population included blood donors, patients for whom HCV infection was resolved following antiviral treatment or spontaneously, patients with cryptogenic chronic liver diseases (LDs), autoimmune hepatitis, thalassemia, hemophilia, lymphoproliferative disorders, or anemia, patients undergoing hemodialysis (HD), HIV positive individuals, and injecting drug users (IDUs). Five surveys had collected data from mixed populations, mainly healthy volunteers as well as those suffering from chronic diseases.

The studied subjects were aged 4 to 89 years and their mean age was between 26 ± 9.31 and 58.9 ± 14.7 years. In 23 studies, 53.2%-98.4% of the participants were males, in 10 surveys, 50.0%-58.1% of them were females, and sex distribution of the samples was not stated in four documents.

As shown in Table 2, the rate of OCI prevalence in this region ranged widely from

Table 1 Selected studies for systematic review and meta-analysis of occult hepatitis C virus infection prevalence in the Middle Eastern countries and Eastern Mediterranean Region

Ref.	Years of data collection	Country	Population	Serostatus	HCV RNA detection method	Sample size	OCI	
							Number	Percent
Makvandi *et al*[21], 2014	2011-2012	Iran	Patients with unexplained abnormal ALT	Seronegative	Is-nested PCR	53	17	32.08
Zaghloul *et al*[22], 2010	2010	Egypt	(1) Patients with unexplained abnormal ALT and AST; (2) Patients with chronic hepatitis C who achieved SVR	Seronegative/seropositive	rRT-PCR	102	11	10.78
El Shazly *et al*[23], 2015	2014	Egypt	Healthy sexual partners of patients with HCV infection	Seronegative	rRT-PCR	50	2	4.00
Bozkurt *et al*[74], 2014	?	Turkey	Hemodialysis patients	Seronegative	rRT-PCR	84	3	3.57
Mohamed *et al*[25], 2017	2017	Egypt	Hemodialysis patients	Seronegative	RT-PCR	60	2	3.33
Donyavi *et al*[26], 2019	2015-2018	Iran	HIV positive injecting drug users	Seronegative/seropositive	RT-nested PCR	77	14	18.18
Ayadi *et al*[27], 2019	2017-2018	Iran	Hemodialysis patients	Seronegative	RT-nested PCR	515	95	18.45
El-Rehewy *et al*[29], 2015	2012-2014	Egypt	Hemodialysis patients	Seronegative	rRT-PCR	75	8	10.67
Sheikh *et al*[31], 2019	2017-2018	Iran	Injecting drug users(negative for HIV)	Seronegative/seropositive	RT-nested PCR	115	11	9.57
Jamshidi *et al*[33], 2020	2015-2019	Iran	HIV positive patients	Seronegative/seropositive	RT-nested PCR	143	14	9.79
Abd Alla *et al*[35], 2017	2015-2017	Egypt	(1) Patients with chronic hepatitis C; (2) Healthy individuals	Seronegative/seropositive	RT-nested PCR	174	41	23.56
Ramezani *et al*[16], 2014	2014	Iran	Hemodialysis patients	Seronegative/seropositive	RT-nested PCR	30	0	0.00
Muazzam *et al*[17], 2011	2007-2009	Pakistan	Patients with chronic hepatitis C who achieved SVR	Seronegative	rRT-PCR	104	0	0.00
Naghdi *et al*[36], 2017	2017	Iran	Hemodialysis patients	Seronegative	RT-nested PCR	198	6	3.03
Abdelrahim *et al*[38], 2016	2013-2014	Egypt	Hemodialysis patients	Seronegative	rRT-PCR	81	3	3.70
Ali *et al*[39], 2018	2014	Egypt	Hemodialysis patients	Seronegative	rRT-PCR	39	9	23.08
Keyvani *et al*[41], 2013	2007-2013	Iran	Patients with cryptogenic cirrhosis	Seronegative	RT-nested PCR	45	4	8.89
Serwah *et al*[42], 2014	2013-2014	Saudi Arabia	Hemodialysis patients	Seronegative	rRT-PCR	84	12	14.29
El-shishtawy *et al*[43], 2015	2015	Egypt	(1) Hemodialysis patients; (2) Healthy volunteers	Seronegative	Strand-specific RT-PCR	63	8	12.70
Nafari *et al*[44], 2020	2017-2018	Iran	Hemophilia patients	Seronegative	RT-nested PCR	450	46	10.22
Eslamifar *et al*[71], 2015	2013	Iran	Hemodialysis patients	Seronegative	RT-nested PCR	70	0	0.00
Bokharaei-Salim *et al*[46], 2011	2007-2010	Iran	Patients with cryptogenic liver disease	Seronegative	RT-nested PCR	69	7	10.14
Rezaee Zavareh *et al*[47], 2014	2012-2013	Iran	Patients with autoimmune hepatitis	Seronegative	RT-nested PCR	35	0	0.00
Ayadi *et al*[48], 2019	2017-2018	Iran	Thalassemia patients	Seronegative	RT-nested PCR	181	6	3.31

Mekky *et al*[50], 2019	2017	Egypt	Patients with chronic hepatitis C who achieved SVR	Seropositive	rRT-PCR	1280	50	3.91
El-Moselhy *et al*[51], 2015	2014-2015	Egypt	Hemodialysis patients	Unknown	RT-PCR/RT-nested PCR	66	18	27.27
Anber *et al*[76], 2016	2015	Egypt	Hemodialysis patients	Seronegative/seropositive	rRT-PCR	63	9	14.29
Abdelmoemen *et al*[53], 2018	2016	Egypt	Hemodialysis patients	Seronegative	rRT-PCR	62	3	4.84
Eldaly *et al*[54], 2016	?	Egypt	Blood donors	Seronegative	RT-nested PCR	138	8	5.80
Youssef *et al*[55], 2012	2010-2011	Egypt	(1) Patients with lymphoproliferative disorders; (2) Healthy volunteers	Seronegative	RT-PCR/RT-nested PCR	87	12	13.79
Bastani *et al*[57], 2016	2015	Iran	Thalassemia patients	Seronegative	RT-nested PCR	106	6	5.66
Farahani *et al*[58], 2013	2010-2011	Iran	Patients with lymphoproliferative disorders	Seronegative	RT-nested PCR	104	2	1.92
Yousif *et al*[59], 2018	2017	Egypt	Patients with chronic hepatitis C who achieved SVR	Seropositive	rRT-PCR	150	17	11.33
Bokharaei-Salim *et al*[60], 2016	2014-2015	Iran	HIV positive patients	Seronegative/seropositive	RT-nested PCR	82	10	12.20
Helaly *et al*[15], 2017	2014-2015	Egypt	(1) Patients with hematologic disorders; (2) Healthy subjects	Seronegative	RT-nested PCR	50	18	36.00
Alavian *et al*[69], 2013	?	Iran	Patients with chronic hepatitis C who achieved SVR	Seropositive	RT-PCR	70	9	12.86
Askar *et al*[49], 2010	?	Egypt	Patients with unexplained persistently abnormal liver function tests	Seronegative	RT-nested PCR	45	20	44.44

ALT: Alanine transaminase; AST: Aspartate aminotransferase; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; Is-PCR: *In situ*-PCR; PCR: Polymerase chain reaction; RT-PCR: Reverse transcription PCR; rRT-PCR: Real time RT-PCR; SVR: Sustained virologic response.

0.0% to 44.44%, with a median of 10.14%. The overall mean prevalence was estimated to be 10.04% (95%CI: 7.66%-13.05%). Across subpopulations, the pooled average OCI rate was highest at 21.70% (95%CI: 11.26%-37.72%) among patients with cryptogenic liver disease, followed closely by 18.18% (95%CI: 11.07%-28.39%) among HIV positive IDUs and 18.15% (95%CI: 10.20-30.20%) in the mixed population. The rate of OCI in Egypt (12.34%; 95%CI: 8.32-17.92%) was higher than in Iran (8.48%; 95%CI: 5.51%-12.84%); however, the difference was not statistically significant (P = 0.157). Subgroup analysis showed that the rate of OCI was probably not related to the disease subpopulations (P = 0.066), year of data collection (P = 0.786), the detection method of HCV RNA (P = 0.507), sample size (P = 0.057), patients' HCV serostatus (P = 0.178) and sex (P = 0.953). Furthermore, meta-regression analysis showed no significant (P = 0.580) time trend in the OCI rate among the total population of this region.

Occult hepatitis C prevalence among healthy populations

Four studies conducted in the M and E area reported OCI prevalence among 300 apparently healthy subjects, such as healthy volunteers, blood donors, and healthy sexual partners of patients with chronic HCV infection. All four studies had been performed among Egyptian HCV-seronegative cases, of which three had studied less than 100 cases, and three had been conducted after 2014. Assessment of HCV RNA in PBMCs had been carried out using RT-nested PCR in three surveys and real-time RT-PCR in another research. Based on the fixed-effect model (Q = 0.77, P = 0.857, I^2 = Zero), the pooled estimation of OCI prevalence among healthy populations was 4.79% (95%CI: 2.86%-7.93%, Figure 2). No evidence was found for a significant trend in the OCI rate in this population over time (P = 0.802).

Occult hepatitis C prevalence among multi-transfused patients

Seventeen studies reported the OCI rate among 2217 multi-transfused patients (MTPs), including 1480 HD patients, 450 hemophilia patients, and 287 thalassemia patients in the region. Fifteen surveys evaluated the presence of viral genome among HCV

Table 2 Subgroup-specific pooled estimates of occult hepatitis C virus infection prevalence across the Middle Eastern and Eastern Mediterranean countries

Prevalence by	Number of studies	Sample sizes	OCI prevalence across studies		Pooled OCI prevalence		Heterogeneity	
			Range (%)	Median	Mean (%)	95%CI	Cochran's Q	I-squared (%)
Studied population								
Blood donors	1	138	-	-	5.80	2.93-11.16	-	-
Hemodialysis patients	13	1427	0-27.27	4.84	9.06	5.83-13.80	64.0[a]	81.2
Healthy sexual partners of patients with chronic hepatitis C	1	50	-	-	4.00	1.00-14.63	-	-
Patients with chronic hepatitis C who achieved SVR	4	1604	0-12.86	7.62	6.70	3.08-13.99	25.4[a]	88.2
Patients with cryptogenic liver disease	4	212	8.89-44.44	21.11	21.70	11.26-37.72	22.6[a]	86.7
Patients with autoimmune hepatitis	1	35	-	-	1.39	0.09-18.67	-	-
Patients with lymphoproliferative disorders	1	104	-	-	1.92	0.48-7.36	-	-
Hemophilia patients	1	450	-	-	10.22	7.74-13.38	-	-
Thalassemia patients	2	287	3.31-5.66	4.49	4.32	2.47-7.46	0.9	0.00
HIV positive individuals	2	225	9.79-12.20	10.99	10.72	7.29-15.50	0.3	0.00
HIV positive injecting drug users	1	77	-	-	18.18	11.07-28.39	-	-
Injecting drug users	1	115	-	-	9.57	5.38-16.45	-	-
Mixed population[1]	5	476	10.78-36	13.79	18.15	10.20-30.20	18.2[b]	78.1
Countries								
Egypt	17	2585	3.33-44.44	11.33	12.34	8.32-17.92	186.2[a]	91.4
Iran	17	2343	0-32.08	9.57	8.48	5.51-12.84	86.5[a]	81.5
Pakistan	1	104	-	-	0.48	0.03-7.15	-	-
Saudi Arabia	1	84	-	-	14.29	8.30-23.49	-	-
Turkey	1	84	-	-	3.57	1.16-10.49	-	-
Temporal duration[2]								
Before 2015	18	1227	0-44.44	9.52	9.58	6.26-14.40	88.5[a]	80.8
2015 and thereafter	19	3973	3.03-36	10.22	10.33	7.21-14.61	192.07[a]	90.6
Method of HCV RNA detection								
RT-nested PCR	22	2833	0-44.44	9.97	11.75	8.52-15.99	159.3[a]	86.8
Real time RT-PCR	12	2174	0-23.08	7.75	7.88	4.96-12.30	58.4[a]	81.2
RT-PCR	2	130	145.71-173.33	159.52	7.75	2.34-22.75	3.3	69.5
Strand-specific PCR	1	63	-	-	12.70	6.48-23.39	-	-
Patients' HCV serostatus								
Seronegative	25	2774	0-44.44	5.80	9.32	6.76-12.73	164.0[a]	85.4
Seropositive	4	1604	6.20-66.50	37.62	6.64	2.94-14.30	25.4[a]	88.2
Seronegative/ Seropositive	7	756	27.27-109.09	73.53	13.58	8.01-22.11	17.8[b]	66.3
Undetermined	1	66	-	-	27.27	17.91-39.19	-	-
Sample size								

Less than 100	23	1440	0-44.44	12.20	12.43	8.87-17.15	102.4[a]	78.5
100 and above	14	3760	0-23.56	7.69	7.43	4.86-11.19	153.0[a]	91.5
Patients' sex								
Female	11	602	0-35.29	8.62	9.92	5.39-17.55	32.4[a]	69.2
Male	11	1785	0-30.56	9.09	10.17	5.85-17.10	70.8[a]	85.9
All studies	37	5200	0-44.44	10.14	10.04	7.66-13.05	284.1[a]	87.3

[a]$P < 0.001$.
[b]$P < 0.01$.
[1]Four studies investigated occult hepatitis C virus infection among healthy volunteers along with hemodialysis patients, patients with chronic hepatitis C, or patients with hematologic and lymphoproliferative disorders. Also one study investigated both the patients with chronic hepatitis C and patients with cryptogenic liver disease.
[2]Based on the last year reported for the data collection. If the collection date was not clear, publication year was considered instead. CI: Confidence interval; Min: Minimum; Max: Maximum; PCR: Polymerase chain reaction; RT-PCR: Reverse transcription polymerase chain reaction; OCI: Occult hepatitis C virus infection.

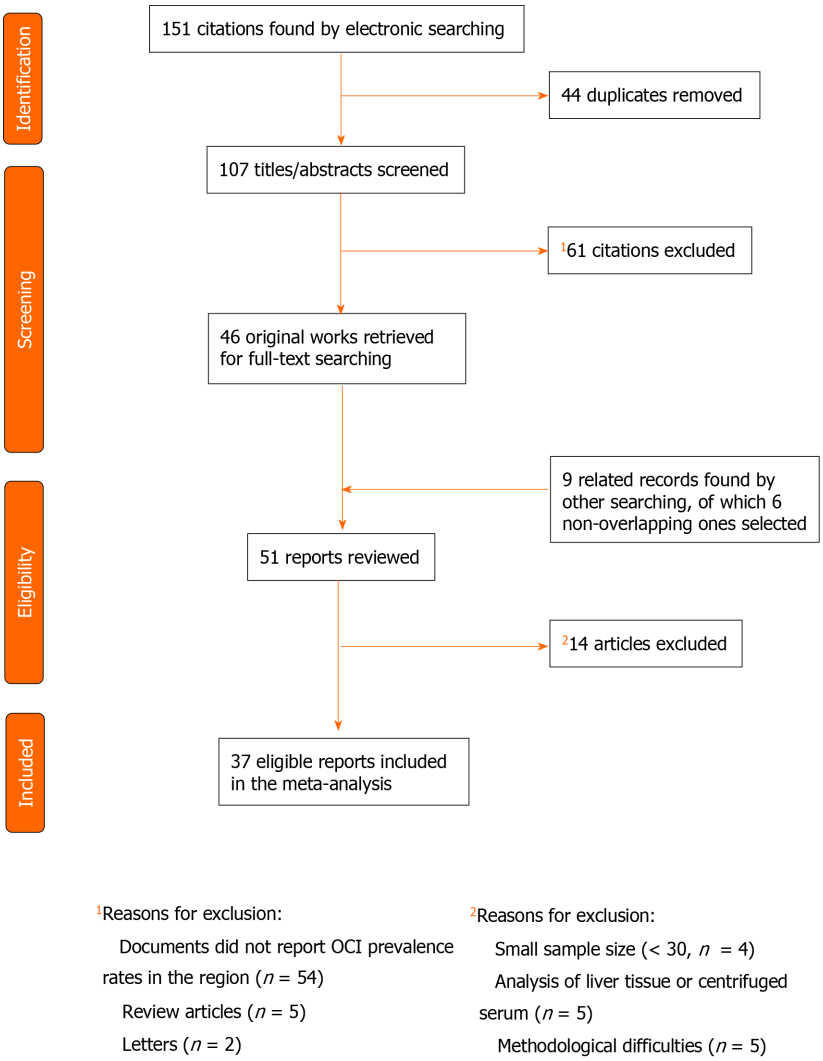

Figure 1 Study selection for the systematic review and meta-analysis of occult hepatitis C prevalence across the Middle East and Eastern Mediterranean countries. OCI: Occult hepatitis C virus infection.

seronegative samples. As shown in Table 3, the prevalence rate was estimated to be 8.71% (95%CI: 6.05%-12.39%) among this population (Figure 3). Based on 14 surveys, the estimated OCI rate among HD patients was 9.52% (95%CI: 6.30%-14.12%).

Subgroup analysis revealed that the rate of OCI in Egypt (11.43%; 95%CI: 6.55%-19.17%) was higher than in Iran (5.93%; 95%CI: 3.09%-11.09%), but the difference was

Table 3 Subgroup-specific pooled estimates of occult hepatitis C virus infection prevalence among multi-transfused patients across the Middle Eastern and Eastern Mediterranean countries

Prevalence by	Number of studies	Sample sizes	OCI prevalenceacross studies		Pooled OCI prevalence (%)		Heterogeneity	
			Range (%)	Median	Mean (%)	95%CI	Cochran's Q	I-squared (%)
Studied population								
Hemodialysis patients	14	1480	0-27.27	7.75	9.52	6.30-14.12	64.0[a]	79.7
Thalassemia patients	2	287	3.31-5.66	4.49	4.32	2.47-7.46	0.9	0.0
Hemophilia patients	1	450	-	-	10.22	7.74-13.38	-	-
Countries								
Egypt	8	499	3.33-27.27	12.48	11.43	6.55-19.17	26.8[a]	73.8
Iran	7	1550	0-18.45	3.31	5.93	3.09-11.09	54.8[a]	89.0
Saudi Arabia	1	84	-	-	14.29	8.30-23.49	-	-
Turkey	1	84	-	-	3.57	1.16-10.49	-	-
Temporal duration[1]								
Before 2015	7	463	0-23.08	3.70	7.89	4.10-14.65	20.6[b]	70.8
2015 and thereafter	10	1754	3.03-27.27	7.94	9.04	5.69-14.09	66.5[a]	86.5
Method of HCV RNA detection								
RT-nested PCR	8	1616	0-27.27	4.49	7.79	4.38-13.47	65.6[a]	89.3
Real time RT-PCR	7	488	3.57-23.08	10.67	9.52	5.26-16.61	17.4[b]	65.4
RT-PCR	1	60	-	-	3.33	0.84-12.37	-	-
Strand-specific PCR	1	53	-	-	15.09	7.73-27.38	-	-
Sample size								
Less than 100	12	767	0-27.27	7.75	9.49	5.85-15.04	40.6[a]	72.9
100 and above	5	1450	3.03-18.45	5.66	7.05	3.61-13.33	47.9[a]	91.6
Participants' sex								
Female	6	322	3.7-33.33	8.86	11.56	6.47-19.81	12.7[c]	60.8
Male	6	506	0-16	6.45	10.74	5.91-18.75	13.5[c]	63.1
All studies	17	2217	0-27.27	5.66	8.71	6.05-12.39	88.56[a]	81.93

[a]$P < 0.001$.
[b]$P < 0.01$.
[c]$P < 0.05$.
[1]Based on the last year reported for the data collection. If the collection date was not clear, publication year was considered instead. CI: Confidence interval; Min: Minimum; Max: Maximum; PCR: Polymerase chain reaction; RT-PCR: Reverse transcription polymerase chain reaction; OCI: Occult hepatitis C virus infection.

not statistically significant ($P = 0.125$). The rate of OCI frequency was not associated with year of study ($P = 0.732$), the detection technique of HCV RNA ($P = 0.618$), sample size ($P = 0.470$), and patients' sex ($P = 0.859$). Moreover, meta-regression analysis showed no significant ($P = 0.520$) changes in the OCI rate among MTPs patients over years.

Occult hepatitis C prevalence among patients with chronic liver diseases

In total, 11 surveys reported the OCI prevalence among 2065 patients with chronic LDs in M and E countries. These patients included 1778 with chronic hepatitis C, including those who achieved sustained virologic response after treatment with antiviral drugs, 252 patients with cryptogenic LDs (persistently abnormal liver tests and/or liver cirrhosis with unknown etiology), as well as 35 patients with autoimmune hepatitis. The rate of OCI prevalence among these patients was estimated to be 12.04% (95%CI:

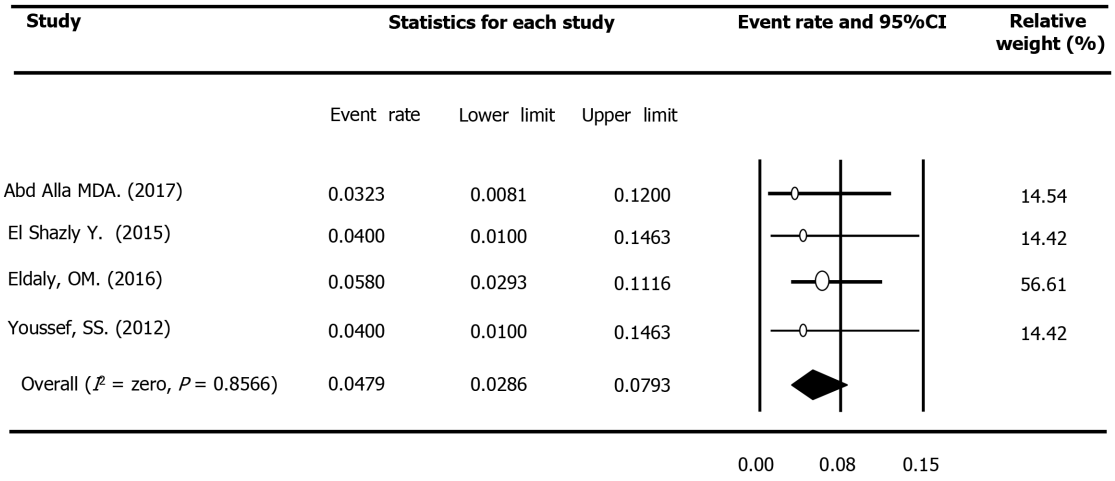

Figure 2 Meta-analysis forest plot of occult hepatitis C among healthy populations across the Middle East and Eastern Mediterranean countries.

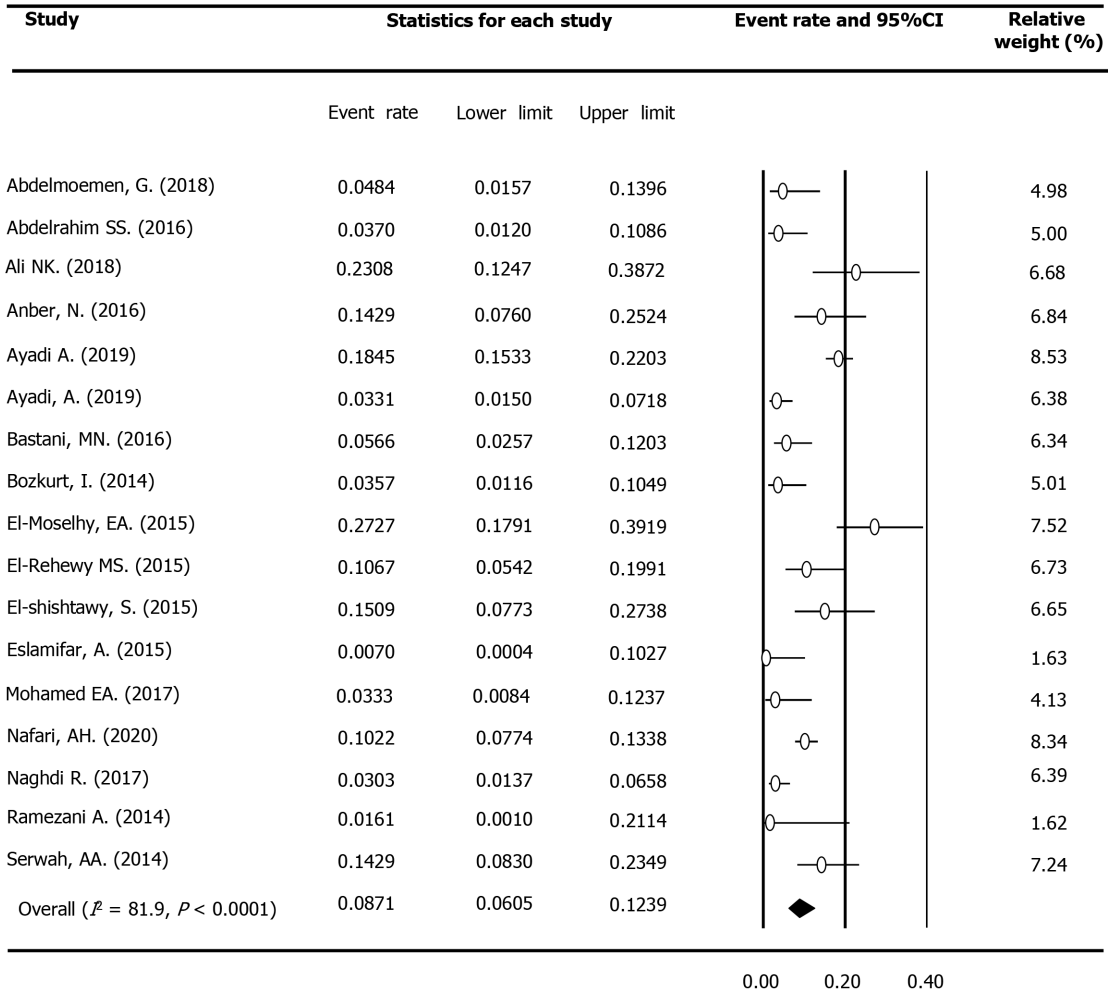

Figure 3 Meta-analysis forest plot of occult hepatitis C among multi-transfused patients across the Middle East and Eastern Mediterranean countries.

5.87%-23.10%, Table 4). The rate in the subgroup of cryptogenic patients (20.81%; 95%CI: 6.87%-48.35%) was double the value calculated for post-HCV non-viremic cases (9.14%; 95%CI: 3.02%-24.53%, P = 0.276). Figure 4 displays the forest plot of OCI among patients with chronic LDs based on the type of the disease. In addition, the rate of OCI among cases detected by RT-nested PCR (21.38%; 95%CI: 11.73%-35.75%) was

Table 4 Subgroup-specific pooled estimates of occult hepatitis C virus infection prevalence among patients with chronic liver diseases across the Middle Eastern and Eastern Mediterranean countries

Variables	Number of studies	Sample sizes	OCI prevalence across studies		Pooled OCI prevalence (%)		Heterogeneity	
			Range (%)	Median	Mean (%)	95%CI	Cochran's Q	I-squared (%)
Countries								
Egypt	5	1689	3.91-44.44	11.33	16.08	5.81-37.35	152.4[a]	97.4
Iran	5	272	0-32.08	10.14	11.46	3.64-30.73	16.6[b]	76.0
Pakistan	1	104	-	-	0.48	00.03-7.15	-	-
Temporal duration[1]								
Before 2015	8	523	0-44.44	10.46	11.83	4.84-26.14	46.0[a]	84.8
2015 and thereafter	3	1542	3.91-34.82	11.33	12.28	3.23-36.99	111.1[a]	98.2
Method of HCV RNA detection								
RT-nested PCR	6	359	0-44.44	21.11	21.38	11.73-35.75	30.5[a]	83.6
Real time RT-PCR	4	1636	0-11.33	7.35	6.29	2.73-13.84	23.9[a]	87.4
RT-PCR	1	70	-	-	12.86	6.83-22.90	-	-
Patients' subpopulation								
Post-HCV non-viremic cases[2]	5	1716	0-34.82	11.33	9.14	3.02-24.53	116.8[a]	96.6
Cryptogenic liver diseases[3]	4	212	8.89-44.44	21.11	20.81	6.87-48.35	22.6[a]	86.7
Chronic HCV infection and Cryptogenic liver diseases[2,3]	1	102	-	-	10.78	6.07-18.43	-	-
Autoimmune hepatitis	1	35	-	-	1.39	0.09-18.67	-	-
Patients' HCV serostatus								
Seronegative	5	247	0-44.44	10.14	16.13	5.41-39.28	27.7[a]	85.6
Seropositive	5	1716	0-34.82	11.33	9.11	2.97-24.69	116.8[a]	96.6
Seronegative/seropositive	1	102	-	-	10.78	6.07-18.43	-	-
Sample size								
Less than 100	6	317	0-44.44	11.50	15.63	6.08-34.67	32.2[a]	84.5
100 and above	5	1748	0-34.82	10.78	8.88	3.04-23.26	116.0[a]	96.5
All studies	11	2065	0-44.44	10.78	12.04	5.87-23.10	179.3[a]	94.4

[a]$P < 0.001$.
[b]$P < 0.01$.
[1]Based on the last year reported for the data collection. If the collection date was not clear, publication year was considered instead.
[2]Including those patients who achieved sustained virologic response after treatment with anti-virals.
[3]Including unexplained persistently abnormal liver enzymes and cryptogenic cirrhosis. CI: Confidence interval; Min: Minimum, Max: Maximum; PCR: Polymerase chain reaction; RT-PCR: Reverse transcription polymerase chain reaction; OCI: Occult hepatitis C virus infection.

considerably higher than cases identified using real-time RT-PCR (6.29%; 95%CI: 2.73%-13.84%, $P = 0.052$). Furthermore, there was no difference in the OCI frequency based on the year of data collection ($P = 0.962$), study location ($P = 0.178$), sample size ($P = 0.416$), patients' HCV serostatus ($P = 0.750$) and sex ($P = 0.749$). According to meta-regression analysis, no significant ($P = 0.943$) link was detected between the OCI rate among this population and data collection time. Figure 4 displays the forest plot of OCI among patients with chronic LDs based on type of the disease.

Occult hepatitis C prevalence among other high-risk categories

Regarding OCI prevalence among HIV-positive subjects in the M and E region, three surveys reported the rate among 417 HCV-seronegative and seropositive samples. All studies had been conducted in Iran after 2014 and evaluated HCV RNA in PBMCs

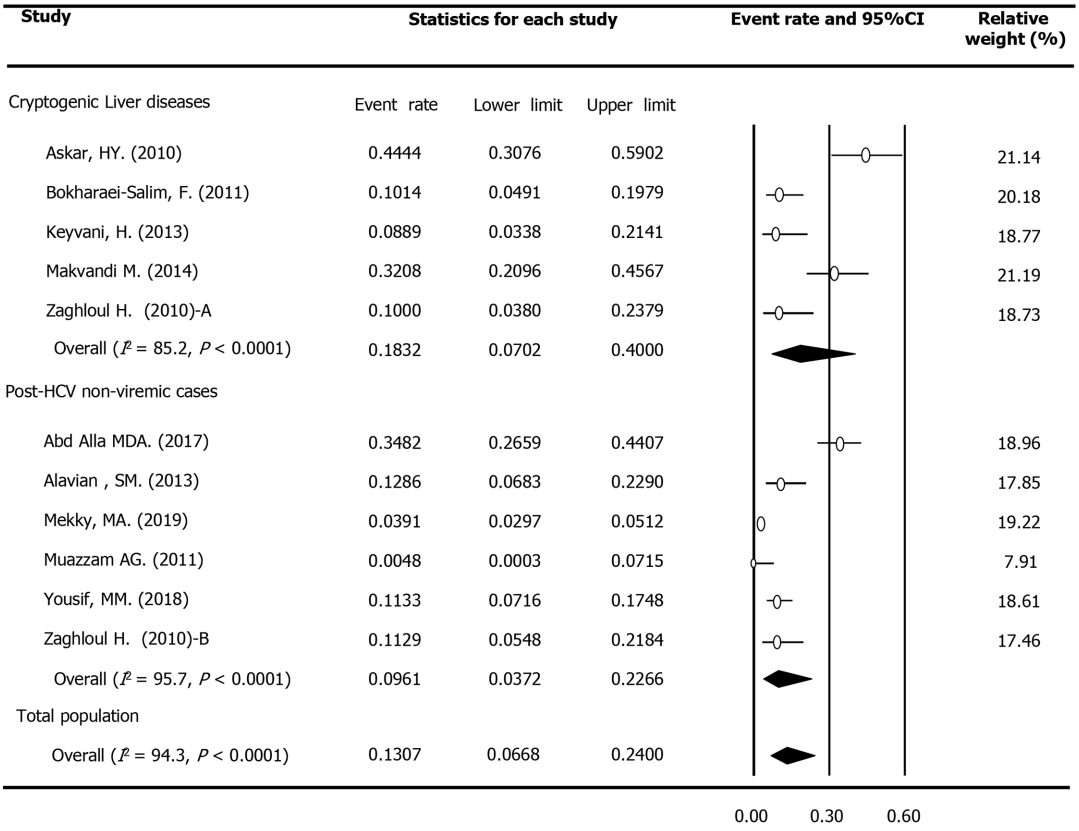

Figure 4 Meta-analysis forest plot of occult hepatitis C among patients with chronic liver diseases across the Middle East and Eastern Mediterranean countries based on type of disease. (Note: Zaghloul's study was considered two surveys, one among patients with cryptogenic liver diseases and one among hepatitis C virus-seropositive patients. Rezaee Zavareh's study among patients with autoimmune hepatitis was not included in this figure).

using the RT-nested PCR method. Using the fixed-effect model (Q = 3.1, P = 0.208, I^2 = 36.3%), the pooled mean prevalence of OCI was estimated at 12.95% (95%CI: 9.56%-17.32%) among this population.

Concerning occult hepatitis C among IDUs, one study recently identified HCV RNA in PBMCs in 18.18% of 77 Iranian HIV-positive IDUs. Moreover, another study from Iran reported an OCI rate of 9.57% among 115 HBV- and HIV-negative IDUs. Both surveys detected HCV genome among both HCV seronegative and seropositive samples by the RT-nested PCR method.

A total of three surveys, including two studies from Egypt and one survey from Iran, focused on OCI among patients with hematologic disorders, such as lymphoma, leukemia, and anemia. All OCI cases were detected by the RT-nested PCR technique among 171 HCV seronegative samples. Using the random-effect model (Q = 29.9, P < 0.001, I^2 = 93.3%), the pooled estimate of OCI among this population was estimated at 19.57% (95%CI: 3.22%-63.99%).

DISCUSSION

Through a comprehensive description and detailed analysis of occult hepatitis C epidemiology among the various populations in M and E countries, we found a considerably high rate of overall OCI prevalence across the region (10.04%; 95%CI: 7.66%-13.05%). The lowest rate (4.79%) was estimated among apparently healthy volunteers and blood donors. On the other hand, the higher rates were estimated for MTPs (8.71%), patients with chronic LDs (12.04%), HIV-positive subjects (12.95%), and those with lymphoproliferative and hematologic disorders (19.57%). Although the rate varied significantly across the studies, the pooled mean rate of OCI was not dissimilar regardless of subpopulation, location and year of study, the detection method of HCV RNA, patients' HCV serostatus or sex. Lastly, meta-regression analysis could not ascertain a declining or rising trend for OCI prevalence as a whole or among the different subpopulations of the region.

The incidence of OCI in each area is affected by various factors, mainly the

prevalence and risk factors of HCV infection in the community as well as in the studied population. Some investigators believed that there is a geographical pattern for OCI that is probably related to HCV endemicity distribution[3]. The majority of all chronically HCV infected people in the M and E region reside in the two countries most affected by the infection, *i.e.* Egypt and Pakistan[78]. In the current review, we noted that the Egyptian population had the highest rates (12.34%; 95%CI: 8.32%-17.92%) of OCI in this region. Likewise, based on four studies from Egypt, we calculated the pooled OCI rate among healthy populations to be 4.79 (95%CI: 2.86%-7.93%). Egypt is one of the countries highly affected by HCV and with high anti-HCV prevalence in almost all population groups[10]. Based on the Egypt Demographic and Health Surveys, anti-HCV prevalence among the adult Egyptian population was 10.0% in 2015[10]. Similarly, a recent systematic review estimated a pooled mean rate of 11.9% (95%CI: 11.1%-12.6%) for anti-HCV prevalence among the general Egyptian population[10]. Another systematic review estimated an average pooled HCV viremic rate of 67.0% (95%CI: 63.1%-70.8%) among anti-HCV positive individuals in this country[14]. In other words, the prevalence of chronic hepatitis C in Egypt is around 8%, which is close to the rate (6.3%) reported previously in 2015[79]. Moreover, four-fifths of hepatocellular carcinoma (HCC) patients in this country are infected with HCV—which ranks first in the world[80]. On the other hand, Iran has one of the lowest rates of HCV infection worldwide, particularly in the M and E region[81]. In this country, where HCV spread is dominated by transmission through injecting drug use[81], the pooled rates of anti-HCV positivity and viremic HCV among the GP have been estimated as low as 0.2%-0.3% and 0.4%-0.6%, respectively[13,79,81,82]. Correspondingly, we identified a lower overall rate of OCI among the Iranian population (8.48%) in comparison with the Egyptian population (12.34%).

Occult hepatitis C is primarily identified among populations at higher risk of health-care-related exposure, such as people who received repetitive transfusions particularly HD patients[66]. Our analysis estimated an average pooled OCI rate among MTPs of 8.7% (95%CI: 6.0%-12.4%); a higher level was calculated for HD patients (9.5%, 95%CI: 6.3%-14.1%) than for thalassemia patients (4.3%, 95%CI: 2.5%-7.5%). The rates of OCI prevalence among HD patients ranged from zero to 45% in different studies across the world[66]. In a survey by Barril *et al*[83], 45% of 109 Spanish HD patients with abnormal serum levels of liver enzymes had detectable HCV-RNA in their PBMCs. The patients with OCI had significantly higher mean levels of serum alanine aminotransferase. In addition, a significantly higher percentage of OCI patients died during the follow-up period compared with patients without OCI (39% *vs* 20%; $P = 0.031$). It is expected that HD patients are at higher risk of HCV infection owing to shared dialysis machines[84]. Some researchers suggested that the duration of dialysis is associated with the increased probability of HCV infection among HD patients[83,85]. In the M and E region, Harfouche *et al*[86] showed that about one-fifth of HD patients are chronic HCV carriers and can potentially spread the infection through the dialysis machine. They suggested that their findings may reflect the higher HCV incidence in the communities along with poor standards of dialysis in this area. Despite the decrease in the prevalence of HCV infection in HD patients, OCI could be the culprit for the constant distribution of HCV among this population[3].

Furthermore, our review estimated a two-fold higher rate of OCI among Egyptian MTPs patients (11.4%, 95%CI: 6.5%-19.2%) than Iranian patients (5.9%, 95%CI: 3.1%-11.1%). These findings were consistent with the reported rates for anti-HCV prevalence among this population in both countries. In a systematic review and meta-analysis of data from 10 countries in the Middle East, the pooled HCV prevalence among HD patients was estimated to be 25.3% (95%CI: 20.2%-30.5%); a much higher rate was reported from Egypt (50%, 95%CI: 46%-55%) in comparison with Iran (12%, 95%CI: 10%-15%)[87]. Indeed, medical care appears to be the main route of both past and new HCV transmission in Egypt[10]. On the other hand, in another recent review, the overall anti-HCV prevalence was estimated at a considerably lower rate (20.0%, 95%CI: 16.4%-23.9%) across Iranian populations at high risk of healthcare-related exposures, such as HD, hemophilia, and thalassemia patients[13].

The rate of occult hepatitis C is considerably higher among populations with liver involvement who were seronegative for HCV RNA[2,61,64]. In a recent survey in Egypt, the rate of OCI among 112 post-HCV non-viremic cases, including 55 non-cirrhotic and 57 cirrhotic patients (34.8%) was significantly higher than 62 healthy control individuals (3.23%)[35]. Likewise, our review indicated a high frequency of OCI among patients with LDs, including individuals with unexplained elevated liver enzymes and cryptogenic hepatitis as well as those with a history of exposure to HCV in the past (12.04%, 95%CI: 5.87%-23.10%); the highest rate was observed in patients with cryptogenic LDs (20.81%; 95%CI: 6.87%-48.35%). High rates of OCI among LD patients

have been reported from countries with both low and high HCV endemicity in the community[21,22,41,48]. Consistently, our analysis revealed that the rate of OCI among LD patients in Egypt (16.08%; 95%CI: 5.81%-37.35%) did not significantly (P = 0.178) differ from Iranian patients (11.46%; 95%CI: 3.64%-30.73%). Regarding active HCV infection among populations with LDs, high rates have also been reported from both countries. A detailed analysis of HCV epidemiology in the Middle East found a pooled mean prevalence of 35.5% (95%CI: 31.7%-39.5%) in all patients with LDs; the highest rates were estimated for HCC (56.9%; 95%CI: 50.2%-63.5%) and hepatic cirrhosis (50.4%; 95%CI: 40.8%-60.0%). The pooled rate was 58.8% (95%CI: 51.5%-66.0%) in Egypt, 55.8% (95%CI: 49.1%-62.4%) in Pakistan, and 15.6% (95%CI: 12.4%-19.0%) in other countries[88]. The rate of HCV infection in each LD population of each country was strongly correlated with HCV prevalence among their GP. The authors concluded that their findings highlight how the role of this infection in liver diseases is a reflection of its background level in the GP[88]. Moreover, in countries like Egypt and Pakistan, high rates of infections among various populations with LDs may support the contribution of HCV to the occurrence of liver disease[9,10]. On the other hand, a significantly lower HCV rate has been reported for Iranian patients with liver-related conditions (7.5%, 95%CI: 4.3%-11.4%)[13]. Another systematic review underlined the different etiology of HCC in countries of the Eastern Mediterranean region; Four-fifths of HCC patients in Egypt and half of the patients in Pakistan were infected with HCV; however, this value was as low as 8.5% for Iranian patients[80].

Our study had several limitations. Almost all of OCI reports (34 of 37) were from Iran and Egypt, and we did not find any data from 21 countries in the M and E region. Our study is also limited by the number of available documents for both healthy subjects and specific at-risk populations, such as IDUs, HIV-positive persons, and thalassemia or hemophilia patients. Other limitations of our review were the quality of retrieved evidence as well as the representativeness of the target populations. The findings of the majority of studies were based on examination of less than 100 consecutive samples selected by a non-random convenience sampling method. There was a wide heterogeneity in OCI rates even within specific subpopulations; nonetheless, there was no evidence that study location, data collection date, the detection technique of HCV RNA, patients' HCV serostatus, and sex-group representation in the sample affected the prevalence rates. Despite these shortcomings, we found a large amount of data in two countries, which contributed to the lowest and highest rate of chronic HCV infections in the region (namely, Iran and Egypt, respectively) that allowed us to conduct an analysis among different population categories and settings.

CONCLUSION

Our systematic review and meta-analysis quantified high levels of OCI prevalence, especially across risk populations in M and E countries. Recommendations include more appropriate OCI screening programs to target individuals who are at high risk for HCV infection, especially the patients undergoing dialysis and those with cryptogenic liver diseases. Besides, further investigations are needed regarding OCI among other risk populations, such as HIV- and HBV-infected subjects, IDUs, and thalassemia and hemophilia patients.

ARTICLE HIGHLIGHTS

Research background

Occult hepatitis C virus (HCV) infection (OCI) is defined as the presence of HCV genome in the liver samples or peripheral blood mononuclear cells despite a negative test for serum viral RNA. OCI, a common condition worldwide, might be associated with significant morbidities such as liver cirrhosis or hepatocellular carcinoma. No review has yet been performed to provide a pooled estimate for the OCI prevalence rate in the Middle East and Eastern Mediterranean (M and E) countries, a region with the highest rates of HCV infection in the world.

Research motivation

In this systematic review and meta-analysis, we tried to characterize a clear feature of OCI epidemiology in 26 countries of the M and E region based on documents found by

searching international and regional electronic sources as well as some local grey literature. We hope our findings help researchers to perform more investigations on diagnosis, management, and control of OCI, particularly in high-risk populations such as patients with chronic liver disease, multi-transfused patients, those infected with HIV, injecting drug users, *etc.*

Research objectives

The main objective of this review is to provide pooled mean estimates of the OCI rate and assess the contribution of potential variables on the between-study heterogeneity in the M and E region. The results would help professionals, investigators and policy makers to organize suitable activities regarding OCI, particularly in high-risk patients.

Research methods

A systematic review and meta-analysis was performed following PRISMA guidelines. A comprehensive search of electronic databases was conducted up to June 2020 in the Web of Science, PubMed, SCOPUS, ScienceDirect, ProQuest, the Index Medicus for the Eastern Mediterranean Region, Scientific Information Database, Iranian Database of publication (Magiran), and Iranian Databank of Medical Literature. Also some conference abstracts and all references from bibliographies of retrieved articles were manually reviewed. Forest plots were applied to demonstrate the point prevalence rates and the 95% confidence intervals, and subgroup and meta-regression analyses were applied to identify the factors contributing to heterogeneity between surveys.

Research results

Thirty-seven studies involving 5200 participants from Egypt, Iran, Pakistan, Saudi Arabia, and Turkey were analyzed. The overall pooled prevalence rate of OCI was 10.04%. The pooled rate among healthy populations was 4.79%, but the rate was much higher among patients with hematologic disorders (19.57%), HIV-positive subjects (12.95%), patients with chronic liver diseases (12.04%), and multi-transfused patients (8.71%). The rate of OCI was not significantly related to the country, disease subpopulations, year of study, the method of HCV RNA detection, sample size, patients' HCV serostatus and sex, and no significant change was detected in the OCI rate over time ($P > 0.05$).

Research conclusions

This review and meta-analysis demonstrates high rates of OCI prevalence, especially across risk populations in the M and E region. Some appropriate OCI screening programs are recommended to target individuals who are at risk of HCV infection.

Research perspectives

According to this systematic review and meta-analysis, further investigations are required in order to collect more data on the OCI frequency in M and E countries other than Egypt and Iran, two nations with the highest and lowest rates of chronic HCV infection in the region, respectively. Moreover, large scale studies are needed to evaluate OCI prevalence among less studied populations such as injecting drug users, HBV-infected patients, and thalassemia and hemophilia patients.

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CASE REPORT

Two-stage hepatectomy with radioembolization for bilateral colorectal liver metastases: A case report

Matteo Serenari, Jacopo Neri, Giovanni Marasco, Cristina Larotonda, Alberta Cappelli, Matteo Ravaioli, Cristina Mosconi, Rita Golfieri, Matteo Cescon

ORCID number:

Matteo Serenari, Matteo Ravaioli, Matteo Cescon, General Surgery and Transplant Unit, IRCCS, Azienda Ospedaliero-Universitaria di Bologna, Sant'Orsola-Malpighi Hospital, Bologna 40138, Italy

Jacopo Neri, Giovanni Marasco, Cristina Larotonda, Matteo Ravaioli, Matteo Cescon, Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Bologna 40138, Italy

Alberta Cappelli, Cristina Mosconi, Rita Golfieri, Department of Radiology, IRCCS, Azienda Ospedaliero-Universitaria di Bologna, Sant'Orsola-Malpighi Hospital, Bologna 40138, Italy

Corresponding author: Matteo Serenari, MD, Academic Research, Surgeon, General Surgery and Transplant Unit, IRCCS, Azienda Ospedaliero-Universitaria di Bologna, Sant'Orsola-Malpighi Hospital, Via Albertoni, 15, Bologna 40138, Italy. matteo.serenari@gmail.com

Author contributions: Serenari M contributed study conception and design; Neri J, Larotonda C, Cappelli A and Mosconi C contributed acquisition of data; Marasco G contributed analysis and interpretation of data; Serenari M contributed drafting of manuscript; Golfieri R, Ravaioli M and Cescon M contributed critical revision.

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Abstract

BACKGROUND

Two-stage hepatectomy (TSH) is a well-established surgical technique, used to treat bilateral colorectal liver metastases (CRLM) with a small future liver remnant (FLR). However, in classical TSH, drop-out is reported to be around 25%-40%, due to insufficient FLR increase or progression of disease. Trans-arterial radioembolization (TARE) has been described to control locally tumor growth of liver malignancies such as hepatocellular carcinoma, but it has been also reported to induce a certain degree of contralateral liver hypertrophy, even if at a lower rate compared to portal vein embolization or ligation.

CASE SUMMARY

Herein we report the case of a 75-year-old female patient, where TSH and TARE were combined to treat bilateral CRLM. According to computed tomography (CT)-scan, the patient had a hepatic lesion in segment VI-VII and two other confluent lesions in segment II-III. Therefore, one-stage posterior right sectionectomy plus left lateral sectionectomy (LLS) was planned. The liver volumetry estimated a FLR of 38% (segments I-IV-V-VIII). However, due to a more than initially planned, extended right resection, simultaneous LLS was not performed and the patient underwent selective TARE to segments II-III after the first surgery. The CT-scan performed after TARE showed a reduction of the treated lesion and a FLR increase of 55%. Carcinoembryonic antigen and CA 19.9 decreased significantly. Nearly three months later after the first surgery, LLS was performed

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Grade D (Fair): 0
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and the patient was discharged without any postoperative complications.

CONCLUSION
According to this specific experience, TARE was used to induce liver hypertrophy and simultaneously control cancer progression in TSH settings for bilateral CRLM.

Key Words: Trans-arterial; Radioembolization; Two-stage hepatectomy; Colorectal liver metastases; Selective internal radiation therapy; Yttrium90; Case report

Core Tip: Two-stage hepatectomy and trans-arterial radioembolization (TARE) are usually used in advanced stage primary liver malignancies. In this case report, two-stage hepatectomy and TARE were combined, for the first time, to treat a patient with bilateral colorectal liver metastases and a small future liver remnant. In particular, TARE was performed to induce liver hypertrophy and at the same time to control tumor growth between stages, thus reducing the risk of tumor progression.

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INTRODUCTION

Two-stage hepatectomy (TSH) has been traditionally advocated for bilateral colorectal liver metastases (CLRM) that could not be resected in a single operation[1]. In TSH, when the future liver remnant (FLR) is considered not enough, contralateral portal vein ligation (PVL) or embolization (PVE) can be performed in the first stage to increase FLR volume. However, 25%-40% of patients will not undergo the second stage due to insufficient liver hypertrophy and/or progression of disease[2]. Trans-arterial radioembolization (TARE) consists of the selective intra-arterial administration of microspheres loaded with a radioactive compound—usually yttrium90—and has been shown to control tumor growth and to induce liver hypertrophy especially in patients with primary liver cancer. More recently, it has been shown to be effective also in CLRM setting[3]. However, the combination of these two techniques has never been explored before.

CASE PRESENTATION

Chief complaints

A 75-year-old female patient presented herself with mild abdominal pain.

History of present illness

Ultrasonography and abdominal computed tomography (CT) detected three unknown hepatic lesions in segment VI-VII (n = 1) and segment II-III (n = 2), respectively. The lesion of the right lobe seemed to infiltrate the right hepatic vein whereas the other two confluent lesions in segment II-III showed a particular intrabiliary growth pattern (Figure 1).

History of past illness

The patient had undergone endoscopic removal of a sigmoid polyp cancer (T1NxMxR0) 3 years before.

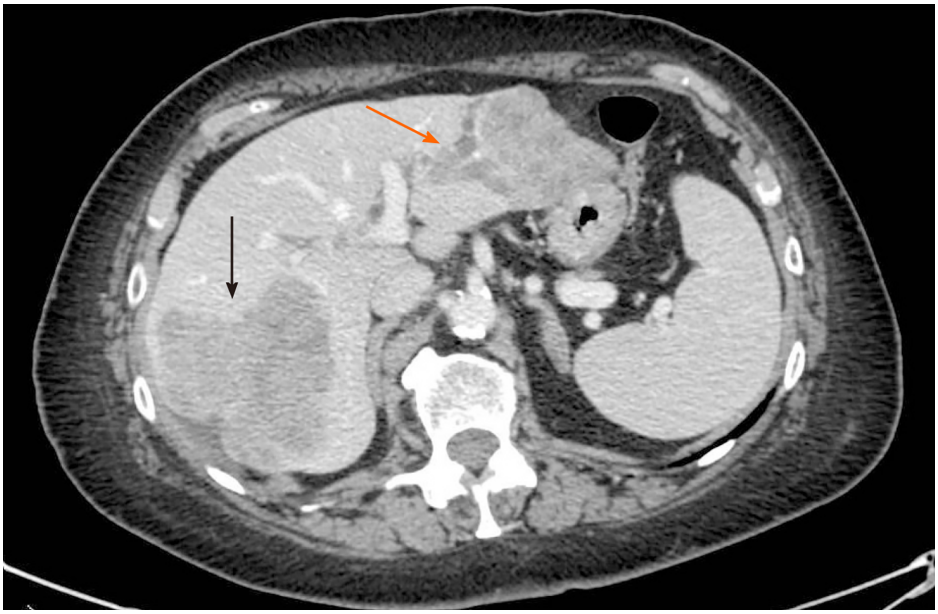

Figure 1 Pre-operative computed tomography-scan. The lesion occupying the right posterior segments of the liver (black arrow) and two other confluent lesions in the left lobe with intrabiliary growth pattern (orange arrow).

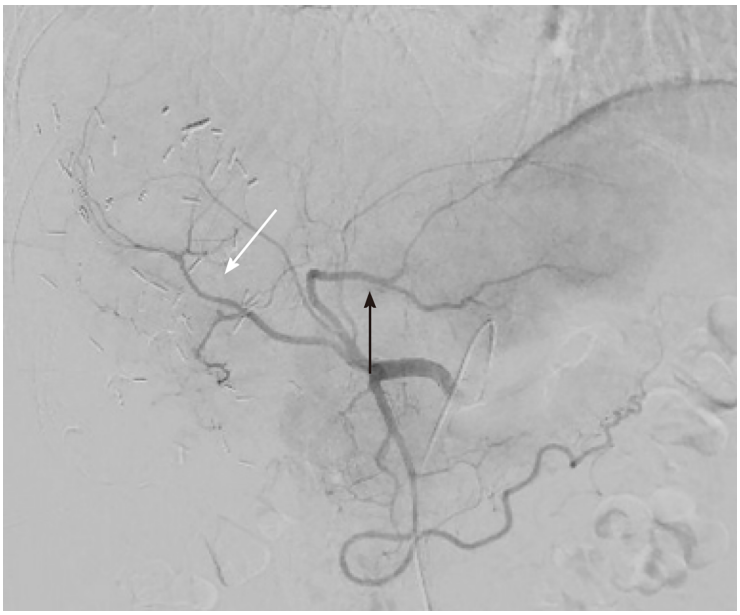

Figure 2 Arterial vascular anatomy during trans-arterial radioembolization procedure treating the tumor lesion in segments II-III (black arrow). The angiography also showed the remaining right anterior portal pedicle (white arrow).

Personal and family history

She suffered from hypertension and hypoparathyroidism. She had no family history of cancer.

Physical examination

The patient had a good performance status. Physical examination was unremarkable with vital signs within the normal range of values. No jaundice was observed.

Laboratory examinations

Liver function tests were normal and tumor markers were increased (carcinoembryonic antigen, CEA = 3284.9 ng/mL; CA 19-9 = 703.9 U/mL).

Imaging examinations

Esophagogastroduodenoscopy and colonoscopy were negative. According to liver volumetry, total functional liver volume (TFLV) measured 1635 mL and FLR (segments I-IV-V-VIII) 621 mL, with a resulting FLR/TFLV of 38%.

FINAL DIAGNOSIS

The final diagnosis of the presented case was suspected bilateral hepatic metastases from colorectal cancer.

TREATMENT

The operation started with a minimally invasive approach, but it was converted to open surgery due to diaphragm infiltration by the lesion located in the right liver. Intraoperative ultrasound showed that part of segment VIII was also involved. After detachment of the lesion from the diaphragm and its suture, a portal branch of segment V was ligated during parenchymal transection. Given the wider than initially planned hepatic surgery (segments V-VI-VII + part of segment VIII) and the difficulties encountered during the first resection, left lateral sectionectomy (LLS) was postponed. As a bridge treatment, TARE was chosen in order to control locally the disease while waiting for FLR increase. According to the CT-scan performed 10 d after surgery, FLR measured 632 mL. A small intrabdominal fluid collection was incidentally detected close to the surgical site as well as an ischemic area in segment V. The patient was discharged home on postoperative day 14, without major complications. The final diagnosis, based on histopathology of surgery specimen, was adenocarcinoma from colorectal cancer (KRAS and BRAF wild-type). TARE was carried out 11 d after discharge and realized with a single treatment (200 Gy) of Selective Internal Radiation (SIR) spheres (Sirtex Medical, Sydney, Australia) without any post-procedural complications (Figure 2). Forty-seven days after TARE, the patient underwent a new CT-scan showing a 32% reduction of the confluent lesion in segment II-III, with a surprisingly final FLR volume of 980 mL (FLR increase = 55%, FLR/TFLV = 77%) (Figure 3). CEA and CA 19.9 decreased to 93.7 ng/mL and 92.5 U/mL, respectively. After resolution of the abdominal collection by percutaneous drainage, we planned the second stage of surgery and 3 mo later after the first operation, the patient underwent LLS.

OUTCOME AND FOLLOW-UP

The postoperative course was uneventful. The patient did not receive any adjuvant chemotherapy and almost two years after the first surgery is still alive and free of disease.

DISCUSSION

According to this specific experience, TARE was used for the first time, combined with classical TSH, to control cancer progression between stages, waiting for adequate liver hypertrophy before the second resection.

TARE has been already shown to produce effective liver parenchyma hypertrophy in patients with primary hepatic malignancies treated with lobar 90Y radioembolization therapy[4]. After TARE, however, compared to PVE/PVL, the hypertrophy is radiation-induced rather than caused by embolization and is reached at a slower rate. According to a recent systematic review[5], the median kinetic growth rate of the controlateral lobe for patients underwent lobar TARE for CRLM was 0.8% per week compared to 6.1% of PVE. Despite a slower increase, however, a hyper-trophy of 26%-47% was obtained at time intervals ranging from 44 d to 9 mo[4], of similar magnitude to that observed after PVE. In addition, Birgin *et al*[5] found that up to 84% of patients affected by primary and secondary hepatic malignancies had a local tumor control following TARE and about 30% of unresectable tumors underwent hepatic resection.

Table 1 Review of the literature including patients submitted to preoperative trans-arterial radioembolization for colorectal liver metastases

Ref.	Year	Type of study	Pts included, *n*	CRLM[1], *n* (%)	Tumor location (*n*)	Bilobar *n* (%)	Prior resection, *n* (%)	Resectability, *n* (%)
Gray *et al*[18]	2001	HAI *vs* HAI + TARE in unresectable CRLM; RCT	74	36 (48.6)	Colon (29), rectum (7)	36/36 (100)	0	1/36 (2.8)
Lim *et al*[6]	2005	TARE after failure of FU in unresectable CRLM; prospective	30	30 (100)	NA	NA	0	1/30 (3.3)
Sharma *et al*[7]	2007	TARE + FOLFOX4 in unresectable CRLM; prospective (phase I)	20	20 (100)	Right colon (4), sigmoid (5), rectum (4), other colon sites (7)	NA	0	2/20 (10)
Cosimelli *et al*[19]	2010	TARE in unresectable CRLM; prospective (phase II)	50	50 (100)	Colon (41), rectum (9)	35/50 (70)	12/50 (24.0)	2/50 (4.0)
Hendlisz *et al*[8]	2010	FU *vs* TARE + FU in unresectable CRLM; RCT	44	21 (47.7)	NA	NA	NA	1/21 (4.8)
Brown *et al*[9]	2011	TARE *vs* CHT *vs* no therapy before hepatectomy; case-control	840	16 (1.9)	NA	NA	NA	16/16 (100)
Whitney *et al*[10]	2011	TARE in unresectable liver disease; retrospective	44	15 (34)	Rectum (15)	0	0	1/15 (6.7)
Vouche *et al*[11]	2013	TARE in unresectable liver disease; retrospective	83	8 (9.6)	NA	0	0	1 (12.5)
Wang *et al*[20]	2013	TARE before liver resection for CRLM; retrospective	24	24 (100)	Sigmoid (1), rectum (1), other colon sites (1), unknown (21)	1/3 (33.3)	0	3/24 (12.5)
Henry *et al*[12]	2015	TARE before liver resection for metastatic cancer; retrospective	9	4 (44.4)	NA	NA	0	4/4 (100)
Justinger *et al*[22]	2015	TARE in marginally resectable CRLM; retrospective	13	13 (100)	Right colon (2), sigmoid (4), rectum (7)	9/13 (69.2)	7/13 (53.8)[2]	11/13 (84.6)
Moir *et al*[13]	2015	TARE in unresectable liver disease; retrospective	44	22 (50)	NA	NA	NA	4/22 (18.2)
Maleux *et al*[14]	2016	TARE in unresectable CRLM; NA	88	71 (80.6)	NA	58/71 (81.6)	10/71 (14.0)	1/71 (1.4)
Lewandowski *et al*[23]	2016	TARE in unresectable right-sided liver disease; retrospective	13	1 (7.6)	NA	0	NA	1/1 (100)
Wright *et al*[15]	2017	TARE in unresectable liver disease; retrospective	465	6 (1.2)	NA	NA	NA	6/6 (100)
van Hazel *et al*[16]	2016	FOLFOX6 *vs* FOLFOX6 + TARE ± Bevacizumab; RCT	530	267 (50.3)	Left colon (141), right colon (72), rectum (45), other colon sites (7), unknown (2)	NA	NA	38/267 (14.2)
Pardo *et al*[21]	2017	TARE before liver resection or transplantation; retrospective	100	30 (30)	NA	44/100	7/30 (23.3)[2]	30/30 (100)
Wasan *et al*[17]	2017	FOLFOX *vs* FOLFOX + TARE; RCT	1103	554 (50.2)	Colon (421), rectum (116), unknown (17)	NA	NA	56/554 (10.1)

[1]Treated with transarterial radioembolization.

[2]Associating liver partition and portal vein ligation for staged hepatectomy. CHT: Chemotherapy; CRLM: Colorectal liver metastases; FU: Florouracil; HAI: Hepatic artery infusion; NA: Not available; RCT: Randomized controlled trial; TARE: Transarterial radioembolization.

Review of the literature, including only studies of patients submitted to hepatectomy for CRLM after preoperative TARE (n = 18)[6-18] (Tables 1 and 2), showed that even though many of them comprised bilateral distribution of CRLM[18-

Table 2 Review of the literature including patients submitted to preoperative trans-arterial radioembolization for colorectal liver metastases

Ref.	FLR increase %	Time TARE-surgery, median (range), mo	Type of hepatic resection (*n*)	Post-operative mortality %	Survival, median (range), mo	Disease free, median (range), mo	Recurrence after surgery, *n* (%)
Gray *et al*[18]	NA	NA	NA	0	96	NA	NA
Lim *et al*[6]	NA	NA	NA	NA	NA	22	1/1 (100)
Sharma *et al*[7]	NA	NA	LLS + S6 (1), RH + S3 (1)	NA	NA	NA	NA
Hendlisz *et al*[8]	NA	NA	RH (1)	0	NA	1.5	1/1 (100)
Brown *et al*[9]	NA	6.5 (4-13)	NA	NA	NA	NA	NA
Whitney *et al*[10]	NA	NA	RT (1)	0	NA	24	1/1 (100)
Vouche *et al*[11]	NA	NA	RT (1)	0	NA	NA	NA
Wang *et al*[20]	NA	NA (4-9)	RH (2), LH + S6 (1)	0	NA	NA	NA
Henry *et al*[12]	NA	5 (2-8)	LLS (1), multiple wedge, HAI pump (1), RT + RFA (1), RT (1)	50	13 (0-27)	6.2 (1.8-10.5)	3/4 (75)
Justinger *et al*[22]	32.9 (ALPPS), 27.1 (no ALPPS)	2 (1-5)	RT (4), RH (5), mesohepatectomy (1), LT (1)	7.6	25 (12-38)[1]	NA	NA
Moir *et al*[13]	NA	4 (2-11)	NA	0	15 (11-19)[1]	NA	NA
Maleux *et al*[14]	NA	NA	S + RFA (1)	0	NA	NA	NA
Lewandowski *et al*[23]	15	1.6 (1-7)	RT (1)	0	4.8	NA	NA
Wright *et al*[15]	NA	9 (3-20)	RT (2), S (1), RH (3)	16.6	25 (NA)	NA	NA
van Hazel *et al*[16]	NA	NA	NA	0	NA	NA	NA
Pardo *et al*[21]	NA	NA	NA	10	NA	NA	NA
Wasan *et al*[17]	NA	NA	NA	3.6	NA	NA	NA

[1]95% confidence interval. ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; HAI: Hepatic artery infusion; LH: Left hepatectomy; LLS: Left lateral sectionectomy; LT: Left trisectionectomy; RFA: Radiofrequency ablation; RH: Right hepatectomy; RT: Right trisectionectomy; S: Segmentectomy; NA: Not available.

20], only Pardo *et al*[21] reported a "two-stage resection" in 10 patients, 7 of whom underwent associating liver partition and portal vein ligation for staged hepatectomy (ALPPS), probably from the cohort of Justinger *et al*[22] In this latter study, resectability of ALPPS + TARE was 85.7%. Increase of FLR in CRLM patients was reported only in few studies[22,23]. In our report, TARE to segment II-III led to a FLR increase of 55%, probably induced by the combined regenerative effect produced by the first

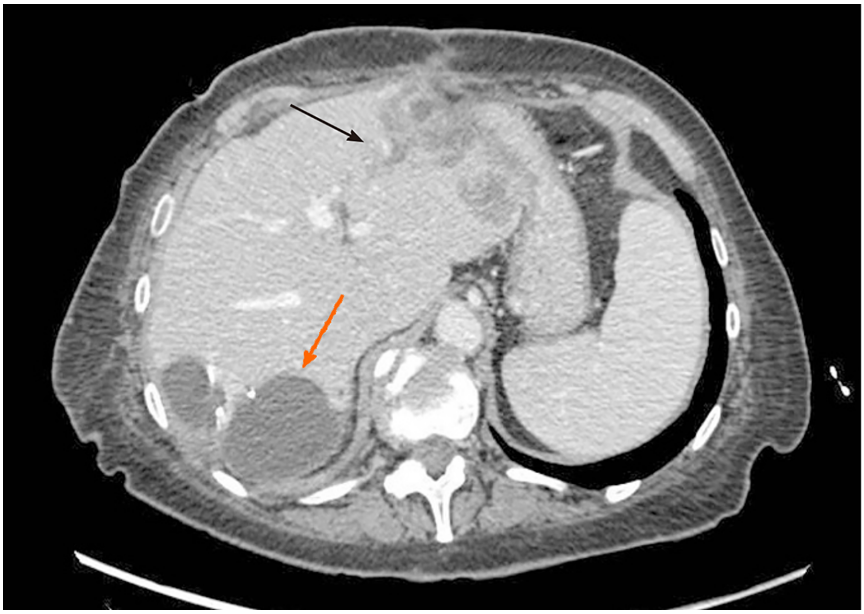

Figure 3 Computed tomography-scan performed one month after trans-arterial radioembolization. The reduction of the lesion of the left lobe and the intrabiliary growth pattern (black arrow). An intrabdominal fluid collection was found close to the surgical site (orange arrow).

liver resection, similar to what happens in ALPPS procedure, and by TARE itself. If a larger FLR hypertrophy was required, the role of TARE in combination also with classical portal vein occlusion techniques such as PVE/PVL or ALPPS, could have been explored. However, in this case, TARE was preferred over PVE or PVL since segment IV had to be preserved being part of the FLR. Furthermore metastasis in segment II-III could have progressed leading to the drop out of the patient. On the other side, the risk of proceeding with a second simultaneous hepatectomy or ALPPS was deemed too high. Last but not least, from the oncological point of view, this strategy may allow surgeons, without dealing with time issue, to select only patients with favorable tumor biology, according to radiological response after TARE[12].

CONCLUSION

TARE in TSH setting may represent a viable option to increase resectability in patients with bilateral CLRM by stimulating liver hypertrophy and controlling locally the disease. Future larger, comparative studies may help answer the questions above.

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