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Contents

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Contents

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Weekly Volume 29 Number 5 February 7, 2023

Contents

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EDITORIAL

Importance of human leukocyte antigen antibodies and leukocyte antigen/killer-cell immunoglobulin-like receptor genes in liver transplantation

Manuel Muro, Isabel Legaz

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Abstract

Many mechanisms have been proposed to explain the hypothetical state of hepatic tolerance, which is described by eventual imbalances or deregulation in the balance of cytokines, mediators, effectors, and regulatory cells in the complex milieu of the liver. In this section, we will comment on the importance of donor-specific anti-human leukocyte antigen (HLA) antibodies (DSA) as well as the compatibility and pairings of HLA and killer-cell immunoglobulin-like receptor (KIR) genotypes in the evolution of liver transplantation. Thus, HLA compatibility, viral infections, and HLA-C/KIR combinations have all been linked to liver transplant rejection and survival. There have been reports of increased risk of acute and chronic rejection with ductopenia, faster graft fibrosis, biliary problems, poorer survival, and even *de novo* autoimmune hepatitis when DSAs are present in the recipient. Higher mean fluorescence intensity (MFI) values of the DSAs and smaller graft size were associated with poorer patient outcomes, implying that high-risk patients with preformed DSAs should be considered for selecting the graft placed and desensitization methods, according to the investigators. Similarly, in a combined kidney-liver transplant, a pretransplant with a visible expression of several DSAs revealed that these antibodies were resistant to treatment. The renal graft was lost owing to antibody-mediated rejection (AMR). The HLA antigens expressed by the transplanted liver graft influenced antibody elimination. Pathologists are increasingly diagnosing AMR in liver transplants, and desensitization therapy has even been employed in situations of AMR, particularly in patients with DSAs in kidney-hepatic transplants and high-class II MFI due to Luminex. In conclusion, after revealing the negative impacts of DSAs with high MFI, pretransplant virtual crossmatch techniques may be appropriate to

improve evolution; however, they may extend cold ischemia periods by requiring the donor to be typed.

Key Words: Acute rejection; Alloantibodies donor-specific antibodies-donor-specific anti-human leukocyte antigen antibodies; Chronic rejection; Human leukocyte antigen matching; Killer-cell immunoglobulin-like receptor matching; Liver transplant

Core Tip: This editorial aimed to raise realities, doubts, and ambiguities in the fundamental role of alloantibodies and the compatibility and association of the proteins encoded by the human leukocyte antigen and killer-cell immunoglobulin-like receptor genes in liver transplantation.

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INTRODUCTION

In the vast majority of transplants performed today, there is a clear demonstration of the role played by the best human leukocyte antigen (HLA) compatibility and the absence of donor-specific anti-HLA antibodies (DSA) in its positive evolution, and it is an increasingly important role. The significance of the role of compatibility and killer-cell immunoglobulin-like receptor (KIR) genotypes (especially in hematopoietic stem cell transplantation) has not been demonstrated in the case of liver transplantation. This suggests that the classic concept of the liver is different and may be an "immunologically privileged" organ. Transplant (even if there is a positive pretransplant crossmatch and DSAs are known) without accounting for donor and recipient typing can lead to antibody-mediated rejection (AMR)[1]. However, there are articles where this has been re-evaluated, and new essential effects of antibodies and compatibility in acute rejection, chronic rejection (CR), fibrosis, and liver transplant survival appear.

The hypothetical state of tolerance of the liver has been explained by many causes (profusely explained in an article of its own), and is explained by eventual imbalances or deregulations in the balance of cytokines, mediator, effectors, and regulatory cells in the complex microenvironment of the liver, including increased or decreased expression of costimulatory or soluble molecules, specific genetic profiles, or even a protective role of Kupffer cells[2-9].

Here we focused on commenting on the role of DSA antibodies and the compatibility and pairings of HLA and KIR genotypes with the evolution of liver transplantation. Thus, HLA compatibility, viral infections, and HLA-C/KIR combinations have been classically related to liver transplant rejection and survival[10-13].

HLA ANTIBODIES AND *HLA*/*KIR* GENES IN LIVER TRANSPLANT IMMUNOLOGY

Regarding the existence of DSA antibodies present in the recipient, there are reports of increased risk of acute rejection and CR with ductopenia, accelerated graft fibrosis, biliary complications, worse survival, and even *de novo* autoimmune hepatitis[2,14]. However, some series and research groups reported different results and disparate causes (Figure 1). However, the literature on the role of DSAs and AMR is limited to clinical cases and small series[15].

Regarding preformed antibodies in the recipient before implantation, there is literature that reveals that patients with preformed DSA presented a worse graft evolution in living donor transplantation [16]. Higher mean fluorescence intensity (MFI) values of the DSAs and small graft size were associated with worse patient outcomes, suggesting to the authors that high-risk patients with preformed DSAs should be considered for selecting the graft implanted and desensitization protocols. Likewise, a pretransplant with the tangible expression of multiple DSAs[17] in a combined kidney-liver transplant showed that these antibodies were refractory to treatment, and the renal graft was lost due to AMR. The elimination of the antibodies depended on the HLA antigens expressed by the implanted liver graft.

In this sense, pathologists diagnose AMR in liver transplants with increasing frequency, and desensitization therapy has even been used in AMR cases, especially in patients with DSAs in kidney-hepatic

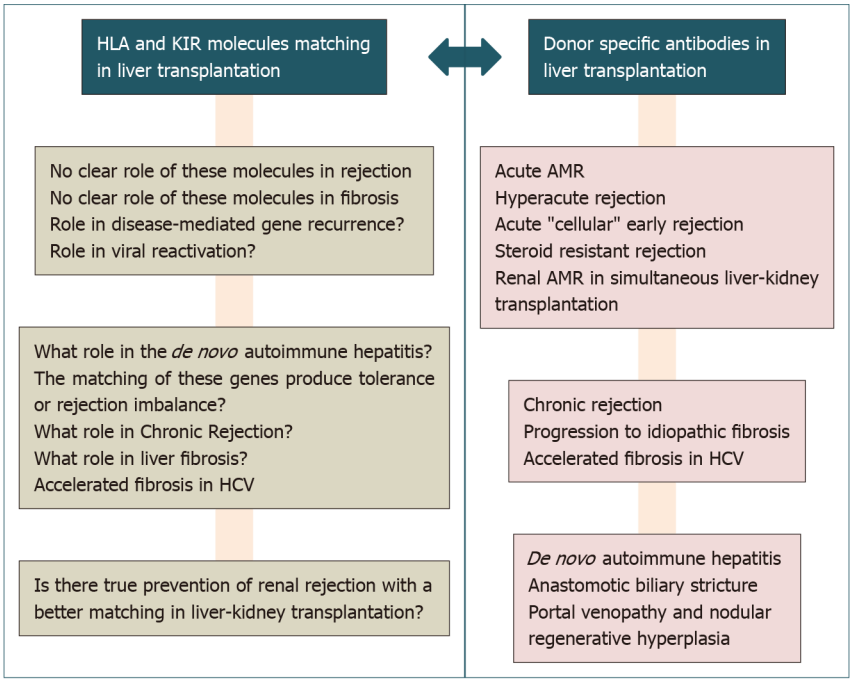

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Figure 1 Potential associations of donor-specific anti-human leukocyte antigen antibodies and human leukocyte antigen and/or matching with the evolution of liver transplantation. AMR: Antibody-mediated rejection; HCV: Hepatitis C virus; HLA: Human leukocyte antigen; KIR: Killer-cell immunoglobulin-like receptor.

transplants with high-class II MFI due to Luminex[18-20].

Although it is daring to assert categorically that the presence of DSAs contraindicates transplantation due to the same scientific literature, which is disparate between series, authors, and transplant centers, it is not well-defined over time (studies of positive, negative, and neutral papers) and the best methods of antibody diagnosis, evaluation of biopsies, and anti-rejection treatments[15]. In this way, regular DSA-post-transplant monitoring cannot as yet be recommended in routine practice but may be helpful in selected cases.

In the case of combined kidney transplants, there is also controversy and disparity between studies and groups. Thus, pretransplant DSAs increase the risk of AMR in the kidney and liver and worsen survival[12], with no data on the case of heart and lung combined with the liver. It has also been observed that the pretransplantation presence of anti-HLA class II antibodies and especially with positive complement fixation C1q or C3d have a risk of early AMR and a worse evolution of the transplant due to association of the graft with deposits of C4d in sinusoidal endothelial cells, increased fibrosis, CR, cirrhosis, and centrilobular fibrosis[2,13,16].

Regarding the development of *de novo* DSA (dnDSA), it has been estimated that immunosuppression may also play a role in the development of dnDSA. Thus, the coefficient of tacrolimus variation and mean tacrolimus levels have been reported to be associated with no dnDSA generation[21].

Other authors found that patients with an immunosuppressive regimen without withdrawal calcineurin inhibitors (mTOR inhibitors and/or maintenance with mycophenolic acid) have a higher prevalence of developing dnDSA post-transplant than patients with a standard regimen[22]. However, dnDSAs with calcineurin-free immunosuppression were associated with normal graft histology. The use of rituximab induction among DSA recipients has also been considered[23]. A dose of rituximab > 300 mg/m^2 was well tolerated and achieved a lower incidence of AMR.

In addition, everolimus combined with tacrolimus was associated with negative HLA and DSA antibody status[24]. Viral etiology of liver disease, hepatocellular carcinoma, and higher degrees of graft steatosis were associated with a lower rate of HLA antibodies. The impact of HLA and DSA antibodies was associated with higher levels of transaminases and bilirubin. In addition, a significant association was detected between higher degrees of inflammation and the presence of HLA and DSA antibodies. Thus, DSA would be associated with histological and biochemical inflammation of the graft after liver transplantation, while fibrosis seems unaffected.

There are also cases in the literature of living donor liver transplants who developed acute AMR after desensitization to perform DSA and were successfully treated with bortezomib and everolimus therapy [25]. In this regard, in sensitized combined liver-kidney transplant recipients, the “delayed” kidney transplant approach was associated with a significant reduction in total and class I DSAs after liver transplantation before kidney transplantation[26], allowing therapeutic interventions such as plasmapheresis, providing optimal results similar to those of crossmatched recipients.

Finally, regarding single or triple-therapy monotherapy, it has been reported that the development of class II DSA occurs more often with immunosuppressive monotherapy and may ultimately result in chronic rejection and graft fibrosis[27].

On the other hand, Shin *et al*[21] found that patients without T-cell rejection in pediatric liver transplantation were more likely to have dnDSAs for HLA-DQ7 and less likely to have these DSAs for HLA-DQ2. Therefore, they deduced that a load of mismatched epitopes predicted the non-generation of these DSAs. At the same time, the specificity of *de novo* DSAs could determine alloimmunity.

Also, references for the location and the importance of the correct detection of these DSAs would corroborate that the existence of intragraft DSA and intragraft union reaction of C3d (using a fluorescent analysis technique of capture of immunocomplexes) harms the outcome of the transplant, unlike DSA present in serum, with no impact[28].

Finally, it has been reported that the incidence of DSA after liver transplantation is higher in children than in adults, that DSAs directed against HLA class II molecules, mainly DQ, occur more often, and that the presence of such anti-class II DSA (DQ/DR), especially of the complement-binding IgG3 subclass, may be associated with endothelial injury, T-cell-mediated rejection (TCMR), inflammation, and fibrosis[29-31].

Regarding the positive, negative, or neutral role of the compatibility of the *HLA* and/or *KIR* genes, it is a subject of almost as much debate as the subject of antibodies. Historical studies have commented on any of the possibilities[10-12,32], and at the moment, there is no consistency in all the studies reviewed in this editorial. Regarding the role of HLA incompatibility and the evolution of the liver allograft, it is not separate from promoting the development of DSAs, with the logical criterion that the more incompatibilities, the more possibilities exist to develop antibodies DSAs *de novo*. Thus, the new molecular HLA incompatibility (MM) improves the prediction of the evolution of the transplant. Thus, in a study by Ono *et al*[33] on liver transplantation from a living donor, the risk of TCMR and the development of dnDSA were evaluated using eplets. MM in HLA-DQB1 eplets was associated with TCMR. The predicted indirectly recognizable HLA epitopes II (PIRCHE-II) score for the *HLA-DQB1* gene was also significantly higher in patients with TCMR. Moreover, DQB1-EpMMs ≥ 9 and DQB1-predicted indirectly recognizable HLA epitopes II score ≥ 3 were predictors of dnDSA formation. Thus, MM analysis may be applied toward tailored immunosuppression based on individual risks.

In this sense, a very recent article[34] on living donor transplants found that the more HLA incompatibilities there are, the worse the patient's survival was (for A + B + DR, A + B + C, DR + DQ, and A + B + C + DR + DQ). For HLA-B + DR mismatches, the risk of a TCMR was more pronounced in adults but not in children. It has also been reported in 1042 liver transplants and 9.38 years of follow-up that HLA-A mismatch was strongly associated with graft failure and mortality, especially with two mismatches [35].

However, other groups commented that incompatibility was not associated with acute rejection, early allograft dysfunction, or survival in living donor liver transplants[36]. The impact of HLA-A and HLA-DR incompatibility on cytomegalovirus reactivation and sepsis were significant but with very low significance and were not conclusive.

There is very little published and consistent literature on KIR compatibility, particularly in liver transplantation[10,16,32,37-41]. From more recent authorship, we know that the incidence of acute rejection does not correlate with HLA compatibility nor with KIR alleles or genotypes of the recipient, but the frequency of C2+ donors did increase in the rejection group and was more frequent when the recipient expressed KIR2DS4[39].

In another study, grafts from donors without *HLA-C2* alleles produced more rejection than in recipients from donors with at least one *HLA-C2* allele[42], consistent with a previous study of ours[32], which showed that *HLA-C2* homozygotes receiving HLA-C1/C2 grafts had a higher risk of rejection than *HLA-C1* homozygotes. Other groups, however, did not find this association in their series[38], so the issue is still under open debate.

CONCLUSION

In conclusion, after demonstrating the adverse effects of DSAs with high MFI, perhaps pretransplant virtual cross match protocols could be appropriate to improve evolution, although they could increase cold ischemia times by having to type the donor. Although today, there is no particular problem as the times of typing results have been shortened, which also allows the optimization of compatibility and *HLA* and *KIR* genotypes[15,43].

In our modest opinion, monitoring of dnDSAs should also be universally adopted in all transplant centers to avoid possible post-transplant complications as much as possible. More extensive cohort studies, including the MFI intensity of each DSA in the donor, the role of the different HLA and KIR compatibility, and particular combinations between donor and recipient, are needed to clarify their actual role in the post-transplant period.

FOOTNOTES

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OPINION REVIEW

Management of gastro-esophageal reflux disease: Practice-oriented answers to clinical questions

Leonardo Frazzoni, Lorenzo Fuccio, Rocco Maurizio Zagari

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Abstract

Gastro-esophageal reflux disease (GERD) is a condition which is frequently faced by primary care physicians and gastroenterologists. Improving management of GERD is crucial to maximise both patient care and resource utilization. In fact, the management of patients with GERD is complex and poses several questions to the clinician who faces them in clinical practice. For instance, many aspects should be considered, including the appropriateness of indication to endoscopy, the quality of the endoscopic examination, the use and interpretation of ambulatory reflux testing, and the choice and management of anti-reflux treatments, *i.e.*, proton-pump inhibitors and surgery. Aim of the present review was to provide a comprehensive update on the clinical management of patients with GERD, through a literature review on the diagnosis and management of patients with GER symptoms. In details, we provide practice-oriented concise answers to clinical questions, with the aim of optimising patient management and healthcare resource use.

Key Words: Gastro-esophageal reflux disease; Diagnosis; Management; Proton-pump inhibitor

Core Tip: Gastro-esophageal reflux disease (GERD) still poses several clinical issues to be faced, from clinical and instrumental diagnosis to medical and surgical therapy. In this review we provide the most updated evidence on the management of GERD. Practice-oriented questions on GERD are answered through a concise review of current literature. The aim is to provide clinicians a practical tool to guide them through the management of patients with GERD.

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INTRODUCTION

Gastro-esophageal reflux disease (GERD) is a complex but common condition[1] that poses several issues to the clinicians. Prompt endoscopy should be reserved only to patients with symptoms of GERD and alarm features or multiple risk factors for Barrett's esophagus. Grade A esophagitis is not sufficient to diagnose GERD, and only patients with grade C and D esophagitis should undergo endoscopic follow-up after proton-pump inhibitors (PPIs). Evidence of posterior laryngitis is not reliable for diagnosing GERD. Reliable selection of patients with PPI-refractory GERD who can benefit from anti-reflux surgery is a critical issue and relies on careful evaluation including impedance-pH monitoring. Prokinetics may be used in patients with concomitant dyspeptic symptoms, whereas potassium-competitive acid blockers (P-CABs) may be an option for erosive esophagitis.

QUESTION 1: SHOULD I PERFORM ENDOSCOPY IN ALL PATIENTS WITH GERD SYMPTOMS?

Answer: According to more recent international guidelines, a clinical response to an empiric 8-wk once-daily PPI therapy is diagnostic for GERD in patients with heartburn or acid regurgitation[2,3]. This pragmatic approach has a sensitivity of 78% and specificity of about 54%[4], which means to avoid unnecessary endoscopy in more than half of patients with symptoms of GERD. In fact, it should be emphasised that most patients with confirmed GERD do not present endoscopic findings of erosive esophagitis[2]. On the other hand, prompt endoscopy is recommended for patients with GERD symptoms and dysphagia or other alarm features (*e.g.*, weight loss, vomiting, or signs of gastrointestinal bleeding). Endoscopy is also recommended in all patients with GERD symptoms and at least 2 of the following risks factors for Barrett's esophagus: Age ≥ 50 years, male gender, Caucasian ethnicity, obesity, family history for Barrett's esophagus or esophageal adenocarcinoma, and smoking[2,5]. Indeed, the prevalence of Barrett's esophagus among patients with GERD symptoms is only about 5%-7%[6,7], therefore endoscopy should be reserved to patients with multiple risk factors for this condition.

QUESTION 2: IS EROSIVE ESOPHAGITIS SPECIFIC FOR DIAGNOSIS OF GERD?

Answer: Traditionally, endoscopic erosive esophagitis is considered specific for the diagnosis of GERD. The Los Angeles (LA) classification is currently the most used one for grading erosive esophagitis and considers 4 degrees: Grade A and B, non-confluent erosions (*i.e.*, mucosal breaks) of longitudinal extension ≤ 5 mm or > 5mm, respectively; grade C and D, confluent erosions between multiple folds affecting < 75% or ≥ 75% of the circumference, respectively[8]. According to recent international guidelines, the presence of grade A erosive esophagitis is not sufficient to diagnose GERD, as it can be present in 5%-8% of healthy subjects who do not experience symptoms of GER nor present complications such as Barrett's esophagus, and can be linked to other factors such as drugs or infections[2,3]. Grade B esophagitis can be considered diagnostic of GERD in the presence of typical symptoms of GERD that respond to PPI therapy, while grade C and D esophagitis are always diagnostic for GERD [2]. Nevertheless, it should be noted that erosive esophagitis is mostly healed by PPI therapy, therefore PPIs should be stopped at least 2 wk before endoscopy[2].

QUESTION 3: WHEN TO PERFORM ESOPHAGEAL BIOPSIES IN PATIENTS WITH GERD SYMPTOMS?

Answer: Esophageal biopsies are currently not considered in patients with GERD symptoms as they are of little value for the diagnosis of GERD. Histopathological findings that are variably associated with GERD, including dilation of the intercellular spaces and inflammatory intraepithelial cells and necrosis, have been described in the literature[9], but are flawed by a suboptimal specificity[10]. In fact, esophageal biopsies should be performed to diagnose eosinophilic esophagitis. This condition might coexist when patients refer also dysphagia and food bolus impaction in the esophagus. In this case, at least 6 biopsies should be performed in multiple esophageal sites[11]. Since PPIs can mask endoscopic and histological features of eosinophilic esophagitis, PPI therapy should be stopped at least 2 wk before endoscopy.

QUESTION 4: SHOULD HIATAL HERNIA ALWAYS BE IDENTIFIED AND MEASURED?

Answer: The systematic identification and measurement of hiatal hernia is important for several reasons: (1) Hiatal hernia is a predisposing factor for GERD; (2) If present, it should be corrected during laparoscopic fundoplication when technically feasible; and (3) Measurement of hiatal hernia presupposes the correct identification of landmarks, *i.e.*, diaphragmatic hiatus and esophago-gastric and squamocolumnar junctions, in turn necessary for the correct diagnosis of Barrett's esophagus. Therefore, although some evidence suggests that endoscopy is not the test of choice for measuring hiatal hernia [12], it is important to standardise this procedure to maximise its accuracy and reliability. First, endoscopy must be performed under sedation to avoid retching that could temporarily displace the gastric fundus. Second, the measurement must be carried out between the diaphragmatic hiatus and the top of the gastric folds (*i.e.*, esophagus-gastric junction). Last, excessive insufflation should be avoided, and the measurement should be always carried out during the same phase of the examination, *i.e.*, during extubation in order to minimise the effect of gastric prolapse following intubation.

QUESTION 5: SHOULD PATIENTS WITH EROSIVE ESOPHAGITIS UNDERGO REPEAT ENDOSCOPY AFTER TREATMENT?

Answer: The rationale for repeating endoscopy after treatment in patients with erosive esophagitis is mainly linked to the possibility that inflammation could obscure the visibility of an underlying Barrett's esophagus. Secondly, in those with more severe erosive esophagitis (LA grade C or D) it is advisable to check for the healing of the lesions and possible occurrence of complications (*e.g.*, peptic stricture) after adequate therapy with PPIs. Barrett's esophagus at repeat endoscopy after PPI treatment for erosive esophagitis has been reported in up to 12% of cases[13]. However, Barrett's esophagus is mostly obscured by LA grade C and D esophagitis, with a lower incidence reported in grades A and B[13]. Therefore, guidelines currently recommend repeating endoscopy after an 8-wk course of PPI therapy only in patients with LA grade C and D erosive esophagitis[14].

QUESTION 6: IS AN INSTRUMENTAL FINDING OF LARYNGITIS A SPECIFIC SIGN OF GERD?

Answer: The extra-esophageal manifestations of GERD are various and their association with GERD cannot always be unequivocally proven. Some findings at laryngoscopy, such as erythema and oedema of the vocal cords or larynx, may be related to GERD, but the specificity of these signs for the diagnosis of GERD is as low as 40%[15]. These findings may be attributable to other conditions, such as post-nasal drip syndrome or exposure to allergens and other environmental irritants[15]. Furthermore, the response to PPI therapy in these patients is unreliable due to the large placebo effect. Therefore, the presence of laryngeal symptoms (*e.g.*, cough, hoarseness), even when associated with an instrumental finding of laryngeal inflammation, is not sufficient for the diagnosis of GERD, and patients should be referred for further diagnostic investigations to confirm this diagnosis, *e.g.*, endoscopy if not previously performed and/or impedance-pH monitoring[2].

QUESTION 7: IS PH MONITORING ALONE INFERIOR TO IMPEDANCE-PH MONITORING TO DIAGNOSE GERD?

Answer: Ambulatory reflux monitoring, including pH-monitoring and impedance-pH monitoring, is the method of choice to confirm or exclude the diagnosis of GERD[2,3]. Impedance detects the movement of fluids and gas inside the esophagus independently from their acidity, thus distinguishing weakly acid from acid refluxes and reliably documenting the total number of reflux events throughout the recording period. Two additional applications need to be briefly mentioned: The post-reflux swallow-induced peristaltic waves (PSPW) index is the ratio between reflux episodes timely followed by a swallow event, and all the reflux episodes; this measure assesses esophageal chemical clearance due to the esophago-salivary reflex and has been shown to be impaired in GERD[16,17]. The mean nocturnal basal impedance (MNBI) is the mean baseline impedance value in three 10-min periods from the most distal impedance channel during nighttime recumbent period; this measure assesses the integrity of esophageal mucosa and is reduced by the chronic inflammation due to GERD[16,17].

Recent evidence has shown that pH monitoring alone, using esophageal acid exposure time (AET) > 6% according to the Lyon consensus[3] confirms the diagnosis of GERD only in 45% of patients with PPI-responsive heartburn[17]. On the other hand, impedance-pH monitoring with the evaluation of total refluxes, MNBI and PSPW index increases the diagnostic yield of about 20%, especially allowing to better characterise patients with inconclusive AET between 4% and 6%[17]. Of note, impedance-pH monitoring can identify ongoing reflux in a much higher proportion of PPI-refractory patients than pH monitoring alone, when performed on-therapy[18]. Therefore, impedance-pH monitoring should be considered the test of choice to confirm or rule out a diagnosis of GERD.

QUESTION 8: HOW SHOULD I MANAGE PPI THERAPY BEFORE IMPEDANCE-PH MONITORING?

Answer: The choice of performing impedance-pH monitoring off-PPI or on-PPI depends on the clinical goal. Impedance-pH monitoring should be performed off-PPI to demonstrate that pathological gastro-esophageal reflux underlies symptoms in a patient with unproven GERD[3]: That is when, for instance, a patient with normal endoscopic findings complains of typical or extra-esophageal symptoms and requires continuous PPI for symptom control or asks for anti-reflux surgery. On the other hand, impedance-pH monitoring should be performed on-PPI to confirm or exclude that ongoing reflux is the cause of inadequate response to double-dosage PPI in a patient with documented GERD[3].

QUESTION 9: WHEN SHOULD I VERIFY PATIENT ADHERENCE TO PPI THERAPY?

Answer: Modality and timing of PPI intake are key factors in obtaining an adequate response. Proton pump inhibitors should be taken at least 30 min before the first meal, preferably in the morning before breakfast, and in case of a second dose in the evening before dinner. This allows to achieve the maximum suppression of gastric acid secretion by inhibiting proton pumps before these are activated by food[19]. However, there is evidence that a large proportion of patients with unresponsive GERD symptoms do not take PPIs 30 min before the first meal[20]. Additionally, two studies found that only about half of patients correctly adhered to PPI therapy prescriptions for more than 80% of the time and that increasing compliance was typically related to symptom improvement[19]. Indeed, patient adherence to PPIs should be always verified in case of PPI-refractory symptoms.

QUESTION 10: WHEN DO I REFER A PATIENT WITH PPI-REFRACTORY SYMPTOMS TO ANTI-REFLUX SURGERY?

Answer: Patients with symptoms suggestive of gastro-oesophageal reflux unresponsive to PPIs should first be investigated about compliance and adherence to therapy. In case of good compliance, they should be referred for off-PPI upper gastrointestinal endoscopy and impedance-pH monitoring to confirm GERD diagnosis. Indeed, PPI therapy is so effective for typical GERD symptoms when properly administered that true PPI-refractoriness should prompt to verify the actual correlation between symptoms and reflux. On the other hand, in case of proven GERD impedance-pH monitoring should be performed on double-dosage PPI therapy started from at least 8 wk, in order to reliably link PPI-refractory symptoms to ongoing reflux and exclude reflux-unrelated symptoms. Indeed, in a recent randomised controlled trial (RCT) evaluating 366 patients referred for PPI-refractory heartburn only 21% of cases showed a clear-cut impedance-pH correlation between heartburn and gastro-esophageal reflux[21]. This highlights the importance to refer for surgical fundoplication only patients with PPI-

refractory GERD confirmed by impedance-pH monitoring. Correct selection of patients is crucial to maximise the outcome of anti-reflux surgery, which can be as high as 90%[18].

QUESTION 11: WHICH IS THE ROLE FOR PROKINETICS IN PATIENTS WITH GERD?

Answer: Dyspeptic symptoms can present in nearly half of patients with GERD, and the probability of dyspepsia in individuals with weekly GER symptoms is nearly 7-fold higher than in subjects without GERD[22]. There is a pathophysiological basis for this association, as prolonged postprandial gastric distention and increased basal intragastric pressure may lead to an increased gastro-esophageal pressure gradient, favoring reflux episodes. Therefore, prokinetics such as metoclopramide and domperidone may be beneficial when added to PPI therapy in patients with concomitant dyspeptic symptoms. However, the caveat is that their use can be limited by side effects including drowsiness, agitation, irritability, depression, dystonic reactions, and tardive dyskinesia for metoclopramide, whereas QT monitoring seems prudential for domperidone due to small risk for ventricular arrhythmia and sudden cardiac death[2].

QUESTION 12: WHICH IS THE ROLE FOR P-CABS IN PATIENTS WITH GERD?

Answer: P-CABs competitively inhibit proton pumps and have been licensed in Japan for the treatment of GERD since 2015[19]. Differently from PPIs, vonoprazan can block both inactive and active proton pumps, resulting in a higher and longer-lasting suppression of gastric acid secretion[19]. Further, its elimination is independent from CYP2C19 metabolism, probably contributing to explain its greater effect[19]. A recent meta-analysis on 19 RCTs found that vonoprazan was superior to PPIs in healing erosive esophagitis, whereas there was no difference in the improvement of GERD symptoms[23]. However, evidence on refractory GERD is scarce, and more studies from Western countries are needed to expand knowledge on the effectiveness of this drug in the setting of erosive reflux disease.

CONCLUSION

GERD is one of the most frequent gastroenterological conditions, yielding a considerable amount of resource consumption in health services[1]. Although several guidelines have been published[2,3], the management of patients with GER symptoms is still controversial. Currently, for example, there is no gold standard for diagnosing GERD, as diagnosis relies on a combination of symptoms, response to PPI therapy, endoscopy, and ambulatory reflux monitoring. Recent evidence-based recommendations provide new insights regarding erosive esophagitis and the management of patients refractory to PPIs [2,3]. This review provides the answers to questions which were selected after collegial discussion between the authors, also taking into account the most debated issues with general practitioners and non-dedicated gastroenterologists, that may help physicians in the management of patients with GERD (see Table 1). The answers are based on the overview of current guidelines and recommendations and on recent evidence provided from systematic reviews and clinical trials.

Table 1 Practice-oriented answers to clinical questions on the management of gastro-esophageal reflux disease

No.	Question	Answer
1	Should I perform endoscopy in all patients with GERD symptoms?	Endoscopy should be reserved for patients with GERD symptoms and either alarm features or multiple risk factors for Barrett's esophagus
2	Is erosive esophagitis specific for diagnosis of GERD?	Only LA grade C and D esophagitis are always specific for GERD
3	When to perform esophageal biopsies in patients with GERD symptoms?	Esophageal biopsies should be performed only when eosinophilic esophagitis is suspected
4	Should hiatal hernia always be identified and measured?	Hiatal hernia should always be identified and measured
5	Should patients with erosive esophagitis undergo repeat endoscopy after treatment?	Only patients with LA grade C and D esophagitis should undergo repeat endoscopy after PPI therapy
6	Is an instrumental finding of laryngitis a specific sign of GERD?	Laryngoscopic findings of laryngitis are not specific signs of GERD
7	Is pH monitoring alone inferior to impedance-pH	Impedance-pH monitoring is the test of choice to confirm or rule out GERD

	monitoring to diagnose GERD?	
8	How should I manage PPI therapy before impedance-pH monitoring?	The choice of performing impedance-pH monitoring off-PPI or on-PPI depends on the clinical goal
9	When should I verify patient adherence to PPI therapy?	Adherence to PPI therapy should be always verified in case of PPI-refractory symptoms
10	When do I refer a patient with PPI-refractory symptoms to anti-reflux surgery?	Only patients with PPI-refractory GERD confirmed by impedance-pH monitoring should be referred to surgical fundoplication
11	Which is the role for prokinetics in patients with GERD?	Prokinetics may be used in patients with GERD and concomitant dyspeptic symptoms
12	Which is the role for P-CABs in patients with GERD?	P-CABs are promising antisecretory drugs, however more evidence is needed

GERD: Gastro-esophageal reflux disease; PPI: Proton-pump inhibitor; LA: Los Angeles; P-CABs: Potassium-competitive acid blockers.

FOOTNOTES

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REVIEW

Transcriptome analysis creates a new era of precision medicine for managing recurrent hepatocellular carcinoma

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Abstract

The high incidence of hepatocellular carcinoma (HCC) recurrence negatively impacts outcomes of patients treated with curative intent despite advances in surgical techniques and other locoregional liver-targeting therapies. Over the past few decades, the emergence of transcriptome analysis tools, including real-time quantitative reverse transcription PCR, microarrays, and RNA sequencing, has not only largely contributed to our knowledge about the pathogenesis of recurrent HCC but also led to the development of outcome prediction models based on differentially expressed gene signatures. In recent years, the single-cell RNA sequencing technique has revolutionized our ability to study the complicated crosstalk between cancer cells and the immune environment, which may benefit further investigations on the role of different immune cells in HCC recurrence and the identification of potential therapeutic targets. In the present article, we summarized the major findings yielded with these transcriptome methods within the framework of a causal model consisting of three domains: primary cancer cells; carcinogenic stimuli; and tumor microenvironment. We provided a comprehensive review of the insights that transcriptome analyses have provided into diagnostics, surveillance, and treatment of HCC recurrence.

Key Words: Recurrent hepatocellular carcinoma; Microarrays; RNA sequencing; Single-cell RNA sequencing; Precision medicine; Tumor heterogeneity; Tumor microenviron-

ment

Core Tip: The high incidence of hepatocellular carcinoma (HCC) recurrence seriously threatens patient outcomes. This review detailed how various transcriptome profiling methods have contributed to our understanding of recurrent HCC with respect to the carcinogenicity of primary cancer cells, carcinogenic stimuli, and tumor microenvironments, which show great promise in improving the management of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 75%-85% of primary liver cancer caused by chronic liver injury[1]. The advance in surgical techniques and locoregional liver-directed therapies contributes to the prognosis of patients suffering from early HCC. However, high-relapse HCC remains a serious burden to patients treated with curative intent, as the annual recurrence rate of HCC following surgery is 50%-70% within 5 years[2-4]. Although recent progress in systemic treatments has led to the modification of treatment strategy for intermediate to advanced HCC[5], early detection of HCC recurrence can provide patients with more treatment options. It is therefore imperative to identify susceptible patients and offer regular monitoring.

Traditionally, post-treatment surveillance of HCC utilized periodic cross-sectional imaging and tumor markers for patient follow-up[6]. The major aim of post-treatment surveillance is early identification of diseases that might be amenable to subsequent local therapy[7]. However, multiphase, contrast-enhanced computed tomography and magnetic resonance imaging suffer from low per-lesion sensitivity[8], difficulty in assessing small HCCs[9], and post-locoregional therapy lesions[10,11]. Ancillary methods like alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin likewise suffer from high false positive and false negative results[12,13]. The imperfection of early HCC recurrence detection urges the need to seek a more reliable armamentarium.

In addition, owing to the inclination to the multifocal occurrence of HCC, it is often challenging to decipher whether the lesions observed after curative liver resection arise from primary HCC or multicentric origin. Differentiation of the two types of multifocal lesions is necessary since their distinct mechanisms may have different effects on the response to treatment[14,15]. Some researchers attempt to distinguish the two conditions based on temporality of tumor occurrence. Early intrahepatic recurrence (IHR), defined as recurrent tumors detected within fewer than 2 years after surgery, has been attributed to residual lesions or intrahepatic micrometastasis from the initial tumor, while late (more than 2 years after surgery) IHR is largely considered a newly developed primary lesion[16,17]. However, such a definition has limited diagnostic accuracy and has not been validated[14,18].

Early studies have analyzed the clonality of multiple HCCs by assessing DNA ploidy, hepatitis B virus (HBV) integration sites, or microsatellite aberration mainly involving loss of heterozygosity and copy number variations (CNV)[19]. The heterogeneity not only exists among multifocal tumors but has also been found within a single lesion[20]. The molecular technologies that have been utilized for these approaches, such as DNA fingerprinting and whole-exome sequencing, are beyond the scope of this review and therefore will not be discussed further. We aimed to emphasize the complexity and heterogeneity behind recurrence widely seen in clinical practice.

The incidence of IHR of HCC after curative resection may be influenced by central factors including the specifics or clonality of primary tumor cells, the microenvironment that offers a susceptible niche for tumor cells to metastasize, and the existence of distinct carcinogens[21,22]. Reviewing past literature, we propose a model of three causations to illustrate the interplay between the factors that determine the recurrence of HCC, analogous to the well-known epidemiologic triangle for infectious disease (Figure 1). The recurrence of HCC results from interactions between primary cancer cells, the tumor microenvironment, and carcinogenic stimuli.

In recent years, transcriptome analysis emerged as a powerful tool to investigate the expression of disease phenotype and its association with genotype[23-25]. The evolution of bench work and laboratory equipment enables assays to be more efficient, enjoy higher throughput, and be more cost-effective. Such advances in molecular biological technology have facilitated the investigation of three causative domains of HCC recurrence described above and yielded abundant results. In the current

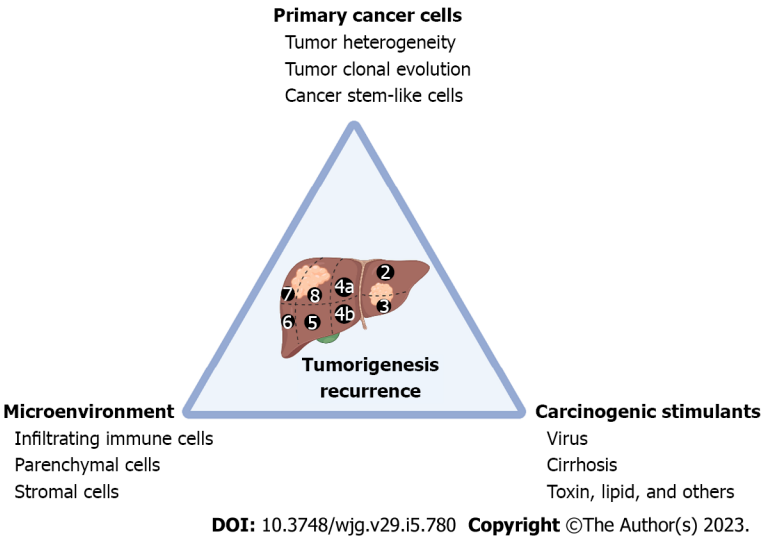

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Figure 1 Causal model of hepatocellular carcinoma recurrence. The figure was created with BioRender.com.

review, we focused on describing the role of transcriptome analyses, including real-time quantitative reverse transcription (RT-q) PCR, microarrays, and RNA sequencing (RNA-seq), as well as the rapidly evolving single-cell transcriptome analysis, among the latest work on this topic. We summarized the major findings of studies that may provide us with a clearer picture of HCC recurrence and give us insight into potential diagnostic targets as well as therapeutic strategies.

QUANTITATIVE RT-QPCR ANALYSIS OF RECURRENT HCC

Kary Mullis invented PCR in 1984. Russel Higuchi and colleagues later exploited fluorescence technology, making it possible to monitor PCR results using fluorescent probes[26,27]. These advances, combined with reverse transcriptase, which had been discovered earlier in 1970, brought about the development of RT-qPCR[28]. Since the late 1990s, RT-qPCR has been widely utilized in the exploration of differential gene expression (DGE) in various diseases, including HCC.

Several studies have utilized RT-qPCR to analyze the DGE in HCC recurrence. To clarify the genes responsible for the hematogenous spreading of HCC cells, one study measuring the expression of matrix metalloproteinase 9 (*MMP9*) and vascular endothelial growth factor in pairs of non-tumor and tumor samples with RT-qPCR found that the expression of *MMP9* in tumors was related to recurrence, while the expression of vascular endothelial growth factor was not. The same study also examined *AFP* mRNA in blood samples and found that the level was associated with recurrence and could serve as a predictor of recurrence or metastasis of HCC[29]. Similarly, another study reported that the mRNA level of *AFP* in peripheral blood samples significantly correlated with postoperative extrahepatic metastasis and disease-free survival[30].

In RT-qPCR, the quantification of complementary DNA (cDNA) from genes of interest is typically compared to that of reference genes, also called housekeeping genes, to allow the normalization of differences seen in different samples. Common reference genes include beta-actin (*ACTB*), beta-2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyltransferase 1, and TATA box binding protein[31]. A suitable reference gene must have stable expression across different conditions of samples. It is noteworthy that both *ACTB* and *GAPDH*, two commonly used reference genes, have been reported to be highly expressed in HCC when compared with non-tumor tissues[32], while TATA box binding protein and hypoxanthine phosphoribosyltransferase 1 were reported to be more suitable reference genes in HCC[33]. However, many currently available data with detection of significant DGE of HCC still used *ACTB* and *GAPDH* as reference genes[34,35], and whether the two genes are reliable for RT-qPCR normalization in HCC specimens requires more investigation.

The step of cDNA amplification endows RT-qPCR with a wide dynamic window and relatively high sensitivity to detect genes expressed with low abundance such as cytokines, and RT-qPCR is regarded as the "gold standard" of transcriptome analysis[36]. However, RT-qPCR also carries several limitations. Technically, various factors may impact the amplification and cause deviation from the ideal mathematical model of PCR, including the RNA quality, the efficiency of RNA-to-cDNA conversion, the primer quality, operator technique, as well as the "Monte Carlo" effect, an inherently and unavoidably high variance in the results from PCR reactions with a low starting template concentration[37]. In terms of its application in transcriptome analysis of clinical diseases, it is relatively low-throughput and can only

test a limited number of genes of interest with known sequences[38].

These limitations largely confine its ability to discover novel DGE of disease status. Only when researchers already know which "suspect" genes or pathways to target can RT-qPCR efficiently identify the disease-related DGE. Without predefined genes of interest and sufficient biological plausibility, it is challenging to identify novel disease-specific DGE solely with RT-qPCR. To achieve the prospective profiling of the transcriptome of HCC and recurrence, a more high-throughput technology that is capable of screening massive numbers of genes with various degrees of probability to have a disease-specific expression in parallel is needed. Thus, in the research of HCC, RT-qPCR is mainly used as a validation tool to confirm the DGE identified by two other technologies that emerged later on: microarrays and RNA-seq. These two research tools have yielded abundant results in all three aspects of HCC recurrence in the causal model we propose (Figure 1).

MICROARRAY ANALYSIS OF RECURRENT HCC

First developed by Schena *et al*[39] at Stanford University in 1995, microarrays have been widely applied in medical research as a high-throughput tool to reveal gene expression in disease status[39]. Microarrays can be divided into two main categories: cDNA microarrays and oligonucleotide microarrays. The surfacing of commercial platforms, such as Human UniGene Set RZPD 1 clone set for cDNA microarrays and Affymetrix Human Genome U95Av2 array for oligonucleotide arrays, have made the technology for both types of arrays widely accessible[40]. Additionally, the oligonucleotide microarrays have further been developed to identify single nucleotide polymorphism (SNP), named SNP arrays, in which the probes designed for harboring the SNP positions are hybridized with fragmented DNA molecules to examine the specific alleles of all SNPs[41]. The advance in microarray technology allows researchers to screen tens of thousands of RNA transcripts simultaneously and makes it possible to identify new genes with DGE related to diseases or specific pathophysiological conditions of interest. In addition to its high-throughput applicability, it has other major advantages including the wide availability of uncomplicated bioinformatics tools, more manageable data, and relatively low cost[38].

Microarray analysis has long played a central role in the field of HCC research. Iizuka *et al*[18] conducted a comprehensive review of the abundant data yielded with this revolutionizing technology [18], in which the authors classified the microarray-based approaches into three groups based on the distinct objectives of the studies, class comparison, class discovery, and class prediction, as proposed by Simon *et al*[42]. Such classification, however, was mainly according to study methodologies but hardly compared findings in the context of biological mechanisms and pathogenesis of HCC. To organize the diverse findings of microarray analyses in an integrated manner and to provide insights with pathobiological plausibility, we summarized the currently available data within the framework of our causal model of HCC, including the carcinogenic profile of primary cancer cells, carcinogenic stimuli, and the tumor microenvironment, in the following paragraphs.

Carcinogenicity of primary HCC cells

Lau *et al*[32] utilized cDNA microarrays to analyze the differential expression of mRNA of 4000 genes in paired HCC and noncancerous tissues[32]. They found that 211 genes were upregulated while 147 genes were downregulated, of which six genes were highly expressed and ten genes were downregulated in more than 30% of pairs. This was the first time when microarray technology was used in humans. Subsequently, either cDNA or oligonucleotide microarray studies, targeting either cell lines or patient-derived samples, have been widely conducted to discover the DGE related to the carcinogenic profile of primary HCC cells. The differentially expressed genes that have been identified include those associated with cell-cell interaction[43,44], transcription factors[18,43,45,46], apoptosis[43,45,47], cytokines[43,45], growth factors and/or growth suppression signals[43,45,48,49], cell proliferation[44,45,47,49,50], the cell cycle[43,45,49], tissue-specific expression proteins related to cell differentiation and development[45, 51], metabolism[49], angiogenesis[43,45,49], and stress-related response[35,50]. With the maturity and extensive application of microarrays, researchers further exploit this powerful technology to identify genes associated with HCC progression and prognosis. For instance, one study found that the upregulation of *ADAR, PSMD4, D9SVA, CCT3, GBAP, RDBP*, and *CSRP2* with downregulation of *IL7R* were associated with dedifferentiation of HCC[52]. Other studies focusing on metastasis and rapid progression of HCC identified differential expression in vimentin[53], granulin-epithelin precursor[54], ephrin-A1[53], and N-Myc downregulated gene 1[55].

It has also been widely known that differential expression of certain genes in primary cancer cells is associated with early recurrence. One study identified the expression profile of claudin-10, along with the pathological tumor-node-metastasis (pTNM) stage, to be independent predictors for HCC recurrence, and the results were validated with RT-qPCR[56]. Another study compared DGE of patients with recurrence *vs* those without, and it was found that four HLA genes (*HLA-DRA, HLA-DRB1, HLA-DG*, and *HLA-DQA*) encoding major histocompatibility complex class II antigens had significantly lower expression in the early IHR group[57]. Furthermore, while the DGE detected by microarrays and RT-

qPCR, as well as pathological tumor-node-metastasis stage and venous invasion were all found to be associated with early IHR in the univariate association study, the multivariate study only identified DGE of *HLA* as an independent predictor for early IHR. Iizuka *et al*[58] compared the gene expression of tumor cells between patient groups with and without recurrence and found that cell adhesion-related genes, including *ITGA6* and *SPP1*, had higher expression levels in HCC with early IHR[58].

Given that portal vein invasion (PVI) is known as a major prognostic factor of HCC recurrence[16,59-61], many studies used microarrays to identify the DGE related to PVI. One study found that cell growth-related genes *TAF4B*, *SLC4A7*, *RAB38*, and *RYR1* were associated with PVI[62]. Another study discovered that upregulation of *MMP14* and downregulation of two cytochrome P450 enzyme (*CYP*) genes, *ADAMTS1*, and *ITGA7* were associated with PVI[63,64]. Moreover, one study identified DGE of 110 sequence tags, *RHOC*, and two small GTPase-related genes (*ARHGAP8* and *ARHGEF6*) to be PVI-associated[63,64]. Lastly, one of the studies listed above successfully used PVI-associated DGE data from the microarray analysis to predict recurrence after surgical resection of HCC[62]. Although not directly analyzing recurrence-associated DGE, these studies offered abundant insight into the carcinogenicity of primary cancer cells defined by PVI.

Knowledge established by microarray studies has commonly been combined and applied to predict the recurrence and outcome of HCC. A data mining study examining the DGE between patients with IHR and those without recurrence based on pre-existing microarray databases generated one set of four differentially expressed genes (*STC1, FOXK2, MMP1*, and *LOXL2*) that promoted either cell cycle advancement or histone modulation could predict the incidence of early recurrence[65]. Another study conducting microarray analysis in human primary HCC tumors developed a 172-gene molecular prediction system for early IHR and tested its performance in independent cases[66]. The value of the predictive system was found to be a significant prognostic factor according to multivariate Cox regression analysis. Thus, DGE related to early IHR can be designed to predict clinical outcomes.

In summary, identification of DGE using microarrays, either by directly comparing recurrence with non-recurrence groups or indirectly looking at surrogate predictors such as PVI, enables us to clarify the carcinogenicity and the propensity of recurrence in primary HCC.

Carcinogenic stimuli

Clinical association studies have identified various risk factors of HCC. Common risk factors for HCC include HBV and hepatitis C virus (HCV) infection, cirrhosis, alcoholic liver disease, and nonalcoholic fatty liver disease. Less common risk factors include exposure to environmental toxins, Wilson's disease, hereditary hemochromatosis, alpha1-antitrypsin deficiency, primary biliary cholangitis, and autoimmune hepatitis[67]. In addition to the carcinogenicity of the primary HCC cells, these carcinogenic factors also play a decisive role in the development and recurrence of HCC. Microarray studies with clustering analysis based on some of these clinicopathological features have been widely performed to provide information about how risk factors contribute to HCC at a molecular level, with the greatest proportion of data coming from studies related to viral hepatitis.

Some studies compared the DGE of HCC cells to that of noncancerous liver tissues in HBV-positive and HCV-positive groups[67,68]. One study directly used oligonucleotide microarrays to compare transcriptomes in HBV-associated *vs* HCV-associated HCC, finding DGE in 83 genes, of which 31 and 52 genes showed increased expression in HBV and HCV-associated HCC, respectively[69]. The genes with DGE found in HBV-positive HCC mainly involved imprinted genes and genes associated with signal transduction, transcription, and metastasis, while in HCV-positive HCC the DGE was mainly found in genes related to detoxification or immune response. Such findings highlight the distinct mechanisms of viral carcinogenesis.

Another study used cassette ligation-mediated PCR to identify the human genome sequence next to the HBV DNA integration site and then conducted a microarray experiment to directly measure the characteristic expression of the affected genes[70]. In addition to viral hepatitis, certain toxins are also known as carcinogenic in favor of HCC, and microarray technology has been utilized to predict the carcinogenicity of chemicals by analyzing changes in gene expression in animal or cell culture models [71,72].

Finally, cirrhosis of any etiology is a major risk factor in the development of HCC. One study compared the gene expression profiles between HCC in patients with cirrhosis and without cirrhosis and identified several genes related to the regulation of inflammation, growth, and invasion of precancerous cells in cirrhotic liver, including C-C motif chemokine receptor and ligand (*CCR7* and *CCL5*), C-X-C motif chemokine ligand, and cytochrome P450 enzymes (*CYP2E1*, *CYP2C9*, and *CYP2A6*)[73].

In terms of recurrence, Kim *et al*[74] developed a risk scoring system with DGE of 65 genes identified with microarrays analyzing primary tumor cells in HBV-positive HCC patients and validated this system in another group of patients, finding that the classifiers successfully predicted early recurrence but failed to predict late recurrence[74]. The same author group further tried another approach by conducting a systemic analysis of gene expression from non-cancerous human liver tissue undergoing hepatic injury and regeneration. They identified a 233-gene signature that was significantly associated with late recurrence of HCC and validated the system in HBV-positive HCC patients who had received curative surgical treatment[75]. Network analysis of the gene signature identified signal transducer and activator of transcription 3/Notch signaling activation to be significantly related to late recurrence of

HCC. With prediction using microarray and multivariate logistic regression analysis, the authors scaled down the system to a four-gene (*RALGDS, IER3, CEBPD,* and *SLC2A3*) model that could successfully predict HCC recurrence. Interestingly, different models targeting cancerous or non-cancerous tissues could predict early and late recurrence of HCC, respectively, which may reflect the distinctive pathogenesis behind early and late recurrence of HCC in HBV-positive individuals.

Similarly, the recurrence of HCV-positive HCC has also been well studied. One study compared the DGE of noncancerous liver tissues from HCV-positive HCC patients with single nodular HCC recurrence and multicentric recurrence. Next, the authors developed a predictive system based on DGE in 36 genes, which was validated to successfully predict multicentric recurrence[76]. Also targeting HCV-positive HCC, another study found that the DGE profile observed in primary HCC biopsy or explant could not predict recurrence-free survival, while those yielded from noncancerous tissues could [77,78], which agreed with other studies on HBV-positive HCC[74,75]. Therefore, regardless of HBV or HCV-positive HCC, late recurrence was more likely to originate from a new clone of cells rather than the original HCC cells.

Another study focusing on genes associated with recurrence of HCC in HCV-positive patients awaiting liver transplantation identified the DGE profile of genes related to viral response as well as transcriptional network regulated by interferons, specifically interferon-α/β-inducible genes (signal transducer and activator of transcription 1, *OAS1*, and *MX1*), to be associated with recurrence-free survival[78]. The study also found that *FAIM3*, an anti-apoptotic gene, and *USP18*, a gene encoding an enzyme of the deubiquitinating protease family, were overexpressed in patients with recurrent HCC. Collectively, these studies exemplified how microarrays contribute to our understanding of carcinogenic stimuli in HCC recurrence.

Tumor microenvironment

Compared to the other two components in our causal model of HCC recurrence, microarray-based studies focusing on the tumor microenvironment and surrounding tissue suffer from less available data. This may be due to the limited ability of microarrays, as one of the "bulk" transcriptome analysis methods, to reveal the status of an individual cell. However, we found one study using cDNA microarrays to compare the gene expression profiles of noncancerous peripheral tissue from two HCC patient groups: those with primary HCC and venous metastases or confirmed extrahepatic metastases by follow-up, termed metastasis-inclined microenvironment samples; and those with HCC without detectable metastases, termed metastasis-averse microenvironment[79]. The authors found DGE in *HLA-DPA1*, *HLA-DRA* (antigen-presenting dendritic cells, B cells, epithelial cells), *PRG1*, and *ANXA1* to be associated with a metastatic phenotype, reflecting a T helper 2-predominant, anti-inflammatory cytokine profile, for which *CSF1* may be responsible. Interestingly, the increased expression of *HLA-DRA* in surrounding noncancerous tissue, as observed in this study, contrasts with one of the studies we cited above in which *HLA-DRA* genes showed lower expression in tumors[57]. The spatial distribution, migration of immune cells, and dynamic nature of the microenvironment may serve as plausible reasons for the discrepancy.

RNA-SEQ ANALYSIS OF RECURRENT HCC

Although microarray technology allows simultaneous measurement of numerous genes in one sample, it still has major drawbacks such as the limited dynamic range, high background noise, and an inability to detect novel transcripts. The advent of next-generation sequencing technology gave rise to a new technology, RNA-seq, that can address these limitations.

In 2008, RNA-seq was initially described by Nagalakshmi *et al*[80] as a new quantitative sequence-based method to map transcribed regions of the yeast genome[80]. Compared to microarray-based methods, RNA-seq enjoys higher genome coverage and better profiling of dynamic transcriptomes, providing information about alternative splicing, allele-specific expression, non-coding RNA, and SNPs. Unlike RT-qPCR and microarrays, which are largely limited by the requirement for *a priori* knowledge of the sequences being interrogated, RNA-seq is exclusively competent to discover novel transcripts [81]. As a result, despite disadvantages such as higher cost and large dataset generation, RNA-seq has been replacing microarrays over the past decade for transcriptome analyses in basic and translational research[82]. A comparison of RT-qPCR, microarrays, and RNA-seq are summarized in Table 1. The typical workflow for RNA-seq and microarray analysis in HCC translational research is illustrated in Figure 2.

Carcinogenicity of primary HCC Cells

RNA-seq enables us to identify specific DGE and expand our knowledge of the pathogenesis of HCC. One study performed pairwise DGE analysis between HCC and non-HCC tissues, finding upregulation of oxidative phosphorylation and higher expression of associated DNA damage-related signals in HCC compared to non-HCC samples. These findings suggest development of HCC may result from oxidative stress generated from overactive oxidative phosphorylation[83]. Another study analyzing poorly differ-

Table 1 Comparisons of real-time quantitative reverse transcription PCR, microarrays, and RNA-sequencing and their applications in hepatocellular carcinoma recurrence

	RT-qPCR	Microarrays	RNA-seq
Basic steps	RNA isolation, genome DNA removal	RNA isolation, mRNA extraction	RNA isolation, mRNA extraction
	cDNA preparation with RT	cDNA library preparation	Quality and quantity check
	Use of primers for amplification	Labeling with fluorescence	cDNA library preparation
	Data analysis	Hybridization with transcript probes on slides	Sequencing
		Scanning	Data analysis
		Image processing and data analysis	Validation
		Validation	
Throughput	Low	High	High
Dynamic range/sensitivity	Widest/high	Narrow/low	Wide (compared to microarrays)/high
Need for reference genome	No	No	Yes
Known sequences of genes of interest	Required	Required	Not required
Cost	Low	Low	High
Advantages	Low cost, simple	High throughput	Ability to detect novel differential transcripts
	Highest dynamic range	Relatively low cost	Splice junctions, SNP, non-coding RNA
	Gold standard	Good bioinformatics and statistical practices	
Downsides	Dependence on pre-existing knowledge of genes of interest	Difficulty to detect novel transcripts, non-coding RNA, splicing, or other dynamic natures of transcriptome	Large data storage
	Low throughput		High cost
		Need for designing probes	
		Low dynamic range	
Applications and main achievements in HCC recurrence-related research	Commonly used as a validation tool for confirming DGE results yielded from other high throughput analyses[56]	Providing abundant information on carcinogenicity of primary HCC cells and carcinogenic stimuli; laid the foundation for our current understanding of the pathogenesis of HCC recurrence[18]	Prospectively discovering DGE as potential novel classifiers for the carcinogenic profile of recurrent HCC cells; elucidating how HBV triggers HCC recurrence by interrupting the human genome[92,94,96]

cDNA: Complementary DNA; DGE: Differential gene expression; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; RNA-seq: RNA-sequencing; RT: Reverse transcription; RT-q: Real-time quantitative reverse transcription; SNP: Single nucleotide polymorphism.

entiated, moderately differentiated, and well-differentiated HCC with RNA-seq found DGE in poorly differentiated HCC to be mostly associated with cell metabolism, cell cycle, translation, and blood coagulation, of which the upregulation of *NOVA1*, *NSMCE2*, and *KIAA0196* and downregulation of *AQP9* were validated with RT-qPCR[84].

Since RNA-seq has a greater dynamic range and is more capable of detecting genes expressed with low abundance, another study analyzed blood samples taken from HCC and non-HCC patients in which the authors first identified 1578 dysregulated genes with RNA-seq and then validated them with RT-qPCR. Six genes (*SELENBP1, SLC4A1, SLC26A8, HSPA8P4, CALM1,* and *RPL7p24*) were differentially expressed, and the *CALM1* expression level was found to decrease along with tumor enlargement and thus had potential as a novel biomarker for tracking HCC[85]. While RT-qPCR also has a wide dynamic window and has been used to analyze peripheral blood during diagnosis[29,30], the ability of RNA-seq to scan many genes at a whole-genome scale makes the identification of candidate biomarkers more efficient. Moreover, RNA-seq is exclusively capable of analyzing non-coding RNA and has been utilized to identify long non-coding RNA (lncRNA) as well as small nucleolar RNA host genes. For instance, *SNHG4*, which is involved in the regulation of ribosomal RNA synthesis, RNA processing, and surveillance pathway, was found to be closely related to the tumorigenesis of HCC[86]. These studies

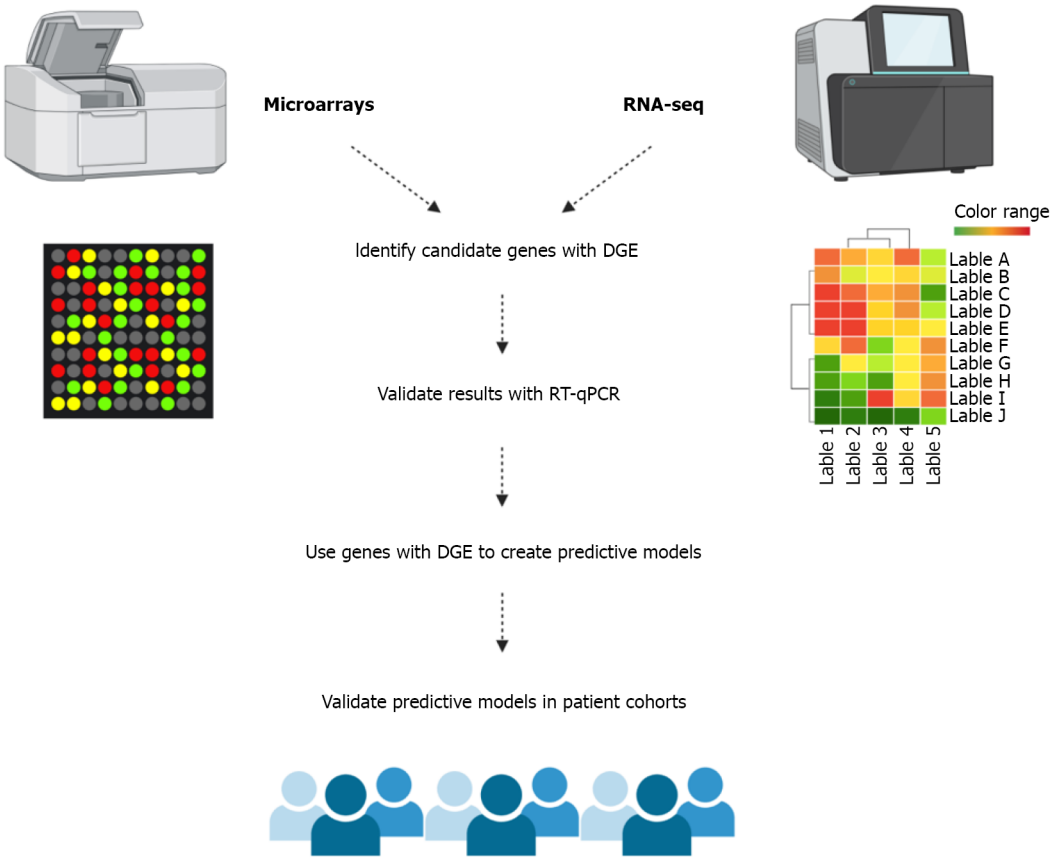

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Figure 2 Typical workflow of bulk transcriptome analysis in translational hepatocellular carcinoma research. The figure was created with BioRender.com. DGE: Differential gene expression; RNA-seq: RNA-sequencing; RT-q: Real-time quantitative reverse transcription.

exemplified the unique advantages of RNA-seq in discovering cancer mechanisms.

RNA-seq has also been used to analyze the carcinogenicity of HCC cells specifically related to recurrence. One study performing transcriptome analysis of 128 post-liver transplant HCC recurrence tissue samples found that the DGE was mainly found in genes involved in DNA synthesis, chromatin segregation, and mitosis, which might facilitate DNA replication and the growth of cancer cells[87]. The authors also performed mutation analysis in this study. Interestingly, the expression of some well-known mutations previously identified in HCC, such as p53, beta-catenin 1, and telomerase reverse transcriptase, did not appear to be significantly associated with HCC recurrence or prognosis. One explanation the authors proposed was that tumors recur after circulating HCC cells present at the time of transplantation traverse through the circulation, survive the turbulent flow environment, proceed through the pulmonary circulation, and finally seed themselves within the new liver. Another possible explanation is that recurrence is influenced by a complex interplay between primary cancer cells and "extratumoral" factors such as neurotransmitters, metabolism, or other constituents. This explanation is consistent with previous results from microarray studies finding that DGE of primary tumor cells failed to predict later recurrence[74,78].

Exploiting the merits of RNA-seq for detecting SNP and CNV, one study comparing liver gene expression in transplant patients with and without recurrence found glutathione S-transferase A2 (*GSTA2*) expression to be associated with early phase systemic injury and reactive oxygen species levels. Moreover, *GSTA2* could serve as a predictor of recurrence. Further, the authors identified that the G335C SNP of the *GSTA2* coding sequence, corresponding to an S112T amino acid substitution, was associated with HCC recurrence and survival[88]. Another study analyzing pairwise DGE analysis between primary and recurrent HCC found SNP variants of *GOLGB1* and *SF3B3* to be significantly related to more aggressive phenotypes[89].

Over the past decade, the popularity of high-throughput sequencing technology has not only made RNA-seq widely available but also brought about a publicly accessible comprehensive repository for genome-wide gene expression data such as the Gene Expression Omnibus and ArrayExpress. The Cancer Genome Atlas (TCGA) collects the results of cancer-related research, and mining of these shared databases provides insights by integrating results from different studies[90]. For example, Wang *et al* [91] identified a 77-gene signature associated with early HCC recurrence by conducting microarray experiments and cross-referencing the results with RNA-seq data from TCGA[91]. Combining data from

an ensemble of transcriptome profiling tools enables researchers to validate and complement results.

Carcinogenic stimuli

Thanks to its ability to capture the dynamic nature of transcriptome profiles, RNA-seq offers a unique advantage in investigating how carcinogenic factors alter transcriptome patterns in HCC development. It is known that HBV DNA can be integrated into the human genome and may result in somatic mutations[92]. One study found DGE of ten matched pairs in HBV-related HCC and non-HCC tissues to be mostly related to cell growth, metabolism, and immune-related pathways, which were significantly enriched at 8q21.3-24.3[93]. Moreover, the authors found a highly upregulated exon-exon junction at the *ATAD2* gene, an important protein that acts as a cofactor for Myc proteins, androgen receptor, and estrogen receptor-alpha. Also using RNA-seq, another study identified contrasting genomic and transcriptomic alterations such as HBV integration, somatic mutation, and CNV by comparing tumor with non-tumor samples[94]. For nonviral carcinogenic factors, one study identified 747 mRNAs and 8 lncRNAs with DGE between HCC and non-HCC cirrhotic tissues, narrowing down the results to 15 hub genes based on an association study with AFP levels in blood samples. Of these, *SPX*, *AFP*, and *ADGRE1* were validated in an independent HCC cohort[95].

With respect to recurrence, one study performed RNA-seq in HBV-related HCC patients to compare tumor and non-tumor tissues with various degrees of fibrosis[96]. HBV host genes overlapped with pathogenic SNPs in tumor suppressor genes of non-tumor tissues. Overlap was more significant in non-tumor tissues among recurrent cases, suggesting that tumor recurrence was highly associated with the integration of HBV genomes into precancerous tumor suppressor genes. Additionally, the difference in pathogenic SNP count between recurrent and non-recurrent patients was much larger in the low fibrosis group compared to the high fibrosis group, indicating that different recurrence risk models are needed for patients with low and high fibrosis. Taken together, these studies show the strength of RNA-seq in investigating the molecular genetic basis of HCC recurrence.

Tumor microenvironment

In addition to primary tumor cells and carcinogenic stimulus, researchers use this powerful tool to investigate the impacts of tumor microenvironment on recurrence. One study performing RNA-seq to analyze DGE between HCC tumors and surrounding cirrhotic tissue showed a gradual suppression of local tumor immunity coinciding with disease progression. In addition, the authors divided tumors into T cell-infiltrated and T cell-excluded based on the localization of CD8+ cytotoxic T lymphocytes visualized by immunohistochemistry staining and performed RNA-seq to examine the DGE between two groups. Twenty-three identified genes were associated with fibrosis and potentially modulated by transforming growth factor beta, platelet-derived growth factors, sonic hedgehog protein, or Notch pathways[97]. Given the emerging evidence suggesting that lncRNAs participate in cancer immunity, another study used RNA-seq to look at immune-related lncRNAs and related mechanisms from the TCGA database, identifying nine immune-related lncRNAs associated with HCC recurrence *via* Cox regression analysis[98]. The authors also created a recurrence prediction model based on their findings that was validated in an independent patient cohort.

The literature reviewed above shows how RNA-seq could be used to reveal molecular features of tumor immune biology in HCC progression and recurrence. However, most of the studies are limited to the discovery of DGE in bulk tissue samples, which may not be able to reflect the highly complicated tumor milieu and immune diversity. Although one study did consider tissue compartmentalization and tried to cluster cell populations to correlate DGE results with histology and immunohistochemistry staining[97], the approach was inefficient. The advent of single-cell RNA-seq (scRNA-seq) could address this issue and has improved our understanding of transcriptomes during cell-cell interaction. ScRNA-seq holds particular promise in research focusing on the tumor microenvironment, as described in the next section.

SINGLE-CELL TRANSCRIPTOME ANALYSIS OF RECURRENT HCC

Conventional bulk RNA-seq inherits the drawback of "averaging out" the data and does not have adequate resolution to delineate cell trajectory and cell-cell interactions. However, HCC is a heterogeneous disease with complex etiologies and tumor milieu[99,100]. There is thus a need to study the heterogeneity of tumoral cells and their ecosystem, particularly the immune cells.

To achieve a high resolution of cell subpopulations in malignant tissue, various single-cell isolation techniques have been developed. Generally, the isolation methods can be categorized into either cell marker-based selection or size-based selection, including fluorescence-activated cell sorting, laser microdissection, manual cell picking, serial dilution, magnetic-activated cell sorting, microfluidics, and CellSearch system[101,102]. When combining single-cell isolation with sequencing techniques, these technologies become promising tools to study intertumoral and intratumoral heterogeneity both spatially and temporally. Since mRNA transcriptome sequencing in a single cell was first reported in 2009[103], advances in sequencing techniques and single-cell selection methods have driven different

applications in the field of cancer biology. In recent years, droplet-based systems for high-throughput scRNA-seq such as inDrop, Drop-Seq, and 10X Genomics[104] have gained attention. Although previous studies used them to profile early HCC and its tumor microenvironment[105-107], studies for recurrent HCC are still limited.

Tumor heterogeneity, including intertumor and intratumor heterogeneity, is responsible for the recurrence of HCC[108]. Previously, researchers stressed the genomic profiling and molecular subclassification of intertumor heterogeneity[109-111]. Nevertheless, cancer cell adaptation, drug resistance, and tumor microenvironment are more closely related to intratumor heterogeneity[108]. To correlate the gene expression landscape of intratumor heterogeneity with HCC patient outcome, Losic *et al*[112] characterized a gene signature composed of 363 genes in the TCGA-HCC database[112]. The gene signature was associated with worse survival and was able to compete with other pre-existing single-biopsy prognostic signatures. The gene signature was also correlated with early tumor recurrence in the Heptromic Cohort as well as with higher levels of the prognostic biomarker AFP. At the single-cell level, the authors found transcriptional factor heterogeneity in the gene regulatory network by analyzing cis-regulatory sequence motifs from seven different locations in two HCC patients.

Another study found cellular heterogeneity in primary tumors similar to portal vein tumor thrombus and metastatic lymph nodes *via* scRNA-seq[113]. Additionally, authors focused on intratumoral T cells, in which they found CD8+ T cell clusters to be more enriched in HBV/HCV-related tumors compared to HBV/HCV-unrelated HCCs, concluding that chronic HBV/HCV infection may lead to CD8+ T cell exhaustion in HCC tumors. This phenomenon reflects the immune checkpoint blockade efficacy of viral-associated HCC in clinical scenarios, as the high checkpoint blockade response rate is related to CD8+ T cell density and programmed cell death protein 1 expression[114-116]. Furthermore, the authors found ligands highly expressed in protumorigenic and prometastatic hepatocytes related to inflammation (*e.g.*, C-X-C motif chemokine ligand 10/CXCR3) and immunosuppression (*e.g.*, macrophage-migration inhibitory factor/CD74), respectively. Distinct functions among malignant hepatocytes shape the immune microenvironment of HCC and provide hints to both tumor progression and immunotherapy.

Tumor recurrence and treatment resistance are partially determined by cancer stem-like cells (CSCs), which consist of a special subset of cells with stemness features and dictate cellular hierarchy and traits of dormancy and plasticity[117-119]. In a previous study, Zheng *et al*[120] combined transcriptome and functional analysis of HCC cells at the single-cell level to assess the degree of CSC heterogeneity as well as relationship to patient prognosis[120]. Discrete CSC subpopulations identified using single-cell surface markers all had a higher self-renewal ability compared to marker-negative cells but demonstrated appreciable biological differences in cell division and response to hypoxic stress in between. In addition, the authors found a 286-gene signature linked to CD133 and epithelial cellular adhesion molecule (EpCAM) are independent predictors of HCC patient survival. Moreover, HCC CSCs display an altered pattern of self-renewal heterogeneity when cultured under normoxia or hypoxia, suggesting a biological plasticity to these cells. Another article utilized scRNA-seq to identify two main HCC populations characterized by differential EpCAM expression[121]. Notably, a CD24+CD44+-enriched subclone within the EpCAM+ population exhibited a specific oncogenic expression signature and indicated the stemness of HCC. These findings were further confirmed by *in vitro* knockdown and *in vivo* tumorigenicity studies.

Sun *et al*[122] combined RNA-seq with single-cell profiling in paired samples from tumor and non-tumor regions of primary or recurrent HCC to unveil the unique immune ecosystem of recurrent HCC. The authors observed decreased regulatory T cells (Treg) and T cell proliferation with an increased proportion of CD8+ T cells and dendritic cells[122]. In addition, the authors concluded that CD8+ T cells in primary tumor and recurrent tumor samples showed the same transition trajectories but displayed considerably different immune and transcriptional states, suggesting that different immune therapy strategies should be considered for the treatment of primary and recurrent HCC. Specifically, CD8+ T cells in recurrent tumor samples, characterized by overexpression of *KLRB1*, revealed an innate dysfunctional state with low cytotoxicity and immunosuppressive phenotypes, which differed from the exhaustion state observed in primary tumor samples. The authors thus provided a model in which CD8+ T cell clones reside in a low proliferative and unresponsive state in the recurrent tumor due in part to tumor selection, suggesting that those cells are unable to recognize and eliminate recurrent tumor cells displaying subclonal neoantigens. In summary, data from this study indicated that malignant cells in the recurrent tumor demonstrated strengthened immune evasion capacities and reduced immune cell proliferation. Recurrent malignant cells could impair antigen presentation in dendritic cells *via* the programmed death-ligand 1-CD80 and cytotoxic T lymphocyte-associated protein 4-CD80 axes. Malignant cells may also recruit innate-like CD161+ CD8+ T cells *via* the CCL20-CCR6 axis, which could compromise anti-tumor immunity in early-relapse HCC.

Differentially expressed genes and pathway enrichment found in single-cell transcriptomes can be further applied to discover candidate drugs for the prevention of HCC recurrence as well as to the study of immune cell-cell communication. To predict the disease-free survival time and postoperative recurrence of HCC, Fu and Lei[123] constructed a risk score based on three immune cell types (effector memory CD8+ T cells, Treg cells, and follicular helper T cells) from the TCGA-HCC database called the T cell risk score[123]. Next, the authors used scRNA-seq data from 12 primary and 6 relapsed HCC samples to identify 645 genes with differential expression in three T cell types. After survival analysis,

the authors established a gene risk score by 15 prognostic genes (*AP000866.1, ATIC, CAPN10, EDC3, EID3, NCKIPSD, OXLD1, PHOSPHO2, POLE2, POLR3G, SEPHS1, SRXN1, TIMM9, ZNF487, and ZSCAN9*), which showed consistency with the T cell risk score in disease-free survival and immune characteristics. The results indicated these 15 hub genes may play a role in the process of immune cells affecting disease-free survival.

Subsequently, these hub genes were screened with CellMiner, a web tool based on the NCI-60 cell line set for identifying potential therapeutic drugs[124]. Pearson correlations between the 15 hub genes and the half-maximal inhibitory concentration of targeted drugs were analyzed, and the studies suggested that postoperative treatment of these drugs, such as imexon, irofulven, and nelarabine, may delay HCC recurrence. Moreover, the authors explored immune cell-cell interactions, finding the strongest communication among these three cell types was from effector memory CD8+ T cells to themselves *via* the granzyme A-coagulation factor 2 receptor signaling pathway as well as effector memory CD8+ T cells to follicular helper T cells and Treg cells *via* the CCL5-CCR4 signaling pathway. These findings illuminate crosstalk among such cell types, which is beneficial in future investigation of effector memory CD8+ T cells, Treg cells, and follicular helper T cells in disease-free survival time and recurrence prevention for patients with HCC.

LIMITATIONS AND FUTURE DIRECTIONS

The current healthcare system is transitioning from the time of evidence medicine towards the era of precision medicine. The omics technologies are evolving quickly and have built up tremendous results. Admittedly, there is still a gap to be bridged between the bench and bedside for translating these technologies.

First, the development of other levels of omics research (*e.g.*, epigenetics, proteomics, and metabolomics) expands cancer research including HCC to a multitude of data. How to incorporate and harness this huge amount of information and confirm its clinical importance (and not just an association) remains a challenge. With the advancement of computing power and artificial intelligence, we have the opportunity to store data and deal with multi-omics in parallel. Researchers can thereby extract significant data, confirm it with mechanistic studies in the laboratory, and translate it into clinical trials.

Second, studies showed that physicians who had lower confidence in genomic or transcriptomic technologies would like to ask for guidelines or training support[125-127]. To popularize transcriptome analysis into clinical practice, we require more physicians who understand the concepts of both omics technologies and tumor biology to stand out. Thus, we can improve medical education, design relevant clinical trials, and formulate health guidelines and policies. In fact, it is not merely a portion of people's responsibility. Multidisciplinary networks to share the collection of patient samples, clinical data, the standard of techniques, and genetic counseling are all indispensable to making medical decisions.

Last but not least, the accessibility of these technologies, especially the cost, is still a burden to patients. We hope in the future that these technologies can be applied more widely, and the price can be affordable for people by following "Moore's law" (*i.e.* higher throughput and lower cost) as in the computer industry (genome.gov/sequencingcosts; accessed November 19, 2022). Meanwhile, health policymakers should recognize the need for providing patients with transparent information and protecting their privacy. Therefore, we can benefit most patients by achieving the P4 discipline (preventive, predictive, personalized, and participatory) in precision medicine[128].

CONCLUSION

The high recurrence rate of HCC remains a serious burden to patients undergoing curative treatments and a major challenge to patient outcome. Over the past few decades, the emergence and evolution of transcriptome profiling methods have benefited the discovery of disease mechanisms, diagnosis, and treatment of HCC recurrence. Our current understanding of HCC pathophysiology is largely based on the fruitful results yielded by transcriptome analysis technology. We found that these abundant studies can be categorized based on the three domains of tumorigenesis, which include carcinogenic profile of primary cancer cells, carcinogenic stimuli, and tumor microenvironment.

We herein summarized the major findings of RT-qPCR, microarray, RNA-seq, and scRNA-seq research under the framework of these three domains (Table 2) and in doing so revealed the strengths and limitations of each technique. Being low-throughput, RT-qPCR is limited in identifying new DGE and is mainly used to validate findings yielded by other high-throughput techniques. Microarrays and RNA-seq have yielded great achievements in the study of carcinogenicity among primary cancer cells and carcinogenic stimuli. Moreover, these technologies contributed largely to our current knowledge on HCC recurrence pathogenesis. To date, a workflow consisting of microarray/RNA-seq search for differential transcripts, RT-qPCR confirmation, predictive model generation, and independent patient cohort validation has become a standard approach in basic HCC science. However, current diagnostic and

Table 2 Representative transcriptomic studies in recurrence of hepatocellular carcinoma

Ref.	Method	Sample comparison	Major findings	Featured research domain
Jiang *et al*[29], 2000	RT-qPCR	Nontumorous liver *vs* tumor samples; peripheral blood from HCC patients	*MMP9* in tumors was related to recurrence. mRNA of *AFP* in blood samples was associated with recurrence	Primary cancer cells
Morimoto *et al*[30], 2005	RT-qPCR	Peripheral blood and bone marrow samples from patients with HCC *vs* benign diseases	*AFP* mRNA level in blood, but not bone marrow, could be useful for predicting postoperative tumor recurrence	Primary cancer cells
Cheung *et al*[56], 2005	Microarray	HCC tumors from patients with post-OP recurrence *vs* without recurrence	*CLDN10*, along with the pTNM stage, were independent predictors for HCC recurrence	Primary cancer cells
Matoba *et al*[57], 2005	Microarray	HCC tumors from patients with *vs* without post-OP early (< 1 yr) recurrence	*HLA-DRA*, *HLA-DRB1*, *HLA-DG*, and *HLA-DQA* had significantly lower expression in the early IHR group	Primary cancer cells
Iizuka *et al*[58], 2006	Microarray	HCC tumors from patients with post-OP IHR *vs* EHR	46 cell adhesion-related genes, including *ITGA6* and *SPP1*, had higher expression levels in HCC with early IHR	Primary cancer cells
Ho *et al*[62], 2006	Microarray	HCC tumors from patients with *vs* without PVI	Differential expression of 14 genes related to the human melanoma gene family, cell growth, DNA glycosylation, and thrombin inhibitors, can be used to predict recurrence	Primary cancer cells
Chen *et al*[63], 2002	Microarray	HCC tumor and corresponding nontumorous tissue with *vs* without PVI	*ARHGAP8* and *ARHGEF6* were PVI-associated.	Primary cancer cells
Okabe *et al*[64], 2001	Microarray	HCC tumor from patients with *vs* without PVI	Upregulation of *MMP14* and downregulation of two *CYP* genes, *ADAMTS1*, and *ITGA7* were associated with PVI	Primary cancer cells
Okamoto *et al*[76], 2006	Microarray	Multicentric *vs* single nodular recurrent HCV-related HCC	36 marker genes were associated with multicentric recurrence and were used to develop a predictive scoring system	Carcinogenic stimulants
Mas *et al*[78], 2007	Microarray	HCV-related HCC from patients with *vs* without disease progression	Upregulation of *FAIM3* and *USP18*, and downregulation of *TFP1*, *HIST1H4E*, and *NRG1* were related to disease-free survival after curative treatment	Carcinogenic stimulants
Nagalakshmi *et al*[80], 2008	Microarray	MIM *vs* MAM	*HLA-DPA1*, *HLA-DRA*, *PRG1*, and *ANXA1* were associated with a metastatic phenotype (Th2-predominant), for which *CSF1* may be responsible	Microenvironment
Yoshioka *et al*[66], 2009	Microarray	HCC tumors from patients with multiple early (< 2 yr) IHR *vs* with DFS > 3 yr	Informative gene sets including *PPARBP*, *RREB-1*, *BCL2*, *HDAC1*, and *BIRC5* were yielded and used for a predictive model, which was validated in independent cases	Primary cancer cells
Kim *et al*[74], 2012	Predictive model construction using microarray database	DGE in 65 genes from pre-existing databases were used for a predictive model for early HCC recurrence and validated in independent HBV-related HCC cohorts	A risk scoring system with 65 differentially expressed genes identified from microarray data successfully predicted overall survival < 3 yr post-OP	Carcinogenic stimulants
Kim *et al*[75], 2014	Predictive model construction using microarray database	DGE of 233 HIR-related genes from preexisting databases were used for a predictive model for late HCC recurrence and validated in independent HBV-related HCC cohorts	Genes related to STAT3/Notch signaling activation were related to late (> 1 yr) recurrence of HCC. *RALGDS*, *IER3*, *CEBPD*, and *SLC2A3* were independent predictors of recurrence.	Carcinogenic stimulants
Nakagawa *et al*[65], 2021	Predictive model construction using microarray database	Validation of intrahepatic metastasis risk signatures created based on a preexisting microarray database in an independent patient cohort	*STC1*, *FOXK2*, *MMP1*, and *LOXL2* that promote either cell cycle advancement or histone modulation could predict the incidence of early recurrence	Primary cancer cells
Liu *et al*[87], 2022	RNA-seq	HCC tumors from patients with *vs* without recurrence	Most altered expression genes are related to DNA synthesis (*MCM8*, *MCM6*, *TOP2A*, and *CDC7*), chromatin segregation (*BUB1* and *CDC6*), and mitosis (*NDC80* and *PPP2R3C*)	Primary cancer cells

Ng *et al*[88], 2021	RNA-seq	Paired tumor tissues *vs* nontumorous tissues from HCC patients	*GSTA2* expression was associated with early-phase systemic injury and reactive oxygen species levels and could serve as a predictor of recurrence	Primary cancer cells
Lachmann *et al* [90], 2018	RNA-seq	Paired primary *vs* recurrent HCC tumor tissues	Mutations of *GOLGB1* and *SF3B3* are potential key drivers for the aggressive phenotype in recurrent HCC	Primary cancer cells
Okrah *et al*[97], 2018	RNA-seq	HBV-related HCC tumor *vs* distant nontumorous liver tissues	More HBV gene integrations correlated with a higher recurrence rate	Carcinogenic stimulants
Wang *et al*[98], 2021	Validation of RNA-seq database	HCC tumors *vs* matched cirrhotic tissues; CD8+ CTL-infiltrated *vs* T cell-excluded tumor tissues	Local tumor immunosuppression coincided with disease progression. Association was found between elevated fibrosis and the T cell-excluded immune phenotype	Microenvironment
Ho *et al*[99], 2021	Predictive model construction using RNA-seq database	Validation of recurrence-associated lncRNAs identified by regression analysis of TCGA database	9 immune-related lncRNAs were tightly associated with recurrence	Microenvironment
Zheng *et al*[120], 2018	scRNA-seq	CSC *vs* non-CSC populations defined by triple+ or triple− surface expression of CD133, CD24, EpCAM	286 signature genes linked to triple+ CSC could predict tumor recurrence in 240 HCC cases with multivariable Cox regression survival risk prediction analysis	Primary cancer cells
Sun *et al*[122], 2021	scRNA-seq	Tumors from primary *vs* early-relapse HCC patients	Decreased Treg and T cell proliferation with an increased proportion of CD8+- T cells and DC were found in early-relapse tumors compared to primary tumors. CD8+ T cells with overexpression of *KLRB1* revealed an innate dysfunctional state with immunosuppressive phenotypes in recurrent tumors	Microenvironment
Fu and Lei[123], 2022	scRNA-seq	Primary *vs* early-relapsed HCC samples	ScRNA-seq analysis of primary *vs* relapsed HCC identified 645 genes with DGE across three T cell types. Univariate and multivariate analysis identified 15 prognostic genes (*AP000866.1*, *ATIC*, *CAPN10*, *EDC3*, *EID3*, *NCKIPSD*, *OXLD1*, *PHOSPHO2*, *POLE2*, *POLR3G*, *SEPHS1*, *SRXN1*, *TIMM9*, *ZNF487*, and *ZSCAN9*)	Microenvironment

AFP: Alpha-fetoprotein; CSC: Cancer stem cell; CTL: Cytotoxic T lymphocyte; *CYP*: Cytochrome P450; DC: Dendritic cell; DFS: Disease-free survival; DGE: Differential gene expression; EHR: Extrahepatic recurrence; EpCAM: Epithelial cellular adhesion molecule; *GSTA2*: Glutathione S-transferase A2; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; HIR: Hepatic injury and regeneration; IHR: Intrahepatic recurrence; lncRNA: Long non-coding RNA; MAM: Metastasis-averse microenvironment; MIM: Metastasis-inclined microenvironment; *MMP9*: Matrix metalloproteinase 9; post-OP: Postoperative; pTNM: Pathological tumor-node-metastasis; PVI: Portal vein invasion; RNA-seq: RNA-sequencing; RT-qPCR: Real-time quantitative reverse transcription; scRNA-seq: Single-cell RNA sequencing; STAT3: Signal transducer and activator of transcription 3; TCGA: The Cancer Genome Atlas; Th2: T helper 2 cell; Treg: T-regulatory cell.

monitoring guidelines are still based on conventional AFP and imaging assessments, having yet to incorporate data from transcriptome analysis.

Whether these gene classifiers can be applied clinically to improve diagnostic accuracy and help identify high-risk patient groups needs further confirmation from population-based studies in groups more representative of the patient population than a limited research cohort. ScRNA-seq, with its remarkably high resolution, outperforms other tools in exploring cell-cell interactions and the tumor microenvironment. Although currently available studies are limited compared to other transcriptome techniques, the high resolution and high-throughput features of scRNA-seq make it a powerful tool with great potential in investigating the tumor environment. In an era when immunotherapy is rapidly advancing, the prospect of being able to decipher the cancer immune ecology serves as a continuous incentive for future scRNA-seq studies in HCC recurrence, which may help us to verify and optimize the efficacy of novel treatments as well as facilitate the inclusion of precision medicine in managing HCC recurrence.

FOOTNOTES

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REVIEW

Impact of chronic liver disease on SARS-CoV-2 infection outcomes: Roles of stage, etiology and vaccination

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Abstract

Since the first identification in December of 2019 and the fast spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, it has represented a dramatic global public health concern. Though affecting mainly the respiratory system, SARS-CoV-2 disease, defined as coronavirus disease 2019 (COVID-19), may have a systemic involvement leading to multiple organ dysfunction. Experimental evidence about the SARS-CoV-2 tropism for the liver and the increasing of hepatic cytolysis enzymes during infection support the presence of a pathophysiological relationship between liver and SARS-CoV-2. On the other side, patients with chronic liver disease have been demonstrated to have a poor prognosis with COVID-19. In particular, patients with liver cirrhosis appear extremely vulnerable to infection. Moreover, the etiology of liver disease and the vaccination status could affect the COVID-19 outcomes. This review analyzes the impact of the disease stage and the related causes on morbidity and mortality, clinical outcomes during SARS-CoV-2 infection, as well as the efficacy of vaccination in patients with chronic liver disease.

Key Words: SARS-CoV-2 infection; COVID-19; Chronic liver disease; Cirrhosis; Non-alcoholic fatty liver disease; Liver injury

Core Tip: It has been observed, since the early months of the pandemic, that pre-existing liver disease was associated with a worsening of clinical outcomes in severe acute respiratory syndrome coronavirus 2 infection. A correlation exists between severity of liver disease and coronavirus disease 2019-related adverse outcomes. The etiology of liver disease could significantly affect mortality rates, as well as vaccination status.

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INTRODUCTION

From December 2019, the fast spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a new virus belonging to the Coronavirus family of respiratory pathogens, has represented a major public health problem worldwide, leading to the declaration of a pandemic status in March 2020 by the World Health Organization (WHO)[1]. Despite the enormous efforts by health personnel and organizations, coronavirus disease 2019 (COVID-19) has caused more than 6.5 million deaths worldwide to date [2]. While the new virus variants show a milder clinical picture with predominant involvement of the upper respiratory tract, the most severe form of SARS-CoV-2 infection characterized by acute respiratory distress syndrome (ARDS) still represents an important cause of morbidity and mortality[3-5]. As observed since the first pandemic phases, organ involvement in COVID-19 is not limited to the respiratory tract, but can result in systemic disease with cardiovascular, renal, neurological and, last but not least, hepatic involvement. In particular, increases in the indices of hepatic cytolysis or cholestasis [mean as an increase of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBIL), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) upper range value] or a more severe acute liver injury (ALI) [defined as ALT and/or AST over 3 × upper limit of normal (ULN) or ALP, GGT, and/or TBIL over 2 × ULN] can be found in 14%-53% of SARS-CoV-2 infection[6]. The mechanism of COVID-19-associated ALI is probably multifactorial, given the combination of direct viral cytopathic effect, cytokine-induced inflammatory damage, hypoxic and drug induced liver injury[7]. ALI has been demonstrated to be a predictor of unfavorable SARS-CoV-2 infection course[8,9]. High levels of AST and TBIL at hospital admission are associated with an increased risk of COVID-19 progression[10]. The coexistence of previous liver disease worsens the outcomes of COVID-19. In fact, if on one hand SARS-CoV-2 infection can determine liver injury[6], on the other hand chronic liver diseases (CLDs) are associated to immune system and hemostasis alterations[11] that are able to accelerate some pathogenetic mechanisms of SARS-CoV-2, as cytokine storm and prothrombotic state, affecting the outcomes significantly[12,13]. In particular, patients with liver cirrhosis are at a high risk of an unfavorable SARS-CoV-2 infection course, with significantly higher mortality rates than the general population[14,15]. The occurrence of ALI during COVID-19 in this population is an additional predictor of all-cause mortality[16]. The risk of adverse outcomes in patients with chronic hepatitis is still poorly understood. Moreover, the etiology of CLD, the disease stage, potential concomitant therapies (*e.g.*, immunosuppressive) and vaccination status could significantly impact the outcomes of COVID-19.

The aim of this review is to describe the role of liver disease during COVID-19, analyzing if and how much the stage of the disease and the related etiology can affect the SARS-CoV-2 infection outcomes and examine what is known on the clinical efficacy of vaccination in patients with CLD to date.

COVID-19 OUTCOMES AND LIVER DISEASE

Despite the prevalence of a pre-existing liver disease in COVID-19 patients appearing low (between 0.6% and 3.4%)[17-19] and not significantly related to the risk of contracting SARS-CoV-2 infection[20], the first studies showed that the presence of CLD was associated with an increase in both hospitalization rate and overall mortality[14]. In this regard, Singh *et al*[14] reported a relative risk (RR) for death in patients with pre-existing liver disease of 2.8 (95%CI: 1.9-4.0). Similarly, also Williamson *et al* [19] and Galiero *et al*[21] found a significant association between liver disease and COVID-19 mortality. A meta-analysis including about 25000 patients confirmed that CLDs were significantly associated with

more severe COVID-19 course [odds ratio (OR): 1.48; 95%CI: 1.17-1.87] and overall mortality (OR: 1.78; 95%CI: 1.09-2.93)[20]. Other studies instead did not show this association[22,23]. For example, Simon *et al*[22] reported that patients with CLD show an increased risk of hospitalization for COVID-19 but not an increased mortality. Furthermore, in a recent retrospective case-control study, patients with CLD did not show more need for invasive mechanical ventilation, as well as admission to intensive care unit (ICU) and overall mortality, compared to patients without liver disease[23].

Therefore, data on the association between liver disease and increase of mortality rates related to COVID-19 are conflicting. However, the spectrum of CLDs is very heterogeneous both for etiology and for stage disease. In fact, the risk appears directly related to the latter. Studies that evaluated all liver disease stages could have been affected by different mortality rates in patients with CLD, greater in cirrhosis stage, particularly if in decompensation. In this regard, Mallet *et al*[15] more recently reported, on a large French cohort, a significant increase in the need for mechanical ventilation (OR: 1.54; 95%CI: 1.44-1.64) and mortality rate (OR: 1.79; 95%CI: 1.71-1.87) in patients with CLD. However, when stratified by disease severity, authors observed that patients with decompensated liver cirrhosis showed a significantly higher mortality rate whereas patients with mild liver disease or compensated cirrhosis were not at increased risk of COVID-19-related mortality[15]. These data have been confirmed by meta-analysis of Middleton *et al*[24], in which liver cirrhosis has been shown to be associated to an increased risk of all-cause mortality in COVID-19 compared to non-cirrhotic patients. Current evidences on the role of disease stage, etiology, and vaccination on COVID-19 outcomes are summarized in Figure 1.

LIVER CIRRHOSIS AND COVID-19 CLINICAL OUTCOMES

Regardless of the etiology, the stage of liver cirrhosis is characterized by a high degree of patient frailty. The impairment of immune system, the concurrent increase of both thrombotic and hemorrhagic risk and the protein-calorie malnutrition make the patient suffering from cirrhosis vulnerable to various kinds of injuries. The frailty of the cirrhotic patient in the setting of COVID-19 is expressed by an excess of mortality and hospitalization rate compared to patients without CLD[14,15,19]. Extracting data of the subgroup of cirrhotic patients, Singh *et al*[14] had already reported that the risk of death in these patients was a 4.6-fold increase compared to ones without liver disease. As already mentioned, the mortality rate appears higher in patients with cirrhosis not only compared to patients without CLD but also when compared to patients with CLD but without cirrhosis (32% *vs* 8%, respectively; $P < 0.001$)[25]. In particular, compared to cirrhotic/SARS-CoV-2 negative patients and to non-cirrhotic/SARS-CoV-2 positive patients, those with liver cirrhosis and COVID-19 had a 2.38- and 3.31-times adjusted hazard ratio of 30-d death, respectively[26]. Similar results were found by Ioannou *et al*[27]. Overall, the 30-d COVID-19-related mortality rate in patients with cirrhosis is very high, ranging from 32% to 47% of cases[25,28,29]. In fact, liver cirrhosis has been proven to be an independent risk factor for COVID-19 related mortality (OR: 3.1)[29]. However, no updated mortality data are available for the new viral variants, with an apparently lower lethality rate than the wild type. Although most studies consider liver cirrhosis as an independent predictor of the risk of COVID-19-related death[15,24,26,29,30], some data would indicate that the high mortality rates in patients with cirrhosis and COVID-19 result from cirrhosis-associated comorbidities and extrahepatic organ failure rather than the liver disease itself[31]. After propensity score matching for age, sex, and extra-hepatic comorbidities, mortality rate during COVID-19 appears to be similar between patients with and without cirrhosis (28.8% *vs* 26.1%, respectively; $P = 0.644$). These results still could have been affected by the typology of matching and the methodology of data collection through registers. Among patients with liver cirrhosis, the coexistence of obesity and diabetes would further worsen the outcomes[16].

Similarly, to what was recently reported for kidney failure[32], COVID-19-related mortality risk was strongly associated with the stage of liver impairment. Overall, the 30-d mortality risk is significantly increased in patients with decompensated liver cirrhosis hospitalized for COVID-19[15,30]. Mortality rates increased according to Child-Pugh (CP) class, raising from 19% of class A (OR: 1.90), to 35% of class B (OR: 4.14) up to 51% of class C (OR: 9.32)[25]. In particular a CP score ≥ 9 at hospital admission would be predictive of high mortality[16]. Compared to patients without liver disease, a CP class B and C cirrhosis would bring an additional mortality rate of +20% and +38.1%, respectively[25]. In this regard, it seems that the Chronic Liver Failure Consortium had better performance in predicting 28-d mortality than CP score and model for end-stage liver disease-Na in patients with cirrhosis and COVID-19[29]. Moreover, in cirrhotic patients, an increasing trend of bilirubin and AST/ALT ratio[16] or the occurrence of liver injury[10,21] would be predictive of mortality.

The most frequent cause of mortality in patients with liver cirrhosis and COVID-19 remains the respiratory failure (71%)[25]. However, acute liver decompensation occurred in up to 46% of patients, even without respiratory symptoms. Liver related complications increased according to stage of liver disease[16]. It is known that infections (bacterial more than viral) may lead to liver decompensation, hepatorenal syndrome and portosystemic encephalopathy, and are one of the most frequent causes of acute-on-chronic liver failure (ACLF) and death in patients with cirrhosis. Conclusive data on the comparison between the SARS-CoV-2 infection outcomes and those of other infectious precipitants are

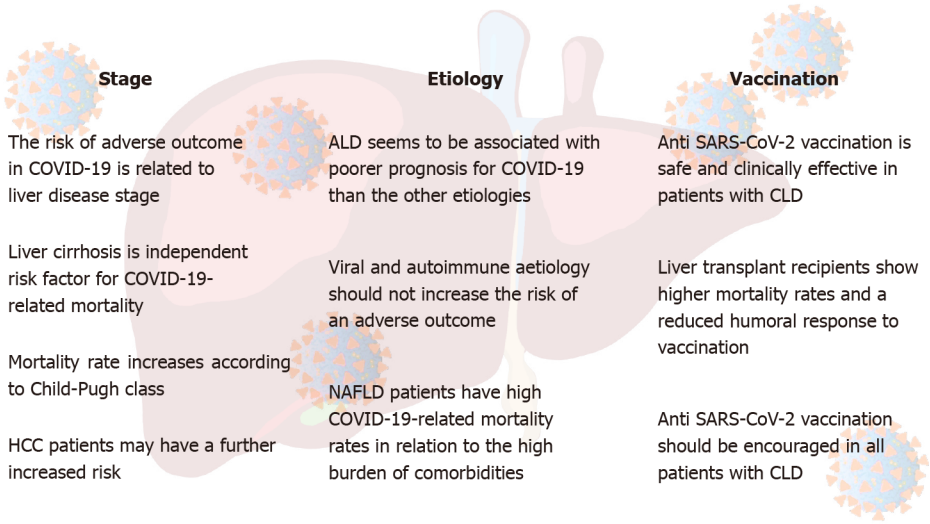

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Figure 1 Impact of chronic liver disease on severe acute respiratory syndrome coronavirus 2 infection outcomes: role of stage, etiology and vaccination. ALD: Alcohol-related liver disease; CLD: Chronic liver disease; COVID-19: Coronavirus disease 2019; HCC: Hepatocellular carcinoma; NAFLD: Non-alcoholic fatty liver disease; SARS-CoV-2: Severe acute respiratory syndrome-coronavirus 2.

currently not available. However, some data indicate that in-hospital mortality rates would be significantly higher in cirrhotic patients with COVID-19 than in those with other bacterial infections [28]. Overall, about 45% of patients with CLD develop ACLF[16]. Higher rates are reported for patients with liver cirrhosis. Moreover, cirrhotic patients with diabetes or obesity had higher ACLF rates than non-diabetic or normal weight patients (OR: 2.1 and 8.9, respectively)[16]. Similarly, to other viral infections, ACLF during COVID-19 could result from an immune-mediated response to viral antigens in the context of a cytokine storm[33], as well as a direct cytopathic effect or iatrogenic injury. Multi-organ damage caused by SARS-CoV-2 is significantly more frequent in immunocompromised patients[34]. The impairment of the immune system in the case of liver cirrhosis due to bone marrow suppression, lower protein synthesis and cirrhosis associated immune dysfunction syndrome, could explain the high rates of ACLF and the severe course in cirrhotic patients.

If in-hospital, COVID-19-related mortality was significantly increased in patients with liver cirrhosis and little is known about post-acute outcomes. Recently, Vaishnav *et al*[35] analyzed the post-discharge mortality of cirrhotic patients with SARS-CoV-2 infection. The data indicate that mortality rates within 2 mo of discharge among COVID-19 survivors are comparable between patients with liver cirrhosis and those without.

Hepatocellular carcinoma

Little data is currently available on SARS-CoV-2 infection clinical course and outcomes in patients with hepatocellular carcinoma (HCC). Indeed, in studies performed during the first pandemic phase on COVID-19-related outcomes in cancer patients, those with HCC were underrepresented[36,37].

Although data are not univocal[25], several studies include the presence of HCC among the independent predictors of COVID-19-related mortality[15,30,38]. Among patients with CLD and COVID-19, HCC patients had 3.31 times the hazard of death for all causes, regardless of the presence of liver cirrhosis[30]. Beyond the association with mortality, according to Mallet *et al*[15] the presence of HCC is also predictive of a severe course of COVID-19 and a greater need for mechanical ventilation. Muñoz-Martínez *et al*[38] evaluated the SARS-CoV-2 infection course in 250 patients with primary liver cancer (218 with HCC and 32 with intrahepatic cholangiocarcinoma). In patients with HCC, a 30-d mortality rate of 18.4% was observed, with a statistically significant trend according to the stage of cirrhosis (assessed by CP) and tumor [assessed by Barcelona Clinic Liver Cancer (BCLC)]. In particular, the mortality rates increased from 6.10% for BCLC-0/A, to 11.76% for BCLC-B, to 20.69% for BCLC-C and 34.52% for BCLC-D[38].

The high COVID-19 related mortality in patients with HCC could result from the link between viral infection and the impairment of the immune-system secondary to active neoplasm, antineoplastic therapy and the frequent coexistence of liver cirrhosis.

Liver transplant recipients

In the analysis of the correlation between SARS-CoV-2 infection and CLD, patients who have undergone liver transplant (LT) represent a separate group due to the effects of chronic immunosuppressive therapy. The hypothesis that this therapy could increase the susceptibility to SARS-CoV-2 has been

suggested from some population studies[39-41]. Observational studies on western populations have reported a mortality of 16%-22% in hepatotransplant patients with SARS-CoV-2 infection[42,43] in liver-transplant patients with SARS-CoV-2 infection, finding an increased survival in the short-term LT recipients (< 2 years), usually treated with full doses of immunosuppressants. This data support the hypothesis that, more than the immunosuppressive effect itself, the main cause of death in these patients is represented by the long-term cardio-metabolic effects induced by immunosuppressive drugs [44].

The study of the correlation between the type of immunosuppressive drug and COVID-19 outcomes in patients who have undergone LT has led to non-univocal results. During the first pandemic wave, Colmenero *et al*[39] reported that mycophenolate mofetil therapy in liver-transplant patients with SARS-CoV-2 infection was associated with an increased risk of a severe course of COVID-19 (RR 3.94, P = 0.003)[39]. Therapies with calcineurin inhibitors or everolimus, instead, have been shown to not be associated with an increased likelihood of adverse outcome. Furthermore, discontinuation of immunosuppressive therapy did not show benefits[39]. On the contrary, Webb *et al*[45] did not find any correlation between the type of immunosuppressant and mortality rate in patient with previous LT and SARS-CoV-2 infection. They highlighted that LT seems to not significantly increase the COVID-19-related mortality rate. These data are supported by the results of a meta-analysis including the main studies performed on LT patients with SARS-CoV-2 infection[46]. In these patients the 30-d mortality was comparable to the mortality rate found in the general population (OR: 0.90, 95%CI: 0.55-1.47). In light of unavailability of univocal data, the European Association for the Study of the Liver suggests to personalize immunosuppressive therapy changes based on patient's medical history, disease severity and the type of ongoing immunosuppressive therapy[47].

CHRONIC HEPATITIS AND COVID-19 CLINICAL OUTCOMES

As mentioned above, if liver cirrhosis is associated with high rates of COVID-19-related mortality, several data indicate that patients with chronic hepatitis do not show an increased risk[15]. However, in addition to the stage of the disease, the different etiology could also affect the COVID-19 outcomes (Figure 1).

Alcohol-related liver disease

During the pandemic, the relationship between alcohol and SARS-CoV-2 infection has been shown to be bidirectional. On one hand, the isolation and socio-economic uncertainties resulting from the COVID-19 pandemic have led to an increase in alcohol consumption[48], already on the rise in the last 20 years [49]. On the other hand, several studies reported that alcohol-related liver disease (ALD) seems to be associated with a poorer prognosis for COVID-19 than the other etiologies[15,25,30,50]. In this regard, Marjot *et al*[25] showed ALD to be an independent risk factor for death from COVID-19 (OR: 1.79; 95%CI: 1.03-3.13). Similar results have been obtained from Mallet *et al*[15] and Kim *et al*[30]. Recently Bailey *et al*[50] confirmed that alcohol use disorders (AUDs) worsen the COVID-19 course and are associated with an increased hospitalization rate and all-cause mortality compared to patients with SARS-CoV-2 infection but without AUDs.

AUDs are already known as a risk factor for ARDS and ARDS-related multiorgan failure[51]. In fact, chronic alcohol consumption has been demonstrated to cause significative alterations in epithelial and endothelial cell function, surfactant synthesis and secretion, lung matrix composition and alveolar-capillary barrier function. Such alterations could increase susceptibility to respiratory pathogens, like SARS-CoV-2, leading to higher ARDS rates and adverse outcomes compared to patients without AUDs. Furthermore, ethanol exposure could stimulate the activity of key inflammatory mediators with a pro-inflammatory response further exacerbated by SARS-CoV-2 infection, resulting in a more severe course of COVID-19[52].

Chronic viral hepatitis

Several studies analyzed the mutual interaction between chronic hepatitis B virus (HBV) and SARS-CoV-2 infection, investigating whether the underlying viral disease could determine a worse prognosis during the COVID-19 course. Numerous data suggest that patients with chronic HBV infection have similar characteristics to HBV-negative patients in prevalence of laboratory abnormalities (changes in cytolysis and cholestasis liver markers), severity of the COVID-19 course and mortality[53-55]. The absence of a significant correlation between COVID-19-related outcomes and chronic viral hepatitis is confirmed by meta-analysis of Sarkar *et al*[56], in which the Authors found no significant impact on overall mortality during SARS-CoV-2 infection. Neither the degree of HBV replicative activity seems to affect the SARS-CoV-2 infection outcomes; inactive carriers or patients with previous infection have ALI and mortality rates comparable to patients with active hepatitis[57,58]. As further demonstration of the absence of correlation between HBV replicative activity and COVID-19-related outcomes, antiviral therapy for HBV is not able to determine a significant impact on mortality, need for admission to the ICU and hospitalization length[59]. Apparently, conflicting with these data, Yang *et al*[60] showed that

the HBeAg-positive chronic HBV hepatitis are associated with a higher rate of hospitalization in ICU and mortality. However, the Authors did not stratify the study cohort in relation to disease stage and the impact of the presence of liver cirrhosis and organ failure on these results is unknown. Finally, the role and safety of immunosuppressive therapies (*e.g.*, corticosteroids, IL-6 pathway inhibitors such as tocilizumab) used in cases of SARS-CoV-2-related ARDS were evaluated for the risk of HBV reactivation in patients with biohumoral signs of previous infection (HBsAg-negative, HBcAb-positive). In these patients, the risk of HBV reactivation following immunosuppressive treatment for COVID-19 appears negligible and not influenced by any antiviral prophylaxis[61].

Little data are available to date on the association between the severity of COVID-19 course and chronic hepatitis C virus (HCV) infection. Some studies report an increase of mortality for patients with chronic HCV infection[62]. However, also in this case, the proportion of patients with liver cirrhosis and the related impact on outcomes is unknown. Butt *et al*[63] showed similar COVID-19-related mortality rates among HCV-positive patients compared to HCV-negatives, despite a higher hospitalization rate. However, in the study population, HCV patients show a higher prevalence of liver cirrhosis than those not with HCV (8.1% *vs* 1.4%, respectively; $P < 0.0001$). Lastly, Cerbu *et al*[64] investigated the differences in outcomes between patients with active HCV infection and those who were under treatment or achieved sustained virological response. They found that patients with active infection showed an overall worse prognosis in terms of hospitalization, severe COVID-19 course, ICU admission and all-cause mortality compared to non-viremic patients. Regarding this, the early treatment with sofosbuvir/velpatasvir combination (used for HCV infection) in patients with COVID-19 has been shown to be effective in speeding up the clearance of SARS-CoV-2 and preventing disease progression [65].

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is currently the most frequent etiology of liver disease worldwide, affecting approximately 32.5% of the global population[66]. It is closely associated to metabolic comorbidities such as obesity, diabetes mellitus, arterial hypertension and chronic kidney failure[67]. Such comorbidities related to NAFLD have been shown to play a predictive role for adverse outcomes in COVID-19, being associated with higher rates of hospitalization, mechanical ventilation and mortality[19,68]. For these reasons, great attention has been paid to determine whether NAFLD itself could represent an independent prognostic factor in COVID-19. However, studies in this setting are affected by the variability in the definition of NALFD patients, using for this purpose clinical-anamnestic or radiological (by ultrasound or computed tomography) criteria or score [hepatic steatosis index (HSI)] in different ways. Data currently available are not univocal. In one of the very first reports, Ji *et al*[69] showed that, net of comorbidities, NAFLD (diagnosed by ultrasound or by a value > 36 of the sums of HSI and body mass index) was an independent predictor of COVID-19 progression (OR: 6.4; 95%CI: 1.5-31.2). Furthermore, NAFLD was associated with higher prevalence of ALI during hospital stay and a slower viral clearance compared to the control group without NAFLD. Mahamid *et al*[70] later confirmed these data, despite the small cohort size. Conversely, in a recent case control study, NAFLD was not found to be associated with higher in-hospital mortality rates, need for ventilatory support, ICU admission, or overall length of hospital stay[71]. Similar results have been obtained by Marjot *et al*[25] and Kim *et al*[30]. Also, in the study by Vrsaljko *et al*[72], NAFLD is not shown to be independently correlated to a severe course of COVID-19 and to mortality rates in the multivariate analysis, while it appears significantly related to the hospitalization length and the incidence of pulmonary thrombosis.

Moreover, the nomenclature of NALFD recently has been changed to metabolic associated fatty liver disease (MAFLD)[73]. At the same time, the diagnostic criteria have been redefined and the results do not overlap with the previous ones. These new criteria have also been recently applied in the setting of patients with SARS-CoV-2 infection, showing conflicting data in this case as well. In this regard, Vá zquez-Medina *et al*[74] reported that patients with MAFLD, but not those with NAFLD, have higher mortality rates (55.0% *vs* 38.3%; $P = 0.02$) than the control group not MAFLD/not NAFLD, whereas both MAFLD and NAFLD are associated with a higher rate of orotracheal intubation. Gao *et al*[75] confirmed that MAFLD increases by 4 times the risk of a severe course of COVID-19 and the association remains even after adjusting for age, sex, and comorbidities. Surprisingly, some preliminary data would indicate that the correlation between MAFLD and severity of COVID-19 course is more significant in patients under 60[76]. In contrast to the above-mentioned studies, Campos-Murguía *et al*[77] observed that fibrosis rather than MAFLD is associated with a severe course of COVID-19 (increased need for mechanical ventilation, increased incidence of acute kidney injury), and higher mortality. However, in most of these studies enrolled patients were not evaluated for the possible presence of liver cirrhosis. This could represent a significant bias with a potential impact on the results. As expected, the presence of intermediate or advanced liver fibrosis in patients with MAFLD is indeed associated with a higher risk of severe COVID-19[78]. In this regard, advanced fibrosis has been shown to determine a significant increase in mortality risk both in patients diagnosed with MAFLD and in those diagnosed with NAFLD compared to patients without CLD[74] and patients with mild or moderate liver fibrosis[79].

Recently, a large-scale 2-sample Mendelian randomization analysis had been carried out in order to provide possible conclusive data on the association between NAFLD and the COVID-19 course[80]. Although NAFLD is associated with a severe course of SARS-CoV-2 infection in univariate analysis, this association disappears after adjustment for age, sex, and comorbidities. Therefore, the Authors conclude that there is no evidence supporting that NAFLD is a causal risk factor for severe COVID-19. The results favoring this association are probably attributable to the correlation between NAFLD and obesity.

Autoimmune liver disease

The incidence of COVID-19 in patients with autoimmune hepatitis (AIH) is comparable to that of the general population[81,82]. Despite that available data are limited, the main studies agree that patients with AIH do not present significant differences in hospitalization rates, disease severity and mortality during SARS-CoV-2 infection compared to patients with non-AIH CLD and the general population[83, 84]. However, steroid treatment during COVID-19, when not indicated, can cause a more severe course of the disease. While steroids represent a cornerstone in the therapy of SARS-CoV-2-related ARDS[85, 86], particularly when associated to antiviral drugs[87], their use is not recommended in the absence of respiratory failure due to lack of benefit and potential worsening of outcomes[85,86]. In this regard, Efe *et al*[88]recently highlighted that the use of corticosteroids or azathioprine for AIH therapy is associated with a significant increase in the risk of severe form of COVID-19 (OR: 4.73; 95%CI: 1.12-25.89), even after adjusting for demographic characteristics, comorbidities and presence of liver cirrhosis. However, in the absence of conclusive data, any remodulation of immunosuppressive therapy during SARS-CoV-2 infection should be personalized after a careful assessment of the risks and benefits[47].

Role and efficacy of vaccination in patients with CLD and liver cirrhosis

The global availability of anti-SARS-CoV-2 vaccines in the last months of 2020 has resulted in a reduction of hospitalization and mortality rates from COVID-19 despite the succession of different viral variants[89]. Given their vulnerability profile in cases of SARS-CoV-2 infection, such vaccines have been strictly recommended in LT recipients and patients with CLD, with priority to cirrhotic patients and those with HCC or ALD (Figure 1)[90-92]. In this setting, vaccines have found to be generally safe[93-95], although sporadic cases of post-vaccinal ALI are reported, with predominantly hepatocellular and immune-mediated injury due to a probable aberrant response of the immune system after vaccine stimulation[96].

Despite the strong indication, patients with CLD (particularly those with liver cirrhosis) appear underrepresented in phase III trials of anti-SARS-CoV-2 vaccines, both for mRNA and viral vector ones [97-99]. In fact, CLD and in particular the presence of significant liver fibrosis could negatively affect the production of innate immunity proteins and the count and performance of B and T lymphocytes[100]. For this reason, in the last 2 years, a growing number of clinical studies have investigated the efficacy of anti-SARS-CoV-2 vaccines in patients with CLD. Anti-SARS-CoV-2 vaccines are able to determine both a T-cell and a humoral response, stimulating the production of antibodies directed against the Spike protein (seroconversion) and neutralizing antibodies[101,102]. Prospective studies compared the immunological response induced by a full course of mRNA vaccines and/or viral vector vaccines in patients with liver cirrhosis and controls[93-95]. Almost all patients with liver cirrhosis showed production of anti-Spike antibodies and a neutralizing antibody activity, similar to patients without liver cirrhosis[93]. A recent meta-analysis confirmed comparable seroconversion rates between cirrhotic patients and patients with CLD without cirrhosis[100]. Despite comparable seroconversion rates, Iavarone *et al*[95] reported a statistically significant difference in the antibody titer developed by patients with liver cirrhosis after vaccination compared to controls (1751 U/mL *vs* 4523 U/mL; P = 0.012). In particular, CP classes B and C and the presence of HCC would appear to be independently associated with low levels of antibody titer. We hypothesize that this suboptimal vaccine response could potentially indicate partial protection against SARS-CoV-2 infection and a reduction in its duration, particularly in patients with decompensated cirrhosis. Furthermore, with regard to the cellular response induced by the vaccine, the production of interferon-gamma after spike-specific stimulation of T lymphocytes is detectable only in 65.4% of patients with liver cirrhosis, against 100% of controls[94].

Beyond humoral response, little data are available on clinical efficacy of anti-SARS-CoV-2 vaccines in patients with CLD. The most important knowledge on this issue have been acquired from Veterans Outcomes and Costs Associated with Liver Disease cohort[103] and National COVID Cohort Collaborative registers[104]. In the first study, John *et al*[103] compared overall and COVID-19-related mortality 60 d after SARS-CoV-2 infection in cirrhotic patients receiving mRNA vaccine and cirrhotic patients not previously vaccinated. Unequivocally, postvaccination COVID-19 was associated with reduced mortality rates (HR: 0.21; 95%CI: 0.11-0.42) compared to unvaccinated patients with liver cirrhosis, with an overall reduction in the risk of death of approximately 80%. The benefit of vaccination is also statistically significant in patients with decompensated cirrhosis and in those who have not completed the vaccination course[103]. Similar results have been reported also by Ge *et al*[104], whose study is available only in "pre-print" version to date. This study, carried out during SARS-CoV-2 alpha and delta variants predominance, describes a 66% reduction in 30-d mortality in vaccinated cirrhotic patients compared to unvaccinated patients with liver cirrhosis. Furthermore, the administration of the third dose of mRNA vaccine in cirrhotic patients seems to lead to an 80% reduction in cases of SARS-

CoV-2 infection (symptomatic or asymptomatic) and a 100% reduction in the severe forms of COVID-19 and related death compared to the administration of two doses, overcoming the hyporesponsiveness to vaccines in these patients[105]. The impact of the third vaccine dose appears stronger among patients with compensated rather than decompensated cirrhosis.

If patients with liver cirrhosis show overall suboptimal but effective seroconversion rates on protection against risk of death and a severe COVID-19 course, data obtained from LT recipients appear less encouraging. In fact, LT recipients show lower vaccine response[94,100,106]. Seroconversion rates in these patients range from 47.5% and 65%, significantly lower than controls[94,107]. Overall, 28% of patients undergoing LT did neither develop a T-cell nor a humoral response after vaccination[94]. An optimal humoral response is developed in only one third of liver transplant patients[107]. Among the factors associated with vaccine response rate in this setting, studies agree in identifying advanced age, alcoholic etiology of liver disease, and immunosuppressive therapy as independent predictors of reduced antibody response[93,106,108]. Conclusive data on the correlation with the specific immunosuppressive regimen are not available to date. Some studies would indicate that the reduced antibody response is particularly significant for patients treated with mycophenolate mofetil[106,108] or high doses of steroids[106]. Other manuscripts reported instead a negative correlation with calcineurin inhibitors compared to other immunosuppressive drugs[94]. Thuluvath *et al*[107] highlights that the use of ≥ 2 immunosuppression drugs is associated with poor immune response.

Finally, at the moment, few data are available about the influence of CLD etiology on vaccine-induced immune responses. Among the various etiologies, despite the high seroconversion rates, AIH patients show a significantly lower antibody titer than both patients with autoimmune cholestatic liver disease and controls, independently from the presence of liver cirrhosis and the ongoing immunosuppressive therapy[109]. Despite this, the clinical benefit of vaccination appears consistently in AIH patients, showing a significative reduction in hospitalization, severe course and COVID-19-related mortality rates (adjusted OR: 0.20)[110].

CONCLUSION

Patients with CLD are more vulnerable to SARS-CoV-2 infection. In particular, patients with liver cirrhosis show higher hospitalization rates, severe COVID-19 course and mortality than the general population. Mortality rates increase according to stage of cirrhosis. Among etiologies, ALD is the most frequently associated to adverse outcomes. Patients with NAFLD have high mortality rates and severe COVID-19 course in relation to the high burden of comorbidities. Anti-SARS-CoV-2 vaccination is safe and effective in patients with CLD: in particular, patients with liver cirrhosis benefit from a complete vaccination course. Patients who have undergone liver transplant show higher mortality rates and a reduced humoral response to vaccination. In any case, vaccination should be encouraged in all patients with CLD, particularly for those at higher risk due to disease stage and related etiology.

FOOTNOTES

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Outcomes of COVID-19 among patients with liver disease

Isidora Vujčić

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Abstract

Coronavirus disease 2019 (COVID-19) is primarily a respiratory disease with multi-organ involvement, including impaired liver function. It has been noticed that a significant proportion of COVID-19 patients have liver dysfunction, especially those with a more severe disease course. The coronavirus causes direct damage to the liver using the angiotensin-converting enzyme 2, a cell-surface receptor for cellular entry, that is expressed in the liver. According to previous research, liver enzyme abnormalities were observed in a considerable proportion of COVID-19 patients, and elevated liver transaminases were found in about 20% of these patients, alkaline phosphatase in 6.1%, and gamma-glutamyl transferase in 21.1%. COVID-19 might trigger a deterioration of liver function in patients with pre-existing chronic liver diseases (CLDs) and also in those without previous liver disorders. The majority of COVID-19 patients who develop liver injury are men, the elderly, and those with a higher body mass index. Compared to the general population, COVID-19 is associated with significant morbidity and mortality in patients with liver disease (cirrhosis and liver transplantation recipients). However, some studies indicate that CLDs have a lesser role in determining patient progression towards higher disease severity.

Key Words: Liver disease; COVID-19; Mortality; Prognosis; Liver function

Core Tip: Drastic lifestyle changes during the coronavirus disease 2019 (COVID-19) pandemic have led to an increase in the incidence of liver disease. Liver damage in COVID-19 infection occurs during disease progression in patients with or without previous liver disorders and represents a risk factor for developing severe illness and death. The prognosis of COVID-19 infection depends predominantly on the patients' characteristics, present comorbidities, severity of clinical symptoms, laboratory parameters, and imaging features. It is important to examine prognostic factors in COVID-19 disease patients with liver disease because it may improve the outcome.

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INTRODUCTION

The coronavirus disease (COVID-19) originated in late 2019 in China and spread with alarming rapidity across the globe[1]. The illness is caused by a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and during the pandemic, more than 600 million cases and more than 6 million deaths were reported worldwide[2]. COVID-19 clinical manifestations vary, and the disease's wide clinical spectrum ranges from mild, self-limiting pulmonary tract infection to progressive severe pneumonia with high mortality rates[3]. Drastic lifestyle changes during the COVID-19 pandemic have led to an increase in the incidence of nonalcoholic fatty liver disease (NAFLD), decompensated cirrhosis, acute alcoholic hepatitis, viral hepatitis, and mortality from liver diseases[4]. Liver dysfunction in COVID-19 patients is a risk factor for severe illness and death[5], and significantly higher morbidity and mortality rates were observed among patients with liver disease and COVID-19, compared to the general population[6]. About 2%-11% of COVID-19 patients had already been diagnosed with chronic liver disease (CLD)[7]. However, liver dysfunction includes a variety of etiologies and heterogeneous groups of patients[8]. In addition, COVID-19 can induce liver injury, especially in those patients with severe forms of the disease[7,9].

GLOBAL BURDEN OF LIVER DISEASES

Globally, two million deaths are attributed to liver diseases, including 1 million due to cirrhosis and 1 million due to viral hepatitis and hepatocellular carcinoma (HCC)[10]. Over the past two decades, the prevalence of CLD has been increasing[11]. CLD includes NAFLD, alcohol-related liver disease (ALD), and chronic viral hepatitis B and C[12], and it can progress to fibrosis, cirrhosis, and HCC[13]. NAFLD, or the recently defined metabolic-associated fatty liver disease (MAFLD), are the most common CLDs, which affect about a quarter of the world's adult population[14]. The global prevalence of MFALD/NAFLD has been rapidly increasing in tandem with the rise in diabetes and obesity prevalence, both of which have been associated with increased mortality in COVID-19[15,16]. Hepatitis B and C are still a major cause of liver disease burden globally, especially in low-income countries in Asia and sub-Saharan Africa, despite the availability of effective preventive measures and treatment[10,17].

LIVER INJURY IN CORONAVIRUS INFECTED PATIENTS

Although coronavirus can cause the worst damage to the lungs, it can also influence the digestive, cardiac, and endocrine systems[18]. Multifactorial causes of liver damage during COVID-19 infection include direct virus cytopathogenic effect, abnormal immune response associated with the cytokine storm, vascular changes due to coagulopathy, hepatic ischemia/hypoxia reperfusion injury, and drug-induced liver injury[19,20]. The coronavirus causes direct liver injury using the angiotensin converting enzyme 2 receptor for cellular entry, which is expressed mainly in the cholangiocytes and less frequently in the hepatocytes[21]. Certain hepatotoxic medications, such as antibiotics (macrolides, quinolones), antivirals (ribavirin), steroids, and other drugs used to treat patients with COVID-19, are connected with drug-induced liver injury and were found in 10.9% of COVID-19 patients[22-24]. However, in COVID-19 patients, liver damage is primarily secondary to ischemic, hypercoagulable, and hyperinflammatory states, which are independent predictors of death rather than liver injury per se [21]. A cytokine storm and a massive acute-phase response are defined by the acute overproduction and uncontrolled release of the proinflammatory cytokines, tumor necrosis factor (TNF), interleukin 1 (IL-1),

and IL-6 paralleled by excessive secretion of C-reactive protein (CRP) and ferritin[25]. Coagulation dysfunction indicates a poor outcome in critically ill COVID-19 patients with hepatic injury, including a significant role of neutrophils and monocytes in amplifying blood clotting[20]. Hepatic apoptosis and elevated liver enzymes are caused by ischemia and reperfusion injury[20].

PROGNOSTIC FACTORS IN COVID-19 PATIENTS WITH LIVER DISEASE

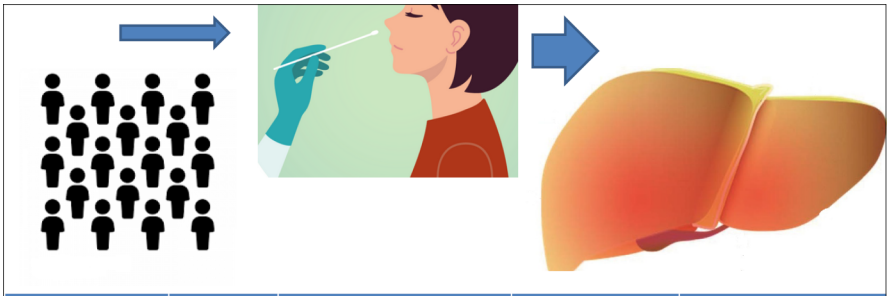
Advanced age and being male are well-established risk factors for severe COVID-19 outcomes[26]. Various medical underlying conditions, such as cardiovascular disease, lung disease, cancer, diabetes, and obesity have also been associated with increased risk[27-29]. However, the prognostic factors in COVID-19 patients with previous liver diseases are not well-defined[9]. In a multicenter cohort study conducted in the United States, comorbidities such as diabetes, hypertension, chronic obstructive pulmonary disease, current smoking and increasing age in patients with ALD, liver cirrhosis decompensation, and HCC predicted a higher mortality when infected with COVID-19[11]. In another study from the United States conducted among CLD patients, it was reported that older age and pre-existing comorbidities were associated with severe COVID-19[30]. Shen *et al*[31] found that COVID-19 patients with liver injury had a significantly poorer prognosis than patients without liver dysfunction, and that male sex and elevated CRP were independent prognostic factors in these patients[31]. Preliminary results of a systematic review and meta-analysis involving 88 studies and 6653207 cases of COVID-19 in Europe showed that liver disease was associated with hospital admission and mortality, after adjustment for age and sex[32]. Liver dysfunction during COVID-19 has been associated with increased disease severity, prolonged hospital stays, ventilatory support and mortality[33].

Liver injury, laboratory findings, and prognosis

The prognosis of the COVID-19 infection depends primarily on the patients' characteristics, present comorbidities, severity of clinical symptoms, laboratory parameters, and imaging features[34] (Figure 1). Liver injury occurs in patients with or without pre-existing liver disorders[24]. The incidence of liver injury manifesting as abnormal levels of liver enzymes ranges from 14.8% to 53.0%[18]. The degree of liver injury is generally mild, and those with digestive symptoms were more likely to present hepatocellular injury[3,35,36]. COVID-19 patients who develop liver injury are more likely to be men, older, and have a higher body mass index (BMI)[37]. Liver enzyme abnormalities are frequent in patients with COVID-19 infection, and they are associated with disease severity[18,38]. The most frequently reported mild to moderate elevations were in aspartate aminotransferase (ALT), alanine aminotransferase (AST), and total bilirubin (tBIL) levels[35], but abnormal gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), and albumin levels have been found in patients with COVID-19 as well[18,22,39]. Liver damage in COVID-19 is usually temporary, and therefore, the enzyme levels of most patients usually return to normal after recovery[18,40]. The systematic review and meta-analysis that included 36 studies and 20724 patients found a 46.9% prevalence of at least one abnormal liver function test, and elevated levels of ALT, AST, and tBIL were independent predictors of COVID-19 severity and in-hospital mortality[41]. A meta-analysis of observational studies revealed that acute liver injury and elevated liver enzymes in COVID-19 patients were significantly associated with disease severity[42]. A study conducted in Hong Kong reported that, ALT/AST elevation at two times the upper normal limit and acute liver injury in patients with COVID-19, were independently associated with poor prognosis, after controlling for diabetes mellitus, hypertension, and albumin level[43]. A systematic review that included 30 articles observed a significantly higher mortality in patients with impaired liver function than in patients with normal function[36]. Wagner *et al*[44] reported that hypoalbuminemia and abnormalities in liver function tests may be prognostic factors for higher COVID-19 severity. Although, there remains controversy in the scientific literature over whether or not liver enzyme abnormalities are associated with worse clinical outcomes, their alteration probably reflects the systemic involvement of the virus and the potential appearance of severe liver complications[45]. However, patients with severe COVID-19 may show a higher risk of post-COVID cholangiopathy, and liver tests in these patients continue to show abnormal results[46].

COVID-19 IN PATIENTS WITH PRE-EXISTING LIVER DISEASE

A number of studies have investigated the impact of CLD on the outcome of COVID-19[8]. COVID-19 patients with CLD account for less than 1% of the reported cases[47]. CLD includes different etiologies and can manifest from mild asymptomatic disease to severe decompensated cirrhosis, so it could be challenging to generalize results from different studies and countries[11,17]. In China, the main cirrhosis etiology was chronic hepatitis B virus (HBV)[48]. Patients with viral hepatitis, ALD, NFALD, liver cirrhosis, and HCC had a higher risk of developing severe COVID-19 and up to a 10-fold higher mortality rate compared to those without any reported comorbidity[49]. Data collected from 13 Asian



Demographic	Habits	Clinical	Laboratory	Chronic liver disease
Advanced age	Alcohol use	Severity of liver disease	Altered liver enzymes	Cirrhosis
Male gender	Smoking	Gastrointestinal symptoms		MAFLD/NAFLD
Higher BMI		Comorbidities		Alcohol-related liver disease
				HBV and HCV

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Figure 1 Prognostic factors in COVID-19 patients with liver disease. BMI: Body mass index; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; MAFLD: Metabolic-associated fatty liver disease.

countries identified that COVID-19 infection induced significant liver damage in CLD patients, and these patients had a higher risk of getting acute liver injury, hepatic decompensation, or acute-on-chronic liver failure (ACLF)[50]. A Danish prospective, population-based cohort study reported that patients with CLD, particularly those with cirrhosis, were at a major risk of severe COVID-19 outcomes and higher mortality[51]. A Swedish nationwide matched cohort study showed that patients with CLD had a higher risk of hospitalization for COVID-19 compared to the general population[52]. However, there was no evidence that these patients were at a higher risk of developing a severe COVID-19 disease course[52]. A study conducted in China reported that COVID-19 patients with CLD showed a prolonged length of stay, slight liver injuries, and higher mortality rates compared to COVID-19 patients without CLD, and that the neutrophil-to-lymphocyte ratio was an indicator of adverse clinical outcomes in this population[53]. A meta-analysis that included fifty studies revealed that pre-existing liver diseases or acute liver injury associated with severe COVID-19 infection were key factors in the prediction of mortality[54]. According to a study conducted in Massachusetts, United States, CLD in patients with COVID-19 was independently associated with higher rates of intensive care unit (ICU) admission, and a need for mechanical ventilation after controlling for comorbidities[12]. Krishnan *et al* [30] found that CLD patients with elevated AST and tBIL levels had a significantly higher risk fora more severe COVID-19 disease course and also reported that ALD was the most important factor associated with the need for mechanical ventilation. A systematic review including 40 studies, mainly from China, reported that CLD was significantly associated with COVID-19 severity and mortality[55]. The risk of getting more severe COVID-19 was 2.44 times higher among patients with CLD compared to those without CLD, and the presence of NAFLD was the most strongly associated with higher COVID-19 severity, followed by MAFLD and cirrhosis. In addition, COVID-19 patients with viral hepatitis were not at higher risk of getting a severe form of COVID-19[55]. After COVID-19 infection, approximately 20% of CLD patients develop progressive cholestasis, particularly patients with NAFLD/non-alcoholic steatohepatitis and metabolic risk factors[56].

COVID-19 and viral hepatitis

There is still insufficient evidence for an association between previous hepatitis B and C infection and COVID-19 outcome, and several studies indicated that these patients were not at increased risk for severe COVID-19[57-59]. Most studies that have examined the influence of HBV on COVID-19 prognosis have been conducted in China due to the high prevalence of HBV in the country[60]. Yu *et al* [61] reported higher in-hospital mortality, more severe disease, and liver function abnormalities in COVID-19 patients infected with HBV compared to COVID-19 patients without HBV. However, the presence of COVID-19 infection or treatment with tocilizumab or corticosteroids could reactivate hepatitis B infection[49,62]. A study conducted in the United States reported that chronic hepatitis C in COVID-19 patients was associated with in-hospital mortality regardless of baseline comorbidities, admission values of laboratory tests, or liver damage induced by COVID-19[63]. The Korean nationwide population-based cohort study reported that after adjusting for age, sex, cirrhosis, and comorbidities, HBV infection itself appears not to influence the prognosis of COVID-19 patients[64].

COVID-19 and NAFLD

The prevalence of NAFLD, or the recently renamed MAFLD, in COVID-19 patients is 31%, which is higher than the prevalence in the general population[65]. Patients with NAFLD had a higher risk of COVID-19 progression, a higher likelihood of liver dysfunction, and a longer viral shedding time than the patients without NAFLD[66]. Mahamid *et al*[16] found an independent association between the COVID-19 severity and NAFLD irrespective of the metabolic syndrome, indicating that NAFLD had a significant impact even in the absence of obesity and/or metabolic syndrome. A systematic review and meta-analysis showed that the proportion of patients with MAFLD and NAFLD ranged from 28% to 50% and from 6% to 38%, respectively, and found that the presence of MAFLD and NAFLD was associated with worse clinical outcomes for COVID-19[67]. Although several studies also showed significant associations between MAFLD and NAFLD and severe COVID-19 outcomes[13,68], there is still no strong evidence that the presence of MAFLD affects its prognosis[65].

COVID-19 and ALD

The COVID-19 pandemic probably had the biggest effect on patients with ALD due to substantially increased alcohol consumption provoked by adverse economic effects, disruptions in work and education, and social isolation[69]. Patients with alcohol use disorders are more likely to develop acute respiratory distress syndrome and have additional comorbidities such as metabolic syndrome, chronic kidney disease, and smoking, all of which are independent predictors of COVID-19 severity[69]. Several studies have identified that ALD is independently associated with COVID-19 mortality after adjustment for important cofactors such as liver disease severity[11,70].

COVID-19 and cirrhosis

COVID-19 patients with cirrhosis are at a greater risk of adverse outcomes than the background population[26,71,72]. Cirrhotic patients have significantly higher all-cause mortality in COVID-19 infection than non-cirrhotic patients, and mortality is probably higher in those with more advanced cirrhosis[8]. A significantly higher COVID-19 related morbidity and mortality had been observed in patients with decompensated cirrhosis compared to those with compensated cirrhosis[6]. Studies conducted in the United States and Europe reported that patients with CLD who had acquired COVID-19 had high rates of hospitalization and mortality[71,73,74]. Marjot *et al*[70] reported that patients with cirrhosis had a higher risk of dying from COVID-19 and that mortality was especially high among patients with more advanced cirrhosis and those with ALD. Hashemi *et al*[12] demonstrated that the presence of cirrhosis was independently associated with COVID-19-related mortality. Similar results were obtained from the United States study, reporting that the presence of decompensated cirrhosis was an independent predictor of mortality in COVID-19 patients[30]. Jeon *et al*[75] reported that the COVID-19 infection in patients with cirrhosis was more likely to cause severe complications in comparison with the cirrhotic patients not infected with COVID-19. Satapathy *et al*[76] found that the development of ACLF was the most important predictor of higher in-hospital mortality in COVID-19 patients with cirrhosis. However, it is still unknown whether the presence of liver disease influences the natural history of COVID-19 infection in cirrhotic patients[21].

COVID-19 and hepatocellular carcinoma

The presence of HCC in patients with CLD and COVID-19 infection was associated with a poor prognosis, including a higher risk of all-cause and COVID-19-related mortality[11]. Most HCC patients have concomitant cirrhosis, and that could potentially increase their risk for severe COVID-19[77]. An international, multicenter, retrospective, cross-sectional study, including two hundred fifty patients from 38 centers, reported that 18.4% of patients with HCC died within the first 30 d from the onset of COVID-19 symptoms, and that the mortality rate in that period was 20.25% in patients with HCC history and 12.96% in those with de novo HCC[78]. COVID-19 in HCC patients tends to be more severe and leads to exacerbation of the liver disease[79]. HCC patients infected with COVID-19 are at a higher risk of complications, ICU admission, and death than the patients without cancer[80].

COVID-19 and autoimmune liver disease

Autoimmune liver disease (AILD) includes primary sclerosing cholangitis, primary biliary cholangitis (PBC), autoimmune hepatitis (AIH), and overlapping syndromes referring to the coexistence of two autoimmune diseases[81]. COVID-19 outcomes in patients with AILD were investigated in international registry studies and retrospective case studies[82]. Combined data from three multinational registries showed that despite the use of immunosuppressive treatment, AIH patients did not seem to have a higher risk of lethal outcomes with COVID-19 compared to patients without liver disease and those with other forms of liver disease[83]. A retrospective study from 34 centers in Europe and the Americas indicated that patients with AIH did not have an increased risk for poor prognosis with COVID-19 than other causes of CLD and that cirrhosis was the most important predictor for high COVID-19 severity in this group of patients[84]. Zecher *et al*[85] indicated that patients with AILD were not at elevated risk for COVID-19. A Spanish nationwide study reported that cumulative incidences of hospitalization and COVID-19 related mortality were greater in patients with PBC than in the general Spanish population,

although the results were not adjusted for other comorbidities[86].

Severity and mortality of COVID-19 among CLD patients

Although the presence of COVID-19 infection in CLD patients is associated with a poor prognosis, including severity and mortality, these results should be interpreted with caution and need to be evaluated in large future studies. Such findings could be explained by overlapping risk factors, therapeutic effort limitations, different etiologies, and the disease spectrum of CLD, which ranges from mild asymptomatic disease to severe decompensated cirrhosis. Cirrhosis severity and older age are the most important predictors of mortality[21]. A French national retrospective cohort study found no increased COVID-19 severity in patients with CLD, alcohol use disorders, cirrhosis, or primary liver cancer, indicating that the COVID-19 outcome in these patients may be more associated with therapeutic effort such as mechanical ventilation and less with liver disease progression or ethanol toxicity[87]. This group of patients was at an elevated risk for mortality from COVID-19 within 30 d after admission but was less likely to need mechanical ventilation[87] in comparison with patients with mild liver diseases, compensated cirrhosis, chronic viral hepatitis, non-viral, non-alcoholic causes of CLD, acquired immunodeficiency syndrome, and liver transplantation, who were not at a higher risk of dying from COVID-19 but were more likely to receive mechanical ventilation[87]. As mentioned earlier, the Swedish nationwide cohort study also did not find an increased risk of getting severe COVID-19 in CLD patients, although they had an increased risk of hospitalization than the background population [52]. The pooled analysis of six studies found that CLD was not related to an elevated risk of a more severe COVID-19 disease course or mortality[88]. Similar results were reported from a nationwide Korean cohort study indicating that LC was not an independent predictor of severe complications, including mortality, in COVID-19 patients and depended on age, hypertension, cancer, chronic obstructive pulmonary disease, and a higher Charlson comorbidity index[75]. After adjustment for age, sex, BMI, cardiac disease, hypertension, diabetes, and respiratory disorders, CLD, and NAFLD were independently associated with ICU admission and the need for mechanical ventilation, but not death [12].

CONCLUSION

Due to the era of the COVID-19 pandemic and the large number of patients with liver disease, it is very important to study the impact of liver damage on the prognosis of patients with COVID-19 and the predictors that may affect the outcome. Identifying predictors of mortality could allow for risk stratification of patients and help improve healthcare delivery[11]. Therefore, it is necessary to improve the understanding of host genetics, behavior, and pre-existing comorbidities and adequately follow-up liver disease patients[17]. Patients with CLD, especially those with cirrhosis or advanced liver damage, should be prioritized for COVID-19 vaccination[89].

FOOTNOTES

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MINIREVIEWS

Bone loss in chronic liver diseases: Could healthy liver be a requirement for good bone health?

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Abstract

Given that the liver is involved in many metabolic mechanisms, it is not surprising that chronic liver disease (CLD) could have numerous complications. Secondary osteoporosis and increased bone fragility are frequently overlooked complications in CLD patients. Previous studies implied that up to one-third of these individuals meet diagnostic criteria for osteopenia or osteoporosis. Recent publications indicated that CLD-induced bone fragility depends on the etiology, duration, and stage of liver disease. Therefore, the increased fracture risk in CLD patients puts a severe socioeconomic burden on the health system and urgently requires more effective prevention, diagnosis, and treatment measures. The pathogenesis of CLD-induced bone loss is multifactorial and still insufficiently understood, especially considering the relative impact of increased bone resorption and reduced bone formation in these individuals. It is essential to note that inconsistent findings regarding bone mineral density measurement were previously reported in these individuals. Bone mineral density is widely used as the "golden standard" in the clinical assessment of bone fragility although it is not adequate to predict individual fracture risk. Therefore, microscale bone alterations (bone microstructure, mechanical properties, and cellular indices) were analyzed in CLD individuals. These studies further support the thesis that bone strength could be compromised in CLD individuals, implying that an individualized approach to fracture risk assessment and subsequent therapy is necessary for CLD patients. However, more well-designed studies are required to solve the bone fragility puzzle in CLD patients.

Key Words: Chronic liver disease; Fracture risk; Hepatic osteodystrophy; Osteoporosis; Bone strength

Core Tip: Secondary osteoporosis and increased bone fragility are frequently overlooked complications in patients with chronic liver disease (CLD). Recent publications agree that CLD-induced bone fragility depends on the etiology, duration, and stage of liver disease, but certain ambiguities are still present. Importantly, etiopathogenetic mechanisms leading to CLD-induced bone loss are still insufficiently clarified. Given that available clinical tools for fracture risk assessment are not entirely reliable, evaluating small-length structural bone properties could improve understanding of the multifactorial nature of bone fragility in CLD patients, which could set a base for the development of more effective preventive and therapeutic strategies.

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INTRODUCTION

The importance of a wide range of liver functions in the human body becomes the most visible in chronic liver disease (CLD). The most commonly known CLD complications are portal hypertension, hepatic encephalopathy, ascites, hepatorenal syndrome, variceal bleeding, and hepatocellular carcinoma [1,2]. However, CLD is also associated with changes in the skeleton, previously known as hepatic osteodystrophy[3,4]. Among CLD patients, substantial heterogeneity of skeletal changes was noted depending on the etiology, duration, and stage of the liver disorder[5,6]. Namely, osteoporosis was initially described as a complication of primary biliary cholangitis and primary biliary cirrhosis (cholestatic liver diseases)[7], while skeletal changes were later described in other (non-cholestatic) hepatic disorders as well[8,9]. It has been reported that approximately every second patient with viral hepatitis, hemochromatosis, and Wilson's disease has osteoporosis or osteopenia[10-12], while up to 55% of patients with alcoholic liver cirrhosis have osteoporotic bone changes[3,13,14]. Interestingly, bone alterations in nonalcoholic fatty liver disease or nonalcoholic steatohepatitis have recently drawn researchers' attention, revealing that up to one-third of these individuals could develop bone alterations [15,16].

Consequently, CLD individuals are at substantial risk for non-traumatic bone fractures[17-19], with a prevalence between 7% and 35%[20]. Recent data suggest that fracture incidence is two to three times higher in end-stage CLD patients compared to healthy controls[19,21], while others reported an eight-fold increase in the risk of bone fractures in these patients[22]. Regarding fracture localization, data suggest that vertebral fractures are most common in patients with end-stage CLD[19,23-26], given that more than one-third of these individuals experienced at least one vertebral fracture during their lifetime [8,23,27]. Moreover, CLD contributes to the age-associated increase in the risk of femoral fracture and subsequently its life-threatening complications[22]. It is important to emphasize that end-stage CLD patients are experiencing fragility fractures at a significantly younger age than most osteoporotic patients[22], considering that the cumulative fracture risk in CLD patients younger than 45 years corresponds to the risk of healthy controls over 75 years of age[22]. It is important to emphasize that CLD likely changes the sex distribution of fracture risk in the aged population, considering that CLD is more frequent in male patients[28], while osteoporosis and osteoporosis-related bone fractures are more likely to develop in older women[29].

Despite the significant number of studies that have assessed various characteristics of bone deterioration in CLD individuals, many unknowns should be elucidated to understand this topic entirely.

OSTEODENSITOMETRY FINDINGS IN CLD PATIENTS

Most studies dealing with bone changes in CLD patients used dual-energy X-ray absorptiometry as the most valuable tool in the clinical assessment of fracture risk[30]. Interestingly, opposite results were yielded. Namely, dual-energy X-ray absorptiometry assessment revealed significantly lower bone mineral density (BMD) in patients with viral, autoimmune, and primary biliary cirrhosis[31-33]. At the same time, other authors failed to show a significant BMD decrease in CLD of the same etiology[34,35]. Multiple studies showed reduced dual-energy X-ray absorptiometry-obtained BMD values, suggesting osteopenia or osteoporotic changes of the lumbar spine and femoral neck in patients with alcohol-induced CLDs[36-38], while other research teams failed to show these bone alterations in individuals prone to chronic alcohol abuse[17,39,40]. Given that the primary source of these contradictory data could be in the study design (cross-sectional study design), selection criteria, and the number of participants included in the study, future well-designed prospective studies are required to fully

understand BMD alterations in CLD patients.

BONE TURNOVER BIOMARKERS IN PATIENTS WITH CLD

As a non-invasive and cost-effective tool for indirect assessment of bone remodeling dynamics, bone turnover biomarkers (BTMs) are a complementary method in the clinical management and follow-up of the treatment effects in patients with osteoporosis and osteoporosis-related bone fragility[41]. Automated or manual immunoassays using blood or urine samples are utilized to measure a specific combination of these protein or protein-derivative biomarkers[42], which are considered indicative of the dynamic relationship between osteoblast activity (bone formation markers) and osteoclast activity (bone resorption markers)[41,43]. The most frequently investigated bone formation markers are osteocalcin, bone alkaline phosphatase, and N-propeptide of type I collagen[41]. On the other side, commonly interpreted bone resorption markers are C-terminal and N-terminal telopeptides of type I collagen, deoxypyridinoline, and tartrate-resistant acid phosphatase isoform 5b[41] (Figure 1).

The interpretation of BTM levels has been of clinical utility in age-related osteoporosis[43], while its role in the clinical management of CLD-induced bone loss is still modest. Some data suggest that serum levels of osteocalcin and bone alkaline phosphatase are decreased in individuals with CLD[25,36,44], while others failed to show significant differences between individuals with CLD and the control group [45,46]. Moreover, contradictory data regarding the level of β-CTX and deoxypyridinoline were noted in CLD patients[36,45,47,48]. It is important to note that liver dysfunction could affect serum concentrations of BTMs, which reveals excessive bone matrix degradation, indicating that its assessment allows only limited conclusions in CLD individuals[10,49]. Multiple limitations of BTM assessment are among the reasons why CLD-induced bone changes are recognized and treated after a patient experiences non-traumatic fracture[10], suggesting that further investigation is required to elucidate the role of BTMs in developing novel, adequate preventive and treatment strategies.

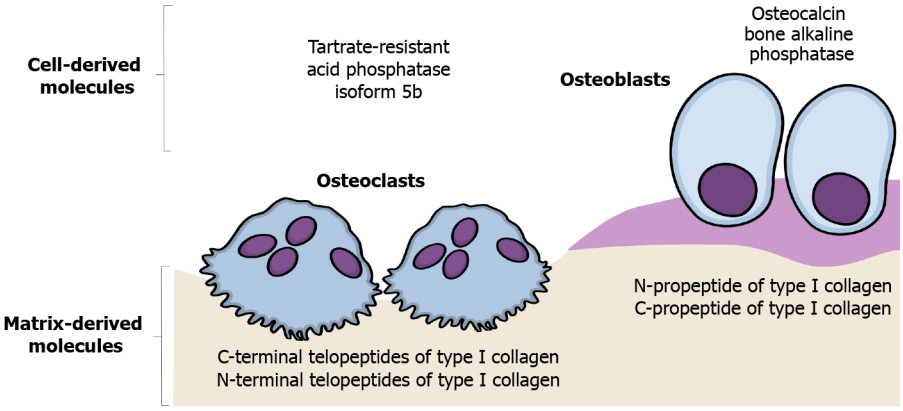
ASSESSMENTS OF MICROSCALE BONE PROPERTIES IN CLD INDIVIDUALS

The World Health Organization recommended BMD as the primary parameter for the diagnosis of osteopenia and osteoporosis and for clinical fracture risk assessment[50]. However, considering that the occurrence of fragility fractures primarily requires the action of several bone strength determinants and their mutual interaction, it is evident that increased bone fragility could not be solely explained by BMD decrease[51,52]. In other words, low BMD should only be considered an applicable and non-invasive clinical surrogate marker of bone fragility[52,53]. Namely, it has been known that only up to one-third of non-traumatic fractures are attributable to low BMD values, indicating that many individuals with bone fractures have BMD in the referent range[54].

Moreover, various bone properties are recognized as important determinants affecting bone strength (ability to resist fracture)[55]. Thus, current studies suggested that multiscale analysis of various bone properties (with respect to the hierarchical structure of the bone, Figure 2) could contribute to a better understanding of increased bone fragility in elderly individuals with chronic comorbidities, including a variety of CLDs[56]. The importance of assessing these bone properties is highlighted by the fact that some pharmaceutical agents were proven to improve bone strength and reduce fracture risk without increasing BMD[57,58], indicating the potential for developing new and effective treatment strategies [52].

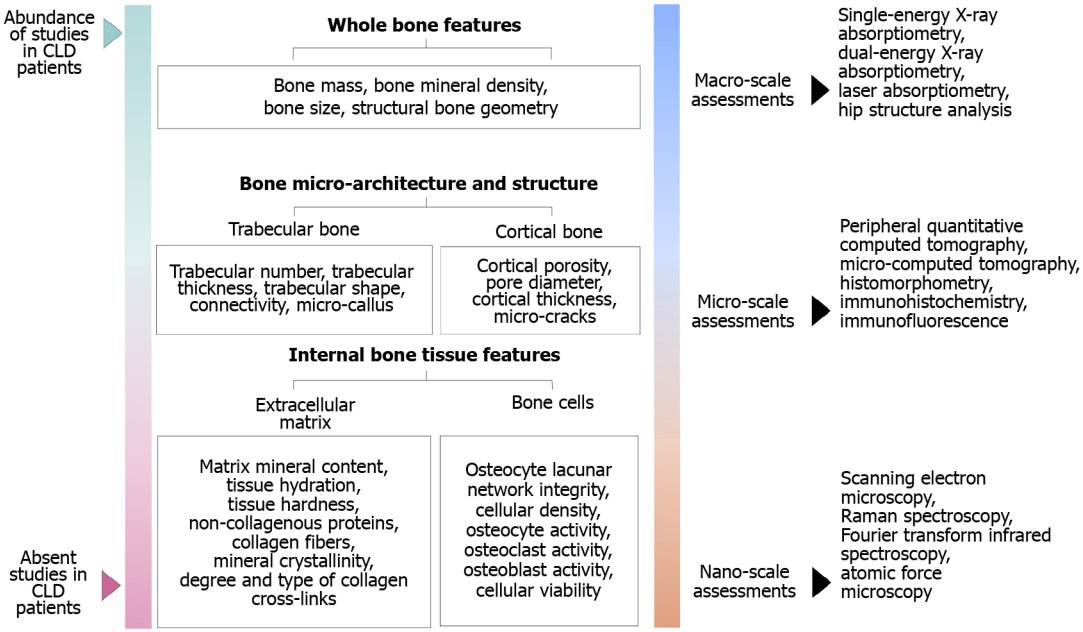
Initially, histomorphometry studies using optic microscopy assessment of iliac bone biopsies showed deteriorated trabecular bone architecture in CLD patients[59,60]. In addition, some novel clinical studies confirmed these results on the tibia and radius of CLD patients, using a newer methodology called peripheral quantitative computed tomography[33,61,62]. Since osteoporosis is not uniform throughout the skeleton[63] it was crucial to assess CLD-induced microstructural decline in lumbar vertebrae and proximal femora[38,64]. Similarly to previous findings, our research group used microcomputed tomography with an isotropic resolution of 10 μm to observe impaired microarchitectural integrity of lumbar vertebrae and proximal femora collected from CLD individuals[9,38,64]. On the trace of altered trabecular and cortical microarchitecture, we demonstrated reduced mechanical bone competence in these individuals[38,65], indicating that altered bone matrix content could be involved in CLD-induced bone fragility.

Future state-of-the-art studies should focus on a precise nanoscale morphostructural estimate of the inorganic (mineral) and organic component of the bone extracellular matrix (collagen fibers) to elucidate its role in increased bone fragility among CLD individuals (Figure 2). Finally, the long-term benefit of small-length bone studies could develop a specific diagnostic algorithm that will help to reliably predict bone strength based on the information available in the clinical context of each patient.



DOI: 10.3748/wjg.v29.i5.825

Figure 1 Schematic representation of the most frequently analyzed bone turnover markers. The emphasis is placed on the difference between bone turnover markers released by catabolic osteoclast activity (bone resorption markers) and anabolic osteoblast activity (bone formation markers).



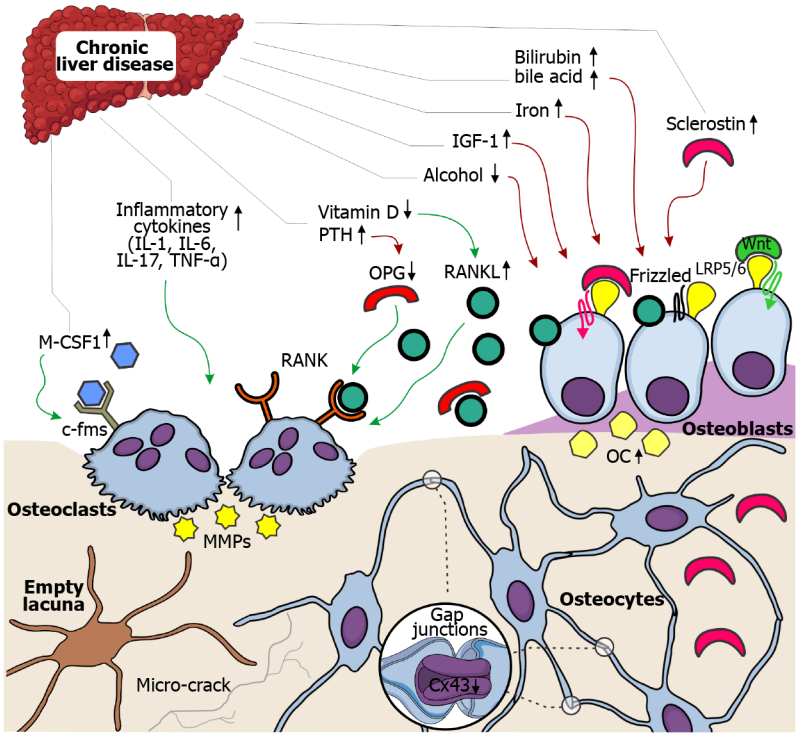
DOI: 10.3748/wjg.v29.i5.825

Figure 2 Multiscale approach in the assessment of bone strength determinants. The importance of the various bone properties that contributes to increased bone fragility, and up-to-date methodologies are used to assess these bone strength determinants. The emphasis is placed on the difference between factors that were previously assessed and those factors that require further investigation in patients with chronic liver disease. CLD: Chronic liver disease.

THE MOLECULAR MECHANISMS INVOLVED IN ETIOPATHOGENESIS OF CLD-INDUCED BONE LOSS

Bone loss in CLD patients is commonly described as a consequence of bone remodeling disturbance[8], but the particular contribution of increased bone resorption and decreased bone formation still needs to be thoroughly explained. Nowadays, a common understanding is that the etiopathogenetic mechanisms of bone loss are dependable on the etiology of liver disease[3,8]. Previous data revealed that osteoblast dysfunction and decreased bone formation play a central role in the etiopathogenesis of bone loss in patients with cholestatic liver disease, Wilson's disease, and hemochromatosis[7,12,48,66]. Conversely, viral CLD displays a more dominant effect on increased osteoclast activity, inducing high-turnover osteoporosis[21,32,67].

On a molecular level, low-turnover osteoporosis in CLD patients is commonly associated with toxic effects of biliary stasis and copper/iron accumulation on differentiation, maturation, and proliferation of osteoblasts (Figure 3)[68-70]. Also, previous studies suggested that osteoblast dysfunction in patients with cholestatic forms of CLD could be mediated by insulin growth factor-1 or oncofetal fibronectin[66,70,71], while direct toxic effects of alcohol on osteoblastic function contribute to bone loss among patients within alcohol-induced CLD[72,73]. During the process of bone formation, osteoblasts become



DOI: 10.3748/wjg.v29.i5.825

Figure 3 Schematic representation of possible pathophysiological mechanisms leading to bone loss in chronic liver disease patients. The role of multiple factors leading to bone loss and osteoporosis in individuals with chronic liver disease places an emphasis on the difference between factors that cause osteoblast dysfunction (reduced bone formation) and factors that stimulate osteoclast activity (increased bone resorption). Green arrows indicate an activating effect, while red arrows indicate a deactivating effect. c-fms: Colony-stimulating factor-1 receptor; Cx43: Connexin 43; IGF-1: Insulin-like growth factor 1; IL: Interleukin; LRP5/6: Low-density lipoprotein receptor-related protein 5/6; M-CSF1: Macrophage colony-stimulating factor 1; MMPs: Matrix metalloproteinases; OC: Osteocalcin; OPG: Osteoprotegerin; PTH: Parathyroid hormone; RANK: Receptor activator for nuclear factor kappa B; RANKL: Receptor activator for nuclear factor kappa B ligand; TNF: Tumor necrosis factor.

embedded within the bone matrix, continuing to function as bone remodeling orchestrators or osteocytes[74]. Osteocytes form a global network throughout the bone tissue by intercellular channels (gap junctions), most frequently formed by connexin 43[75]. Reduction in osteocytic expression levels of connexin 43 and minor disruptions in the osteocyte lacunar network was noted in CLD individuals (Figure 3), suggesting that the mechanosensing potential and molecular transduction might be defective in those patients with CLD[65,76]. In addition, increased bone expression levels of sclerostin (an osteocyte-derived negative regulator of bone formation) were noted in CLD individuals[65,76], which was in accordance with previous clinical studies[77,78]. These data indicate that treatment targeting sclerostin may be an interesting strategy to fight osteoporosis in CLD patients[10]. Still, possible therapeutical utilities in CLD patients are yet to be thoroughly investigated in the years ahead.

Previous studies revealed that bone loss in CLD individuals could be explained by a strong link between systemic hyperproduction of inflammatory mediators and increased bone resorption (Figure 3)[21,32,67]. Most commonly, it is understood that tumor necrosis factor-α, interleukin (IL)-1, IL-6, IL-7, IL-11, IL-13, IL-15, and IL-17, produced by immune cells, could directly activate osteoclast precursors or display an indirect effect by osteoblasts[8,10,72]. Namely, increased secretion of receptor activator for nuclear factor kappa B ligand (RANKL), the disturbed ratio between RANKL and osteoprotegerin, matrix metalloproteinases activity, and cathepsin K are described as contributing factors in CLD-induced bone loss *via* increased bone resorption (Figure 3)[10,79-81]. The recent recommendation for therapy targeting RANKL advocates the importance of the RANK-RANKL-osteoprotegerin system in bone loss among CLD patients[20,82]. In addition, increased circulating macrophage colony-stimulating factor 1 in CLD patients could promote bone resorption due to its role in priming a larger number of monocytes to form osteoclasts in these patients[6].

Lastly, low vitamin D levels, unbalanced diet (low calcium and protein intake), malabsorption, disruption in the homeostasis of the intestinal microbiome, coupled with a variety of hormonal and metabolic disruptions (such as increased levels of parathyroid hormone, hypogonadism, and hypercorticism) were identified as factors that contribute to bone loss in CLD individuals[20,72,83]. Based on these data, new nutritional support guidelines were recently introduced by the European Association for the Study of the Liver[20,84]. However, given that bone changes in CLD patients are undoubtedly present, it is vital to further investigate more direct etiopathogenetic mechanisms involved in the relationship between liver and bone disorders.

CONCLUSION

Bone alterations are a common complication in patients with CLD, especially in those with liver cirrhosis. Over the previous period, numerous studies have contributed to understanding bone fragility in CLD patients. However, numerous ambiguities are still present due to the modest reliability of clinical diagnostic methods, which could lead clinicians to doubt whether or when it is necessary to start treating CLD-induced skeletal alterations. Thus, evaluating small-length structural bone properties could improve understanding of the multifactorial nature of bone fragility in CLD patients. All these data could set a base for developing a patient-specific diagnostic algorithm that will reliably predict bone strength based on the information available in a clinical context. Additionally, specific clinical guidelines for preventing, diagnosing, and treating skeletal disorders in patients with CLD need to be established in the near future.

FOOTNOTES

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MINIREVIEWS

Liver involvement in patients with COVID-19 infection: A comprehensive overview of diagnostic imaging features

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Abstract

During the first wave of the pandemic, coronavirus disease 2019 (COVID-19) infection has been considered mainly as a pulmonary infection. However, different clinical and radiological manifestations were observed over time, including involvement of abdominal organs. Nowadays, the liver is considered one of the main affected abdominal organs. Hepatic involvement may be caused by either a direct damage by the virus or an indirect damage related to COVID-19 induced thrombosis or to the use of different drugs. After clinical assessment, radiology plays a key role in the evaluation of liver involvement. Ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI) may be used to evaluate liver involvement. US is widely available and it is

considered the first-line technique to assess liver involvement in COVID-19 infection, in particular liver steatosis and portal-vein thrombosis. CT and MRI are used as second- and third-line techniques, respectively, considering their higher sensitivity and specificity compared to US for assessment of both parenchyma and vascularization. This review aims to the spectrum of COVID-19 liver involvement and the most common imaging features of COVID-19 liver damage.

Key Words: Liver; Fatty liver; Hepatomegaly; Hepatic infarction; Liver diseases; Liver failure; Biliary tract diseases; COVID-19; SARS-CoV-2; Infection; X-Ray computed tomography; Magnetic resonance imaging; Ultrasonography; Adults; Pediatrics

Core Tip: Coronavirus disease 2019 (COVID-19) infection has an impact not only on lung involvement but also in other systems, in particular the gastrointestinal one, with a special focus on the liver. Hepatocytes express the receptor of angiotensin-converting enzyme which is the main door of the entrance of severe acute respiratory syndrome coronavirus 2. Consequently, different mechanisms can lead to different hepatic scenarios, such as hepatomegaly, steatosis, steatohepatitis, and drug-induced liver injury. As for lung involvement, the infection can lead to hepatic vascular involvement, especially portal vein thrombosis. Finally, it has been demonstrated a possible biliary involvement in COVID-19 patients.

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, named coronavirus disease 2019 (COVID-19), represents an epoch-making global healthcare crisis, with 603711760 confirmed cases and 6484136 deaths caused to date worldwide[1].

Although the lung represents the most affected organ, COVID-19 may present as a multiorgan disease. Clinical manifestations may vary from flu-like symptoms, such as fever, dry cough, myalgia, and fatigue, often coupled with hypo-/anosmia and ageusia[2-4], to more severe conditions with dyspnea and respiratory impairment requiring admission to the intensive care unit (ICU) and advanced respiratory assistance[5]. A severe course of the disease has been reported in 5%-22 % of COVID-19 patients[3,5].

In this scenario, we focused our attention on hepatic manifestations of COVID-19 infection. Hepatic involvement in patients with COVID-19 infection is not negligible. Liver damage can occur in different ways, ranging from hepatomegaly, acute hepatitis, steatosis and steatohepatitis, portal vein thrombosis (PVT) and liver infarction, biliary and gallbladder involvement, up to drug-induced liver injury (DILI), with chronic liver disease that needs further long-term studies to be understood (Figure 1).

In this review, we aim to describe the spectrum of COVID-19 liver involvement and the most common imaging features of COVID-19 liver damage with a descriptive correlation to the underlying pathogenesis.

IMAGING TECHNIQUES

The standard radiological approach for liver assessment (*i.e.* anatomy, focal liver lesions, or diffuse diseases) has been widely described and does acknowledge the use of ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI). Technical advances in liver imaging have also been conducted over the last decades, with lots of research on quantitative and functional assessments in different liver pathologies[6].

US

Brightness-mode (B-mode) transabdominal US generally represents the first-line approach in patients with suspected liver disease[7]. US is widely available, non-invasive, low cost, safe, ionizing radiation-free[6] and can be performed at the bedside, particularly in ICU or isolated patients. Anatomical and

vascular imaging and lesion detection are feasible, although limited by the field of view and are dependent on operator experience[7].

During the last decades, different US developments have been introduced, including elastography, contrast-enhanced US (CEUS), and novel doppler techniques[6-9].

Moreover, an advanced multiparametric US approach for the evaluation of the liver could be an option, particularly for long-term follow-up of COVID-19 patients. The multiparametric US includes elastography, share wave dispersion, and attenuation imaging. The evaluation by the 2D-shear wave elastography technique allows quantification of the increase in liver stiffness related to the evolution toward fibrosis, but it can be altered in the inflammatory early stages (*e.g.*, steatohepatitis)[10]. Shear-wave dispersion is a measure of liver viscosity that is changed during inflammatory processes in the liver. Finally, hepatic attenuation imaging is a useful tool for quantifying steatosis.

Elastography enables the assessment of liver fibrosis[6]. Usually, a quantitative assessment of liver stiffness is obtained by applying an external force by means either of a US-induced focused impulse (point shear wave elastography) or a mechanically induced impulse (transient elastography)[7]. More recent developments of US elastography include a volumetric assessment of liver stiffness and its real-time variations. Clinical use of US elastography is mainly limited by cutoff values for fibrosis staging that vary across US systems from different vendors[6].

CEUS is accepted as a second-line imaging modality for the characterization of focal liver lesions after inconclusive baseline US, and its cost-effectiveness is higher compared to CT or MRI[6,7,11]. CEUS interpretation is similar to CT and MRI, relying on the similar post-contrast phases (arterial, portal-venous, delayed), vascular architecture, and phase-specific enhancement of the lesion compared with the adjacent liver parenchyma[7]. CEUS is useful for lesion detection and characterization in several clinical settings, without the use of ionizing radiations and with higher temporal resolution compared to CT or MRI[6]. It can be useful in non-oncologic, non-cirrhotic patients[6,9], for the assessment of incidental focal lesions, in cirrhotic patients, allowing characterization of contrast enhancement patterns of hepatocellular carcinoma (HCC) with good sensitivity and specificity[6], and in oncologic patients, providing higher sensitivity compared to the standard US for liver metastases detection and indeterminate CT or MRI lesions characterization[12]. CEUS can also be used to guide, in real-time, both focal lesions procedures[7,8] and locoregional ablative therapies, as well as for treatment response assessment[13,14]. According to the latest guidelines[14], US contrast agents can be safely administered in various applications, with minimal risks to patients. The reported rate of anaphylactoid-type reactions is extremely rare (0.014%).

Among novel third-generation doppler developments, US manufacturers have introduced techniques such as superb microvascular imaging (SMI) that has improved the sensitivity and accuracy of Doppler US in the assessment of hepatic vascular anatomy[9] and the detection of liver tumors vascularity with a safe, inexpensive, and readily available modality[6]. SMI is based on an adaptive algorithm that separates low flow signals from overlaying tissue motion artifacts; thereby SMI allows visualization of microscopic vessels (either native or within lesions), with no need for contrast agents injection[9].

CT

CT represents the mainstay technique for liver imaging, with the majority of acquisitions performed with multiphase acquisition protocols, and standardized assessment based on size and density measurements. Due to its wide availability, CT is generally preferred to MRI in daily clinical practice, despite its overall lower sensitivity. Moreover, reproducibility and high temporal and spatial resolutions allow its employment in both standard and emergency settings[6,15-17].

Contrast-enhanced CT allows the characterization of liver lesions deemed indeterminate on US in non-oncologic and non-cirrhotic patients. In cirrhosis, while the US remains the standard technique for follow-up, contrast-enhanced CT and MRI are the currently recommended techniques for the characterization of US-detected nodules, diagnosis and post-treatment follow-up of HCC[6,18]. In oncologic patients, CT is generally used for staging and follow-up. Accurate timing of image acquisition during the various dynamic phases is critical to enable a correct determination of liver lesion characteristics and enhancement features[11]. In these scenarios, CT acquired during the portal-venous phase is the most common performed study in oncologic patients[6], but its main limitation is the detection and characterization of small hypoattenuating lesions and lesion detection in the background of liver steatosis[6,19]. On the other hand, multiphase scans (arterial, portal-venous, and delayed phases) are generally used in cirrhotic patients, for focal liver lesions characterization, and in trauma patients[6,8].

More advanced and emerging techniques include perfusion CT, dual-energy CT (DECT) and photon-counting detector CT (PCD-CT)[6,17].

DECT is based on CT data acquisition by using X-rays generated at two different energy spectra; therefore, allowing for superior materials discrimination and characterization. Images are obtained either with dual-source, ultra-fast kV switching, or sandwich detector[6,16]. Then, DECT data post-processing generates several types of images: monochromatic image reconstructions, useful to improve iodine contrast visualization; attenuation maps of different elements according to their atomic number, including iodine, calcium, and water[6]. Moreover, the possibility of generating virtual unenhanced (VUE) images may help reduce radiation dose exposure. DECT improves the delineation of hypo- and hypervascular liver lesions by increasing the lesion to parenchyma contrast. Given the possibility of

material decomposition, DECT can also allow distinguishing contrast from calcifications, and noninvasively quantifying fat, iron, and other moieties, compensating for the high cost and examination time length of MRI and invasiveness of biopsy[6,16,17]. However, DECT is affected by some shortcomings, including technical limitations (limited field-of-view, reduced spectral separation depending on vendor or scanner) and software challenges (lack of enough research comparing vendors and scanners' variability on VUE and iodine attenuation values)[16].

PCD-CT is the most recent promising technique but nowadays is still mainly limited to preclinical or small *in vivo* studies in volunteers. More clinical and validation research is therefore needed over a longer time[6].

MRI

MRI is fundamental in the workup of patients with liver disease[6,17,20] and has been addressed as the preferred imaging modality for the characterization of equivocal focal lesions detected by other imaging modalities[17]. Along with appropriate clinical information, MRI can also allow a definitive diagnosis, avoiding in most cases invasive procedures such as biopsy[21].

A properly dedicated MRI liver protocol requires it to be short, comprehensive, standardized, and reproducible[6,17,21]. Pre-contrast MRI, given its higher contrast resolution compared to CT, provides information about tissue and lesion composition (*i.e.* solid or liquid; iron, fat, glycogen, blood products) and lesions cellularity, either neoplastic or inflammatory[6,11,17,22]. Diffusion-weighted imaging (DWI) has been reported to improve the detection and characterization of focal liver lesions, also allowing differentiation of cysts from solid masses. Moreover, by combining hyperintensity on high b-value DWI (hypercellular lesions) with dynamic multiphasic studies, improvement in lesions detection and characterization (in particular for tumors < 2 cm) can be achieved[11].

Contrast-enhanced MRI represents a relevant component of any liver MRI protocol. It provides reliable information about focal lesions characterization, vascular and biliary anatomy, and more recently organ function[17,20,21]. Both gadolinium-based extracellular (ECA) and hepatobiliary (HBA) contrast agents can be used for multiphase imaging[6,17,21]. Morphologic and vascular-related information are obtained with ECA and HBA through the dynamic study[21].

Moreover, HBA provides the ability to acquire images in the hepatobiliary phase (HBP), offering information about hepatocytes uptake and excretion in the biliary system[6,11,20,21]. Therefore, HBP may provide functional information[21]. Indeed, lesions, or abnormalities without hepatocytes or with non-functioning hepatocytes, appear as hypointense compared to the surrounding liver parenchyma [20]. Among reported HBA advantages, it is worth mentioning higher lesion conspicuity with increased sensitivity in lesion detection, and improved lesion characterization with increased ability in the differential diagnosis.

One of the most used advanced MRI techniques useful to detect and characterize focal or diffuse liver disease is DWI. Highly cellular tissues or those with cellular swelling exhibit lower diffusion coefficients, and these aspects can be useful for the evaluation of liver diseases[22].

The evaluation of the biliary tree can be easily made using highly weighted T2 sequences in different planes. In this setting, magnetic resonance cholangiopancreatography (MRCP) is nowadays considered the reference standard for noninvasive biliary evaluation. Thanks to the improvement of MRI techniques, it is now possible to acquire 3D images that can be reformatted in every plane of space by post-processing techniques[23].

LIVER DISEASE INVOLVEMENT

COVID-19 liver injury is defined as any liver involvement that occurs during the course of COVID-19, whether there is a known history of liver disease or not[24]. The presence of liver damage from a laboratory point of view is very common: an increase in liver enzymes is described in around 40% of patients[24], it is greater with severe COVID-19 and at the same time a predictor of adverse events[25,26].

SARS-CoV-2 primarily infects respiratory epithelial cells *via* angiotensin-converting enzyme 2 (ACE2). ACE2 is also expressed at high levels in the endothelium layer of tiny blood arteries, cholangiocytes and in hepatocytes. Furthermore, the SARS-CoV-2 virus may use the gut-liver route *via* the hepatic reticular system to reach the liver[25]. Finally, other organ systems and drugs have a significant influence on the liver. As a result, the causative mechanisms of liver damage in COVID-19 infection are many, including direct cytotoxicity caused by active SARS-CoV-2 replication, immune-mediated liver injury, vascular impairment caused by coagulopathy, endothelium, or cardiac congestion, hypoxic changes caused by respiratory failure, DILI, and exacerbation of the underlying chronic liver disease[25] (Figure 1 and Table 1).

Hepatomegaly and steatosis

COVID-19 causes twice as much liver damage at the cellular level. First, hepatocellular damage occurs, resulting in mild steatosis, lobular and portal inflammation, and areas of apoptosis and necrosis. This

Table 1 Summary of the most common findings in liver involvement due to coronavirus disease 2019 infection

Liver involvement	Ultrasound	Computed tomography	Magnetic resonance
Hepatomegaly	Qualitative criteria: The right lobe extends inferiorly over the lower pole of the right kidney; Rounding of the hepatic inferior border Quantitative criterion: Length of the right liver lobe > 16.5 cm		
Steatosis	Hyperechoic liver in comparison to the spleen or neighboring kidney; Absence of the normal echogenic walls of the portal and hepatic veins; Poor visualization of deep portions of the liver	Unenhanced: Relative hypoattenuation: liver attenuation more than 10 HU less than that of spleen; Absolute low attenuation: liver attenuation lower than 40 HU; C+: Does not add information	IP/OOP imaging: Signal drop out on OOP; T2W: Isointense; T1: Isointense; T1 C+: Does not add significant information
Acute hepatitis	Hepatomegaly; Reduced echogenicity; Steatosis; Peri-portal edema; Reduced Doppler signal in the hepatic artery; Thickening of the gallbladder wall	Hepatomegaly; Homogeneous/heterogeneous hypoattenuation (steatosis); Peri-portal edema; Thickening of the gallbladder wall; Periportal/hepatoduodenal enlarged nodes	IP/OOP imaging: Steatosis can be present; T2W: Diffuse mild increase in signal; Increased signal around the portal system (periportal edema); T1 C+: Periportal enhancement; Thickening of the gallbladder wall; Hilar enlarged nodes
DILI	Nonspecific findings: Hepatomegaly, steatosis, and peri-portal edema can be present		
Portal vein thrombosis	Absent or reduced flow in the portal vein on Color Doppler; Presence of heterogeneous material (focal or diffuse) in the portal vein lumen	Unenhanced: Higher attenuation into the portal vein lumen; Dilation of the portal trunk; C+: Hypoattenuating material into the lumen; Enhancement of vein walls	T2W: Iso- to hyperintense clot according to the phase (acute or subacute); T1: Hyperintense clot; T1 C+: Hypointense material into the lumen; Enhancement of vein walls
Biliary involvement	Focal or diffuse bile duct dilatation (with/without intrahepatic or extrahepatic stones)	Unenhanced: Does not add information; C+: Focal or diffuse bile duct dilatation (with/without intrahepatic or extrahepatic stones); Heterogeneous enhancement of parenchyma; Peri-portal edema	MRCP: Focal or diffuse bile duct dilatation with/without intrahepatic or extrahepatic stones; Stone(s) in the biliary lumen (hypointense); Multifocal biliary strictures alternated with dilated tracts (beaded appearance); T2W: Hypointense stone; Increased T2 signal around the portal system (periportal edema); T1: Hyperintense stone; T1 C+: Heterogeneous enhancement of peri-biliary parenchyma
Acute cholecystitis	Gallbladder wall thickening (> 3 mm); Pericholecystic fluid; Gallbladder distension; Possible sludge	Unenhanced: Gallbladder distension; Possible sludge (hyperattenuating); Pericholecystic fluid; C+: Gallbladder wall thickening (> 3 mm); Inhomogeneous gallbladder wall; Mural or mucosal hyperenhancement	MRCP: May show an impacted stone in the gallbladder neck or cystic duct; T2W: Gallbladder wall thickening (> 3 mm); Inhomogeneous gallbladder wall due to edema; T1: Sludge (hyperattenuating); T1 C+: Inhomogeneous gallbladder wall; Mural or mucosal hyperenhancement

HU: Hounsfield unit; IP: In-phase; OOP: Out-of-phase; C+: Contrast-enhanced; DILI: Drug-induced liver injury; MRCP: Magnetic resonance cholangiopancreatography.

type of damage raises aspartate aminotransferase (AST) and alanine transaminase (ALT) levels. Later on, the damage is direct to cholangiocytes, with bile duct damage and increase in gamma-glutamyl transferase (GGT) and bilirubin[27].

When there is a clinical suspicion of liver involvement in COVID-19, bedside US is the first imaging technique used in the diagnostic workup. A quick and targeted bedside US may be critical in referring selected patients to second-level imaging techniques, to reduce unnecessary exams and diagnostic delays. US can detect morphological or structural changes in the liver: the most encountered findings in COVID-19 patients are hepatomegaly[28,29] and steatosis[29,30].

According to Abdelmohsen *et al*[28], the most common morphological change in the liver in critically ill COVID-19 patients is hepatomegaly (about 55% of patients), which is also consistent with autopsies in COVID-19 patients[29-31]. Spogis *et al*[29] found hepatomegaly associated with gallbladder wall thickening and decreased echogenicity (*i.e.* signs of acute hepatitis) in 33% of COVID-19 patients with elevated liver cytolysis indices (> 10-fold). Hepatomegaly is usually identified subjectively during imaging: the "qualitative criteria" include the inferior extension of the right lobe to the lower pole of the right kidney and the rounding of the hepatic inferior border. Otherwise, the quantitative criteria are based on the length of the right liver lobe, with a cutoff of 16.5 cm[31].

On the other side, the most common liver structural change associated with COVID-19 is the presence of hepatic steatosis[28,29] (Figure 2). Coagulation activation can produce hepatic steatosis, and this could be a unique mechanism that leads to both thrombosis and steatosis that are common findings in COVID-19 patients[32]. US B-mode is the most used technique for diagnosing and classifying hepatic steatosis[33], particularly in the moderate or severely affected liver. It has an overall sensitivity and specificity of 85% and 93%, respectively[33]. However, the detection of a moderate degree of steatosis remains poor, with about 60% sensitivity[34]. B-mode US imaging is mostly used to analyze the liver

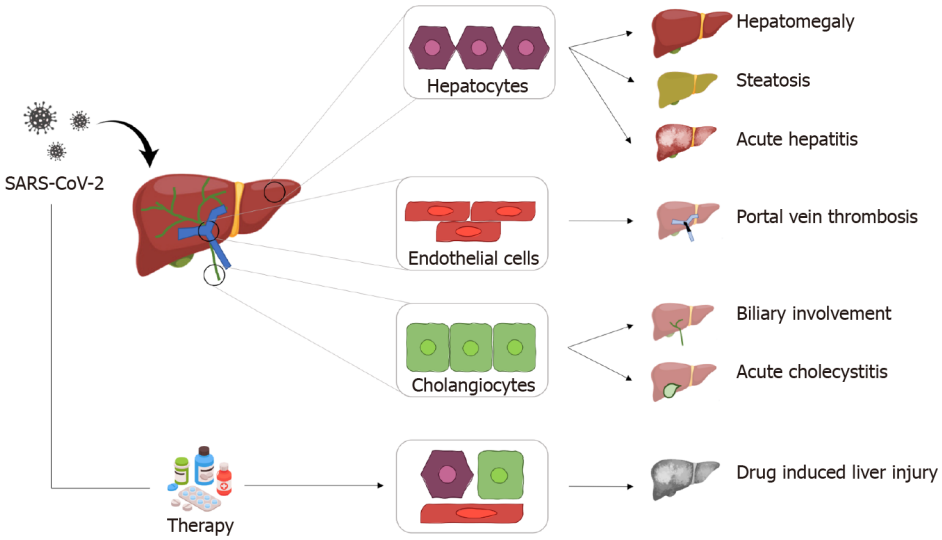

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Figure 1 Graphical summary of the most common hepatic pathological findings in coronavirus disease 2019. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

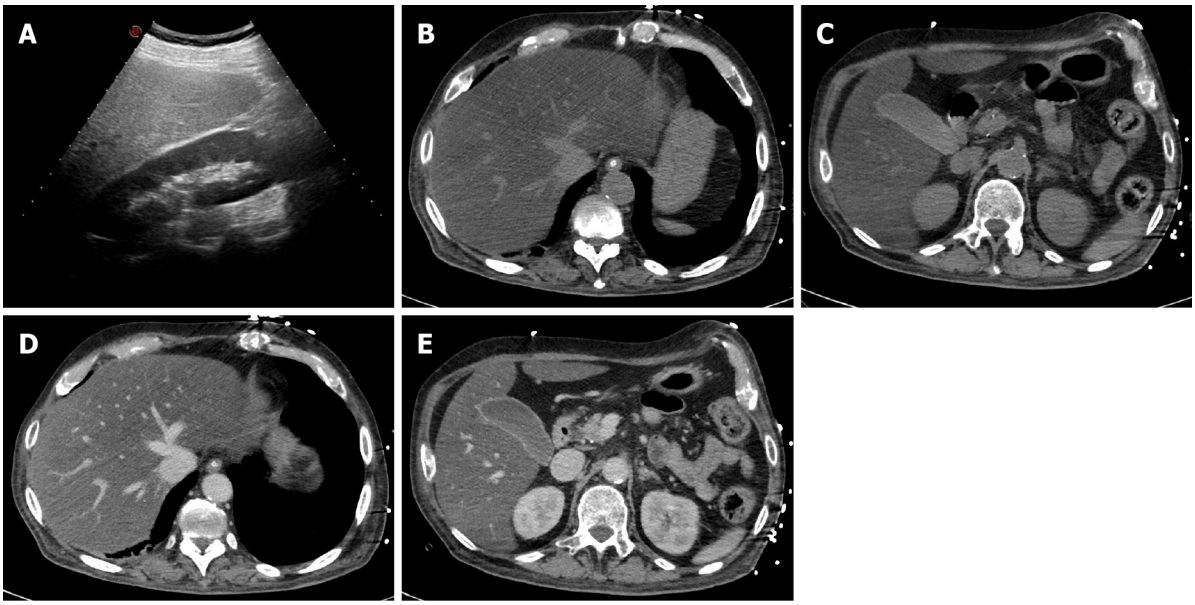

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Figure 2 A 45-year-old woman with coronavirus disease 2019 infection underwent abdominal ultrasonography due to elevated liver enzymes. A: As shown on ultrasonography, the liver is hyperechoic in comparison to the renal parenchyma, suggesting marked steatosis; B-E: The patient underwent contrast-enhanced computed tomography; B and C: On unenhanced images, the liver is diffusely hypoattenuating while its main vessels (*i.e.* portal vein and hepatic veins) appear brighter than the parenchyma due to a marked hepatic fatty infiltration; D and E: On post-contrast images, the liver is homogeneous without any focal lesion; C and E: The gallbladder is filled by sludge, with normal wall thickness. Finally, the combination of clinical and radiological findings allowed the final diagnosis of steatohepatitis.

qualitatively, searching for characteristic markers of steatosis (*i.e.* hyperechoic liver in comparison to the spleen or neighboring kidney)[29]. US findings can be confirmed on abdominal CT. According to Lei *et al*[35], the most common CT abnormalities in COVID-19 patients include diffuse hypoattenuation of the liver (26%) – more common in severe patients (59%) – and the CT-quantified liver/spleen attenuation that can predict prognosis in COVID-19 patients[35,36]. Furthermore, because the liver is partially included in every chest CT, liver data from chest CT scans performed in many COVID-19 patients can be easily retrieved.

Using a multiparametric US approach, Radzina *et al*[30] evaluated 90 patients affected with COVID-19 in the previous 3-9 mo demonstrating how liver elasticity, viscosity, and steatosis are altered after COVID-19 and that these alterations well correlate with liver enzyme abnormalities, even better than CT or MRI findings.

Other consequences of chronic liver disease, such as nonalcoholic fatty liver disease (NAFLD), must be considered in addition to COVID-19-induced liver damage[37]. Obesity and other components of metabolic syndrome have been linked to COVID-19 severity. The impact of NAFLD in COVID-19 patients is controversial in the literature[37]. In a meta-analysis of 1851 patients, Singh *et al*[38] found that, while there was an increase in the course severity of COVID-19 with a 2.60 odds ratio (OR), the adjusted OR (aOR) for mortality risk was 1.01; however, these data should be considered with caution due to the significant heterogeneity among the included studies. Interestingly, Ghoneim *et al*[39] analyzed 8885 patients with common comorbidities known to be linked with COVID-19 and found out that the cumulative incidence of disease was higher if metabolic syndrome was the primary diagnosis (OR 7.0). COVID-19 patients who were African Americans (aOR 7.45), hypertensive (aOR 2.53), obese (aOR 2.20), diabetic (aOR 1.41), hyperlipidemic (aOR 1.70) or had non-alcoholic steatohepatitis (NASH) (aOR 4.93) had higher aORs. These findings demonstrated that, among all concomitant metabolic disorders, NASH had the strongest connection with COVID-19. In support of these data, Roca-Ferná ndez *et al*[40] analyzed 41791 people who underwent MRI for assessment of liver fat, liver fibro-inflammatory disease, and liver iron with proton density fat fraction calculation before February 2020 and found that people with fatty liver (> 10%) had a higher likelihood of testing positive (OR 1.35), and people with obesity and fatty liver had a 5.14 times higher risk of hospitalization. Obese people who did not have a fatty liver can have an increased risk (OR 1.75). According to the findings, obese people with fatty liver disease are at a higher risk of COVID-19 infection and hospitalization.

To summarize, COVID-19 patients have a remarkable risk of liver damage, with the main morphologic and structural changes being hepatomegaly and steatosis, which have a significant impact on patients' prognosis and can be easily studied with US (Table 1). Epidemiologically, people with NAFLD/NASH appear to be at a higher risk of severe COVID-19 infection. However, it is unclear how much of this rise is due to hepatic steatosis or the presence of overlapping risk factors and comorbidities.

Acute hepatitis in COVID-19

Liver test abnormalities are frequently encountered in COVID-19 patients at admission, and their increase is associated with the severity of the disease[41,42]. In the majority of cases, COVID-19-induced hepatitis occurs as benign new transient hepatitis with gradual onset, elevated AST and ALT levels, and lack of any radiological changes[29,42,43]. Occasionally, COVID-19-induced hepatitis may occur in otherwise asymptomatic patients as the sole manifestation of COVID-19 infection[44]. Liver damage in COVID-19-induced hepatitis may be the result of viral infection of hepatocytes or cholangiocytes, hypercoagulability with both microangiopathy and local thrombus formation, immune-mediated damage, systemic inflammation, or hypoxic hepatitis due to the respiratory disease[45-49]. Regarding the first mechanism, the virus enters the hepatocytes, and then viral replication results in rupture of cells, generating elevated serum liver enzymes[48,50]. Thrombotic complications in COVID-19 patients are likely to occur due to a pro-coagulant effect or a progressive endothelial thrombo-inflammatory syndrome[49]. Interestingly, in a systematic review of pathology studies, hemodynamic compromise and thromboembolic disease in the liver were demonstrated in 48.3% and 39.4%, respectively, while liver microthrombi were not identified[48].

Radiological hallmarks of acute hepatitis in COVID-19 patients are encountered in approximately 8% of patients with mild-to-moderated liver test abnormalities, and the occurrence increases in patients with severe elevation of liver enzymes. Radiological hallmarks of COVID-19-induced hepatitis – particularly in the most severe cases – include thickening of the gallbladder wall, hepatomegaly, reduced echogenicity of the liver on the US or homogeneous/heterogeneous liver hypoattenuation on CT, and reduced Doppler signal in the hepatic artery[29,35,51]. Liver hypoattenuation is significantly more common in the most severe cases, with the decrease of the liver to spleen CT attenuation ratio being significantly correlated with the severity of pulmonary lesions and the overall COVID-19 severity[35] (Figure 3). A pathology-radiology correlation study demonstrated that the histological features in patients with sonographic changes included macrophage activation, centroacinar necrosis, granulocytic and histiocytic infiltrate, endothelial damage, and severe cholestasis[29,52]. Among these, macrophage activation was particularly interesting as it may represent the histopathologic correlate of a hyperinflammatory syndrome[29]. In a case of COVID-19-induced hepatitis being the sole symptom and without any other cause for liver damage, pathology demonstrated periportal and interstitial inflammation with predominantly lymphocytes, rare plasma cells, and neutrophils, hepatocyte rosette formation, apoptotic bodies, centrilobular congestion, and mildly increased portal and pericellular fibrosis[52] (Table 1).

DILI

DILI is a liver dysfunction caused by drugs used as a treatment for COVID-19 disease. Therapeutic choices for COVID-19 have rapidly expanded and changed over time with the increased understanding of the virus and the disease[53]. The various therapeutic treatments used over time included antiviral drugs, antibiotics, antimalarials, immunomodulator agents, antipyretic agents, adjunctive treatments, and several investigational treatments including convalescent plasma administration from COVID-19 recovered patients[54]. Different studies reported that liver injury in patients with COVID-19 infection

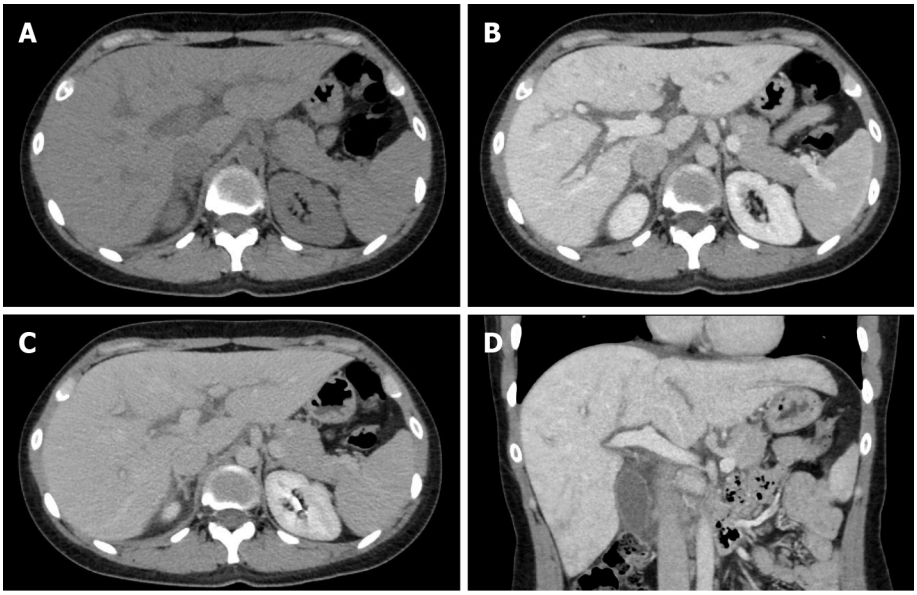

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Figure 3 A 33-year-old woman with coronavirus disease 2019 infection underwent contrast-enhanced computed tomography due to a suspicion of portal vein thrombosis. A: On unenhanced phase liver is enlarged, homogeneous and without any focal lesion; B: On the portal venous phase the liver enhancement is slight inhomogeneous associated with peri-portal edema, a typical finding of acute hepatitis; C and D: These aspects can be clearly observed also on the coronal reconstruction acquired on the portal venous phase (D) and the delayed phase (C).

could be a direct consequence of the administration of different drugs, such as antivirals and monoclonal antibodies, with most patients showing elevation of AST and ALT levels, and some also of bilirubin, lactate dehydrogenase, and C-reactive protein[55-59]. However, in most cases, elevated levels of AST and ALT do not lead to severe liver injury and the outcome is favorable[59].

The mechanisms underlying DILI in COVID-19 patients are not yet fully understood and they seem to vary depending on drug type[59,60]. A hepatocellular injury has been more often reported than cholestatic or mixed injury in COVID-19 patients with DILI[59]. On one hand, the drug-induced injury may lead to microvesicular steatosis as a result of drug interference with β-oxidation of fatty acids, mitochondrial respiration, or both which leads to the accumulation of non-esterified fatty acids which are subsequently converted into triglycerides[61,62]. On the other hand, there could be a downregulation of cytochromes p450 or CYPs family enzymes involved in oxidative biotransformation of many drugs, thus altering the metabolism of several COVID-19 drugs[60,61]. DILI could be enhanced by the production of reactive oxygen species by inflammatory cells, in addition to immune mechanisms shown in a small subset of DILI cases[63,64].

The radiological manifestations of DILI in COVID-19 patients are non-specific. One of the most frequent radiological signs of DILI is hepatic steatosis[65]. Hepatic steatosis is seen in the US as a bright liver echo pattern with the markedly increased liver to kidney contrast, and on CT as a reduction of liver attenuation below 40 UH as an absolute value or liver attenuation reduced of more than 10 HU compared to the spleen[66]. MRI is the gold standard for detection and quantification of liver steatosis: new quantitative techniques are now available, and others are still being investigated in US and CT[6]. DILI may occasionally lead to acute hepatitis and, therefore, radiological signs in these cases include hepatomegaly with decreased parenchymal enhancement, periportal edema, gallbladder wall thickening, and ascites[29]. However, the role of traditional and new quantitative techniques for assessing hepatic steatosis and liver injury occurring as a manifestation of DILI in COVID-19 is poorly investigated, and it seems quite difficult to analyze: COVID-19 patients may have more commonly hepatic steatosis and liver injury not related to DILI and there are not specific signs allowing us to differentiate between steatosis and liver injury caused by drugs or by other causes (*e.g.*, steatohepatitis, viral infection)[30] (Table 1).

PVT

The pandemic taught us the great impact of COVID-19 infection on the development of coagulation disorders, especially disseminated intravascular coagulation-like massive intravascular clot formation [67]. In this setting, it has been partially explained that the cytokines' cascade and endothelial damage can lead to the development of intravascular coagulation in the whole body, as reported by Cui *et al*[68]: critically ill patients showed a significantly higher incidence of thrombosis, up to 25%.

One of the most important visceral districts to consider in a setting of an altered vascular and endothelial environment is the portal vein, considering its importance in blood drainage from the

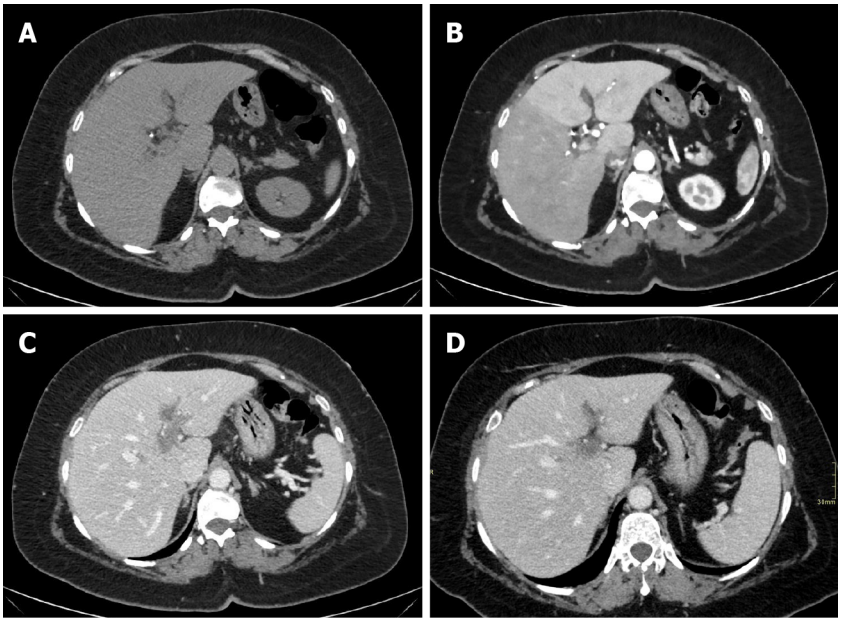

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Figure 4 A 40-year-old men with coronavirus disease 2019 infection and marked respiratory symptoms, underwent contrast-enhanced computed tomography due to elevated liver and biliary enzymes. A and B: On unenhanced phase (A) the liver is within normal limits. After the injection of contrast agent, on arterial phase (B) liver parenchyma shows inhomogeneous enhancement, with hypervascularization of the left liver lobe, as in case of transient hepatic attenuation differences; C: On the portal venous phase, the arterial hypervascularization fades to homogeneous enhancement and diffuse thrombosis of the left branch of the portal vein is demonstrated; D: After 6 mo the patient underwent contrast-enhanced computed tomography that demonstrated persistent portal vein thrombosis without venous collaterals.

gastrointestinal tract.

In non-cirrhotic patients, acute PVT may present with pain, even though the majority are found incidentally[69] (Figure 4). Different causes have been demonstrated as key roles in the development of PVT, with infection and inflammation being the most common. Rajani *et al*[70], reported that gastrointestinal inflammation accounts for about 14% of all causes of PVT. As for other viral and bacterial infections, COVID-19 can manifest with different gastrointestinal manifestations, including diarrhea, nausea, vomiting, and abdominal pain, which typically present after the respiratory symptoms (9 d *vs* 7.3 d)[71].

Even if it is well known that the inflammatory environment can lead to the development of micro- and macro-thrombosis, the current literature has not been focused on the impact of PVT in COVID-19 patients. In fact, by searching medical databases, few studies were published regarding this topic, the majority being case reports.

A meta-analysis published in 2020[72] included 18 studies and reported that all COVID-19 patients were over 15-years-old, and the majority were male (62%). The authors found a pooled prevalence of vascular thrombosis of 29.4%, one of the most representative signs in the autoptic series. Similarly, Kheyrandish *et al*[73] (2021) reviewed all cases of PVT published in the literature, confirming the higher incidence in males during infection and in females after vaccination. Thrombocytopenia was the most common laboratory finding, followed by high D-Dimer values, and abnormal coagulation tests.

Radiology plays a key role in the diagnosis of PVT. The first imaging technique useful to determine portal vein patency is US, which can be performed at bedside, especially in critically ill or isolated patients. Acute PVT can manifest as the presence of heterogenous material in the portal vein lumen, which can be partial or complete[74]. Occasionally, the portal vein thrombus can be iso- or hypoechoic on US; in this setting, the use of color doppler can support the final diagnosis, showing the lack of flow in all or some parts of the portal vein lumen[75]. Nowadays, CT is the reference standard imaging technique to evaluate PVT and its extension, both intra-hepatic and into the whole mesenteric venous system. On the unenhanced images, a higher attenuation into the portal vein lumen, due to the fresh clot, can be appreciated. The injection of an intravenous iodinated contrast agent is necessary to evaluate the lack of enhancement in the lumen, more evident in the portal-venous phase. Liver enhancement can be inhomogeneous due to areas of hypervascularization during the arterial phase, then becoming homogenous in the portal-venous and delayed phases[74]. The portal trunk can be dilated, and, sometimes, it is possible to appreciate the enhancement of vein walls due to inflammatory response[76].

Due to the different imaging spectrum of COVID-19 infection, patients may undergo MRI of the upper abdomen. In these settings, acute PVT is represented by an inhomogeneous intraluminal area(s) both on T1- and T2-weighted sequences. On T1-weighted sequences, PVT can manifest as hyperintense to the muscle if it is recent (acute), while isointense if subacute. On T2-weighted sequences PVT can

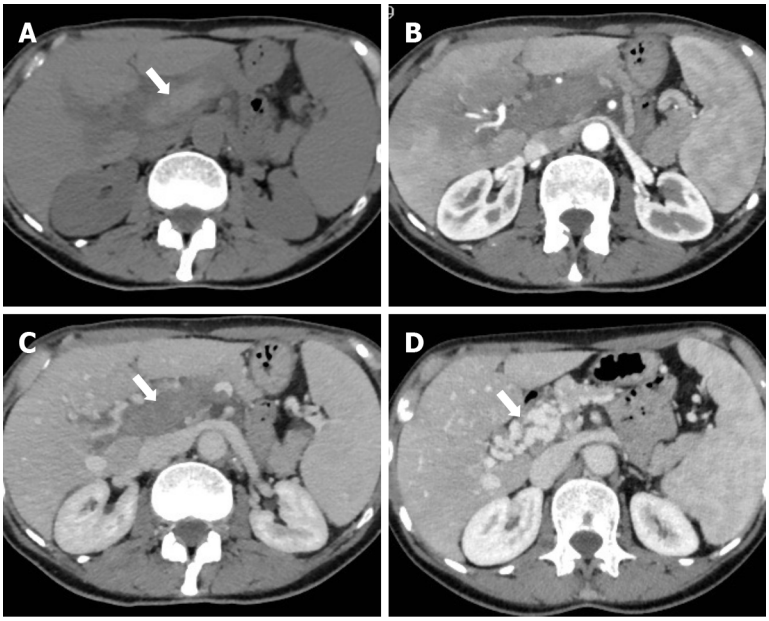

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Figure 5 A 62-year-old woman with acute portal vein thrombosis after coronavirus disease 2019 infection. A-C: Computed tomography (CT) images show acute thrombosis with hyperintense thrombus on unenhanced phase (A, arrow), heterogeneous enhancement of the liver parenchyma on hepatic arterial phase (B), and complete portal vein thrombosis on portal venous phase (C, arrow); D: Contrast-enhanced CT at 6-mo follow-up demonstrates chronic findings of portal cavernoma with multiple collateral vessels at the hepatic hilum (arrow).

manifest with different grades of hyperintense signal according to the phase (acute or subacute). If MRI is performed also after intravenous injection of contrast agents, the appearance is superimposable to the above-mentioned CT scan (Table 1).

If the PVT is not treated, cavernous transformation can occur (Figure 5): the main portal venous trunk is not appreciable and the development of periportal venous collaterals can help the drainage of venous flow from the gastrointestinal tract to the liver. Considering that chronic PVT is not reported in any patients with COVID19 infection, its findings are out of the scope of the present review.

Biliary and gallbladder involvement

The development of biliary injury in patients with COVID-19 represents an important complication, associated with poor prognosis and clinical outcome[77]. Biliary involvement in patients with COVID-19 often demonstrates clinical and biochemical features similar to sclerosing cholangitis in critically ill patients (SSC-CIP), manifesting as increased cholestasis indexes (GGT and total bilirubin) in patients with prolonged admission to the ICU and no history of biliary or liver disease nor signs of mechanical obstruction[77,78]. Severe cholestasis has been reported in up to 27% of patients with COVID-19 admitted to the ICU[79]. The mechanism of the cholestatic injury in patients with COVID-19 is not completely understood and it is likely multifactorial, with direct viral damage due to the expression of ACE2 on cholangiocytes, immune or inflammatory damage associated with liver injury, toxic bile injury, and ischemic or hypoxic injury of the biliary epithelium[80]. Cholangiopathy has also been observed after chronic exposure to ketamine, a general anesthetic used for sedation of patients with COVID-19 and acute respiratory distress syndrome[81]. The prognosis of patients with SSC-CIP is poor, with mortality in up to 50% of cases due to the development of biliary complications and worsening of liver function[82]. Particularly, patients with pre-existing chronic liver disease have an increased risk of SSC-CIP and higher mortality during COVID-19 infection[83].

Imaging is important to guide the diagnosis of cholangiopathy in patients with COVID-19 in conjunction with laboratory markers and to exclude other causes of biliary obstruction. US and CT can be performed as first-line imaging examinations and can reveal the presence of bile duct dilatation with intrahepatic stones[84] (Figures 6 and 7). Heterogeneous enhancement of the liver parenchyma with periportal edema can also be observed on contrast-enhanced CT[85]. MRI with MRCP should be performed in patients with persistent cholestasis and elevated liver function tests to assess the extension of biliary damage. MRCP can demonstrate features of secondary sclerosing cholangitis characterized by multifocal biliary strictures alternated with dilated tracts, with a "beaded" appearance[79,85]. Biliary strictures can be complicated by biliary cast, presenting as intraductal filling detects on MRCP and T2-weighted images with corresponding linear hyperintensity on unenhanced T1W images. In a recent study by Ghafoor *et al*[86], MRCP findings associated with COVID-19 cholangiopathy included intrahepatic bile duct strictures associated with upstream dilatation in 58% of patients and the presence of biliary casts in 11.8% of cases (Figure 8). Peribiliary changes characterized by hyperintensity on T2W

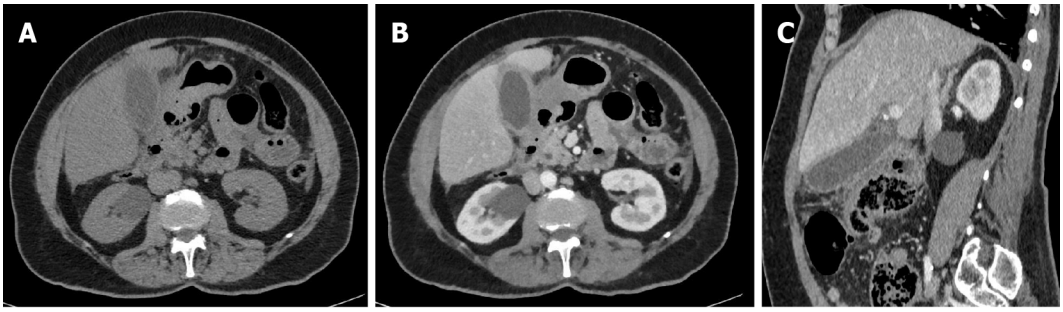

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Figure 6 A 66-year-old men with coronavirus disease 2019 infection, associated with abdominal pain in the upper quadrants. A-C: Contrast-enhanced computed tomography showed diffuse thickening of the gallbladder walls (A), with homogeneous contrast enhancement (B), better appreciable on the sagittal reconstruction (C). The gallbladder lumen is filled with biliary sludge (A) without calcified stones. The final diagnosis was acute acalculous cholecystitis.

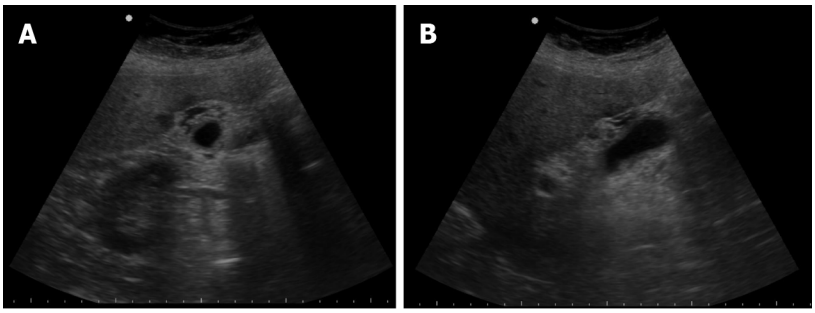

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Figure 7 A 53-year-old woman with coronavirus disease 2019 infection and markedly elevated biliary enzymes and C reactive protein levels. A and B: Ultrasonography demonstrates diffuse thickening of the gallbladder wall, associated with multiple small anechoic components into the walls, as in case of intramural abscesses. No stone is appreciated. The final diagnosis was acute acalculous cholecystitis.

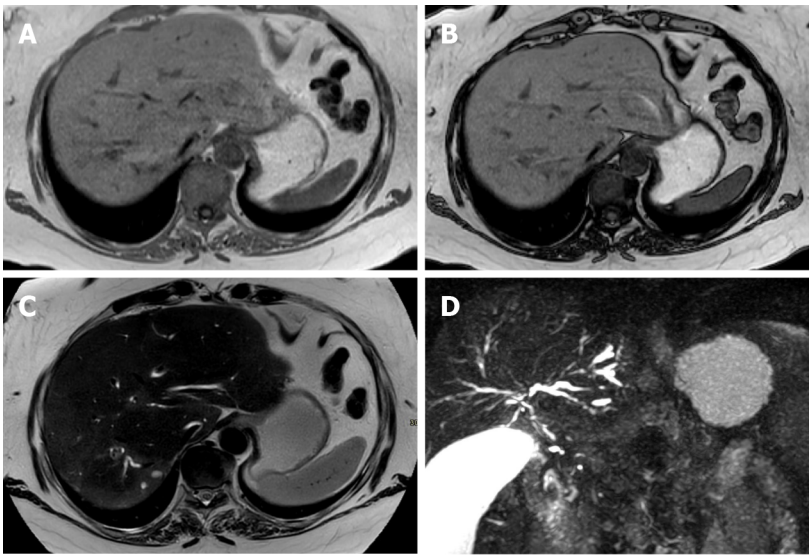

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Figure 8 A 44-year-old woman with coronavirus disease 2019 infection underwent magnetic resonance cholangiopancreatography due to elevated biliary enzymes with negative findings on ultrasonography and contrast-enhanced computed tomography. A and B: In-phase (A) and out-of-phase (B) imaging demonstrates hepatic homogeneous parenchyma without areas of fatty infiltration; C: On the T2-weighted image liver inflammation, especially in the right lobe, is characterized by areas of slight hyperenhancement, located nearby the biliary ducts; D: Finally, magnetic resonance cholangiopancreatography allows the evaluation of the biliary tree, showing multiple and diffuse focal biliary stenosis, in particular in the right lobe, with upstream dilation of small biliary ducts. This radiological aspect is typical of sclerosing cholangitis and the final diagnosis was a biliary involvement due to severe acute respiratory syndrome coronavirus 2.

images and DWI restriction were reported in 70.6% of patients, while peribiliary enhancement was observed in 23.1% of cases[86]. Extrahepatic bile duct involvement is rare[86]. Other complications include sepsis with the possible development of hepatic abscesses and progressive liver disease with morphologic features of biliary cirrhosis. Endoscopic retrograde cholangiopancreatography can be performed to confirm the diagnosis of biliary strictures and stones in selected cases and allow treatment of biliary obstruction.

Acalculous cholecystitis has been reported as the most common gallbladder involvement in patients with COVID-19[87,88]. Despite the pathogenesis of acalculous cholecystitis in COVID-19 is still under investigation, the presence of SARS-Cov-2 virus was demonstrated in samples from the gallbladder wall, probably due to the presence of ACE2 receptors in the gallbladder[87]. Other possible causes include mechanical ventilation and prolonged total parenteral nutrition[89]. The US is the first-line imaging modality in patients with suspected acalculous cholecystitis and it may reveal thickened gallbladder wall with peri-cholecystic fluid collection and gallbladder distension, in the absence of gallstones. Contrast-enhanced CT may be performed in case of suspected complications, such as gallbladder perforation, fistula, or necrosis (*i.e.* gangrenous cholecystitis)[90]. CT findings include distended gallbladder with wall thickening, hyperenhancement of the gallbladder wall during postcontrast phases, and pericholecystic fluid[91] (Table 1).

Chronic findings

The current literature is lacking studies evaluating the chronic findings of COVID-19 on liver imaging. The type of hepatic chronic findings should be related to the sequelae of acute liver damage during COVID-19 infection after recovering from the acute disease. Severe liver cholestatic injury can progress into chronic liver disease with the development of cirrhosis, manifesting as abnormal liver morphology with associated imaging features of portal hypertension and ascites. On US, a prospective multiparametric assessment of post-COVID-19 patients observed increased liver stiffness and steatosis at 3-9 mo after COVID-19 compared to normal controls[30]. Complete PVT can progress to portal vein cavernoma if not promptly recanalized and resulting in chronic findings of noncirrhotic portal hypertension and risk of variceal bleeding[49].

Further studies are still needed to assess the evolution of hepatic findings and the possible long-term sequelae of liver damage in patients recovering from COVID-19.

CONCLUSION

Even if COVID-19 is extensively reported as a disease that mainly affects the lungs, the viral infection may cause an involvement of abdominal parenchymal organs and the gastrointestinal tract, increasing the risk of both acute and long-term health problems, especially of liver parenchyma. The liver damage may be caused by different mechanisms, including direct hepatocytes involvement from the viral infection, indirect response to the systemic inflammatory status, or hepatotoxicity due to drugs used to manage the infection. In this setting, the diagnostic imaging workup plays a crucial role for early detection of liver manifestations and assessment of long-term complications.

US should be considered as the main diagnostic option for the first evaluation of liver, biliary tree, and vascular district, in patients with abdominal symptoms, or with altered blood test, while abdominal contrast enhanced CT seems to be the most useful diagnostic tool for the overall abdominal assessment offering useful information regarding not only the liver itself, but also other parenchymal organs and vascular system. Finally, MRI should be considered the tool that better clarifies liver alterations in patients with COVID infection deemed indeterminate on US and CT.

It's important to underline that the main limitation in this field which should still be considered is the difficulty to understand the main COVID-19 pathological mechanisms and their related consequences. Further studies should be more focused on the evaluation of COVID-19 patients, in particular those with liver involvement, to quickly address the diagnosis and the best management possible.

A comprehensive knowledge of COVID-19 hepatic involvement assessed through the different diagnostic imaging modalities can help clinicians in addressing the correct treatment and long-term management of the disease.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Saccharomyces cerevisiae prevents postoperative recurrence of Crohn's disease modeled by ileocecal resection in HLA-B27 transgenic rats

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Abstract

BACKGROUND

Postoperative recurrence (POR) after ileocecal resection (ICR) affects most Crohn's disease patients within 3-5 years after surgery. Adherent-invasive *Escherichia coli* (AIEC) typified by the LF82 strain are pathobionts that are frequently detected in POR of Crohn's disease and have a potential role in the early stages of the disease pathogenesis. *Saccharomyces cerevisiae* CNCM I-3856 is a probiotic yeast reported to inhibit AIEC adhesion to intestinal epithelial cells and to favor their elimination from the gut.

AIM

To evaluate the efficacy of CNCM I-3856 in preventing POR induced by LF82 in an HLA-B27 transgenic (TgB27) rat model.

METHODS

Sixty-four rats [strain F344, 38 TgB27, 26 control non-Tg (nTg)] underwent an ICR at the 12th wk (W12) of life and were sacrificed at the 18th wk (W18) of life. TgB27 rats were challenged daily with oral administration of LF82 (10^9 colony forming units (CFUs)/day (d), n = 8), PBS (n = 5), CNCM I-3856 (10^9 CFUs/d, n = 7) or a combination of LF82 and CNCM I-3856 (n = 18). nTg rats receiving LF82 (n = 5), PBS (n = 5), CNCM I-3856 (n = 7) or CNCM I-3856 and LF82 (n = 9) under the same conditions were used as controls. POR was analyzed using macroscopic (from 0 to 4) and histologic (from 0 to 6) scores. Luminal LF82 quantifications were performed weekly for each animal. Adherent LF82 and inflammatory/regulatory cytokines were quantified in biopsies at W12 and W18. Data are expressed as the median with the interquartile range.

RESULTS

nTg animals did not develop POR. A total of 7/8 (87%) of the TgB27 rats receiving LF82 alone had POR (macroscopic score ≥ 2), which was significantly prevented by CNCM I-3856 administration [6/18 (33%) TgB27 rats, P = 0.01]. Macroscopic lesions were located 2 cm above the anastomosis in the TgB27 rats receiving LF82 alone and consisted of ulcerations with a score of 3.5 (2 - 4). Seven out of 18 TgB27 rats (39%) receiving CNCM I-3856 and LF82 had no macroscopic lesions. Compared to untreated TgB27 animals receiving LF82 alone, coadministration of CNCM I-3856 and LF82 significantly reduced the macroscopic [3.5 (2 - 4) *vs* 1 (0 - 3), P = 0.002] and histological lesions by more than 50% [4.5 (3.3 - 5.8) *vs* 2 (1.3 - 3), P = 0.003]. The levels of adherent LF82 were correlated with anastomotic macroscopic scores in TgB27 rats (r = 0.49, P = 0.006), with a higher risk of POR in animals having high levels of luminal LF82 (71.4% *vs* 25%, P = 0.02). Administration of CNCM I-3856 significantly reduced the levels of luminal and adherent LF82, increased the production of interleukin (IL)-10 and decreased the production of IL-23 and IL-17 in TgB27 rats.

CONCLUSION

In a reliable model of POR induced by LF82 in TgB27 rats, CNCM I-3856 prevents macroscopic POR by decreasing LF82 infection and gut inflammation.

Key Words: Crohn's disease; Recurrence; *Escherichia coli*; Probiotic; *Saccharomyces cerevisiae*; Colorectal surgery

Core Tip: Gut dysbiosis plays a main role in the postoperative recurrence (POR) of Crohn's disease (CD). CD dysbiosis is characterized by a lower microbiota diversity with an increase in pathogenic species. Among them, adherent-invasive *Escherichia coli* (AIEC) has been linked to POR. *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 is a probiotic yeast that specifically targets AIEC by preventing the bacterial adhesion process and inhibiting its persistence within the bowel. This study confirmed the capacity of *S. cerevisiae* CNCM I-3856 to prevent AIEC-induced POR by decreasing the infection in a transgenic HLA-B27 rat model of POR after ileocecal resection.

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INTRODUCTION

Crohn's disease (CD) is a complex chronic inflammatory bowel disease that requires surgical resection of macroscopic lesions in approximately 30%-50% of patients in their lifetime[1]. Unfortunately, surgery is not curative, and endoscopic recurrence at the anastomotic site occurs in up to 70% of patients in the first year after surgery, followed by clinical recurrence a few years later[2]. Postoperative management of these patients is crucial to identify those at highest risk of recurrence to begin rapid prophylactic treatments targeting mainly tumor necrosis factor α (TNFα)[3], interleukins 12/23 and α4β7 integrins on leukocytes[4]. Given the high rate of recurrence after intestinal resection for CD and the cost and potential adverse effects of biologic therapies used in prophylaxis, there is a clear need to identify the mechanisms leading to postoperative recurrence (POR), to develop noninvasive methods predicting

recurrence and to propose new evidence-based therapeutic strategies.

The physiopathology sustaining POR of CD remains partially unknown. Abnormal interactions between the mucosal/mesenteric immune system and the intestinal microbiota favored by surgical techniques and environmental factors are pivotal hallmarks in POR dynamics[5]. Recently, ileal transcriptome analyses of CD patients found a gene signature of POR characterized by an upregulation of the interleukin (IL)-23 and IL-17 pathways together with abnormal JAK/STAT activation[6]. Numerous changes in the microbial composition and a reduction in species diversity have been observed in the intestinal flora of CD patients[7], and a few studies have identified an intestinal microbial signature associated with POR. Recolonization of the neoterminal ileum by *Escherichia coli* (*E. coli*), Bacteroides, and Fusobacteriaceae and the depletion of Streptococcaceae, Actinomycineae and *Faecalibacterium* are associated with endoscopic recurrence of CD[8]. Among these microorganisms, adherent-invasive *E. coli* (AIEC) isolated more than 20 years ago by Darfeuille-Michaud *et al*[9] from the ileal mucosa of a patient with CD[10] remains one of the most prominent and influential strains associated with CD. AIEC are pathobionts found in approximately 30% of CD patients and in 10% of healthy controls[11]. They are not strictly pathogenic bacteria, and their influence on CD physiopathology remains incompletely understood. However, AIEC is associated with the early stages of CD and is predictive of endoscopic POR at 6 mo[12], reinforcing the need for interventional studies targeting these bacteria to better understand their direct impact on mucosal inflammation and to find new opportunities to treat CD patients.

Several therapeutic strategies, including the use of antibiotics[13], pre/probiotics[14] and fecal microbiota transplantation[15], have been proposed to target the intestinal flora in CD. Due to side effects or limited efficacy, their routine utilization cannot be recommended[16,17]. Other strategies to inhibit adhesion or to specifically erase AIEC using FimH blockers[18,19] or specific bacteriophages[20,21] are ongoing and seem more promising in preclinical studies. In this context, *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 is a probiotic yeast with good tolerance and beneficial effects on gastrointestinal symptoms[22,23] that has been shown to agglutinate the LF82 AIEC strain and to prevent its adhesion to intestinal epithelial cells *in vitro*, favoring LF82 elimination from the gut of mice [24]. Among the thousands of strains belonging to the AIEC family and identified from European and USA isolates, LF82 remains the most studied reference strain that can both adhere to and invade epithelial cells and, moreover, survive and replicate within macrophages without inducing cellular death[25,26].

In the present study, we developed a new animal model of POR of CD occurring 6 wk after ileocecal resection (ICR) in HLA-B27 transgenic (Tg) rats[27,28] infected by the LF82 AIEC strain[29] to better evaluate the causal role of LF82 on the early steps of CD lesions and the effectiveness of a rationally selected *S. cerevisiae* CNCM I-3856 probiotic to prevent recurrence of the disease.

MATERIALS AND METHODS

Animals

HLA-B27 transgenic (Tg) and nontransgenic (nTg) control Fisher rats (strain F344) were provided by Professor M. Breban (Cochin Institute, INSERM U1016, Paris, France). Sixty-four rats were maintained in a specific pathogen-free facility at the Institut Pasteur (Lille, France) and were fed a standard diet with free access to water. Animals were maintained at a constant temperature with a 12-hour light/dark cycle. Intragastric gavage administration was carried out with conscious animals using straight gavage needles appropriate for the animal size. Surgery was performed under general anesthesia, and postoperative analgesia by opioid treatment was provided. All animals were euthanized by cervical dislocation under general anesthesia. Experiments were realized according to the European directive 2016/63/UE enforced by the decree n°2013-118 and authorized by the departmental ethics committee (No. CEEA 01292-01).

AIEC LF82 and S. cerevisiae CNCM I-3856 strains

The streptomycin-kanamycin-resistant AIEC strain LF82 isolated from an ileal biopsy of a patient with CD was provided by Professor Nicolas Barnich (Clermont-Auvergne University, France) and used as an AIEC reference strain[30]. Bacteria were routinely grown at 37 °C in Brain-Heart broth or on Drigalski agar plates. The dry *S. cerevisiae* CNCM I-3856 yeast strain was provided by Lesaffre International (Marcq-en-Baroeul, France). The LF82 and *S. cerevisiae* CNCM I-3856 strains were rehydrated at room temperature in PBS (pH = 7.2, 2 × 10^9 colony forming units (CFUs)/mL) before gavage.

Experimental design

ICR with end-to-end anastomosis was performed at 12 wk (W) of life (W12) in 64 rats (38 Tg, 26 nTg) (Figure 1). ICR was performed blindly by two operators (Caroline Dubuquoy and Caroline Valibouze) in Tg and nTg animals. Tg rats were challenged daily by oral gavage in the morning with PBS (n = 5), *S. cerevisiae* CNCM I-3856 alone (10^9 CFUs/day (d)) (n = 7), LF82 alone (10^9 CFUs/d) in the afternoon (n = 8), or the combination of *S. cerevisiae* CNCM I-3856 (10^9 CFUs/d) and LF82 (10^9 CFUs/d) (n = 18) given

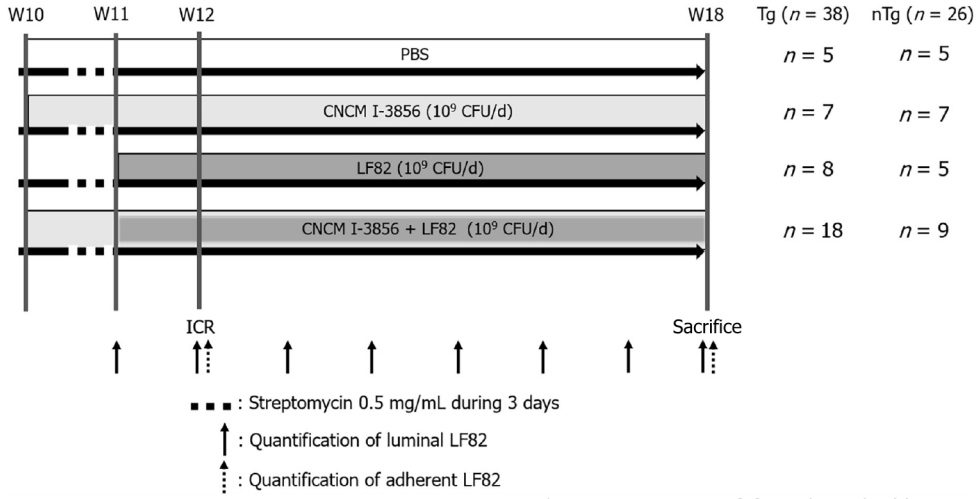

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Figure 1 Study design. HLA-B27 transgenic rats (Tg) and wild-type rats (nTg) were randomized to receive phosphate buffered saline (*n* = 10), *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 (*n* = 14), adherent-invasive *Escherichia coli* strain LF82 (*n* = 13), or *S. cerevisiae* CNCM I-3856 and LF82 (*n* = 27) by oral gavage from week (W) 10 or 11 to W18. Ileocecal resection was performed at W12, and animals were sacrificed at W18. Streptomycin (dotted line) was given on the last 3 d of W10 in all rats. Luminal (arrows) and/or adherent (dotted arrows) LF82 was quantified weekly during the 8-wk study. CFU: Colony-forming unit; PBS: Phosphate buffered saline; ICR: Ileocecal resection; d: Day.

in the morning and in the afternoon, respectively. Age-matched nTg rats receiving PBS (*n* = 5), *S. cerevisiae* CNCMI-3856 alone (*n* = 7), LF82 alone (*n* = 5), or the combination of *S. cerevisiae* CNCM I-3856 and LF82 (*n* = 9) under the same conditions were used as controls. LF82 was administered from W11 to W18, and *S. cerevisiae* CNCM I-3856 was similarly administered from W10 to W18 in Tg and nTg rats. Streptomycin was given in drinking water at 0.5 mg/mL for the last 3 d of W10 in Tg and control animals. The rats were followed during the eight-week procedure for weight changes (% of change compared to initial body weight at W11), diarrhea and the presence of macroscopic bloody stools and were killed at W18.

Macroscopic and histologic lesions

At W18, the whole intestine was excised and photographed. Anastomotic macroscopic lesions (± 2 cm above anastomosis) were assessed blindly using a macroscopic grading scale adapted from the Rutgeerts score ranging from 0 to 4 (Figure 2)[2]. By analogy with endoscopic recurrence after surgery in patients with CD (25), POR was defined by a macroscopic score of ≥ 2 corresponding to the presence of ulcerations ± stenosis. The results were expressed as the median with the interquartile range (IQR).

Transparietal biopsies of anastomotic areas were collected during surgery at W12 and W18. Tissues were fixed in 4% buffered formaldehyde, embedded in paraffin and stained by May-Grunwald Giemsa for scoring (from 0 to 6) using the adapted score of Geboes (Table 1)[31]. Identical areas of each section of the different biopsy specimens were examined at 10× magnification by two blinded observers familiar with the scoring system (Caroline Dubuquoy and Caroline Valibouze). Anastomotic histologic scores were expressed as the median score with IQR when an interobserver coefficient of variation < 15% was obtained.

Luminal and adherent quantification of LF82

Feces (10 - 600 mg) were collected weekly from W11 to W18 for each animal after abdominal massage for the quantification of luminal LF82. Mucosal anastomotic swabs (10 - 100 mg) were performed at W12 during surgery and at sacrifice (W18) in all animals for the quantification of anastomotic adherent LF82. Fresh feces and swabs were collected in 1.5 mL of sterile cysteinated Ringer's solution. After serial dilutions, samples were incubated for 24 - 48 h at 37 °C in Drigalski agar containing 100 μg/mL streptomycin to select and quantify LF82 expressed as log10 CFUs *per* gram of feces. The results are expressed as the median with the IQR.

mRNA quantification in anastomotic biopsies at W12 and W18

Anastomotic biopsies were frozen at -80 °C, and total RNA was extracted using a Nucleospin RNA kit (Macherey Nagel). After RNAse inactivation, genomic DNA was suppressed from the samples by DNAse treatment, and total RNA was extracted in RNAse-free water. The RNA content was measured using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Retro-transcription of total RNA was achieved using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Random primers, RT buffer and reverse transcriptase were added to 1 μg of

Table 1 Anastomotic histologic score (0-6)

Score	Histologic lesions
0	None
1	Inflammatory infiltrate and mucosal erosions < 30% of the section
2	30% < inflammatory infiltrate and mucosal erosions < 70% of the section
3	Inflammatory infiltrate and mucosal erosions > 70% of the section
4	Mucosal ulceration < 30% of the section
5	30% < mucosal ulceration < 70% of the section
6	Mucosal ulceration > 70% of the section

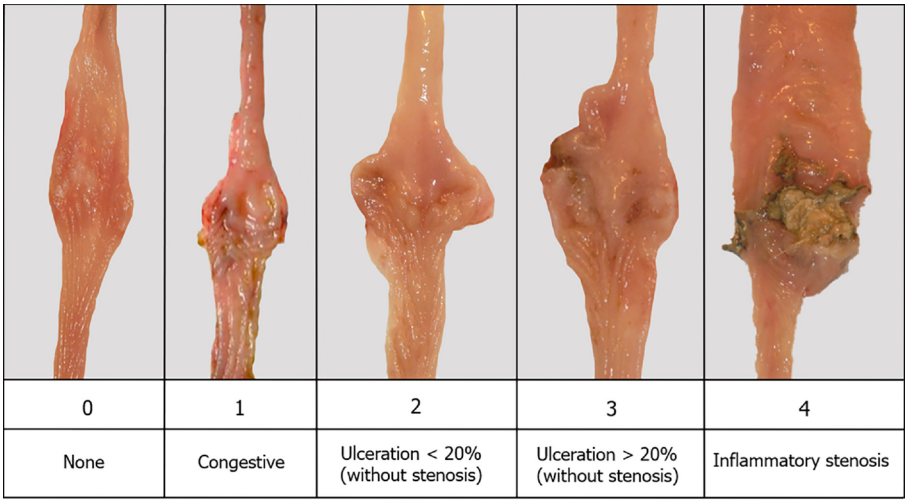

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Figure 2 Anastomotic macroscopic score (0-4).

total RNA, and the samples were incubated for 10 min at 25 °C, then 2 h at 37 °C and finally 5 min at 85 °C in the Gene AmpPCR System 9700 automaton (Thermos Fisher Scientific, Waltham, Massachusetts, USA). All kits were used according to the manufacturers' protocols. IL-1β, IL-6, TNFα, interferon (IFN)γ, IL-17, IL-23 and IL-10 were quantified by quantitative polymerase chain reaction (PCR) in real time for 40 cycles in the StepOnePlus™ Real Time PCR system (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using SYBR Green PCR Master Mix (Thermo Fisher Scientific). qPCR signal quantification was expressed relative to the expression of β-actin as the reference gene. The results are expressed as the median with IQR.

Statistical analysis

Data are expressed as the median with IQR. Comparisons were performed using the nonparametric Mann-Whitney test for unmatched data and the Wilcoxon signed-rank test for matched data. Pearson's chi-square test was used for contingency analysis. The correlation between macroscopic scores and the number of LF82 was tested using Spearman's test. To classify animals with low or high quantities of LF82, a cutoff value was determined using the receiver operating characteristic (ROC) curve. The risk of recurrence for low and high producers was compared using Pearson's chi-square test. All statistical tests were two-tailed and considered statistically significant if $P < 0.05$. Statistical analyses were conducted using the GraphPad Prism 5.00 (GraphPad Software, San Diego, CA) software package for PCR and Xlstat 2020.1 version for the ROC curve.

RESULTS

Effect of S. cerevisiae CNCM I-3856 on clinical signs

No mortality, diarrhea or bloody stools were observed in any animals receiving PBS, LF82 alone, *S. cerevisiae* CNCM I-3856 alone or *S. cerevisiae* CNCM I-3856 and LF82 during the 8-wk observation study.

A similar pattern of weight evolution was observed in nTg (Figure 3A) and Tg animals (Figure 3B), with significant weight loss occurring one week after surgery followed by a weight recovery phase. More important weight loss was transiently observed at W13 in Tg rats receiving LF82 *vs S. cerevisiae* CNCM I-3856 and LF82 (95.7, IQR: 92 - 97 *vs* 85.4, IQR: 81 - 94, $P = 0.007$). The global weight changes assessed by the relative difference in weight variation between W11 and W18 were similar in the 4 groups of Tg and nTg animals.

Effect of S. cerevisiae CNCM I-3856 on macroscopic anastomotic lesions and POR

No intestinal lesions were present at W12 in any animal. No macroscopic lesions (or therefore POR) were observed at W18 in control nTg animals receiving PBS, *S. cerevisiae* CNCM I-3856 alone, LF82 alone, or *S. cerevisiae* CNCM I-3856 and LF82 (Figure 4A). In contrast, anastomotic macroscopic lesions corresponding mainly to edema and ulcerations on more than 20% of the anastomotic area without stenosis were observed in Tg rats receiving LF82 (3.5, IQR: 2 - 4), leading to 87.5% POR in this group of animals (Figure 4A and B). Compared to untreated Tg rats receiving LF82 (3.5, IQR: 2 - 4), coadministration of *S. cerevisiae* CNCM I-3856 and LF82 significantly reduced the macroscopic score (1, IQR: 0 - 2, $P = 0.002$) and POR (87.5% *vs* 33.3%, $P = 0.01$) by more than 60% (Figure 4A and B). Anastomotic macroscopic lesions were similar in Tg rats receiving PBS or *S. cerevisiae* CNCM I-3856 alone or *S. cerevisiae* CNCM I-3856 and LF82, without a difference compared to those of control nTg animals (Figure 4A).

Effect of S. cerevisiae CNCM I-3856 on anastomotic histologic lesions

No histologic lesions were present at W12 in any animal (data not shown). At W18, no significant and only mild histologic lesions characterized by neutrophil infiltration not exceeding 30% of lamina propria cells were observed in control nTg animals receiving either PBS, LF82 alone, *S. cerevisiae* CNCM I-3856 alone or *S. cerevisiae* CNCM I-3856 and LF82 (Figure 5). In contrast, erosions and mucosal ulcerations associated with moderate neutrophil infiltration were observed at W18 in Tg rats receiving LF82 (4.5, IQR: 3.3 - 5.8) (Figure 5). Compared to untreated Tg animals receiving LF82, coadministration of *S. cerevisiae* CNCM I-3856 and LF82 significantly reduced the histological lesions by more than 50% (4.5, IQR: 3.3 - 5.8 *vs* 2, IQR: 1.3-3, $P = 0.003$) (Figure 5). No significant lesions were observed in Tg rats receiving PBS or *S. cerevisiae* CNCM I-3856 alone, which was not different from the findings in control nTg animals (Figure 5).

Effect of CNCM I-3856 on luminal and adherent LF82 Levels (W12-W18)

At W12, *i.e.*, one week after the beginning of LF82 administration (10^9 CFUs/d), the quantities of luminal (Figure 6A) and adherent (Figure 7A) LF82 were similar in Tg and nTg rats receiving LF82 alone or *S. cerevisiae* CNCM I-3856 and LF82. The levels of luminal (4.4, IQR: 2.5 - 5.2 *vs* 3.4, IQR: 1.7 - 5.5) and adherent (2.7, IQR: 2.4 - 3 *vs* 3.1, IQR: 2.3 - 5) LF82 remained similar between W12 and W18 in Tg rats receiving LF82 alone (Figures 6 and 7), while a significant decrease in luminal (4.6, IQR: 3.5 - 5.2 *vs* 1.8, IQR: 1.7 - 2.3, $P = 0.0002$) and adherent (3.1, IQR: 2.5 - 3.6 *vs* 2.5, IQR: 2.3 - 2.6, $P = 0.0005$) LF82 was observed between W12 and W18 in paired Tg animals receiving *S. cerevisiae* CNCM I-3856 and LF82 (Figures 6 and 7).

In addition, the global persistence of viable luminal LF82 after surgery and during the last 5 wk of the study was significantly higher in the stools of Tg rats receiving LF82 alone (0.22, IQR: 2.071e-008 - 0.7) compared to Tg rats receiving *S. cerevisiae* CNCM I-3856 and LF82 (-0.6, IQR: -0.7 - 0.3, $P = 0.0004$) (Figure 8).

Correlation between LF82 Levels and macroscopic lesions in Tg rats

A correlation was found between the levels of adherent LF82 and the scores of anastomotic macroscopic lesions observed at W18 in Tg animals receiving LF82 alone or in combination with *S. cerevisiae* CNCM I-3856 ($r = 0.49$, $P = 0.006$) (Figure 9A). These levels of anastomotic adherent LF82 were correlated at W12 ($r = 0.81$, $P = 0.02$) and W18 ($r = 0.79$, $P = 0.03$) with the levels of luminal LF82 in paired Tg animals receiving LF82 alone (Figure 9B and C). Next, we analyzed whether luminal LF82 Levels at W14 may be predictive of POR in the 26 Tg rats receiving LF82 alone ($n = 8$) or in combination with *S. cerevisiae* CNCM I-3856 ($n = 18$). Using a cutoff value of 2.262 Log10 CFUs of luminal LF82 per gram of stool determined by the ROC curve, 14 animals at W14 were classified as highly infected by LF82, and 12 were classified as mildly infected (Figure 10A). POR was significantly more frequent in the highly infected Tg rats than in the mildly infected Tg rats (71.4% *vs* 25%, $P = 0.02$) (Figure 10B). A value of 2.262 Log10 CFUs luminal LF82 *per* gram of stool at W14 had an 80% sensitivity, 69.2% specificity, 71.4% positive predictive value and 75% negative predictive value for POR.

Anastomotic cytokine mRNA quantification

The levels of IL-1β, IL-6, TNFα and IFNγ mRNA were variable and similar in all Tg and nTg animals at W12 and W18, regardless of the presence of POR, LF82 administration or treatment with *S. cerevisiae* CNCM I-3856 (data not shown).

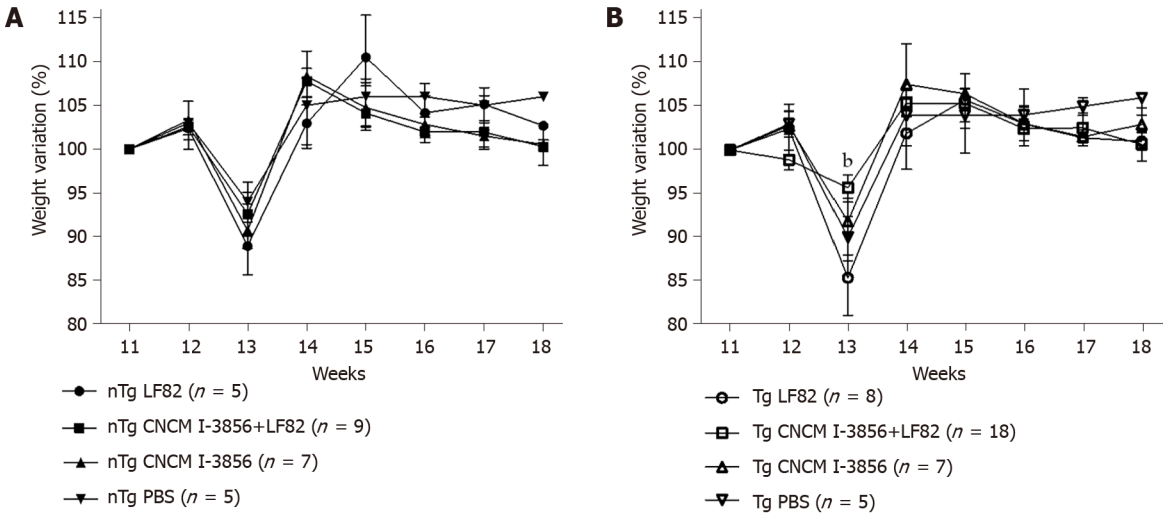

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Figure 3 Body weight evolution. A: Evolution of weight changes compared to body weight at W11 in nontransgenic (nTg) rats; B: Evolution of weight changes compared to body weight at W11 in transgenic (Tg) rats. $^{b}P < 0.01$. LF82: Adherent-invasive *Escherichia coli* strain LF82; CNCM I-3856: *Saccharomyces cerevisiae* CNCM I-3856; PBS: Phosphate buffered saline.

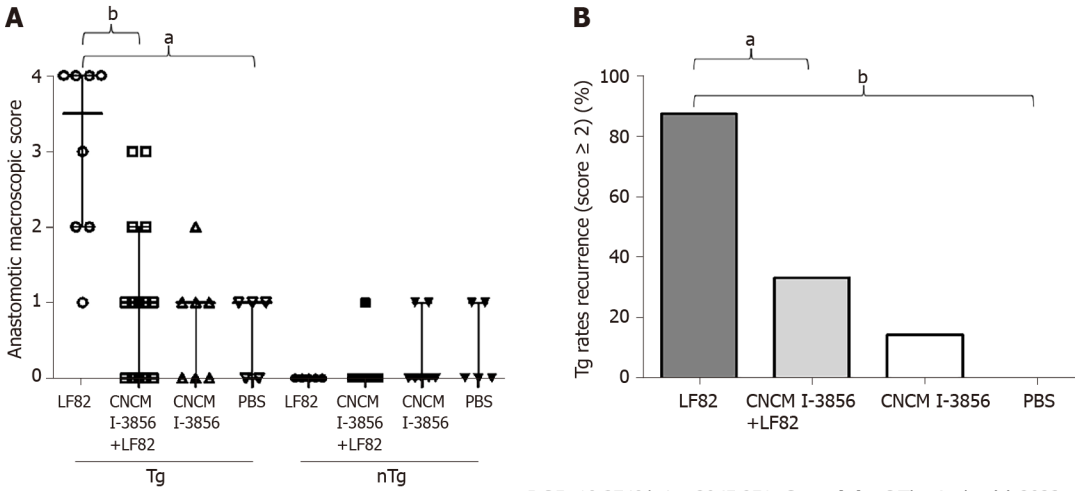

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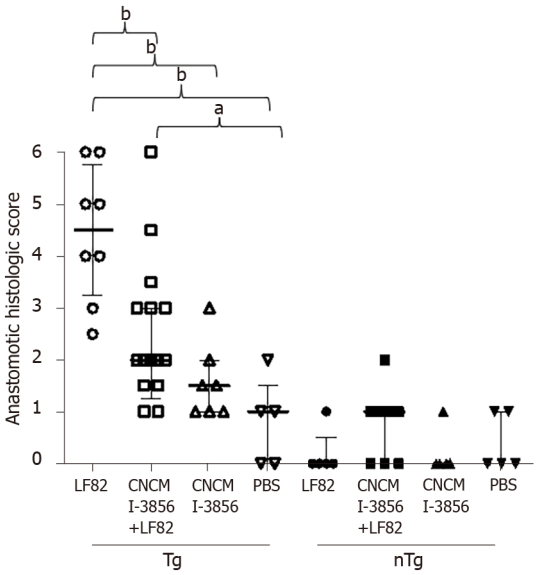
Figure 4 Anastomotic macroscopic lesions and postoperative recurrence at sacrifice. A: Anastomotic macroscopic scores in the different groups of HLA-B27 transgenic (Tg) rats and wild-type (nTg) rats at sacrifice; B: % postoperative recurrence (anastomotic macroscopic score ≥ 2) at sacrifice in HLA-B27 Tg rats. $^{a}P < 0.05$, $^{b}P < 0.01$. LF82: Adherent-invasive *Escherichia coli* strain LF82; CNCM I-3856: *Saccharomyces cerevisiae* CNCM I-3856; PBS: Phosphate buffered saline.

Concerning IL-10 mRNA levels, the only significant difference found by paired analysis in the different groups of animals revealed higher levels of IL-10 mRNA at W18 compared to W12 in animals receiving *S. cerevisiae* CNCM I-3856. In the Tg groups, administration of *S. cerevisiae* CNCM I-3856 with or without the coadministration of LF82 induced a significant increase in IL-10 production between surgery and sacrifice (2.5×10^5, IQR: 1.7×10^5 - 2.6×10^5 *vs* 4.9×10^5, IQR: 3.3×10^5 - 9×10^5, $P = 0.017$ and 2.6×10^5, IQR: 1.5×10^5 - 3.9×10^5 *vs* 7.4×10^5, IQR: 5.3×10^5 - 0.4×10^5, $P = 0.031$, respectively), while similar IL-10 Levels were found in animals receiving LF82 alone (Figure 11A-C).

Concerning IL-23 mRNA levels, a significant increase was observed at W18 in Tg animals receiving LF82 alone in comparison to the groups of rats treated with *S. cerevisiae* CNCM I-3856 with or without administration of LF82 ($P = 0.04$ and $P = 0.006$, respectively) (Figure 12A). Additionally, using a paired t test, a significant increase in inflammatory IL-23 production was observed between surgery and sacrifice in the Tg group receiving LF82 alone (2.2×10^4, IQR: 1.8×10^4 - 8×10^4 *vs* 26.9×10^4, IQR: 6.1×10^4 - 6×10^4, $P = 0.008$), while no significant difference was observed in the Tg groups treated with *S. cerevisiae* CNCM I-3856 with or without administration of LF82 (Figure 12B-D).

Analysis of IL-17 mRNA levels found significantly higher rates at W18 in Tg rats receiving LF82 in comparison with the Tg group receiving *S. cerevisiae* CNCM I-3856 and LF82 (2.7×10^4, IQR: 0.8×10^4 - $9,5 \times 10^4$ *vs* 0.4×10^4, IQR: 0.2×10^4 - 0.6×10^4, $P = 0.015$) (Figure 13).



DOI: 10.3748/wjg.v29.i5.851

Figure 5 Anastomotic histologic lesions at sacrifice. Anastomotic histologic scores in the different groups of HLA-B27 transgenic (Tg) rats and wild-type (nTg) rats at sacrifice. [a]*P* < 0.05, [b]*P* < 0.01. LF82: Adherent-invasive *Escherichia coli* strain LF82; CNCM I-3856: *Saccharomyces cerevisiae* CNCM I-3856; PBS: Phosphate buffered saline.

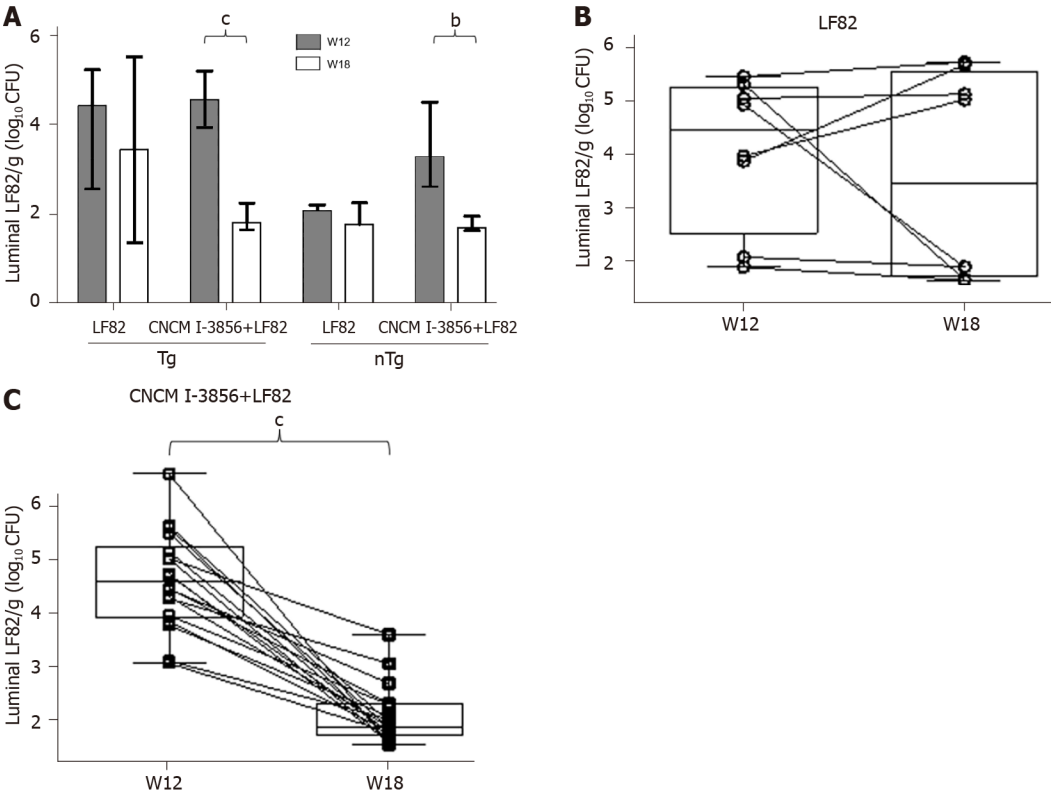


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Figure 6 Levels of luminal adherent-invasive *Escherichia coli* LF82 at surgery and sacrifice. A: Luminal levels of adherent-invasive *Escherichia coli* strain LF82 at surgery [week (W) 12] and sacrifice (W18) in the different groups of HLA-B27 transgenic (Tg) rats and wild-type (nTg) rats; B: Luminal levels of LF82 at W12 and W18 in paired Tg rats receiving LF82 alone; C: Luminal levels of LF82 at W12 and W18 in paired Tg rats receiving *Saccharomyces cerevisiae* CNCM I-3856 and LF82. [b]*P* < 0.01, [c]*P* < 0.001. CFU: Colony-forming unit; log10: Decimal logarithm.

DISCUSSION

The role of the intestinal microbiota composition and diversity in POR of CD is important. Among intestinal microorganisms potentially involved in POR, many studies support the roles of AIEC in early

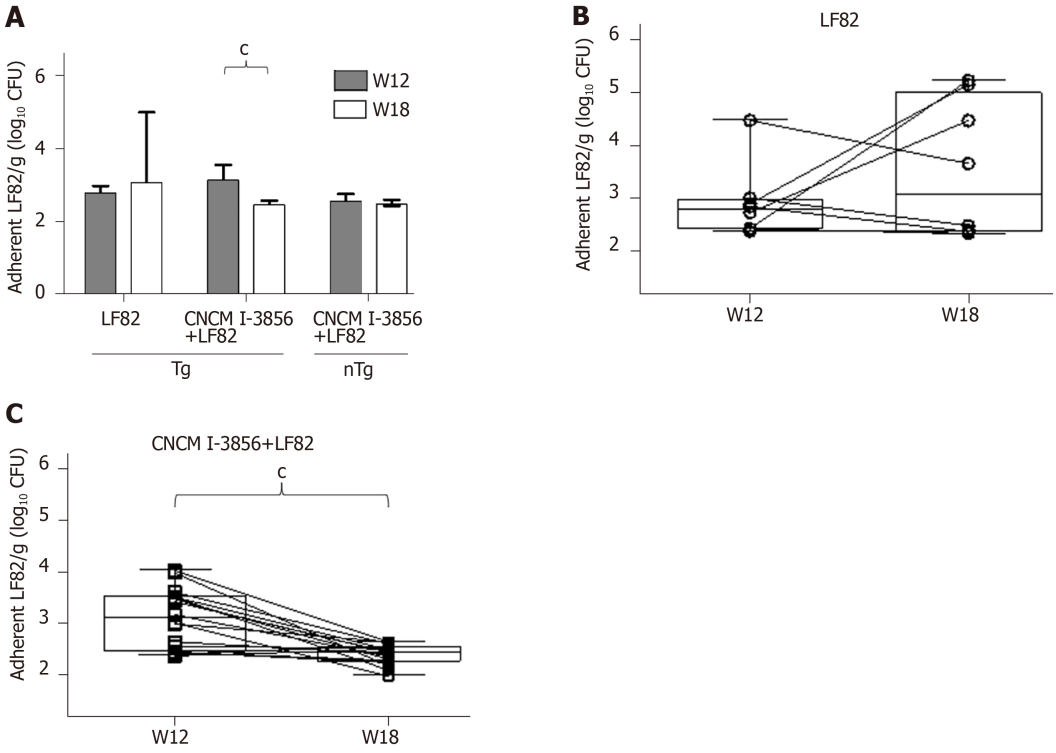

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Figure 7 Levels of anastomotic adherent adherent-invasive *Escherichia coli* LF82 at surgery and sacrifice. A: Adherent levels of adherent-invasive *Escherichia coli* strain LF82 at surgery [week (W) 12] and sacrifice (W18) in the different groups of HLA-B27 transgenic (Tg) rats and wild-type (nTg) rats; B: Adherent levels of LF82 at W12 and W18 in paired Tg rats receiving LF82 alone; C: Adherent levels of LF82 at W12 and W18 in paired Tg rats receiving *Saccharomyces cerevisiae* CNCM I-3856 and LF82. °P < 0.001. CFU: Colony-forming unit; log10: Decimal logarithm.

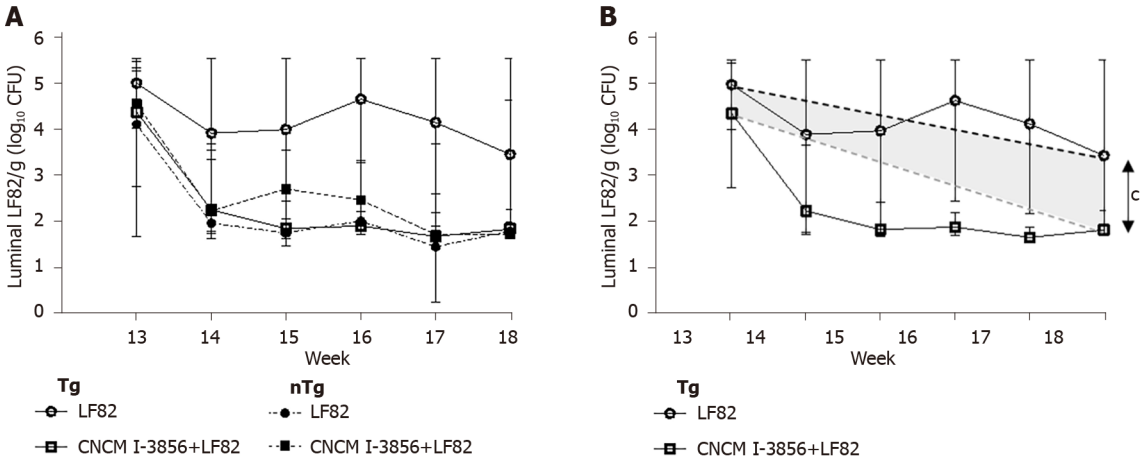

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Figure 8 Evolution of the levels of luminal adherent-invasive *Escherichia coli* LF82 after surgery. A: Weekly evaluation of the luminal LF82 Levels after surgery in HLA-B27 transgenic (Tg) rats and wild-type (nTg) rats receiving adherent-invasive *Escherichia coli* strain LF82 alone or *Saccharomyces cerevisiae* CNCM I-3856 and LF82; B: Global persistence of viable luminal LF82 after surgery and during the last 5 wk of the study in Tg rats receiving LF82 alone or CNCM I-3856 and LF82. °P < 0.001. CFU: Colony-forming unit; log10: Decimal logarithm.

ileal lesions of CD and particularly in endoscopic POR occurring 6 mo after CD-related ileocolonic resection[12]. In the present study, we show that the probiotic *S. cerevisiae* CNCM I–3856 prevents LF82-induced POR occurring 6 wk after ICR in susceptible HLA-B27 Tg rats. In our model, oral administration of the LF82 AIEC strain induced POR in 85% of HLA-B27 Tg rats raised in a controlled pathogen-free facility. The lesions developed in a concentration-dependent manner to the amount of adherent LF82; moreover, they shared many similarities with CD lesions, including erosions and ulcers that could lead to stenosis, transparietal neutrophil infiltration, and a shift in cytokine profiles toward the IL-23/IL-17 axis. The goal of the postoperative management of CD is to identify patients at highest risk of recurrence to begin prophylactic treatment with biotherapies[8]. In our study, a high fecal concentration

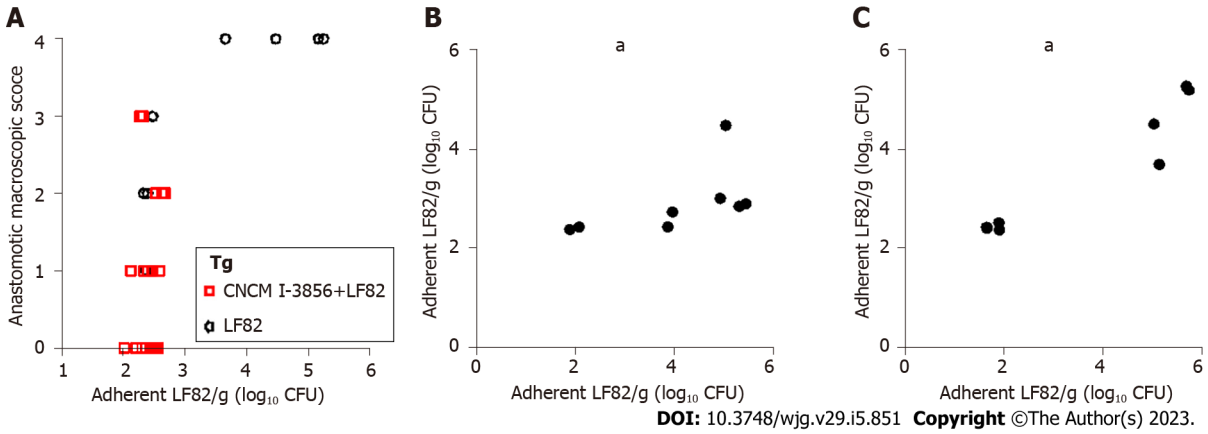

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Figure 9 Correlation between anastomotic macroscopic scores and adherent and luminal adherent-invasive *Escherichia coli* LF82 levels. A: Adherent levels of adherent-invasive *Escherichia coli* strain LF82 at sacrifice [week (W) 18] were correlated with anastomotic macroscopic scores at sacrifice in paired transgenic (Tg) animals receiving LF82 alone or in combination with *Saccharomyces cerevisiae* CNCM I-3856; B: At surgery (W12), the levels of adherent LF82 were correlated with luminal LF82 Levels in paired Tg animals receiving LF82 alone; C: At W18, the levels of adherent LF82 were correlated with luminal LF82 Levels in paired Tg animals receiving LF82 alone. $^{a}P < 0.05$, $^{b}P < 0.01$. CFU: Colony-forming unit; log10: Decimal logarithm.

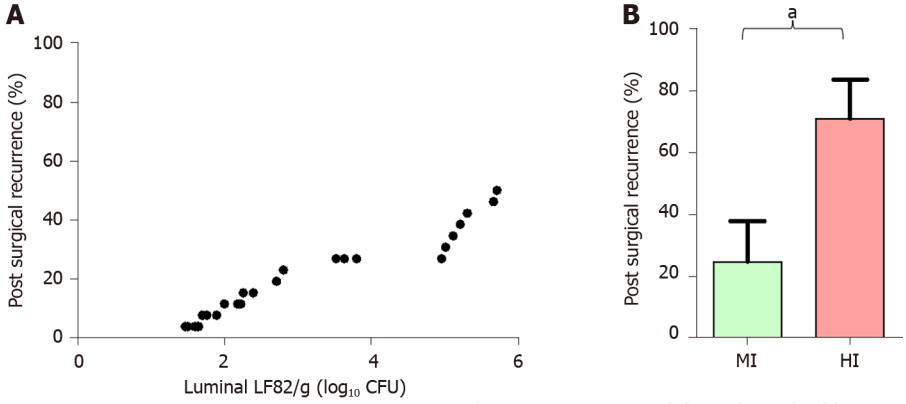

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Figure 10 Prognostic value of luminal adherent-invasive *Escherichia coli* LF82 levels in postoperative recurrence. A: Correlation between luminal adherent-invasive *Escherichia coli* strain LF82 Levels at week 14 and the risk of postoperative (POR) recurrence at W18 in transgenic (Tg) animals receiving LF82 alone or *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 and LF82; B: Higher frequency of POR in highly infected (HI) Tg animals receiving LF82 alone or *S. cerevisiae* CNCM I-3856 and LF82 as defined by a cutoff value of 2.262 Log_{10} CFUs (colony-forming units) of luminal LF82 per gram of stool at W14 in comparison with mildly infected (MI) Tg rats (71.4% *vs* 25%, $P = 0.02$). $^{a}P < 0.05$. CFU: Colony-forming unit; log10: Decimal logarithm.

of LF82 had a 70% positive predictive value for POR occurring 4 wk later. The utility of this noninvasive diagnostic biomarker for predicting POR should be considered in future clinical studies evaluating the postoperative management of CD patients.

S. cerevisiae CNCM I-3856[22] is a probiotic yeast that has already been evaluated in large-scale clinical studies showing the safety and efficacy of this strain for abdominal pain management in patients with irritable bowel syndrome[22,32-34]. In the present study, daily oral administration of *S. cerevisiae* CNCM I-3856 at 10^{9} CFU/d was perfectly tolerated and reduced the severity and frequency of POR by more than 60% in HLA-B27 Tg rats. Moreover, an absence of LF82-induced POR without any macroscopic lesions was observed in 40% of transgenic animals treated preventively with *S. cerevisiae* CNCM I-3856. To our knowledge, this is the first time that a probiotic treatment showed such efficacy in preventing POR in a rodent preclinical model of POR of CD.

Different mechanisms of action may be involved in the therapeutic preventive effect of *S. cerevisiae* CNCM I-3856 against POR. Specific fractions of β6-glucan and α4-glucan expressed by *S. cerevisiae* CNCM I-3856 represent the strongest anti-adhesive yeast cell wall components against AIEC adhesion [24,35]. In our study, prevention of LF82-induced POR by *S. cerevisiae* CNCM I-3856 was associated with a significant decrease in adherent LF82 in the intestinal mucosa of animals together with a decrease in the persistence of luminal LF82, demonstrating the ability of *S. cerevisiae* CNCM I-3856 to decolonize AIEC from the gut of rats. Additional preclinical studies will be performed in our model using specific soluble glucan fractions of *S. cerevisiae* CNCM I-3856 to avoid the constraints of a live probiotic and to optimize the therapeutic efficacy. Another possible mechanism by which *S. cerevisiae* CNCM I-3856

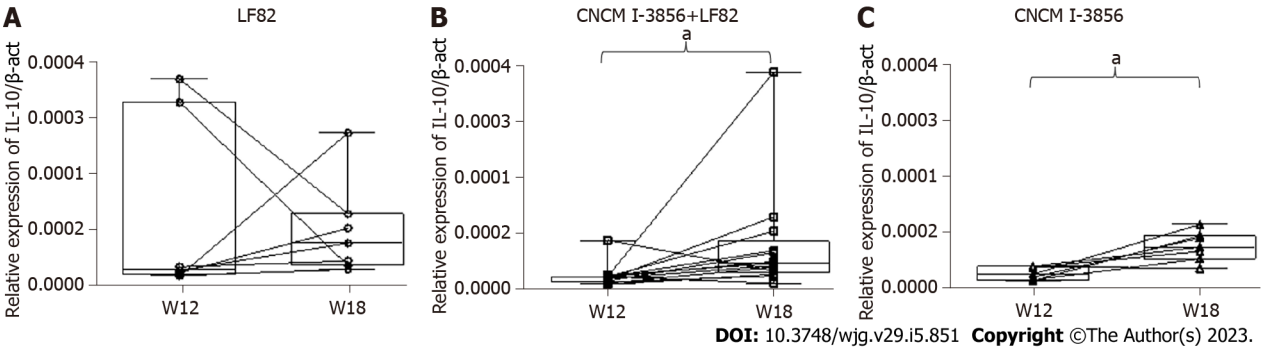

Figure 11 Interleukin-10 mRNA expression in the anastomotic mucosa. A: Interleukin (IL)-10 mRNA expression between surgery (week (W) 12) and sacrifice (W18) in paired Tg rats receiving adherent-invasive *Escherichia coli* strain LF82 alone; B: IL-10 mRNA expression between W12 and W18 in paired Tg rats receiving *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 and LF82; C: IL-10 mRNA expression between W12 and W18 in paired Tg rats receiving *S. cerevisiae* CNCM I-3856 alone. $^{a}P < 0.05$. β-act: β-actin.

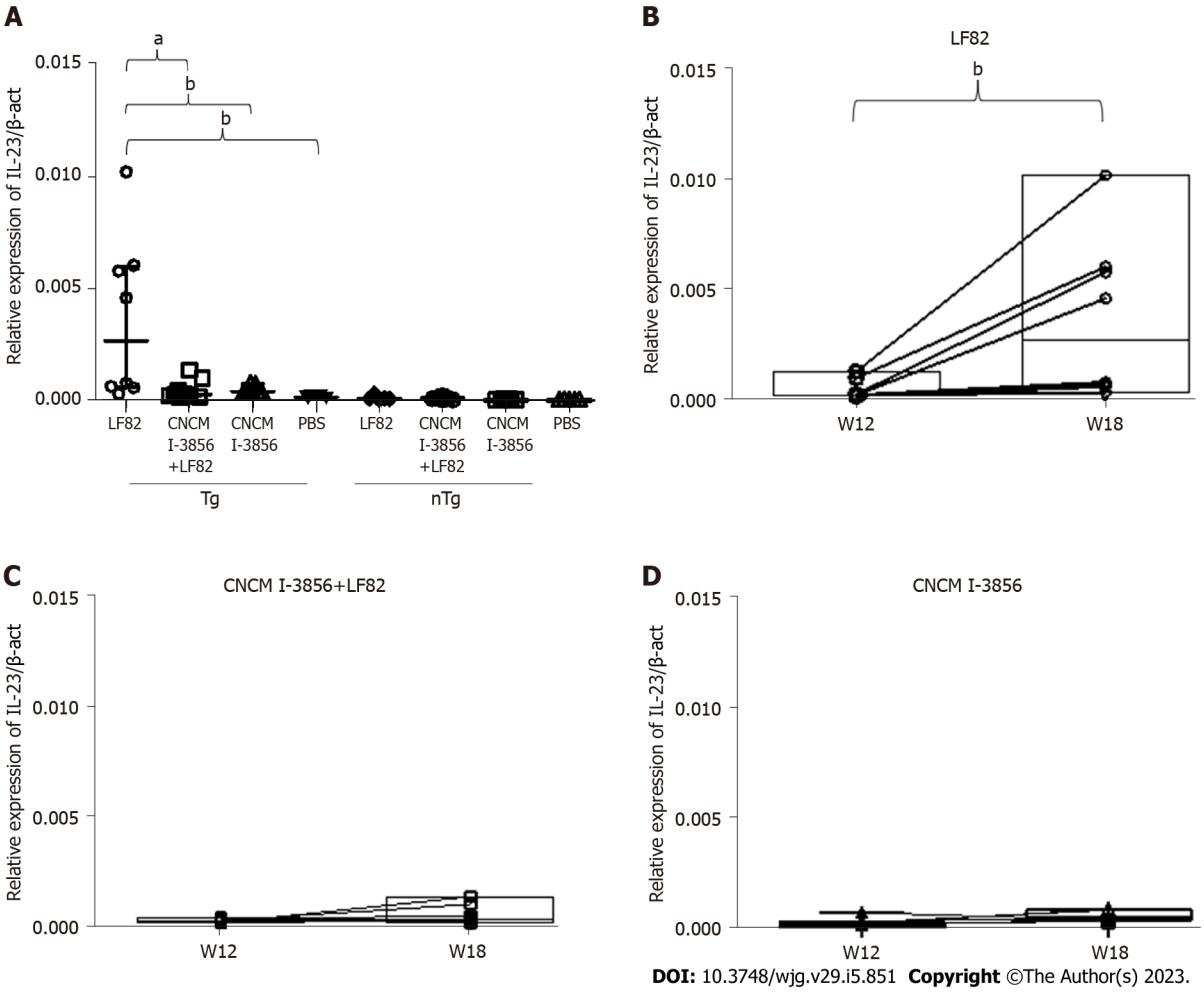

Figure 12 Interleukin-23 mRNA expression in the anastomotic mucosa. A: Expression of interleukin (IL)-23 mRNA in the perianastomotic mucosa in all transgenic (Tg) and nontransgenic (nTg) groups at sacrifice; B: IL-23 mRNA expression between surgery [week (W) 12] and sacrifice (W18) in paired Tg rats receiving adherent-invasive *Escherichia coli* strain LF82 alone; C: IL-23 mRNA expression between W12 and W18 in paired Tg rats receiving coadministration of *Saccharomyces cerevisiae* (*S. cerevisiae)* CNCM I-3856 and LF82; D: IL-23 mRNA expression between W12 and W18 in paired Tg rats receiving *S. cerevisiae* CNCM I-3856 alone. $^{a}P < 0.05$, $^{b}P < 0.01$. β-act: β-actin; PBS: Phosphate buffered saline.

prevents POR resides in its immunomodulatory and anti-inflammatory capacities[36]. We observed that the administration of *S. cerevisiae* CNCM I-3856 significantly increased IL-10 production in the intestine of rats and restored the local upregulation of IL-17 and IL-23 associated with LF82-induced POR in transgenic animals. The capacity of *S. cerevisiae* to induce IL-10 production has already been highlighted *in vitro* in bone-marrow dendritic cells and in porcine jejunal epithelial cells[36,37]. In the gut, IL-10 is

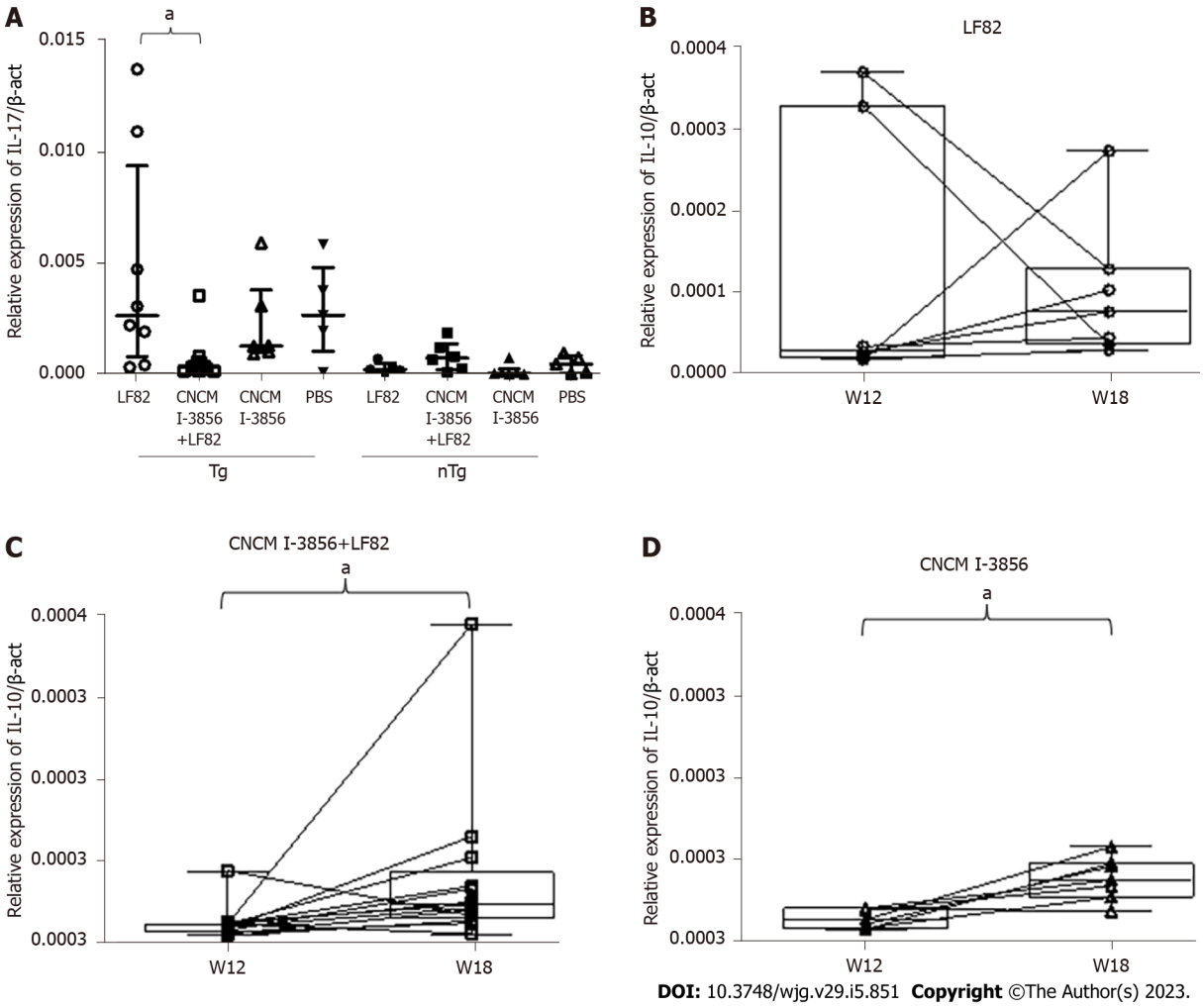

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Figure 13 Interleukin-17 mRNA expression in the anastomotic mucosa. A: Expression of interleukin (IL)-17 mRNA in the perianastomotic mucosa in all transgenic (Tg) and nontransgenic (nTg) groups at sacrifice; B: IL-17 mRNA expression between surgery [week (W) 12] and sacrifice (W18) in paired Tg rats receiving adherent-invasive *Escherichia coli* strain LF82 alone; C: IL-17 mRNA expression between W12 and W18 in paired Tg rats receiving coadministration of *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 and LF82; D: IL-17 mRNA expression between W12 and W18 in paired Tg rats receiving *S. cerevisiae* CNCM I-3856 alone. $^{a}P < 0.05$. β-act: β-actin; PBS: Phosphate buffered saline.

produced by leukocytes and intestinal epithelial cells and plays important roles in maintaining gut homeostasis and harmonizing the interaction between host immunity and luminal microorganisms[38]. In a previous study of 79 patients with CD undergoing a first ileocolectomy and ileocolonic anastomosis, we reported that a low ileal IL-10 mRNA concentration was predictive of endoscopic recurrence occurring 3 mo later[39]. Thus, the ability of *S. cerevisiae* CNCM I-3856 to induce the intestinal production of IL-10 could be a key factor in preventing POR in our model.

CONCLUSION

In conclusion, our results identified *S. cerevisiae* CNCM I-3856 as a new and original candidate for the prevention of POR in selected AIEC-infected CD patients. In a reliable model of ICR in HLA-B27 Tg rats mimicking POR of CD, *S. cerevisiae* CNCM I-3856 was found to prevent macroscopic and histologic POR through a pathobiont AIEC-targeted mechanism and through its ability to induce intestinal IL-10 production. Given that the majority of patients with CD wish to have safe, natural, nonchemotherapeutic treatment, the *S. cerevisiae* CNCM I-3856 probiotic, which is already an alternative solution for the management of patients with irritable bowel syndrome because of its ability to alleviate abdominal pain and to improve quality of life, should represent a promising therapeutic solution in the management of postoperative CD.

ARTICLE HIGHLIGHTS

Research background

The presence of adherent-invasive *Escherichia coli* (AIEC) in intestinal flora is associated with postoperative recurrence (POR) of crohn's disease (CD). *Saccharomyces cerevisiae* (*S. cerevisiae*) CNCM I-3856 is a safe and effective probiotic yeast that has already been evaluated in randomized placebo-controlled studies in patients with irritable bowel syndrome. Preclinical studies demonstrate the capacity of *S. cerevisiae* CNCM I-3856 to agglutinate invasive *Escherichia coli* strains and to prevent their adhesion to intestinal epithelial cells, favoring AIEC elimination from the gut of mice.

Research motivation

To demonstrate that *S. cerevisiae* CNCM I-3856 should be considered as a postoperative prophylactic medical therapy in CD patients harboring AIEC bacteria.

Research objectives

To evaluate the beneficial effect of *S. cerevisiae* CNCM I-3856 and its mechanisms of action in preventing AIEC-induced POR in an HLA-B27 transgenic (TgB27) rat model of CD.

Research methods

TgB27 and control rats underwent an ileocecal resection at the 12th wk of life and sacrificed 6 wk later to assess POR using macroscopic and histological scores and quantification of mucosal inflammatory/regulatory cytokines. Animals were challenged daily with an oral administration of AIEC and were treated orally with *S. cerevisiae* CNCM I-3856 (10^9 colony forming units/day). Luminal and adherent AIEC were regularly quantified throughout the duration of the study.

Research results

Eighty-seven percent of TgB27 rats developed POR characterized by anastomotic macroscopic ulcerations, transparietal neutrophil infiltration and a shift in the cytokine profile toward the interleukin (IL)-17/IL-23 axis. Oral administration of *S. cerevisiae* CNCM I-3856 reduced this POR by more than 60%, increased AIEC elimination from the gut, induced intestinal IL-10 production and restored the local upregulation of IL-17/IL-23. A high concentration of AIEC quantified in the stool of rats after surgery had a 70% positive predictive value for POR occurring 4 wk later.

Research conclusions

Ileocecal resection in TgB27 rats is a novel, useful, reliable model mimicking POR of CD and aided the discovery of new therapeutic targets. Oral administration of *S. cerevisiae* CNCM I-3856 safely prevented POR of CD through AIEC decolonization and immunomodulatory/anti-inflammatory capacities.

Research perspectives

The probiotic *S. cerevisiae* CNCM I-3856, which is already an alternative solution for the management of patients with irritable bowel syndrome to improve abdominal pain and quality of life, should represent a promising prophylactic natural nonchemotherapeutic solution in the management of postoperative CD. Monitoring AIEC levels in stool after surgery for CD should be considered as a companion test to identify patients at high risk of POR and to monitor treatment response.

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FOOTNOTES

Author contributions: Desreumaux P, Dubuquoy C and Valibouze C designed the study; Valibouze C, Dubuquoy C, M'Ba L, Schneider L and Neut C acquired the data; Genin M supervised the statistical analysis; all authors interpreted the data; Valibouze C and Desreumaux P drafted the article; All authors critically reviewed the manuscript and approved the final version for submission.

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Institutional animal care and use committee statement: Animal experiments were authorized by the departmental

ethics committee (No. CEEA 01292-01).

Conflict-of-interest statement: Mourey F is an employee of Lesaffre. Desreumaux P reports personal fees from Abbvie, personal fees from Abbott, personal fees from Amgen, personal fees from Biocodex, personal fees from Biofortis, personal fees from Biogen, personal fees from Biokuris, personal fees from Ferring, personal fees from Fresenius, personal fees from Janssen, personal fees from Kitozyme, personal fees from Lesaffre, personal fees from MSD, personal fees from Norgine, personal fees from Pfizer, personal fees from Sandoz, personal fees from Shire, personal fees from Takeda, personal fees from Tillotts, and personal fees from UCB, outside of the submitted work. In addition, Dr. Desreumaux has a patent (WO2009103884) issued. All other authors have nothing to disclose.

Data sharing statement: The dataset is available from the corresponding author at caroline.valibouze@chu-lille.fr.

ARRIVE guidelines statement: The authors read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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ORIGINAL ARTICLE

Basic Study

Impact of endothelial nitric oxide synthase activation on accelerated liver regeneration in a rat ALPPS model

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Abstract

BACKGROUND

Although the associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) induces more rapid liver regeneration than portal vein embolization, the mechanism remains unclear.

AIM

To assess the influence of inflammatory cytokines and endothelial nitric oxide synthase (eNOS) activation on liver regeneration in ALPPS.

METHODS

The future liver remnant/body weight (FLR/BW) ratio, hepatocyte proliferation, inflammatory cytokine expression, and activation of the Akt-eNOS pathway were evaluated in rat ALPPS and portal vein ligation (PVL) models. Hepatocyte proliferation was assessed based on Ki-67 expression, which was confirmed using immunohistochemistry. The serum concentrations of inflammatory cytokines were measured using enzyme linked immune-solvent assays. The Akt-eNOS pathway was assessed using western blotting. To explore the role of inflammatory cytokines and NO, Kupffer cell inhibitor gadolinium chloride ($GdCl_3$), NOS inhibitor N-nitro-arginine methyl ester (L-NAME), and NO enhancer molsidomine were administered intraperitoneally.

RESULTS

The ALPPS group showed significant FLR regeneration (FLR/BW: 1.60% ± 0.08%, $P < 0.05$) compared with that observed in the PVL group (1.33% ± 0.11%) 48 h after surgery. In the ALPPS group, serum interleukin-6 expression was suppre-

ssed using $GdCl_3$ to the same extent as that in the PVL group. However, the FLR/BW ratio and Ki-67 labeling index were significantly higher in the ALPPS group administered $GdCl_3$ (1.72% ± 0.19%, $P < 0.05$; 22.25% ± 1.30%, $P < 0.05$) than in the PVL group (1.33% ± 0.11% and 12.78% ± 1.55%, respectively). Phospho-Akt Ser^{473} and phospho-eNOS Ser^{1177} levels were enhanced in the ALPPS group compared with those in the PVL group. There was no difference between the ALPPS group treated with L-NAME and the PVL group in the FLR/BW ratio and Ki-67 labeling index. In the PVL group treated with molsidomine, the FLR/BW ratio and Ki-67 labeling index increased to the same level as in the ALPPS group.

CONCLUSION

Early induction of inflammatory cytokines may not be pivotal for accelerated FLR regeneration after ALPPS, whereas Akt-eNOS pathway activation may contribute to accelerated regeneration of the FLR.

Key Words: Hepatectomy; Nitric oxide; Liver regeneration; Cytokines; NG-Nitroarginine methyl ester; Molsidomine

Core Tip: In extended hepatectomy for hepatobiliary tumors, adequate future liver remnant (FLR) is essential to prevent postoperative liver failure. Portal vein embolization (PVE) and associated liver partition and portal vein ligation for staged hepatectomy (ALPPS) are performed to increase the FLR. Although ALPPS induces more rapid liver regeneration than PVE, the mechanism remains unclear. In this study, we compared ALPPS with portal vein ligation (PVL) in a rat model and found that activation of the Akt-endothelial nitric oxide synthase pathway promotes liver regeneration. The combination of PVL and nitric oxide-producing agents may induce liver regeneration comparable to ALPPS in a non-invasive manner.

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INTRODUCTION

Hepatectomy is the most curative treatment for hepatobiliary carcinoma[1,2]. Extended hepatectomy is occasionally performed to achieve R0 surgical margins. However, postoperative liver failure may occur in these cases because of an inadequate volume of the future liver remnant (FLR)[3,4]. To resolve this issue, portal vein embolization (PVE) is widely performed before major hepatectomy to obtain a sufficient FLR volume[5,6]. Although PVE results in a 10%-45% increase in FLR, it requires a waiting period of 2-8 wk[6-8]. Hepatectomy cannot be performed in some cases because of tumor progression, inadequate volume increase, or both in the FLR, even after PVE. Therefore, the resection rate after PVE has been reported as only 70%[7,8]. Furthermore, it has been reported that hepatocellular carcinoma (HCC) is nourished by abnormal vessels in the hepatic artery (HA). Thus, PVE may reduce blood flow in the portal vein and increase blood flow in the HA of the liver to be resected, which may result in rapid progression of HCC[9]. As described above, PVE has limited indications and therapeutic effects. Therefore, the development of new surgical or therapeutic methods is desired to promote further liver regeneration in the short term.

As an alternative to PVE, associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) was reported in 2012[6]. This method enables the FLR to increase by 70%-80% within 10 d[6]. ALPPS promotes a much faster increase of FLR than PVE[6,9], but the mechanism of this rapid liver regeneration remains unclear. Although increases in inflammatory cytokines, such as interleukin-6 (IL-6), which is an inducer in the early stage of liver regeneration, have been reported as a cause of rapid liver regeneration[10-13], it remains controversial[14]. However, in previous studies on the mechanism of liver regeneration after liver resection and portal vein ligation (PVL), shear stress caused by blood viscosity, blood flow velocity, and endothelial nitric oxide synthase (eNOS) activation, followed by NO induction, has been reported to promote liver regeneration[15,16]. This study aimed to explore the mechanism of promoting liver regeneration in ALPPS and investigate the involvement of inflammatory

cytokines and eNOS activation using PVL and ALPPS rat models.

MATERIALS AND METHODS

Animals

Eight-week-old male Wistar rats (CLEA Japan, Kanagawa, Japan) weighing 230-300 g were used in this study. The animals were housed in wood-chip-bedded cages in an air-conditioned room (24 ± 1 °C) with a 12 h light/dark cycle under specific pathogen free condition. There were no diet restrictions. Based on national and institutional regulations and guidelines, all procedures for animal experiments were reviewed by the Committee for Animal Experiments and approved by the President of Shinshu University (Approval numbers 270018 and 019067).

Surgical procedures and study design

Rats were divided into two groups, PVL and ALPPS, and examined 72 h after surgery. A midline laparotomy was performed under isoflurane-induced anesthesia. In the PVL model, the portal vein branches to the caudate lobe, left lobe, left side of the median lobe, and right lobes were ligated with 7-0 silk (Figure 1A). In the ALPPS model, in addition to PVL, liver parenchymal transection between the right lobe and the left side of the middle lobe was performed based on the gross morphology and demarcation line after PVL. The Glisson flowing into the left side of the median lobe was ligated with 7-0 nylon (Figure 1B). Little bleeding occurred during the liver parenchymal transection because the parenchyma on either side of the dissection line was ligated with 6-0 Prolene before parenchymal transection to control intraoperative bleeding. The abdomen was then closed in layers.

The rats were sacrificed to collect blood samples and liver tissue from the right side of the median lobe (RML) at 1, 4, 6, 24, 48, and 72 h after surgery (n = 5 for each group per time point). Blood samples were collected from the inferior vena cava at the time of liver removal and centrifuged at 2600 × g for 5 min. The serum was stored at -80 °C. Liver tissue samples were frozen in liquid nitrogen and stored at -80 °C. The remaining liver tissue was fixed with 4% paraformaldehyde.

The weight of the FLR, that is, the RML, and body weight (BW) were measured before surgery and at 24, 48, and 72 h after surgery. The BW (FLR/BW) ratio (%) was used as the liver regeneration index. In western blotting analysis and volumetric blood flow analysis, the PVL and ALPPS groups were compared based on the control group, in which only open and closed abdomens were performed.

ELISAs of serum inflammatory cytokines and hepatocyte growth factor

Serum concentrations of IL-6, tumor necrosis factor-α (TNF-α), and hepatocyte growth factor (HGF) were measured at 1, 4, 6, and 24 h after surgery using ELISA kits (R&D Systems, Minneapolis, MN, United States). IL-6 concentration in the RML tissue was also quantified 1 h after surgery.

Immunohistochemistry

The liver tissues were fixed with paraformaldehyde and embedded in paraffin. After deparaffinization, antigen retrieval, and quenching of endogenous peroxidases, the sections were incubated overnight at 4 °C with a mouse monoclonal anti-Ki-67 antibody (1:200 dilution; Dako, Glostrup, Denmark; 1:200 dilution, Abcam, Cambridge, United Kingdom), followed by incubation for 30 min at room temperature with a peroxidase-labeled anti-mouse antibody (Histofine Simplestain Max PO; Nichirei). The sections were immersed in diaminobenzidine solution for visualization and counterstained with hematoxylin. To evaluate hepatocyte proliferation 48 h after surgery, the average percentage of Ki-67-positive cells to total hepatocytes in three random high-power fields was used as the Ki-67 labeling index.

Kupffer cell inhibition in the ALPPS model

To explore the role of inflammatory cytokines in liver regeneration, the Kupffer cell inhibitor gadolinium chloride ($GdCl_3$; Sigma-Aldrich, St. Louis, MO, United States) was used. Another set of animals was used for the Kupffer cell inhibition experiments. We prepared an ALPPS model for $GdCl_3$ administration (n = 3). $GdCl_3$ (10 mg/kg) was administered intraperitoneally 24 h before surgery. In the control group, physiological saline was administered. All rats were sacrificed 48 h after surgery to obtain liver samples.

NOS inhibition in the ALPPS model and NO enhancement in the PVL model

To explore the role of NO in liver regeneration, the NOS inhibitor NG-nitro-arginine methyl ester (L-NAME; Sigma-Aldrich) and the NO enhancer molsidomine (Cayman Chemical, MI, United States) were used. Another set of animals was used for the NOS inhibition and NO enhancement experiments. We prepared the ALPPS model for L-NAME administration, the PVL model for molsidomine administration, and the corresponding control PVL and ALPPS models (n = 5 for each group). L-NAME (100 mg/kg) or molsidomine (10 mg/kg) was administered intraperitoneally 24 h before and during surgery. In each control group, physiological saline was administered. All rats were sacrificed 24, 48, and 72 h

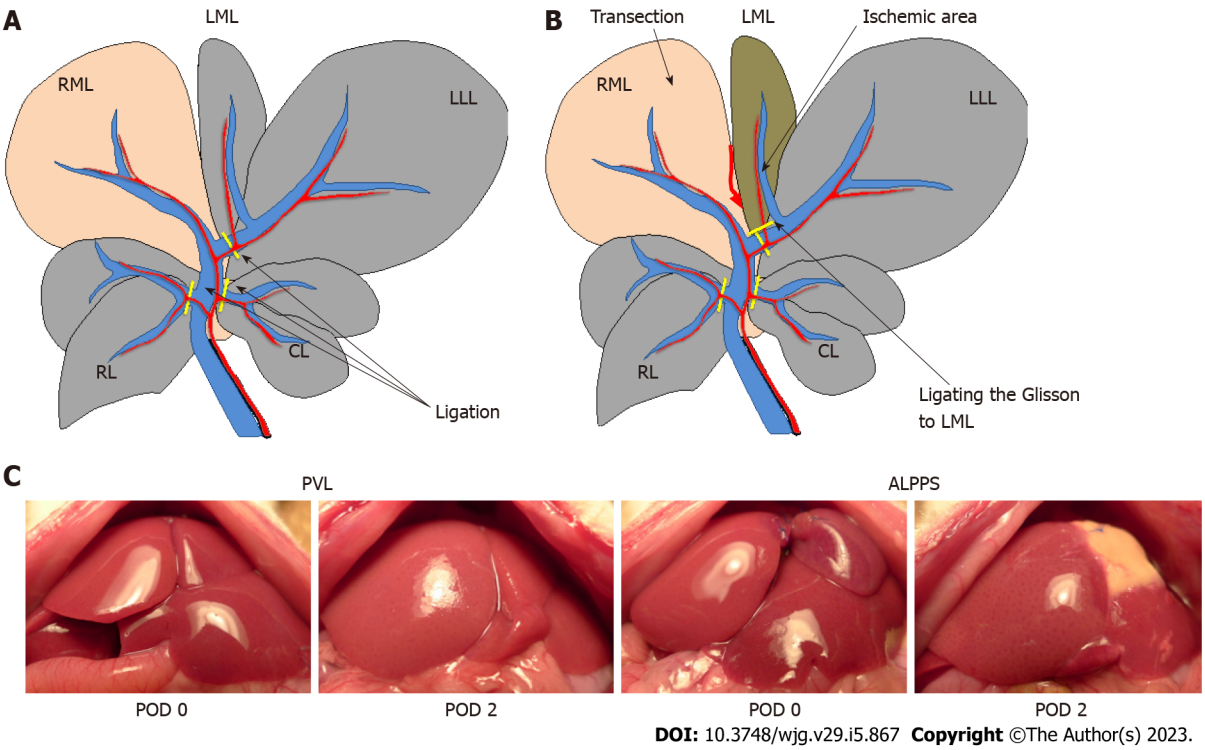

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Figure 1 Schema of experimental models. A: Portal vein ligation (PVL) group. Portal vein branches were ligated, other than the right median lobe; B: Associating liver partition and PVL for staged hepatectomy group. In addition to ligating the portal vein as performed in the PVL group, the median lobe was transected, and the left Glisson was ligated; C: Macroscopic findings after operations in each group. ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; PVL: Portal vein ligation; RML: Right median lobe; LML: Left median lobe; LLL: Left lateral lobe; RL: Right lobe; CL: Caudate lobe; POD: Postoperative day.

after surgery to obtain liver samples.

Western blot analysis

The RML tissue proteins were collected at 1, 4, and 6 h after surgery using radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Inc., CA, United States). The protein concentration was measured using the bicinchoninic acid assay method. Samples of 10 μg proteins from FLRs of PVL and ALPPS models were separated on 4%-12% NuPAGE Gels and transferred onto nitrocellulose membranes. After blocking with 5% dry skim milk for 1 h, the membranes were incubated with primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. The blots were developed with ECL Select western blotting Detection Reagent (Amersham, GE Healthcare Life Sciences, Chicago, IL, United States) and photographed using a Molecular Imager ChemiDoc XRS device (Bio-Rad Laboratories, Inc., Hercules, CA, United States). The density of the bands in the immunoblots was analyzed using Image Lab Software (Bio-Rad Laboratories, Inc.). The results are expressed as a percentage of the β-actin internal control. The anti-human antibodies used were rabbit monoclonal antibodies against p-Akt (Ser 473) (cat. no. 4060), p-eNOS (Ser1177) (Cat. no. 9570), p-eNOS (Thr495) (Cat. no. 9574), total eNOS (Cat. no. 32027) (Cell Signaling Technology, Inc., Danvers, MA, United States), and mouse monoclonal antibody against β-actin (Cat. no. A5441; Sigma-Aldrich). Anti-β-actin antibody was used at a 1:3000 dilution, and the other antibodies were used at a 1:1000 dilution.

Volumetric blood flow analysis

Before the estimation of volumetric blood flow in the HA and PV of the FLR, blood velocity and vascular diameter (r) were measured using ultrasonography (Vevo2100, Primetech, Tokyo, Japan). Volumetric blood flow was estimated from the blood velocity and vascular cross-sectional area (πr^2) (volumetric blood flow = blood velocity × πr^2) in mm^3 per second.

Statistical analysis

The collected data were evaluated statistically using the JMP software, version 13.2 (SAS Institute, Cary, NC, United States). Data are expressed as mean ± SD. Statistical analysis was performed using an unpaired student's *t*-test. Statistical significance was defined as $P < 0.05$.

RESULTS

Differences in liver regeneration in PVL and ALPPS models

The FLR/BW ratio increased over time in both groups. At 48 h after surgery, the FLR/BW ratio in the ALPPS group was significantly higher (1.60% ± 0.08%, $P < 0.05$) than that in the PVL group (1.33% ± 0.11%) (Figures 1C and 2A). However, no significant difference was observed between the two groups at 24 and 72 h after surgery. The Ki-67 labeling index of the RML at 48 h after surgery was significantly increased in the ALPPS group (22.1% ± 4.01%, $P < 0.05$) compared with that in the PVL group (12.8% ± 1.73%), which was consistent with the FLR/BW ratio (Figures 2B and 2C).

Association between serum inflammatory cytokines and liver regeneration

The serum concentrations of IL-6, TNF-α, and HGF in RML were measured at 1, 4, 6, and 24 h after surgery. Serum IL-6 and TNF-α levels increased in both groups after surgery compared with the levels before surgery. However, no difference was found in the two groups at 1, 4, and 6 h after surgery. At 24 h after surgery, IL-6 and TNF-α concentrations were significantly higher in the ALPPS group (25.91 ± 6.05 pg/mL, $P < 0.05$ and 1.52 ± 0.68 pg/mL, $P < 0.05$) compared with that in the PVL group (4.11 ± 3.99 pg/mL and 0.54 ± 0.38 pg/mL). Serum HGF concentration at 1 h after surgery was significantly higher in the ALPPS group (68.86 ± 4.89 ng/mL, $P < 0.05$) compared with that in the PVL group (55.34 ± 9.97 ng/mL). However, no significant difference was observed in serum HGF concentration at 4, 6, and 24 h (Figures 3A-C).

Liver regeneration in the ALPPS model under the suppression of IL-6 using $GdCl_3$

To evaluate the effect of IL-6 on liver regeneration, ALPPS rats were administered $GdCl_3$, which suppressed the activation of Kupffer cells in the liver. In the $GdCl_3$-ALPPS group, the IL-6 concentrations in serum (40.3 ± 11.3 pg/mL, $P < 0.05$) and the RML tissue (3.27 ± 0.54 ng/TP 1 g, $P < 0.05$) 1 h after surgery were significantly decreased compared with the concentrations in the corresponding groups without administration of $GdCl_3$ (Figures 4A and 4B). However, there was no significant difference in the FLR/BW ratio or Ki-67 labeling index at 48 h after surgery in the ALPPS group with or without administration of $GdCl_3$ (Figures 4C and 4D).

Short-term postoperative liver regeneration induced by eNOS

Phosphorylation of Akt and eNOS in RML tissue at 1, 4, and 6 h after surgery was evaluated using western blotting (Figure 5A). Phospho-Akt Ser^{473} and phospho-eNOS Ser^{1177} levels increased in the ALPPS group compared with those in the PVL group. The quantitative measurement revealed that the phosphorylation levels of eNOS Ser^{1177} in the ALPPS group was significantly higher than that in the PVL group at 1 and 4 h after surgery. However, there was no significant difference at 6 h after surgery (Figure 5B).

L-NAME, an NOS inhibitor, was administered to rats to examine whether suppression of eNOS affected liver regeneration. The FLR/BW ratio and Ki-67 labeling index at 48 h after surgery in the L-NAME-ALPPS group were significantly lower than those in the ALPPS group without L-NAME administration and were comparable to those in the PVL group (Figures 6A and 6B).

Additionally, molsidomine, which induces eNOS activation, was administered to the rats to examine whether eNOS activation affects liver regeneration. The FLR/BW ratio and Ki-67 labeling index at 48 h after surgery in the molsidomine-administered PVL (molsidomine-PVL) group were significantly higher than those in the PVL group without molsidomine administration and comparable with those in the ALPPS group (Figures 6C and 6D). However, there was no significant difference in the long-term FLR/BW ratio on a postoperative day 7 between the PVL, ALPPS, and molsidomine-administered PVL groups (data not shown).

Increased HA blood flow in the ALPPS model

PV flow in the PVL and ALPPS groups was significantly faster than that in the control group; however, there was no significant difference in PV flow between the PVL and ALPPS groups (180.1 ± 54.4, 216.6 ± 71.4 mm^3/s) (Figure 7A). HA flow in the PVL group was significantly slower than that in the control group without surgical intervention (1.73 ± 1.14 *vs* 3.66 ± 0.74 mm^3/s, $P < 0.05$), whereas that in the ALPPS group was significantly faster (11.32 ± 2.40 mm^3/s, $P < 0.05$) than that in control and PVL groups (Figure 7B). The total blood flow, that is, the sum of PV and HA, was not significantly different between the PVL and ALPPS groups (Figure 7C).

DISCUSSION

Hepatectomy is the most curative treatment for HCC and intraductal cholangiocarcinoma[1,2]. Additionally, major hepatectomy is the standard operative procedure for perihilar cholangiocarcinoma [17,18]. Extended hepatectomy may be required, depending on the location of the cancer. Postoperative

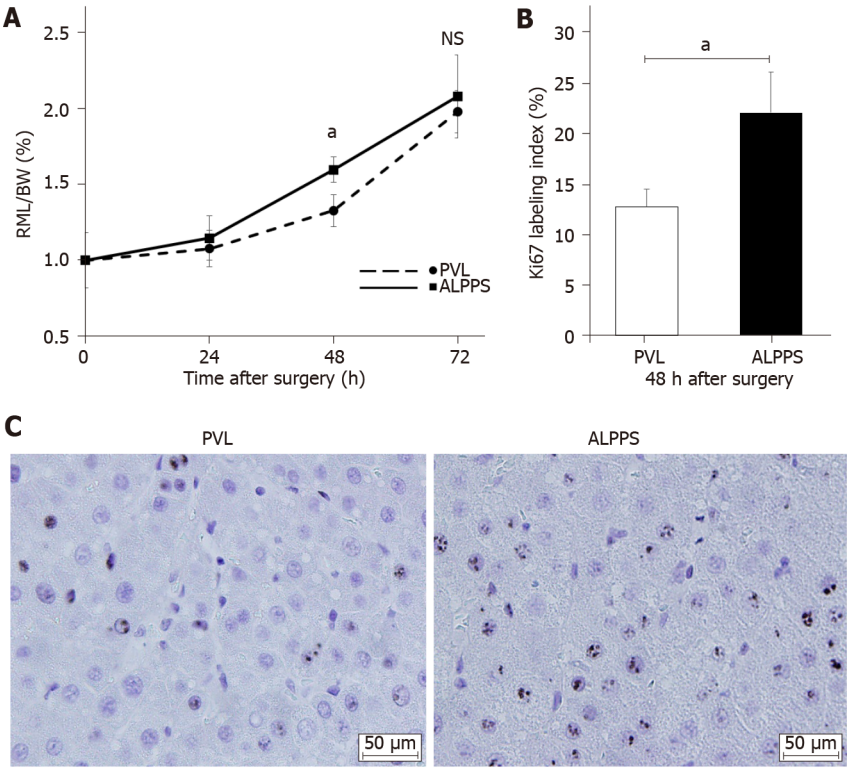

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Figure 2 Changes in the right side of the median lobe weight to body weight ratio and Ki-67 index after surgery. A: Future liver remnant/body weight ratio up to 72 h after surgery; B: Immunohistochemistry of Ki-67 at 48 h after the operation; C: Ki-67 labeling index at 48 h after the surgery. Values are expressed as the mean ± SD; $n = 5$ for each group; $^{a}P < 0.05$; NS: Not significant; RML/BW: Right side of the median lobe weight/body weight; PVL: Portal vein ligation; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy.

liver failure that results from insufficient residual liver volume is a fatal complication of hepatectomy. PVE and ALPPS were developed with the aim of pre-operative liver enlargement to avoid postoperative liver failure[5,6,18]. ALPPS leads to the rapid regeneration of FLR compared with PVE, although high mortality (90-d mortality of 9%) and morbidity (grade IIIb of 40% in the Clavien-Dindo classification) are limitations[19]. Elucidation of the mechanism of rapid liver regeneration after ALPPS may contribute to improving surgical outcomes for patients who undergo extended hepatectomy for hepatobiliary malignancies and to the development of novel alternative treatments that provide effective and safe regeneration of the FLR.

In this study, we obtained two crucial findings regarding the mechanism of liver regeneration in ALPPS. First, the induction of inflammatory cytokines, such as IL-6, might not be pivotal for the rapid regeneration of FLR after ALPPS in the early phase. Second, activation of the Akt-eNOS pathway may be an important factor in promoting liver regeneration after ALPPS.

The mechanism of liver regeneration has been studied in animal models of partial hepatectomy. The regeneration process is distinctive, complex, and well-coordinated and depends on the interactions of several signaling pathways, cytokines, and growth factors. Additionally, endocrine hormones, such as norepinephrine, growth hormone, insulin, and thyroid hormones, have been reported to influence these pathways and factors[20-22]. Since Schnitzbauer *et al*[6] reported ALPPS in 2012, there have been several reports to elucidate the major factors in liver regeneration of ALPPS, which promote rapid liver regeneration compared with PVL[10-13,23]. Activation of downstream signals, such as c-Jun N-terminal kinase-Indian hedgehog signaling from stellate cells by inflammatory cytokines[24], activation of the Janus kinase 2/signal transducer and activator of transcription 3 pathway *via* regenerating islet-derived 3α/3β, and hypoxia-induced stabilization of hypoxia-inducible factor-α subunits by hypoxia[14,25,26], have been reported as major factors. However, the mechanism of liver regeneration in ALPPS has not yet been completely elucidated.

Previous studies have reported that the peak of cell proliferation is 48 h after surgery, and inflammatory cytokines and their downstream signal enhancement cause liver regeneration in ALPPS[11,12,23]. Although the peak liver regeneration in this study was consistent with previous studies, the relationship between the early induction of inflammatory cytokines and liver regeneration was not consistent. In this study, serum concentrations of inflammatory cytokines, such as IL-6 and TNF-α, in the short term (1, 4, and 6 h) after surgery did not differ between the ALPPS and PVL groups. However, the ALPPS group showed a greater increase in FLR and a higher Ki-67 labeling index than in the PVL group. Additionally, suppression of inflammatory cytokines using $GdCl_3$ did not suppress liver

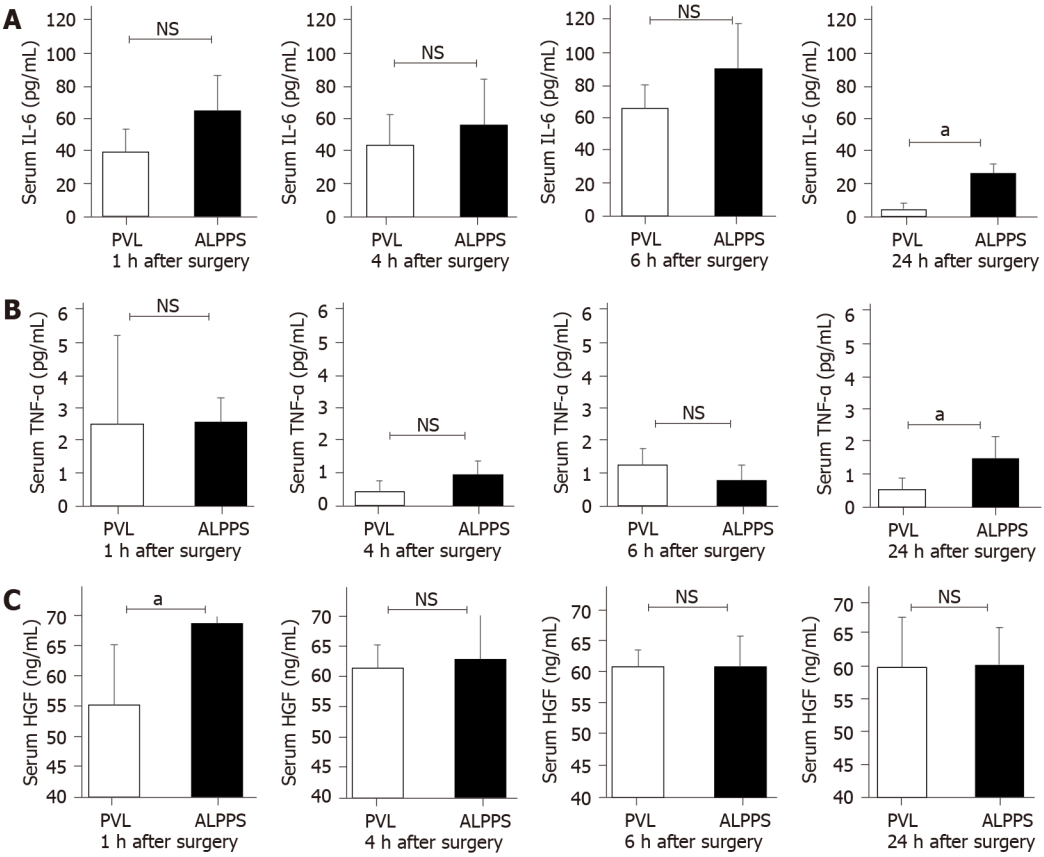

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Figure 3 Expression of inflammatory cytokines and hepatocyte growth factor in serum and right side of the median lobe tissue. A: Serum interleukin-6 concentrations at 1, 4, 6, and 24 h after surgery; B: Tumor necrosis factor-α concentrations at 1, 4, 6, and 24 h after surgery; C: Hepatocyte growth factor concentration at 1, 4, 6, and 24 h after surgery. Values are expressed as the mean ± SD; $n = 5$ for each group; $^{a}P < 0.05$; NS: Not significant; PVL: Portal vein ligation; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; IL: Interleukin; TNF: Tumor necrosis factor; HGF: Hepatocyte growth factor.

regeneration. These results suggest that the induction of inflammatory cytokines in the early phase after ALLPS is not necessarily a major factor in accelerating liver regeneration. The reason why no difference was observed in the expression of inflammatory cytokines may be the site of liver resection, setting of FLR, or differences in animal models. The timing of specimen collection may have influenced the results, as specimens collected 24 h after surgery had higher concentrations in the ALPPS group.

Activation of eNOS and NO induction have been reported to be a mechanism of liver regeneration other than inflammatory cytokines[15,16]. In this study, we focused on the effect of eNOS activation on liver regeneration after PVL and ALPPS. Evaluation of eNOS activation in the liver tissue showed that eNOS Ser^{1177} phosphorylation was significantly increased in the ALPPS model at 1 and 4 h after surgery. Thus, the FLR/BW ratio and Ki-67 labeling index in the ALPPS model were increased compared with those in the PVL model. Furthermore, the activation of Akt, which is upstream of eNOS, was observed, suggesting that the Akt-eNOS pathway contributes to the mechanism of liver regeneration in ALPPS. The administration of L-NAME, which suppresses NO, inhibits liver regeneration. The administration of molsidomine, which activates eNOS, promotes liver regeneration. Molsidomine is a nitrate drug used as a coronary vasodilator for the treatment of angina pectoris; its intermediate metabolite, SIN-1 (ionidamine chlorohydrate) produces NO[27]. When endothelial cells are stimulated by shear stress or vascular endothelial growth factor, phosphoinositide 3-kinase (PI3K) is activated and PIP3 is produced, which activates the PI3K-Akt pathway and activates downstream signals such as eNOS[28,29]. An increase in shear stress, which has been reported to cause NO production[30], is due to hemodynamic changes in the residual liver caused by hepatectomy, which is expected to affect liver regeneration in ALPPS. To evaluate the effect of increased shear stress on liver regeneration, we examined the blood flow exchange after PVL and ALPPS. Contrary to our expectations, there was no difference in PV or total blood flow, which might be associated with shear stress, between the PVL and ALPPS groups; however, HA flow in the ALPPS group was significantly higher than that in the PVL and control groups. Therefore, the difference in oxygenation of the FLR, rather than the shear stress between ALPPS and PVL, might be associated with the difference in liver regeneration. However, Schadde *et al*[25] reported that hypoxia due to reduced HA flow in the FLR promotes hepatic regeneration in patients who underwent ALPPS and in the rat ALPPS model. However, in their study, HA flow was evaluated

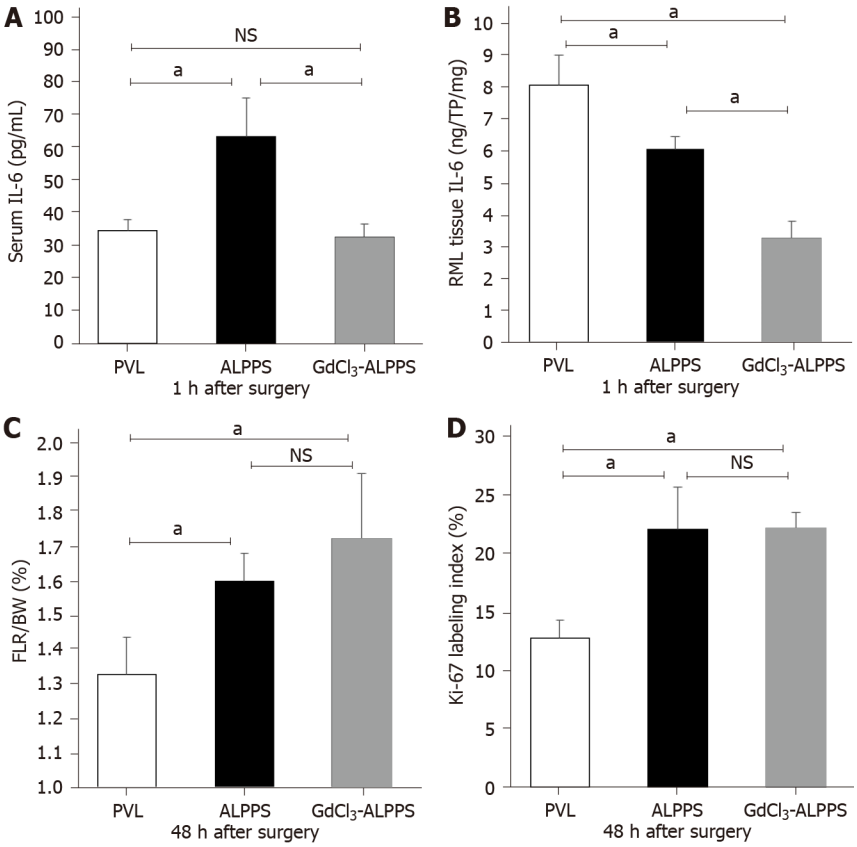

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Figure 4 Interleukin-6 expression and liver regeneration in the gadolinium chloride model. A: The serum concentration of interleukin (IL)-6 at 1 h after surgery; B: IL-6 concentration in right side of the median lobe tissue at 1 h after surgery; C: Future liver remnant/body weight ratio at 48 h after surgery; D: Ki-67 labeling index. Values are expressed as mean ± SD; n = 3 or 5 for each group; $^{a}P < 0.05$; NS: Not significant; PVL: Portal vein ligation; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; IL: Interleukin; RML: Right median lobe; FLR/BW: Future liver remnant/body weight; $CdCl_3$: Gadolinium chloride.

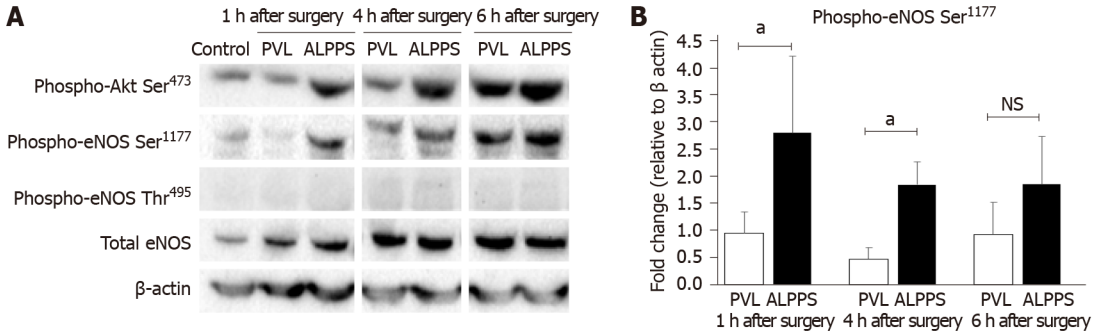

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Figure 5 Western blotting of Akt-endothelial nitric oxide synthase pathway-related proteins. A: Western blotting was used to evaluate the expression of phosphorylated Akt and endothelial nitric oxide synthase (eNOS) in right side of the median lobe at 1, 4, and 6 h after surgery in portal vein ligation (PVL) and associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) groups; B: Comparison of the expression of P-Akt Ser^{473} and P-eNOS Ser^{1177} in PVL and ALPPS groups (quantification of western blots, n = 5 for each group). Values are expressed as the mean ± SD; n = 5 for each group; $^{a}P < 0.05$; NS: Not significant; eNOS: Endothelial nitric oxide synthase; PVL: Portal vein ligation; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy.

only in patients who underwent ALPPS, and this evaluation was not compared with that in patients who underwent PVE. Furthermore, the transition of HA flow before and after ALPPS has not been evaluated in a rat model. In the rat ALPPS model, liver transection between the right and left median lobes with ligation of the Glisson of the left median lobe caused a necrotic change in the left median lobe, which is synonymous with liver resection of the left median lobe considering hemodynamics. These results suggest that both hemodynamic changes and differences in oxygenation of the FLR affect regeneration rates in the ALPPS and PVL models. The increased HA flow to the RML observed in the

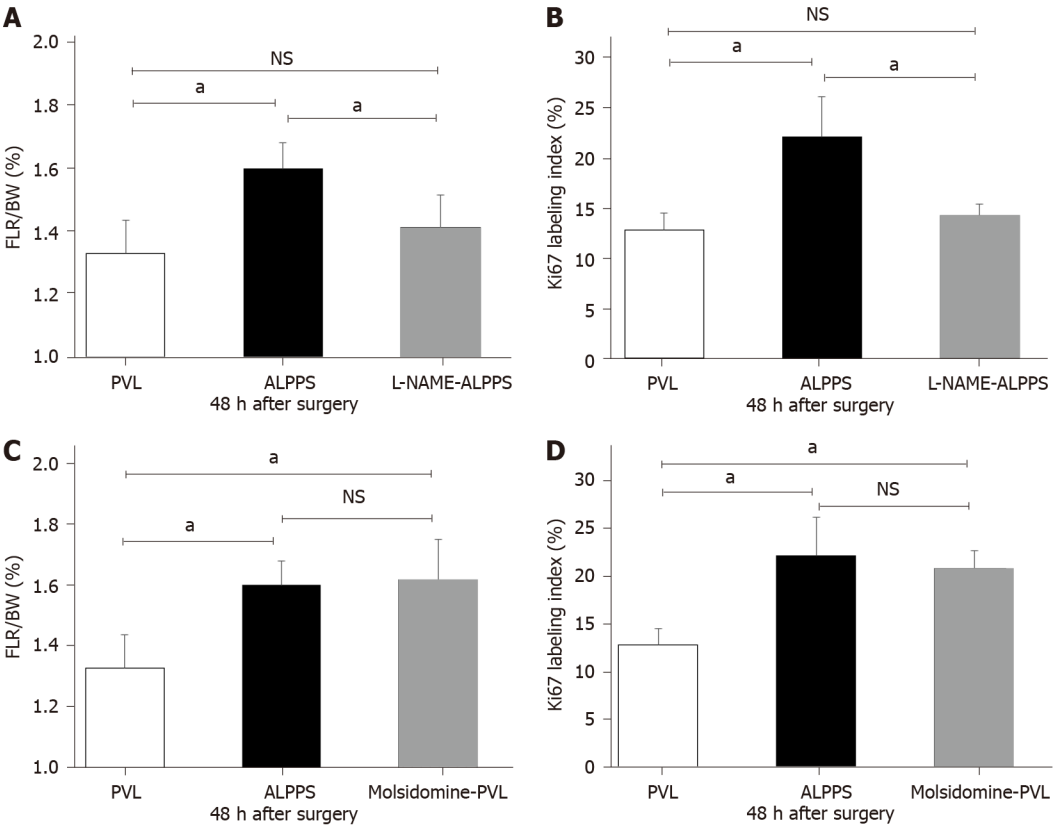

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Figure 6 Changes in liver regeneration and cell proliferation due to drug administration. A: Future liver remnant/body weight (FLR/BW) ratio in the N-nitro-arginine methyl ester (L-NAME)-administered associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) group; B: Ki-67 labeling index in the L-NAME-administered ALPPS group; C: FLR/BW ratio in the molsidomine-administered portal vein ligation (PVL) group; D: Ki-67 labeling index in the molsidomine-administered PVL group; n = 5 for each group; $^{a}P < 0.05$; NS: Not significant; PVL: Portal vein ligation; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; FLR/BW: Future liver remnant/body weight; L-NAME: N-nitro-arginine methyl ester.

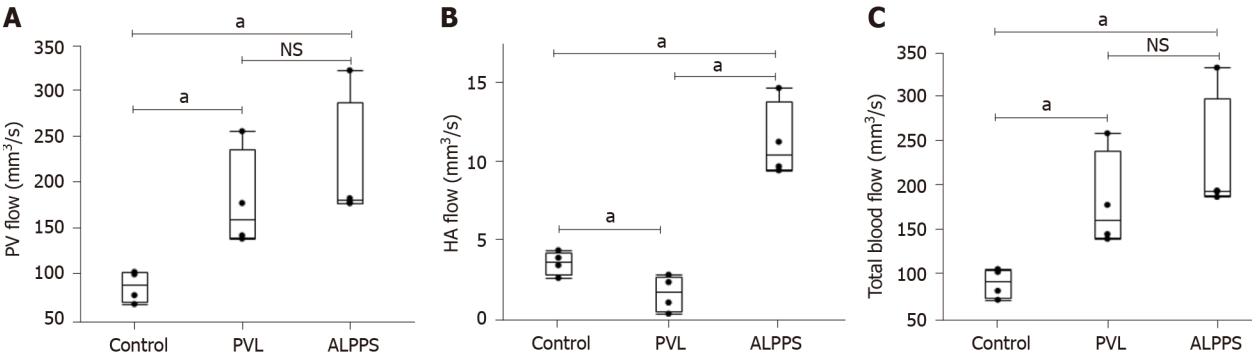

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Figure 7 Evaluation of hepatic artery and portal vein volumetric blood flow in the future liver remnant after surgery. A: Portal vein (PV) flow in control, PV ligation (PVL), and associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) groups; B: Hepatic artery flow in control, PVL, and ALPPS groups; C: Total blood flow in control, PVL, and ALPPS groups. Values are expressed as the mean ± SD; n = 4 for each group; $^{a}P < 0.05$; NS: Not significant; PVL: Portal vein ligation; ALPPS: Associating liver partition and portal vein ligation for staged hepatectomy; HA: Hepatic artery.

ALPPS group may have been due to a hepatic arterial buffer response derived from collateral blood flow blockage by hepatectomy. In contrast, the reason for the observed decrease in HA flow to the RML in the PVL group might be the effect of HA influx from the RML to the left median lobe (LML) *via* collateral circulation after the PV blockade to the LML.

This study had some limitations. First, because we observed short-term changes in rat models, it is unknown whether NO activation promotes clinically meaningful liver regeneration in humans. Second, the mechanism underlying the activation of the Akt-eNOS pathway is unclear and requires further investigation that includes real-time monitoring of oxygenation in the FLR. Despite these shortcomings,

we believe that our results are of interest because few reports have focused on the relationship between eNOS activation and liver regeneration after ALPPS.

CONCLUSION

The activation of the Akt-eNOS pathway in ALPPS may be an important factor in promoting early liver regeneration. If a combination of NO-producing agents and PVL or PVE enables liver regeneration within a short time after surgery, it may be an alternative to ALPPS and is expected to be applied clinically as a less invasive procedure.

ARTICLE HIGHLIGHTS

Research background

Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) has already been clinically applied in various countries. Although it has been reported that ALPPS offers faster and larger liver regeneration compared to portal vein embolization (PVE), the mechanism of this phenomenon is still unclear.

Research motivation

The aim of this study was to investigate the underlying mechanism of rapid liver regeneration after ALPPS focusing on inflammatory cytokines and endothelial nitric oxide synthase (eNOS) activation.

Research objectives

Activation of eNOS was considered one of key points on mechanism of rapid liver regeneration after ALPPS.

Research methods

Liver regeneration was compared between the rat portal vein ligation (PVL) model and the rat ALPPS model. In addition, impact of administration of gadolinium chloride ($GdCl_3$, Kupffer cell inhibitor), NG-nitro-arginine methyl ester (L-NAME, NOS inhibitor), and molsidomine (NO enhancer) on liver regeneration after PVL and/or ALPPS.

Research results

Administration of $GdCl_3$ before ALPPS provided no significant negative influence of liver regeneration after ALPPS. Administration of L-NAME before ALPPS suppressed liver regeneration after ALPPS, while administration of molsidomine before PVL accerelated liver regeneration after PVL as well as ALPPS.

Research conclusions

ALPPS is an alternative to PVE for reducing posthepatectomy liver failure after major hepatectomy.

Research perspectives

Combination of NO-producing agents and less invasive procedure can be an alternative to ALPPS procedure in the future.

FOOTNOTES

Author contributions: Masuo H, Motoyama H, Yoshizawa T, Hosoda K, and Yasukawa K contributed to the acquisition and analysis of experimental data and drafting of the manuscript; Shimizu A, Kubota K, Notake T, Kobayashi A, and Soejima Y contributed to the conception and design of the study and made critical revisions related to the important intellectual content of the manuscript; and all authors have provided final approval for the version of the manuscript for submission.

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Institutional animal care and use committee statement: Based on national and institutional regulations and guidelines, all procedures for animal experiments were reviewed by the Committee for Animal Experiments and approved by the President of Shinshu University (Approval numbers 270018 and 019067).

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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ORIGINAL ARTICLE

Retrospective Study

Convolutional neural network-based segmentation network applied to image recognition of angiodysplasias lesion under capsule endoscopy

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Abstract

BACKGROUND

Small intestinal vascular malformations (angiodysplasias) are common causes of small intestinal bleeding. While capsule endoscopy has become the primary diagnostic method for angiodysplasia, manual reading of the entire gastrointestinal tract is time-consuming and requires a heavy workload, which affects the accuracy of diagnosis.

AIM

To evaluate whether artificial intelligence can assist the diagnosis and increase the detection rate of angiodysplasias in the small intestine, achieve automatic disease detection, and shorten the capsule endoscopy (CE) reading time.

METHODS

A convolutional neural network semantic segmentation model with a feature fusion method, which automatically recognizes the category of vascular dysplasia under CE and draws the lesion contour, thus improving the efficiency and accuracy of identifying small intestinal vascular malformation lesions, was proposed. Resnet-50 was used as the skeleton network to design the fusion mechanism, fuse the shallow and depth features, and classify the images at the pixel level to achieve the segmentation and recognition of vascular dysplasia. The training set and test set were constructed and compared with PSPNet, Deeplab3+, and UperNet.

RESULTS

The test set constructed in the study achieved satisfactory results, where pixel accuracy was 99%, mean intersection over union was 0.69, negative predictive value was 98.74%, and positive predictive value was 94.27%. The model parameter was 46.38 M, the float calculation was 467.2 G, and the time length to segment and recognize a picture was 0.6 s.

CONCLUSION

Constructing a segmentation network based on deep learning to segment and recognize angiodysplasias lesions is an effective and feasible method for diagnosing angiodysplasias lesions.

Key Words: Artificial intelligence; Image segmentation; Capsule endoscopy; Angiodysplasias

Core Tip: Small intestinal vascular malformation (vascular dysplasia) is a common cause of small intestinal bleeding. Herein, we proposed a semantic recognition segmentation network to recognize small intestinal vascular malformation lesions. This method can assist doctors in identifying lesions, improving the detection rate of intestinal vascular dysplasia, realizing automatic disease detection, and shortening the capsule endoscopy reading time.

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INTRODUCTION

Small intestinal vascular malformations (angiodysplasias) are common causes of small intestinal bleeding[1,2]. Angiodysplasias are degenerative lesions that manifest as abnormalities of arteries, veins, or capillaries of the original normal blood vessels. Occasionally, the term angiodysplasias include various synonymous disease concepts, such as angioectasia (AE), Dieulafoy's lesion (DL), and arteriovenous malformation. According to the Yano-Yamamoto classification, small bowel vascular lesions are classified into four types under endoscopy[3]. AE includes small erythemas and can be defined as type 1a: punctuate (< 1 mm), or type 1b: patchy (a few mm). They are characterized by thin, dilated, and tortuous veins lacking smooth muscle layers, which explain their weakness and tendency to bleed. Typically, DLs consist of small mucosal defects and can be classified as type 2a: punctuate lesions with pulsatile bleeding or type 2b: pulsatile red protrusions without surrounding venous dilatation[4]. Some arteriovenous malformations and pulsatile red protrusions with dilated peripheral veins are defined as type 3. Congenital intestinal arteriovenous malformations manifest as polypoid or cluster type[5,6] and are classified as type 4. Nevertheless, the Yano-Yamamoto classification cannot fully reflect the histopathological findings.

Capsule endoscopy (CE) is a painless and well-tolerated approach that can achieve complete visualization of the small intestine[7]. It captures images for > 8 h[8]. Previous studies have demonstrated the probability of CE diagnosis of angiodysplasias was 30%-70%, and > 50% of obscure gastrointestinal bleeding patients have angiodysplasias[9-11]. The detection rate of CE was reported to be higher than other diagnostic methods, such as small bowel computed tomography, mesenteric angiography, and enteroscopy. Therefore, using CE as a first-line inspection tool for the diagnosis of angiodysplasias is recommended[12]. Nonetheless, CE has some limitations, and only 69% of angiodysplasias can be diagnosed by gastroenterologists[13]. Less relevant lesions, such as erosions or tiny red spots, are regarded as negative results; however, distinguishing highly relevant lesions from less relevant lesions could be challenging. In addition, the diagnostic efficiency of CE decreases when the presence of bile pigments, food residues, or bubbles affects the observation of the intestinal mucosa. The doctor's manual reading of the entire gastrointestinal tract is time-consuming, and the heavy workload affects the accuracy of the diagnosis. Therefore, making diagnosis of angiodysplasias solely based on CE is challenging.

The detection rate of angiodysplasias in the small intestine can be increased by using artificial intelligence (AI) to assess the effect of automatic diagnosis, which has been successfully applied for the recognition and diagnosis of gastrointestinal endoscopic images[14]. AI assists in the recognition and diagnosis of CE images, eliminates errors in manual reading, reduces the workload of doctors, and

improves diagnosis efficiency. The clinical application of AI-based deep learning technology in wireless CE has been a research focus, which has gained increasing interest in the past two years[15-32]. Several studies[15,23-26] have used deep learning to identify ulcers from CE data. Pogorelov *et al*[27] used the color texture features to detect small intestinal bleeding in CE data. Blanes-Vidal *et al*[28] constructed a classification network to identify intestinal polyp lesions in CE data. Kundu *et al*[29] and Hajabdollahi *et al*[30] identified small bowel bleeding in CE data using a classification neural network. Obscure gastrointestinal bleeding is the main indication for small intestinal CE, and the potential risk of bleeding from vascular malformations is high[14]. Therefore, we focused on AI-assisted recognition technology for angiodysplasias in the present study. Hitherto, there are few semantic segmentation networks based on deep learning to segment and recognize angiodysplasias lesions in CE, which prompted us to introduce a segmentation model in the study. Compared with the classification model and target detection model in deep learning, the segmentation model based on deep learning can more accurately locate the focus of small intestinal vascular malformation, better assist doctors in diagnosing small intestinal vascular malformation, and improve the accuracy and efficiency of doctors' diagnosis.

Currently, significant progress has been made in semantic segmentation in the field of deep learning. To the best of our knowledge, this is the first paper that proposed using a semantic segmentation network to solve the pixel-level small intestinal vascular malformation focus recognition and location. Resnet-50 was used as the skeleton network, and the fusion mechanism based on shallow features and deep features was introduced so that the segmentation model could accurately locate the location and category of lesions. Shallow features can perceive the texture details of lesions, while deep features can perceive the semantic information between lesions. By combining these two features to segment the image, the phenomenon where the lesion area is divided into uncorrelated small areas is reduced, the pixel accuracy (PA) is improved, and the missed detection rate of the lesion is reduced. This paper introduced the proposed network structure in detail and compared three common segmentation models, *i.e.*, PSPNet[31], Deeplabv3+[32], and UperNet[33]. The obtained results confirmed that the model proposed in this paper had high-performance indicators.

MATERIALS AND METHODS

ResNet was introduced in 2015 and won first place in the classification task of the ImageNet competition on account of being "simple and practical". Afterward, many methods, which were based on ResNet50 or ResNet101, have been widely used in detection, segmentation, recognition, and other fields. This method makes a reference (X) for the input of each layer, learning to form residual functions rather than learning some functions without reference (X). This residual function is easier to optimize and can greatly deepen the number of network layers. Moreover, the extracted image features have strong robustness. ResNet50 is faster than ResNet100. Therefore, ResNet50 is selected as the skeleton network of the semantic segmentation network in this paper. Based on the fusion of shallow and deep features, Resnet-50 was used as the skeleton network to construct an improved convolutional neural network (CNN) segmentation network model that automatically recognizes the type of angiodysplasias under CE and draws the outline of the lesion in the study. The present study aimed to assist doctors in diagnosing angiodysplasias lesions with CE.

The model proposed in this study was composed of three sub-units, *i.e.*, down-sampling, up-sampling, and classifier. CE small intestine data were used as input in the module, and the final output was image lesion category information and lesion boundary information.

Research data set

In order to train and evaluate the segmentation model, 378 patients with angiodysplasias who underwent OMOM CE (China Chongqing Kingsoft Technology Co., Ltd) at the Ruijin Hospital between January 2014 and December 2020 were recruited in this study. The sampling frequency of OMOM capsules of 2fps, the working time of > 12 h, and the apex field of view of 150° were used to diagnose the patients. A total of 12403 pictures were identified with an image resolution of 256 × 240. The patient data were anonymized, any personal identification information was omitted, and examination information (such as examination date and patient name) was deleted from the original image. All patients provided written informed consent, and the ethics committee approved the study [the certification number was (2017) provisional ethics review No. 138]. The annotated data were marked by an experienced endoscopy group that included three experts from Ruijin Hospital Affiliated to Shanghai Jiao Tong University. The average age of the experts was 35 years, and their average CE reading experience was 5 years, with an average of 150 CE cases each year. The five types of lesions of vascular malformation were annotated, and 12403 image data and 12403 annotated mask image data were generated. The data sample map is shown in Figure 1.

This project used the image data of 178 cases as the training set and the remaining 200 cases as the test set. The training set was divided into training and verification data at a ratio of 7:3 during the training process. The test set contained 1500 images without lesions and 1500 images with lesions. The training set and test set image data are summarized in Table 1.

Table 1 Details of the training set data and test set data

Lesion type	Lesion morphology	Number of pictures/pieces	
		Training set	Test set
Telangiectasia	Red cluster	838	38
	Red spider nevus	162	4
Venous dilatation	Red branched	752	38
	Blue branched	2583	1088
Vein tumor	Blue cluster	3058	332

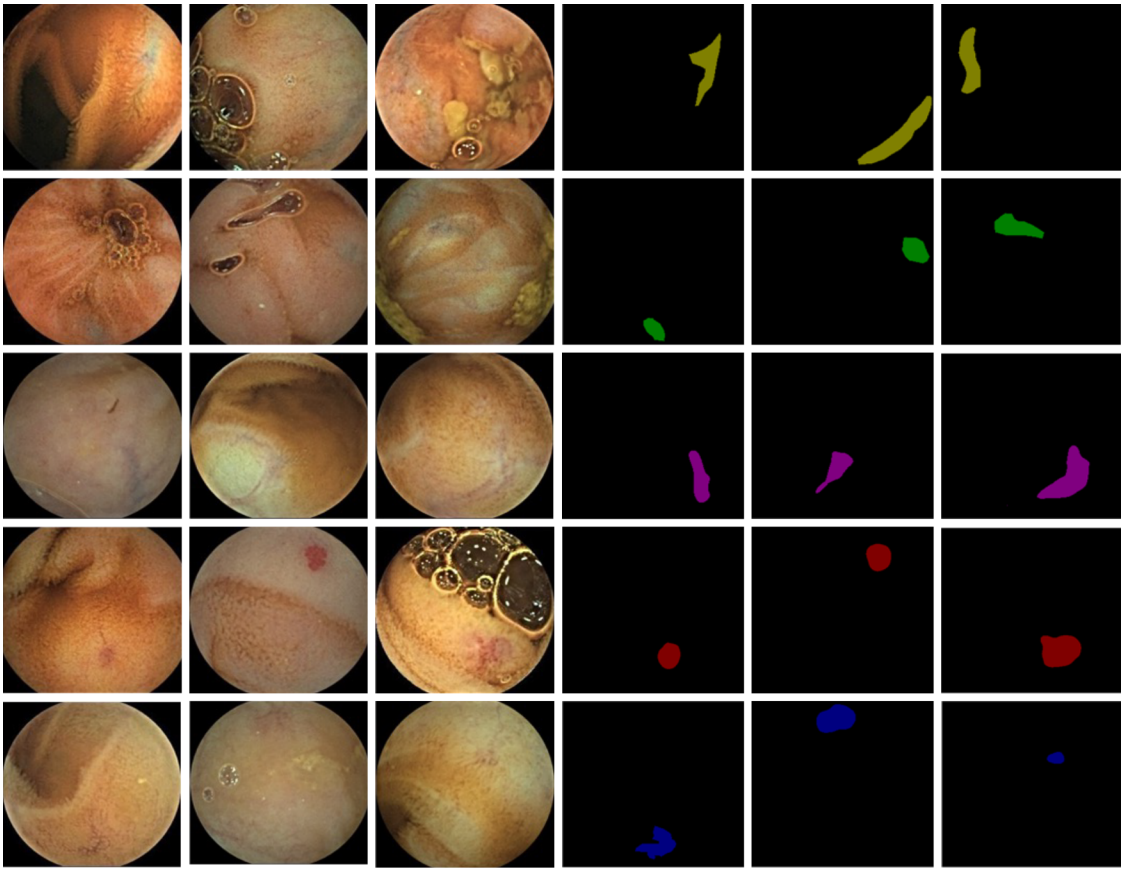

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Figure 1 Sample image of training data. The left three columns are the original image of the capsule, and the right three columns are the manual annotation results.

Data preprocessing

The training data were preprocessed to meet the requirements of the deep learning model. The preprocessing steps of the model constructed in the study were as follows: (1) Resizing the image to 256 × 240 × 3; (2) using enhancement methods (rotation, flip, and tilt) on the resized image; and (3) normalizing all images. In order to train a deep learning model, the dataset was split. The dataset image was randomly divided into two parts: 70% for training and 30% for verification.

Segmentation network details

The network structure proposed in this study is shown in Figure 2. The construction of the network model was inspired by the UperNet model. ResNet-50 was used as the skeleton network. The fusion mechanism of shallow features and deep features were introduced. Subsequently, the feature with the same size as the original image was obtained through the down-sampling operation. Finally, the classifier was connected to realize the pixel-level segmentation task of the image.

Based on the new semantic segmentation recognition network framework, a single end-to-end network could be trained to capture and analyze the semantic information of the CE small intestine

data. In order to fuse the shallow features and deep feature information, the last feature mapping set output by each stage in ResNet was expressed as C1, C2, C3, and C4, and the two-by-two fusion of features were utilized as down-sampling operation input, where the down-sampling rates were 4, 8, 16, and 32, respectively. The texture features of the lesion were captured at the highest layer, and the pixel-level segmentation of the lesion was completed based on the lowest layer features.

The last down-sampling operation generated a feature map with the same resolution as the original image, with a size of 256 × 240. After the feature was operated by Flatten, a classifier composed of a fully connected layer was connected to complete the segmentation and recognition tasks of the capsule data.

In order to assess the fusion of features of different scales, bilinear interpolation was used to adjust them according to the size, after which a non-evolutionary layer was applied to fuse the features of different levels and reduce the channel size. All non-classifier convolutional layers underwent batch normalization Relu operations after output. The learning rate of the current iteration was equivalent to the initial learning rate multiplied by (1-iter/max-iter_size)^{power}, and the initial learning rate and power were set to 0.02 and 0.9, respectively.

RESULTS

Evaluation index

The performance of the segmentation model of angiodysplasias lesions in CE was evaluated based on the following indicators: Positive predictive value (PPV), negative predictive value (NPV), mean intersection over union (mIOU), and PA. PPV and NPV were calculated using formulae 1 and 2, respectively.

$$PPV = \frac{TP}{TP + FP} \quad (1)$$

$$NPV = \frac{TN}{TN + FN} \quad (2)$$

Where true positive (TP) and true negative (TN) are the true number of positive samples and the true number of negative samples, respectively; FP and FN are false positives and false negatives, respectively. IOU and mIOU calculation formulae are shown as formulae 3 and 4, respectively.

$$OU = \frac{\text{Target area} \cap \text{Prediction area}}{\text{Target area} \cup \text{Prediction area}} \quad (3)$$

$$mIOU = \frac{1}{k+1} \sum_{i=0}^{k} \frac{P_{ii}}{\sum_{j=0}^{k} P_{ij} + \sum_{j=0}^{k} P_{ji} - P_{ii}} \quad (4)$$

Supposedly, there were K+1 categories (including an empty category or background) in semantic segmentation, which indicated that class i is predicted as i, and class j is predicted as j. The PA is calculated by formula 5.

$$PA = \frac{\sum_{i=0}^{k} P_{ii}}{\sum_{i=0}^{k} \sum_{j=0}^{k} P_{ij}} \quad (5)$$

Experimental design

Python 3 is a good deep-learning programming language that supports multiple deep-learning frameworks. The model was implemented using Python 3 and Torch framework. The training server has a graphics processing unit. All images were first passed to the image data generation class in Pytorch, and the preprocessing operations were performed, including enhancement, resize, and normalization operations. Then, the generated images were sent to the model to start the training. The layers in the backbone network ResNet-50 used pre-trained weights on ImageNet. An optimizer (SGD) was used to train the model, after which a weight decay of 0.0001 and a momentum of 0.9 were applied. Each model ran approximately 25000 Epochs; each Epoch iterated eight times, and the batch size was 8. In the model training process, the loss change, PA index, and mIOU changes were detected (Figure 3).

Comparison results of multiple models

On a test set consisting of 3000 image data, the following test indicators were compared on the four models: PPV, NPV, mean IOU, PA, parameter quantity, float calculation quantity, and duration. The results are shown in Table 2.

Based on the method of fusion of shallow and deep features, the CNN segmentation network model was improved and optimized, and the segmentation and recognition of five types of angiodysplasias lesions, *i.e.*, blue branch, blue cluster, red branch, red cluster, and red spider nevus, were realized. This method fully uses the shallow and deep features extracted from the skeleton network to perceive the global information and lesion texture information of the small intestine capsule image data as a whole. Thus, it significantly improves the PPV and NPV of the segmentation model in the angiodysplasias lesion image. In order to obtain the highest PPV, the NPV has to be the highest. The unified perception of the global and local information of the small intestine capsule data was completed through a CNN,

Table 2 Comparison of model accuracy

Network type	PPV (%)	NPV (%)	mIOU	PA (%)	Parameter (M)	Float calculation amount (G)	Time (s)
PSPNet	85.14	98.62	0.64	98	51.43	829.10	0.9
DeeplabV3+	45.07	99.75	0.59	89	59.34	397.00	0.95
UperNet	92.55	95.69	0.69	98	126.08	34.94	0.9
Our model	94.27	98.74	0.69	99	46.38	467.2	0.6

PPV: Positive predictive value; NPV: Negative predictive value; mIOU: Mean intersection over union; PA: Pixel accuracy.

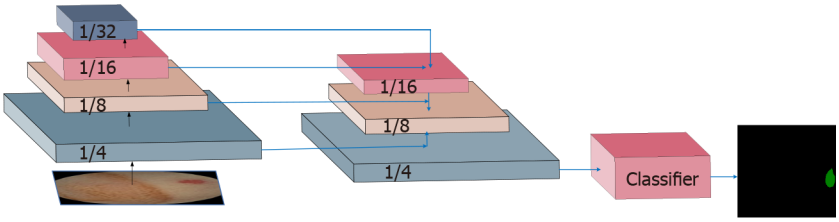

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Figure 2 Network structure diagram. This figure shows the structure of semantic segmentation network, in which the first two modules are shallow and deep feature fusion, the third module is pixel classifier, and finally the network output results.

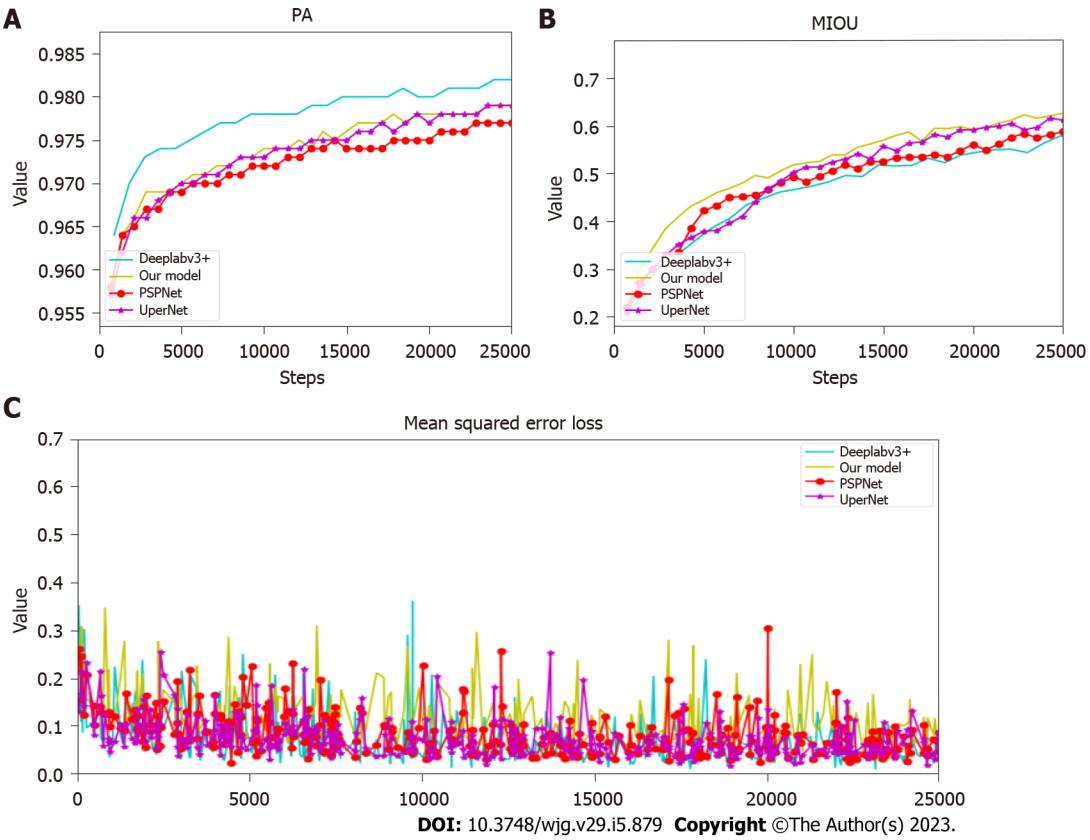

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Figure 3 Change process of loss, pixel accuracy and mean intersection over union. A: Comparation of the pixel accuracy (PA) values of each model during training. The abscissa represents the number of training iterations, and the ordinate represents the value of PA; B: Comparation of the mean intersection over union (mIOU) values of each model during training. The abscissa represents the number of training iterations, and the ordinate represents the value of mIOU; C: Comparation of the loss value of each model in the training process. The abscissa represents the number of training iterations, and the ordinate represents the loss value. PA: Pixel accuracy; mIOU: Mean intersection over union.

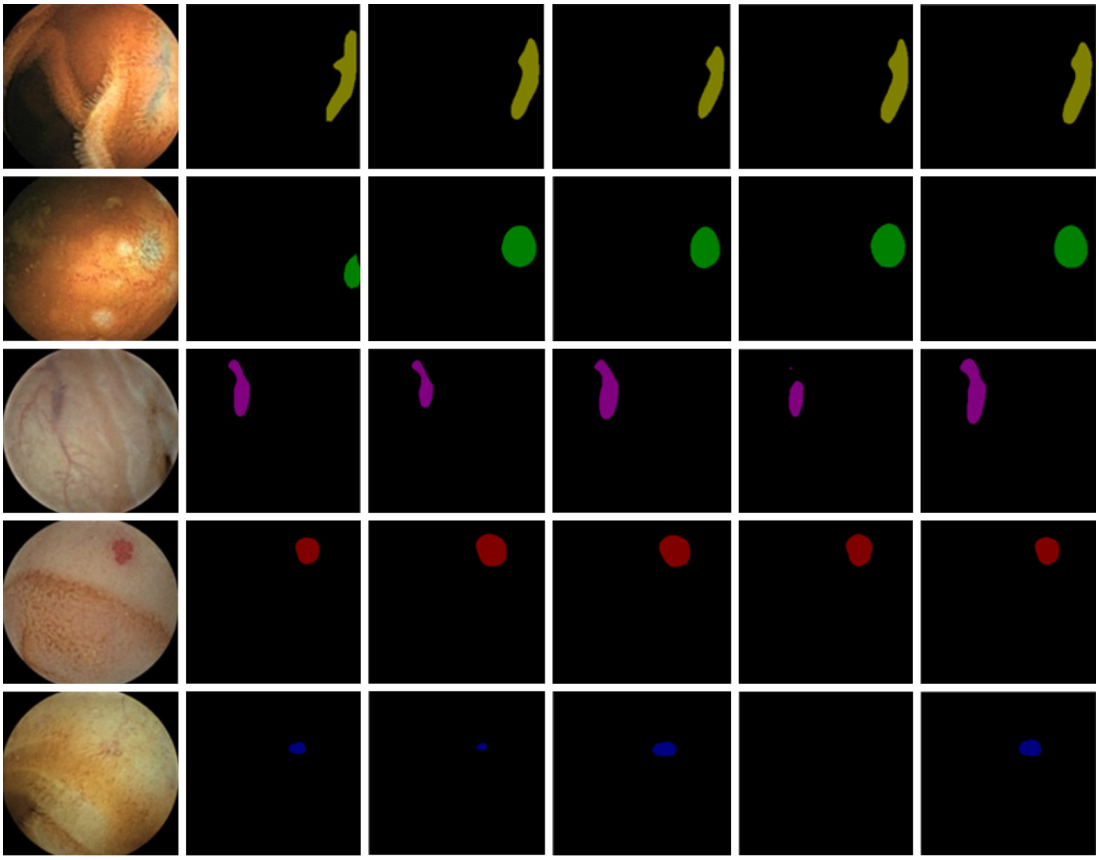

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Figure 4 Five rows from top to bottom: blue branch, blue lumpy, red branch, red lumpy and red spider nevus. The first column on the left is the original image, the middle four columns are the results of the current excellent segmentation network, and the last column is the results of the model we proposed.

which reduced the number of network model parameters, the number of float calculations, and the inference time of the deep learning model. Furthermore, a comparative experiment was designed and compared to the current advanced segmentation network models: PSPNet, DeeplabV3+, and UperNet. Our model showed that the NPV reached the highest 98.74% when the PPV was the highest.

The comparison of the segmentation and recognition effects of the four models on the vascular aberration lesions of the CE small intestine data is shown in Figure 4. The model proposed in the study was similar to that of the expert's annotation results.

Compared with relevant literature, Leenhardt *et al*[19] applied technology for segmentation, achieving the highest level of lesion detection, with an NPV value of 96%. However, the algorithm presented in this paper had some advantages in the test set. Also, our NPV value was 98%.

DISCUSSION

The classification network and the target detection network are the mainstream network structure that combines the deep learning model and the CE diagnosis method. In the present study, we introduced the segmentation network in deep learning, segmented and identified the angiodysplasias lesions, and completed the pixel-level segmentation task of the angiodysplasias lesions. The semantic segmentation network model had clinical practicality application as assessed using the training and test sets in comparative experiments.

The segmentation networks have been obviously developed in the field of deep learning. PSPNet uses the prior knowledge of the global feature layer to understand the semantics of various scenes, combined with the deep supervision loss to develop an effective optimization strategy on ResNet and embed difficult-to-analyze scene information features into the functional connectivity networks prediction framework to establish a pyramid. The pooling module aggregates the contextual information in different regions and improves the ability to obtain global information. This system was used for scene analysis and semantic segmentation and was 83% accurate on the COCO data set. The DeepLabV3+ model was based on an encoder-decoder structure, which improved the accuracy and saved the inference time; an accuracy rate of 89% was obtained in the COCO dataset. UperNet used

unified perception analysis to build a network with a hierarchical structure to ensure that multiple levels were resolved at visual concepts, learn the differentiated data in various image datasets, achieve joint reasoning, and explore the rich visual knowledge in the images. Finally, 79.98%-PA was obtained on the ADE20K data set. UperNet used a unified perception analysis module from scenes, objects, parts, materials, and textures to simultaneously analyze the multilevel visual concepts of images, such that many objects could be segmented and recognized, and the rate of missed objects could be reduced. The CE small intestine image data has a simple scene and fewer semantic levels. The use of large segmentation network models would cause over-fitting in training and high computational complexity. This study was inspired by UperNet and optimized basic CNN segmentation network, which led to the creation of a network model suitable for the segmentation and recognition of angiodysplasias with CE.

On the other hand, a case-based dataset encompassing typical vascular malformation images, atypical angiodysplasias images, and normal images was constructed, including pictures with poor intestinal cleanliness. According to the color and morphology of the angiodysplasias lesions in the cases, the five types of angiodysplasias lesions were summarized as blue branched, blue cluster, red branched, red cluster, and red spider nevus. The dataset constructed in this study verified the clinical applicability of the semantic segmentation model. Thus, the dataset was essential in diagnosing CE small bowel vascular malformation based on the deep learning model.

CONCLUSION

The deep learning model constructed in this study showed high PPV and NPV for the segmentation and recognition of angiodysplasias lesions. In the future, it could be used to assist capsule endoscopists in the real-time diagnosis of angiodysplasias lesions. Deep learning does not require prior knowledge, as it can directly learn the most predictive features from image data, as well as segment and recognize the image. The larger the amount of data, the higher the advantages of deep learning and the higher the recognition accuracy. AI facilitates grassroots' CE to obtain the same diagnosis effect as senior experts. However, the current uneven distribution of medical resources and the technical level of grassroots CE are the driving forces for the development of AI. In conclusion, the segmentation model based on deep learning can assist doctors in identifying the lesions of small intestinal vascular malformations.

ARTICLE HIGHLIGHTS

Research background

Small intestinal vascular malformations (angiodysplasias) commonly cause small intestinal bleeding. Therefore, capsule endoscopy has become the primary diagnostic method for angiodysplasias. Nevertheless, manual reading of the entire gastrointestinal tract is a time-consuming heavy workload, which affects the accuracy of diagnosis.

Research motivation

The doctor's manual reading of the entire gastrointestinal tract is time-consuming, and the heavy workload affects the accuracy of the diagnosis. Also, significant progress has been made in semantic segmentation in the field of deep learning.

Research objectives

This study aimed to assist in the diagnosis and increase the detection rate of angiodysplasias in the small intestine, achieve automatic disease detection, and shorten the capsule endoscopy (CE) reading time.

Research methods

A convolutional neural network semantic segmentation model with feature fusion automatically recognizes the category of vascular dysplasia under CE and draws the lesion contour, thus improving the efficiency and accuracy of identifying small intestinal vascular malformation lesions, was proposed.

Research results

The test set constructed in the study achieved satisfactory results: pixel accuracy was 99%, mean intersection over union was 0.69, negative predictive value was 98.74%, and positive predictive value was 94.27%. The model parameter was 46.38 M, the float calculation was 467.2 G, and the time needed to segment and recognize a picture was 0.6 s.

Research conclusions

Constructing a segmentation network based on deep learning to segment and recognize angiodysplasias

lesions is an effective and feasible method for diagnosing angiodysplasias lesions.

Research perspectives

The model detects the small intestinal malformation lesions in the capsule endoscopy image data and draws the lesion area through segmentation.

FOOTNOTES

Author contributions: Chu Y read and reviewed capsule endoscopy imagines, and participated in the design, writing, and revision of the manuscript; Huang F performed AI algorithm research, designed the segmentation recognition protocol, and verified the algorithm; Gao M organized the experimental results, visualized the experiment, and wrote the draft of the paper; Zou DW designed and revised the protocol of the paper; Zhong J reviewed capsule endoscopy images; Wu W and Wang Q participated in the reading of CE and preparation of the material; Shen XN and Gong TT partial writing of the manuscript; Li YY contributed to the coding and debugging of interface between algorithm and software; Wang LF designed and modified the protocol of the paper.

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Informed consent statement: The informed consent statement was waived by the Ruijin Hospital Ethics Committee.

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

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at lifuwang@sjtu.edu.cn. Participants gave informed consent for data sharing.

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ORIGINAL ARTICLE

Clinical Trials Study

Efficacy of dexamethasone and N-acetylcysteine combination in preventing post-embolization syndrome after transarterial chemoembolization in hepatocellular carcinoma

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Abstract

BACKGROUND

Conventional transarterial chemoembolization (cTACE) is the current standard treatment for intermediate-stage hepatocellular carcinoma (HCC). Post-embolization syndrome (PES) is complex clinical syndrome that presents as fever, abdominal pain, nausea, and vomiting. Either dexamethasone (DEXA) or N-acetylcysteine (NAC) is used to prevent PES; however, the synergistic effect of their combined therapy for preventing PES and liver decompensation has not been determined.

AIM

To evaluate the efficacy of DEXA and NAC combination in preventing PES and liver decompensation after cTACE.

METHODS

Patients with Barcelona Clinic Liver Cancer stage A or B HCC who were scheduled for TACE were prospectively enrolled. All patients were randomly stratified to receive NAC and DEXA or placebo. The dual therapy (NAC + DEXA) group received intravenous administration of 10 mg DEXA every 12 h, NAC 24 h prior to cTACE (150 mg/kg/h for 1 h followed by 12.5 mg/kg/h for 4 h), and a continuous infusion of 6.25 mg/h NAC plus 4 mg DEXA every 12 h for 48 h after cTACE. The placebo group received an infusion of 5% glucose solution until 48 h after procedure. PES was defined by South West Oncology Group toxicity code grading of more than 2 that was calculated using incidence of fever, nausea, vomiting, and pain.

RESULTS
One-hundred patients were enrolled with 50 patients in each group. Incidence of PES was significantly lower in the NAC + DEXA group compared with in the placebo group (6% *vs* 80%; $P < 0.001$). Multivariate analysis showed that the dual treatment is a protective strategic therapy against PES development [odds ratio (OR) = 0.04; 95% confidence interval (CI): 0.01-0.20; $P < 0.001$). Seven (14%) patients in the placebo group, but none in the NAC + DEXA group, developed post-TACE liver decompensation. A dynamic change in Albumin-Bilirubin score of more than 0.5 point was found to be a risk factor for post-TACE liver decompensation (OR = 42.77; 95%CI: 1.01-1810; $P = 0.049$).

CONCLUSION
Intravenous NAC + DEXA administration ameliorated the occurrence of PES event after cTACE in patients with intermediate-stage HCC.

Key Words: Hepatocellular carcinoma; Post-embolization syndrome; Transarterial chemoembolization; Liver decompensation

Core Tip: Conventional transarterial chemoembolization (TACE) is the current standard treatment for intermediate-stage hepatocellular carcinoma (HCC). A combination of N-acetylcysteine and dexamethasone ameliorated the occurrence of post-embolization syndrome event after TACE in patients with intermediate-stage HCC. A dynamic change in Albumin-Bilirubin score of more than 0.5 point was found to be a risk factor for post-TACE liver decompensation.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major-public health concern and the fourth common cause of cancer-related deaths worldwide[1]. In Thailand, HCC is the second most common tumor type and the most common cause of cancer-related death. Without treatment, patients with HCC have a one-year overall survival rate of less than 20%. Conventional transarterial chemoembolization (cTACE) has been established as a standard treatment for HCC with Barcelona Clinic Liver Cancer (BCLC) stage B. Systemic reviews and meta-analyses have demonstrated that cTACE therapy improved the survival of patient at this stage. HCC patients who underwent super-selective TACE had a 5-year survival rate of 40%-48%. cTACE involves embolization of vessel supply to tumors, causing ischemia in not only tumor cells but also normal hepatocytes, along with targeted chemotherapy; the systemic effects of chemotherapeutic agents result in the occurrence of post-embolization syndrome (PES)[2-5].

PES is a complex clinical syndrome manifested as fever, abdominal pain, nausea, and vomiting[6,7] with a South West Oncology Group (SWOG) score > 2[7]. PES is self-limited and either resolves within 24 h or exhibits sustained symptoms for up to two weeks based on various factors such as tumor size, tumor numbers, dosage of chemotherapy, and performance status of the patient[6,8,9]. Management of PES mainly includes supportive treatment such as with analgesic, antiemetic, and antipyretic administration[9,10]. Depending on its pathogenesis, PES may be related to systemic inflammation, resulting in toxic and ischemic effects on tumor cells and hepatocytes[9]. Steroids and antioxidants may play an important role in the prevention and treatment of PES. Dexamethasone (DEXA) and prednisolone are recommended by the National Comprehensive Cancer Network as effective medications for preventing chemotherapy-induced nausea/vomiting. Moreover, a few randomized control trials have demonstrated the effect of DEXA in terms of PES prevention[11-14]. N-acetylcysteine (NAC), an established glutathione precursor and a potent antioxidant, was found to ameliorate ischemic liver injury by improving the systemic hemodynamic parameter and tissue oxygen delivery in animal models[14-16]. Further, a pilot study reported that NAC can reduce the incidence of PES after cTACE but cannot reduce that of post-TACE liver decompensation[17]. Currently, there are no standard prevention guidelines for PES. Our study aims to evaluate the dual effect of DEXA and NAC therapy in preventing

PES or liver decompensation after cTACE in patients with HCC.

MATERIALS AND METHODS

Study design

A randomized, double blind, placebo-controlled trial was conducted at the Gastroenterology and Liver Unit, Vajira Hospital, Navamindradhiraj University, Bangkok, Thailand from November 2020 to January 2022.

Study population

Eligible patients were those aged 18-80 years with diagnosed early- or intermediate-stage HCC, according to BCLC classification, and had a good performance status, defined by the Eastern Cooperative Oncology Group. Diagnosis of HCC was based on either histological or radiological typical hallmark criteria according to the American Association for the Study of Liver Disease[18] and European Association for the Study of the Liver[19]. The exclusion criteria were as follows: (1) Decompensated liver cirrhosis (Child-Pugh score ≥ 9) or cirrhosis with main portal vein invasion; (2) Congestive heart failure and/or respiratory failure; (3) Severe comorbid illness, such as end-stage renal disease, persistent poorly-controlled diabetes mellitus or hemoglobin A1C ≥ 8.5, uncontrolled hypertension (systolic blood pressure ≥ 180 mmHg or diastolic blood pressure ≥ 120 mmHg), with a life expectancy of < 6 mo; (4) Severe allergy or anaphylaxis/anaphylactoid to NAC; (5) Pregnancy; and (6) History of non-steroid anti-inflammatory drugs, steroids, or NAC use within 21 d of trial initiation. All enrolled patients agreed on receiving cTACE treatment and provided informed consent before participating in the study.

Sample size calculation

Based on previous results, the incidence of PES among patients with HCC after receiving cTACE was > 60%[12]. Superiority of the DEXA regimen over the control regimen was defined as a 25% decrease in PES. Intravenous DEXA was hypothesized to reduce the incidence of PES by 20%. This study used a two-tailed test that calculated the requirement of at least 44 patients in each group to obtain a P value < 0.05 with alpha and beta errors of 5% and 20%, respectively.

Ethical approval

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of Faculty of Medicine, Vajira Hospital (COA 051/2564).

Randomized strategy and intervention

All patients were admitted at least 24 h before the prescheduled cTACE procedure and were randomly (1:1) assigned to either NAC–DEXA or placebo group. The randomization sequence was computer-generated in blocks of four and stratified according to Child-Pugh classification (class A or B). All patients were blinded to the treatment assignment. Both groups underwent therapy initiation 24 h prior to the procedure. The specific dosage of the NAC-DEXA protocol was based on a previously reported recommended dosage[11,17]. The NAC-DEXA group received intravenous infusion of 5% dextrose with NAC, with an initial loading dose of 150 mg/kg/h over 1 h followed by 12.5 mg/kg/h for 4 h and 10 mg of intravenous DEXA every 12 h; this was followed by continuous intravenous infusion of 6.25 mg/kg/h NAC and 4 mg of intravenous DEXA every 12 h for the remaining 48 h post-TACE. The placebo group received 5% glucose in normal saline for 48 h at an infusion rate of 60 mL/h post-TACE (as shown in Supplementary Figure 1). If mild-to-moderate allergic symptoms developed (*e.g.*, urticarial rash or bronchospasm), treatment was temporarily stopped for 1 h and intravenous antihistamine was immediately administered; treatment was resumed after the symptoms subsided. If severe allergic or anaphylactoid reaction occurred, treatment was permanently stopped, and the patient was treated according to standard protocol for severe allergic reaction. cTACE was performed by two interventional radiologists (Tanasoontrarat W and Claimon T) who were blinded to the randomization assignment. Pre-procedure single intravenous dose of ceftriaxone (1 g) or amoxicillin-clavulanic acid (1.2 g) along with single intravenous dose of ondansetron (8 mg) was administered to all patients. The femoral artery was catheterized under local anesthesia. A thorough angiographic examination was performed to locate all of the tumor-feeding arteries. An emulsion of lipiodol (2.5-15 mL) and chemotherapeutic agent (mitomycin, 5-20 mg) was infused into the feeders at an optimal dose determined by the interventional radiologist to be sufficient for tumor control. Thereafter, gelatin sponge particles were injected through the tumor-feeding branch. Selective cTACE was defined as occlusion of the segmental or subsegmental arterial feeder.

Post-procedural assessment

After completion of the procedure, all patients were admitted in the hospital for at least 72 h. During

hospitalization period, the following parameters were recorded: Symptoms (nausea, vomiting, fever, abdominal pain, and anorexia), vital signs, and other adverse events. Laboratory parameters, including liver function test, erythrocyte sedimentation rate, and C-reactive protein, were assessed at 24 and 48 h post-procedure. Hemoculture, urinalysis, complete blood count, and chest X-ray were performed if body temperature was > 38 °C.

Outcome measurement

The primary outcome was the development of PES after TACE within 48 h. In the present study, PES was identified using three different definitions: (1) SWOG toxicity coding score characterized by fever, nausea, vomiting, and/or abdominal pain within 48 h post-procedure, defined as calculated sum score more than two point (Supplementary Table 1)[7]; (2) Criteria defined by Ogasawara *et al*[11] in a randomized double-blind control study of DEXA, based on Common Terminology Criteria for Adverse Events (CTCAE) [version 5 (Supplementary Table 2) with symptoms other than those of grade I]; and (3) Criteria defined by Siramolpiwat *et al*[17] in a randomized controlled trial of single NAC dose (temperature ≥ 38.5 °C and 3-fold higher alanine transaminase level from baseline within 48 h post-procedure). The secondary outcome was the development of post-TACE liver decompensation, defined as an increase in Child-Pugh score of more than two points or newly developed decompensating events, such as ascites, hepatic encephalopathy, or serum total bilirubin > 2 mg/dL. Other cumulative adverse events (classified by CTCAE) and length of hospital stay were compared between the two groups. In patients with suspected infection or > 38 °C body temperature, laboratory testing and septic work-up were performed; moreover, these patients were treated with empirical antibiotic therapy until fever subsided or hemoculture was negative. All patients were followed up after 7 d to evaluate other post-procedure events.

Statistical analysis

Continuous data are presented as mean with standard deviation (SD) or median with range. Student's *t* test or the Mann-Whitney *U* test was performed to compare between two groups. For categorical data, Chi-square test or Fisher's exact test was applied. Statistical significance was set at $P < 0.05$. For analysis of factors that impact PES development, a logistic regression analysis was performed. Data are reported as odds ratio (OR) with 95% confidence interval (CI). All serious adverse events were reported to the Institutional Review Board of the Faculty of Medicine, Vajira Hospital, Navamindradhiraj University.

RESULTS

Patient characteristics

A total of 124 patients with HCC were screened from November 2020 to January 2022. Eighteen patients who refused to participate in this study, four patients who progressed to Child-Pugh class C, and two patients with main portal vein thrombosis were excluded. The remaining 100 patients who underwent TACE randomly received NAC-DEXA ($n = 50$) or placebo ($n = 50$) treatment. Figure 1 illustrates a flow chart of patients enrolled in this study. The mean age of patients was 60.6 years, with a male predominance (89%). The prevalence of comorbid diabetes was not different between the NAC-DEXA and placebo groups (34% *vs* 32%, $P = 0.83$). All patients exhibited cirrhosis, the majority of which with an etiology of chronic hepatitis B and alcoholic cirrhosis, followed by chronic hepatitis C, and a minority showing non-alcoholic steatohepatitis etiology. Most patients (83%) were classified as Child-Pugh class A, with no difference in mean Child-Pugh scores between the two groups (5.5 ± 0.8 in NAC-DEXA *vs* 5.5 ± 0.9 in placebo). Almost all patients (91%) were in BCLC stage B. Nine patient in BCLC stage A were justified for TACE as it would serve as a bridging therapy before curative treatment. Nearly half of the patients exhibited alpha fetoprotein (AFP) level > 200; however, the distribution of AFP level was not statically significant in both groups. Per tumor characteristics, no difference in the median tumor diameter between the two groups (NAC-DEXA, 5.5 cm; range 1.4-19 cm *vs* placebo, 7.95 cm; range 1.4-17.2 cm; $P = 0.39$) was observed. More than 50% of patients in both groups had multiple nodules (Table 1). Approximately 34% of the patients underwent their first TACE session. The type of chemotherapy and the volume of lipiodol did not differ between the two groups. Level of embolization was selected based on tumor position. In the same TACE episode, more than one-third (36%) of patients in the NAC-DEXA group had embolization of more than two branches.

Primary outcome

According to the various pre-defined criteria mention above, PES was detected in 43% of the patients. Most patients with PES had fever (93.0%) and nausea (72.1%), while only five (11.6%) patients had abdominal pain. The occurrence of PES after TACE was significantly lower in the NAC-DEXA group than in the placebo group in all PES-defining criteria (SWOG score more than 2 point, 6% *vs* 80%; Siramolpiwat *et al*[17] criteria, 2% *vs* 54%; and Ogasawara *et al*[11] criteria, 10 % *vs* 84 %; all $P < 0.001$) as shown in Figure 2A. The NAC-DEXA group had a lower mean SWOG PES score than the placebo group

Table 1 Baseline characteristics of patients with intermediate-stage hepatocellular carcinoma

	N-acetylcysteine + dexamethasone	Placebo	*P* value
Sex			
Male	44 (88%)	45 (90%)	0.75
Age (mean ± SD)	60.8 ± 10.52	60.46 ± 11.27	0.88
Underlying disease			
Diabetic mellitus	17 (34%)	16 (32%)	0.83
Hypertension	22 (14%)	28 (56%)	0.23
Dyslipidemia	14 (28%)	18 (36%)	0.40
CKD	1 (2%)	2 (4%)	0.56
HIV	1 (2%)	1 (2%)	1.00
Tumor characteristic			
Size			
Median (range)	5.5 (1.4-19)	7.95 (1.4-17.2)	0.39
> 3 cm	40 (80%)	42 (84%)	0.65
Number			
Median (range)	2 (1-10)	2 (1-10)	0.21
1	23 (46%)	17 (34%)	0.56
≥ 2	27 (54%)	33(66%)	
Etiology			
Hepatitis B	23 (46%)	28 (56%)	0.31
Hepatitis C	15 (30%)	12 (24%)	0.50
Alcoholic	22 (44%)	20 (40%)	0.67
Non-alcoholic steatohepatitis	3 (6%)	4 (8%)	0.70
Staging			
BCLC-A	6 (12%)	3 (6%)	0.23
BCLC-B	44 (88%)	47 (94%)	
Child-Pugh Score			
A (5-6)	40 (80%)	43 (86%)	0.42
B (7-8)	10 (20%)	7 (14%)	
ALBI score (mean ± SD)	-2.61 ± 0.58	-2.54 ± 0.53	0.54
Alpha fetoprotein (ng/mL)			
< 20	18 (36%)	19 (38%)	0.50
20-200	14 (28%)	9 (18%)	
201-1000	10 (20%)	9 (18%)	
> 1000	8 (16%)	13 (26%)	
Episode of TACE			
1	16 (32%)	18 (36%)	0.67
2-5	34 (68%)	32 (64%)	
Embolization agents			
Mitomycin (mg)			
5-10	1 (2%)	3 (6%)	0.07
10.1-15	20 (40%)	10 (20%)	

15.1-20	29 (58%)	37 (74%)	
Lipiodol (mL)			
< 5	1 (2%)	1 (2%)	0.47
5-10	13 (26%)	8 (16%)	
> 10	36 (72%)	41 (82%)	
Level of embolization			
Left hepatic artery	17 (34%)	14 (28%)	0.51
Right hepatic artery	46 (92%)	44 (88%)	0.50
Middle hepatic artery	5 (10%)	3 (6%)	0.46
≥ 2 major branches	18 (36%)	8 (16%)	0.02

ALBI: Albumin-bilirubin; BCLC: Barcelona Clinic Liver Cancer; CKD: Chronic kidney disease; HIV: Human immunodeficiency virus; SD: Standard deviation; TACE: Transarterial chemoembolization.

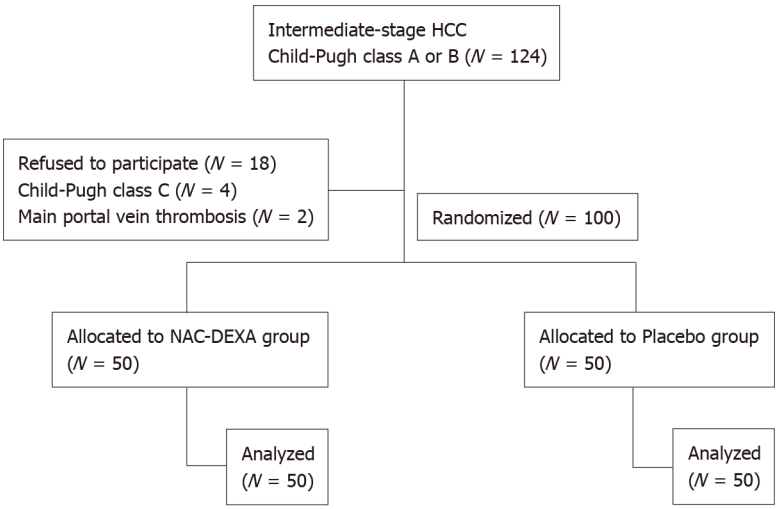

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Figure 1 Flow chart of patient screened, recruited, and analyzed in the study (consort diagram). HCC: Hepatocellular carcinoma; NAC: N-acetylcysteine; DEXA: Dexamethasone.

(0.38 ± 1.1 *vs* 4.04 ± 2.2; $P < 0.001$). Baseline characteristic comparison of patients with or without PES after TACE is shown in Table 2. The PES group had a higher proportion of patients with large tumor size (> 5 cm) (67.4% *vs* 47.4%; $P = 0.045$) and massive tumor burden (up to 12 criteria; 48.8% *vs* 26.3%; $P = 0.02$). Neither volume of embolizing agents nor level of vessels embolization influenced PES occurrence. Nevertheless, most (86%) patients in the PES group underwent TACE with single vessel embolization technique.

Secondary outcomes

Interestingly, post-TACE liver decompensation occurred only in the placebo group (14% *vs* 0%; $P < 0.006$). All cases were accompanied by PES. A higher proportion of patients with baseline Child-Pugh class B was observed in the post-TACE liver decompensation group, but no statistical significance was found (28.6% *vs* 16.1%, $P = 0.39$). A higher proportion of patients with abnormal liver function test, except albumin levels, was observed in the liver decompensation group. Neither tumor burden nor number of cTACE episodes influenced the occurrence of post-TACE liver decompensation. Multiple vessel embolization was performed for more patients in the post-TACE liver decompensation group compared with the group without liver decompensation (57.1% *vs* 28%, $P = 0.02$). A shorter median duration of hospital stay was observed in the NAC-DEXA group (4 *vs* 6 d; $P < 0.001$) as seen in Figure 2B. Most patients with PES were febrile, requiring empirical antibiotics therapy that was provided until negative hemoculture was obtained; this was the main reason for increased duration of hospitalization. Acute kidney injury was observed in three patients with baseline chronic kidney disease. All of them showed improved creatinine level and glomerular infiltration rate and received standard intravenous fluids at a rate of 60 mL/h from 24 h pre-TACE till 48 h post-TACE.

Table 2 Comparison between patients based on the occurrence of post-embolization syndrome and liver decompensation after conventional transarterial chemoembolization

	PES after cTACE		*P* value	Liver decompensation after TACE		*P* value
	Yes (*n* = 43)	No (*n* = 57)		Yes (*n* = 7)	No (*n* = 93)	
Male	40 (93%)	49 (86%)	0.26	7 (100%)	82 (88.2%)	0.34
Age (mean ± SD)	60.72 ± 11.11	60.56 ± 10.74	0.26	63.57 ± 7.91	60.41 ± 11.04	0.35
Child-Pugh score	5.35 ± 0.69	5.61 ± 0.9	0.09	5.86 ± 0.9	5.47 ± 0.82	0.24
A (5-6)	40 (93%)	43 (75.4%)	0.02	5 (71.4%)	78 (83.9%)	0.4
B (7-8)	3 (7%)	14 (24.6%)	0.02	2 (28.6%)	15 (16.1%)	0.4
ALBI (median; IQR)	-2.72 (-3.05, -2.29)	-2.65 (-2.93, -2.2)	0.47	-1.95 (-2.74, -1.77)	-2.69 (-3.04, -2.29)	0.09
MELD (mean ± SD)	11.67 ± 3.54	12.05 ± 3.78	0.61	12.71 ± 3.86	11.83 ± 3.66	0.54
Staging						
BCLC-A	3 (7%)	6 (10.5%)	0.54	1 (14.3%)	8 (8.6%)	0.61
BCLC-B	40 (93%)	51 (89.5%)	0.54	6 (85.7%)	85 (91.4%)	0.61
Tumor characteristics						
AFP ≥ 200 ng/mL	17 (39.5%)	27 (47.4%)	0.44	3 (42.9%)	28 (30.1%)	0.48
Median (range)	9.6 (4,13.2)	5 (3.2,10)	0.05	5.7 (3.2,15)	7 (3.2,13)	0.8
Large tumor ≥ 5 cm	29 (67.4%)	27 (47.4%)	0.045	4 (57.1%)	52 (55.9%)	0.95
Number						
1	18 (41.9%)	22 (38.6%)	0.74	4 (57.1%)	36 (38.7%)	0.34
≥ 2	25 (58.1%)	35 (61.4%)		3 (42.9%)	57 (61.3%)	
Size plus number						
Up to 7	32 (74.4%)	37 (64.9%)	0.31	4 (57.1%)	65 (69.9%)	0.48
Up to 12	21 (48.8%)	15 (26.3%)	0.02	3 (42.9%)	33 (35.5%)	0.7
cTACE episode						
1	15 (34.9%)	19 (33.3%)	0.87	4 (57.1%)	30 (32.3%)	0.18
≥ 2 (2-5)	28 (65.1%)	38 (66.7%)		3 (42.9%)	63 (67.7%)	
Embolization agent						
Mitomycin (mg)						
< 10	2 (4.7%)	2 (3.5%)	0.44	0 (0%)	4 (4.3%)	0.51
10-15	10 (23.3%)	20 (35.1%)		1 (14.3%)	29 (31.2%)	
15.1-20	31 (72.1%)	35 (61.4%)		6 (85.7%)	60 (64.5%)	
Lipiodol (mL)						
< 5	1 (2.3%)	1 (1.8%)	0.32	0 (0%)	2 (2.2%)	0.82
5-10	6 (14%)	15 (26.3%)		1 (14.3%)	20 (21.5%)	
> 10	36 (83.7%)	41 (71.9%)		6 (85.7%)	71 (76.3%)	
Level of embolization						
Left hepatic artery	10 (23.3%)	18 (31.6%)	0.36	3 (42.9%)	28 (30.1%)	0.32
Right hepatic artery	38 (88.4%)	44 (77.2%)	0.15	7 (100%)	83 (89.2%)	0.37
Middle hepatic artery	1 (2.3%)	7 (12.3%)	0.07	0 (0%)	8 (8.6%)	0.43
1 vessel	37 (86%)	34 (59.6%)	0.004	3 (42.9%)	67 (72%)	0.25
≥ 2 vessels	6 (14%)	23 (40.4%)	0.007	4 (57.1%)	26(28%)	0.02
Baseline laboratory						

(median; IQR)						
Hct (%)	36.8 (32.3, 40.3)	35 (32.6, 38.9)	0.33	39.8 (36.6, 40.6)	35.2 (32.2, 39)	0.04
WBC (× 10^9/L)	5.83 (4.50, 7.18)	5.48 (4.13, 6.97)	0.18	5.49 (4.14, 6.08)	5.58 (4.29, 7.07)	0.57
Platelet (× 10^9/L)	186 (125, 241)	144 (103, 204)	0.09	105 (65, 226)	171 (117, 230)	0.39
PTT (s)	12.9 (12.3, 13.8)	13.5 (12.7, 14.7)	0.02	13 (12.1, 13.2)	13.1 (12.4, 14.2)	0.34
AST (U/L)	51 (35, 102)	47 (37, 79)	0.78	163 (102, 169)	49 (36, 77)	0.003
ALT (U/L)	35 (20, 80)	33 (21, 51)	0.29	106 (80, 139)	31 (20, 51)	< 0.001
ALP (U/L)	124 (104, 290)	120 (93, 153)	0.211	290 (121, 386)	120 (94, 169)	0.111
Albumin (g/dL)	4 (3.6, 4.2)	3.9 (3.5, 4.2)	0.569	3.7 (3.2, 4.1)	3.9 (3.6, 4.2)	0.360
Total bilirubin (mg/dL)	0.59 (0.39, 1)	0.69 (0.51, 1.3)	0.163	1.59 (0.82, 2.11)	0.59 (0.42, 1.01)	0.005
Direct bilirubin (mg/dL)	0.35 (0.24, 0.65)	0.39 (0.26, 0.78)	0.415	0.91 (0.49, 1.76)	0.35 (0.24, 0.65)	0.004
Presence of PES, *n* (%)	-	-	-	7 (100%)	36 (38.7%)	0.002
Fever	40 (93.0%)	5 (8.8%)	< 0.001	7 (100%)	38 (40.9%)	0.002
Vomit	25 (58.1%)	2 (3.5%)	< 0.001	3 (42.9%)	24 (25.8%)	0.327
Pain	5 (11.6%)	0 (0.0%)	0.008	0 (0.0%)	5 (5.37%)	0.529
Anorexia	21 (48.8%)	2 (3.5%)	< 0.001	4 (57.1%)	17 (18.3%)	0.015

AFP: Alpha fetoprotein; ALBI: Albumin-bilirubin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BCLC: Barcelona Clinic Liver Cancer; cTACE: Conventional transarterial chemoembolization; Hct: Hematocrit; IQR: Interquartile range; MELD: Model of end-stage liver disease; PES: Post-embolization syndrome; PTT: Prothrombin time; SD: Standard deviation; WBC: White blood cell.

Predictors of PES

In univariate analysis of tumor volume, calculated increase in Albumin-Bilirubin (ALBI) score of more than 0.5 and a dynamic change in liver function (more than 20-fold and 1.5-fold increase from baseline in transaminase and bilirubin levels, respectively) within 48 h post-TACE were associated with the development of PES. Multivariate analysis showed that only intravenous NAC-DEXA pre-procedure could reduce the incidence of PES (OR = 0.04; 95%CI: 0.01-0.2; P < 0.001) (Table 3).

Predictors of liver decompensation

In univariate analysis, an occurrence of PES after TACE with an SWOG score > 4, more than 0.5 point increase in ALBI score, and a 1.5-fold increase from baseline in bilirubin level were associated with the development of liver decompensation (Table 3). In multivariate analysis, only a dynamic change in ALBI score > 0.5 point was considered an important risk for occurrence of liver decompensation with an OR of 42.77 (95%CI: 1.01-1810; P = 0.049).

Safety

Only two patients in the NAC-DEXA group developed a minor allergic skin reaction; in which, we disrupted treatment immediately. Six hours after drug discontinuation and administration of anti-allergic medication, the symptoms resolved. Subsequently, both patients completed the NAC-DEXA protocol with a lower infusion rate. No serious adverse event was reported in the NAC-DEXA group (Figure 2C). One patient in the placebo group died within 90 d post-procedure due to severe sepsis with liver decompensation. Although most patients were febrile post-TACE procedure, none contracted intrahospital secondary bacterial infection. The incidence of hyperglycemia did not differ between the NAC-DEXA and placebo groups (34% *vs* 32%, P = 0.83). Only three patients experienced grade 3 CTCAE hyperglycemia that was managed with antidiabetic therapy. According to changes in the liver function test at 48 h post-TACE, the 3-fold increase in total bilirubin level from baseline, but not transaminase, was more pronounced in the placebo group compared with that in the NAC-DEXA group (58 % *vs* 18 %, P = 0.006; Supplementary Table 3).

DISCUSSION

Pathogenesis of PES is conceivably related to multiple factors, such as direct toxic effects of chemotherapeutic agents and release of inflammatory cytokines related to tumor cell necrosis or ischemic hypoxic injury of normal hepatocytes[13,17,20]. The incidence of PES after TACE, per previous reports, ranges

Table 3 Univariate and multivariate analyses of patients with post-embolization syndrome and liver decompensation after conventional transarterial chemoembolization

Parameter	PES after cTACE						Liver decompensation after cTACE					
	Crude OR	95%CI	*P* value	aOR	95%CI	*P* value	Crude OR	95%CI	*P* value	aOR	95%CI	*P* value
NAC + DEXA	0.02	0-0.06	< 0.01	0.04	0.01-0.2	< 0.01	0	0-1	1.00	0	0-1	1.00
Size + number up to 12	1.09	1-1.19	0.04	1.08	0.92-1.27	0.33	0.99	0.84-1.16	0.88	-	-	-
AST rise > 20 folds in 48 h	1.51	1.09-2.09	0.01	1.35	0.72-2.52	0.35	1.7	0.95-3.03	0.07	1.3	0.56-3.02	0.54
ALBI change > 0.5	7.58	1.96-29.36	0.003	3.03	0.39-23.67	0.29	122.52	5.6-2681	0.002	42.77	1.01-1810	0.049
Total bilirubin rising > 1.5 folds	3.83	1.8-8.14	< 0.01	1.43	0.42-4.87	0.57	2.79	1.23-6.31	0.01	1.46	0.52-4.1	0.48
South west oncology grading > 4	1	0-1	0.99	-	-	-	1.43	1.04-1.96	0.03	0.94	0.51-1.76	0.86

ALBI: Albumin-bilirubin; aOR: Adjusted odds ratio; AST: Aspartate aminotransferase; CI: Confidence interval; DEXA: Dexamethasone; NAC: N-acetylcysteine; OR: Odds ratio; PES: Post-embolization syndrome; TACE: Transarterial chemoembolization.

widely (45%-83%). Studies that did not provide a strategic prophylaxis for PES reported an incidence of up to 80%. The incidence of PES in the present study was 43%. PES was verified by the occurrence of symptoms such as fever, nausea, vomiting, and abdominal pain, using SWOG toxicity code score of more than two point[7]. Fever is the most common symptom exhibiting a heterogeneity of prevalence (11.6%-74%), followed by nausea (11.6%-80.6%) and vomiting (16.2%-58.9%)[6,9,12,13,21]; in this study, the reported prevalence is 90%, 66%, and 54% for fever, nausea, and vomiting, respectively. Amelioration of nausea and vomiting by ondansetron premedication (8 mg) and selection of the less adverse chemotherapeutic events-causing agent, mitomycin C, may explain the lower incidence of nausea/vomiting in the present study than that in previous studies. Despite the application of super-selective single vessel embolization technique by radio-intervention at our center, a large tumor burden and a trend of higher mitomycin dose (> 10 mg) was observed and may have affected both tumor and normal liver cell necrosis, resulting in a higher PES occurrence.

NAC not only lowers free-radical levels, attributed to its antioxidant properties, but also acts as an indirect antioxidant by increasing the glutathione level and anti-inflammatory effect[22]. Therefore, many gastrointestinal guidelines recommend NAC for the treatment of alcoholic hepatitis[23], acetaminophen overdose[24], and non-acetaminophen acute liver failure[25]. Other favorable effects could be linked to its hepatoprotective activity. Interestingly, Siramolpiwat *et al*[17] demonstrated this protective effect; intravenous NAC minimized PES compared with the placebo group even though criteria for PES diagnosis was defined as only occurrence of fever and elevated serum alanine transaminase, without reference to clinical symptoms.

The beneficial effects of DEXA were presumably attributed to its antiemetic and inflammation dampening properties. Kogut *et al*[26] demonstrated that patients receiving prophylactic DEXA tended to require lower doses of antiemetic agents than those who do not. Ogasawara *et al*[11] reported that intravenous administration of a combination of DEXA and antiemetics (total 36 mg) for 3 d ameliorated PES by 52.5%. Recently, a randomized controlled trial by Sainamthip *et al*[12] demonstrated that a single

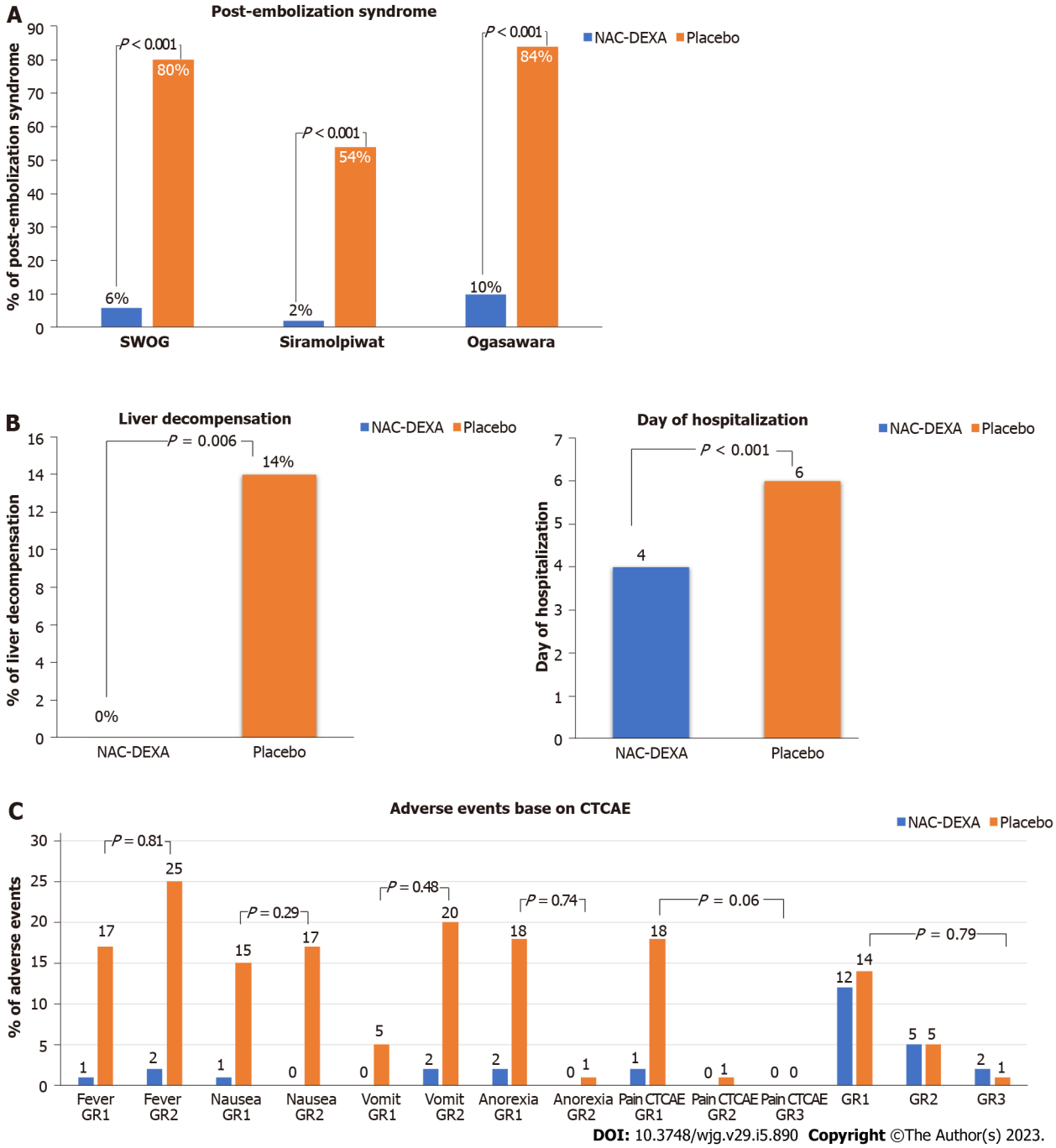

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Figure 2 Post-embolization syndrome, liver decompensation and length of hospital stay and adverse events after conventional transarterial chemoembolization. A: Post-embolization syndrome after conventional transarterial chemoembolization; B: Post-conventional transarterial chemoembolization liver decompensation and length of hospital stay; C: Post-conventional transarterial chemoembolization adverse events. NAC: N-acetylcysteine; DEXA: Dexamethasone.

dose of intravenous DEXA (8 mg) can prevent PES, achieving a negative PES rate of 63.3%.

We decided to maximize the protective effect of DEXA and NAC in prevention of PES by combining the two, with intravenous administration of DEXA (cumulative dose of 36 mg) and NAC 24 h before and continuous infusion until 48 h after cTACE. Interestingly, our study found that pre-TACE therapy with NAC-DEXA regimen led to a lower PES occurrence of less than 10%, which is the lowest incidence compared with those reported in previous publications (24.6% in a NAC study[17] and 37%-47% in DEXA studies[11,12]. The synergistic effect of NAC and DEXA can diminish systemic inflammatory response and ischemic hepatitis[27-29]. Hence, this study emphasizes the advantage of NAC-DEXA combination due to its synergistic PES-reducing effect.

Post-TACE liver decompensation is one of the most important complications and is associated with significant morbidity and mortality[21,30]. It is characterized by an increase in Child-Pugh score of more than 2 points, ≥ 2 mg/dL rise in serum total bilirubin level, newly developed ascites, or hepatic encephalopathy within two weeks post-procedure. In the present study, seven patients developed post-TACE

liver decompensation. Previous studies reported that portal vein thrombosis, poor baseline liver status, high serum AFP, and PES were associated with this complication[17,27,28]. Correspondingly, all patients with liver decompensation in our study had concurrent PES. In patients who underwent post-TACE without any prophylaxis treatment, we observed a lower incidence (14%) of liver decompensation compared with those in previous reports (13.4%-17.3%)[28,29]; the lower incidence in our study is attributed to the fact that a majority of patients had good liver reserve with Child-Pugh class A. Because all eligible cTACE patients in the present study fulfilled BCLC staging criteria, neither Child-Pugh nor Model for End-Stage Liver Disease score influenced the outcome. Further, more than half of our decompensated patients received cTACE intervention with multiple vessel embolization technique, which may have compromised the vessels; however, owing to the small sample size in this group, further studies are warranted.

ALBI score is the only objective parameter to stratify patients into different grades and is used to predict the prognosis of all HCC stages. This new model outperforms the Child-Pugh score for evaluation of liver function reserve. In a previous study, evaluation of baseline ALBI score in HCC patients who underwent cTACE not only predicted survival but also estimated liver decompensation and liver failure[31]. In our study, the mean pre-treatment ALBI score was not significantly different between the NAC-DEXA and placebo groups. Our findings indicate that the proportion of preserved liver, defined by ALBI grade 1, was comparable between the two groups. Moreover, all patients who developed post-TACE liver decompensation had a baseline ALBI grade 1 or 2 with a mean pre-treatment ALBI score of -2.57. The increase in ALBI score was hypothesized to be a novel non-invasive tool for earlier prediction of post-TACE liver decompensation than Child-Pugh score. Our study demonstrated that a dynamic increase in ALBI score of more than 0.5 point had a marked impact on liver decompensation. Thus, the application of dynamic increase in ALBI score, but not albumin level, for a prediction of early post-TACE liver decompensation requires further research.

Limitation

Our study had some limitations. First, we did not perform dose optimization for DEXA and NAC in order to minimize PES. Second, this dual treatment was not compared with single DEXA. The dynamic changes in cytokine levels due to this dual treatment is an interesting topic for further study.

CONCLUSION

A combination of DEXA and NAC can not only maximize the reduction in PES incidence but also shorten hospitalization period in HCC patients undergoing cTACE procedure. Dynamic alterations in ALBI score, but not CPS, may predict liver decompensation.

ARTICLE HIGHLIGHTS

Research background

Transarterial chemoembolization (TACE) is the current standard treatment for intermediate-stage hepatocellular carcinoma (HCC). Post-embolization syndrome (PES) is a complex clinical syndrome which may occur after conventional TACE (cTACE). Either N-acetylcysteine (NAC) or dexamethasone (DEXA) is used to prevent PES.

Research motivation

The synergistic effect of the combined therapy for preventing PES and liver decompensation has not been determined.

Research objectives

The aim of this study was to evaluate the efficacy of NAC and DEXA combination in preventing PES and liver decompensation after cTACE.

Research methods

A single-center randomized controlled clinical trial.

Research results

Our study provides clinical evidence that intravenous NAC plus DEXA administration ameliorates the occurrence of post-TACE PES in patients with intermediate-stage HCC. Interestingly, we found that a dynamic change in Albumin-Bilirubin (ALBI) score was a risk factor for post-TACE liver decompensation.

Research conclusions

A combination of NAC and DEXA ameliorated the occurrence of PES after cTACE in patients with intermediate-stage HCC.

Research perspectives

The application of dynamic increase in ALBI score for a prediction of early post-TACE liver decompensation requires further research.

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FOOTNOTES

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Informed consent statement: All participants provided informed written consent prior to study enrollment.

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LETTER TO THE EDITOR

Timing of biliary decompression for acute cholangitis

Jian Yang, Ying Liu, Shi Liu

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Abstract

Severe acute cholangitis (AC) exacerbates the risk of death because of the rapid progression of the disease. The optimal timing of biliary decompression (BD) as a necessary intervention in patients with severe AC is controversial. A recently report titled "Timing of endoscopic retrograde cholangiopancreatography in the treatment of acute cholangitis of different severity" in the *World Journal of Gastroenterology* that the optimal time of endoscopic retrograde cholangiopancreatography for treating patients with severe AC is ≤ 48 but not ≤ 24 h, providing clinical evidence for selecting the optimal time for implementation of BD. Here, we discuss the controversy over the optimal timing of BD for AC based on guidelines and clinical evidence, and consider that more high-level clinical researches are urgent needed to benefit the management of patients with different severity of AC.

Key Words: Acute cholangitis; Biliary decompression; Endoscopic retrograde cholangiopancreatography; Severity; Optimal time; Clinical evidence

Core Tip: Severe acute cholangitis (AC) exacerbates the risk of death because of the rapid progression of the disease. The optimal timing of biliary decompression (BD) as an intervention for severe AC is controversial. The purpose of this letter is to highlight the controversy surrounding the existing clinical evidence regarding BD for the treatment of AC of varying severity and to suggest that clinical studies providing higher levels of evidence will improve the therapeutic benefits.

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TO THE EDITOR

Acute cholangitis (AC) originates as an infection of the extrahepatic biliary system and is usually characterized by rapid progression leading to systemic sepsis. The morality rate of severe AC may reach 30% [1]. In most cases, biliary decompression (BD) is necessary to treat patients with severe AC, regardless of the treatment modality chosen [endoscopic retrograde cholangiopancreatography (ERCP), percutaneous transhepatic biliary drainage, or stone removal][2]. However, the optimal time at which to implement BD to obtain the maximum therapeutic benefit for patients with AC remains uncertain.

We recently became extremely interested in a retrospective study by Huang *et al*[3] published in the October 2022 issue of the *World Journal of Gastroenterology*. This was a high-quality observational study with a Newcastle-Ottawa Quality Assessment Scale score of 7 (3, 2, 2)[4]. It was independently assessed by two of our authors, and disagreements were resolved by a third author. Based on a retrospective analysis of 683 patients with AC, the conclusions drawn by the authors properly summarize the data in the study. The authors' data indicated that 30-d mortality in patients with AC was not significantly different between ERCP performed at > 24 and ≤ 24 h. However, patients with AC had lower 30-d mortality and a shorter length of stay when ERCP was performed at ≤ 48 h. Additionally, patients with grade III AC had lower 30-d mortality rates than patients with grade I and II AC, although they had higher intensive care unit admission rates and longer lengths of stay[3]. Huang *et al*[3] suggested that a ≤ 48-h duration from the patient's presentation to initiation of ERCP therapy, rather than a ≤ 24-h duration, provided the best survival benefit for patients with AC, especially for patients with grade III AC. This unique insight breaks with the traditional treatment concept that earlier performance of BD is associated with better outcomes in patients with severe AC. We thank Huang *et al*[3] for their study, which provides clinical evidence for the optimal timing of BD in patients with grade III AC.

The 2018 revised Tokyo guideline (TG18) currently serves as the most influential guideline for assessing AC severity. It delineates three grades of assessment of AC severity and indicates that the impact of the BD implementation time on the therapeutic benefit is significantly correlated with AC severity[5]. Therefore, we consider that the AC severity characterizes the urgency of the BD implementation time.

Reference Citation Analysis (RCA) (https://www.referencecitationanalysis.com/) is an artificial intelligence technology-based open multidisciplinary citation analysis database. We searched the RCA database for articles in cutting-edge fields in the last 3 years using the search terms "endoscopic retrograde cholangiopancreatography", "biliary decompression", and "acute cholangitis". Recent guidelines and clinical evidence suggest that the optimal timing of BD implementation remains controversial. First, controversy regarding the timing of BD implementation in the guideline exists primarily for patients with grade II and III AC. The American Society for Gastrointestinal Endoscopy (ASGE) 2021 guideline states that important outcome indicators for evaluating the survival benefit in patients with AC are 30-d mortality, inpatient mortality, length of stay, and organ failure[2]. The guideline also suggests that performance of ERCP at ≤ 48 h may be associated with lower 30-d mortality and a shorter length of stay[2]. However, the ASGE 2021 guideline does not report the correlation between the time to BD implementation and AC severity because the available clinical evidence is insufficient[2]. TG18 states that BD in patients with grade II AC should be performed within 24 h, and although clinical data do not indicate the optimal time for BD in patients with grade III AC, urgent decompression (within 24 h) may improve the prognosis of patients with grade III AC[5]. The European Society of Gastrointestinal Endoscopy 2019 guideline states that for patients with severe AC (grade III), implementation of BD is recommended within 12 h; for patients with moderate AC (grade II), it should be performed within 48 to 72 h[6]. Second, recent clinical studies have produced controversial results in terms of early implementation of BD for patients with grade III AC. In addition to the study by Huang *et al*[3] discussed herein (BD at ≤ 48 but not ≤ 24 h), another retrospective study by Lu *et al*[7] showed that BD is recommended at 24 h of admission for patients with grade III AC and within 12 h for those with AC accompanied by neurological or cardiovascular dysfunction. However, a study by Becq *et al*[8] using a propensity score matching approach indicated that the use of BD within 6h or 12 h of AC onset was not associated with better clinical outcomes, but possibly reduced readmission rates. Finally, the results of three recent systematic reviews and meta-analyses suggest that the controversy over the optimal timing of BD is mainly focused within 24 h and 48 h based on data analysis of superior outcomes within their respective time zones[9-11]. However, the three studies did not report that the optimal timing of BD affects survival outcomes in populations with different severities of AC (*i.e.*, grades I, II, and III)[9-11].

Based on the above-mentioned current controversies, we present the following future outlook. First, because of the controversy in the current guidelines and among recent clinical studies regarding the optimal timing of BD for patients with grade II and III AC, a multicenter prospective cohort study or randomized controlled trial should be conducted. Second, the medical community is called upon to pay attention to the clinical studies that have been reported and to perform systematic reviews and meta-analyses on the optimal time to implement BD for the treatment of patients with grade II and III AC.

In conclusion, a higher level of clinical evidence regarding the optimal time to implement BD in patients with different severities of AC is needed to improve the therapeutic benefit.

FOOTNOTES

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