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## Cost of postoperative complications: How to avoid calculation errors

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### Abstract

Postoperative complications (PC) are a basic health outcome, but no surgery service in the world records and/or audits the PC associated with all the surgical procedures it performs. Most studies that have assessed the cost of PC suffer from poor quality and a lack of transparency and consistency. The payment system in place often rewards the volume of services provided rather than the quality of patients' clinical outcomes. Without a thorough registration of PC, the economic costs involved cannot be determined. An accurate, reliable appraisal would help identify areas for investment in order to reduce the incidence of PC, improve surgical results, and bring down the economic costs. This article describes how to quantify and classify PC using the Clavien-Dindo classification and the comprehensive complication index, discusses the perspectives from which economic evaluations are performed and the minimum postoperative follow-up established, and makes various recommendations. The availability of accurate and impartially audited data on PC will help reduce their incidence and bring down costs. Patients, the health authorities, and society as a whole are sure to benefit.

**Key words:** Morbidity; Postoperative complications; Costs and cost analysis; Economic evaluation; Healthcare costs; Opportunity cost; Clavien-Dindo classification; Comprehensive complication index

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**Core tip:** No surgery service in the world registers and/or audits the postoperative complications (PC) of all the surgical procedures it performs. Most economic studies of PC are of poor quality; without an accurate registration of PC, their costs cannot be reliably determined. The article describes ways of quantifying and classifying PC, discusses the perspectives from which their economic evaluation and monitoring can be approached, and makes recommendations. An accurate assessment of PC and their costs will allow us to determine which areas need investment in order to lower the incidence of PC, improve surgical results, and reduce economic costs.

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## INTRODUCTION

Expenditure on health as a proportion of gross domestic product in OECD countries in 2017 ranged from 17.2% in the United States to 4.2% in Turkey<sup>[1]</sup>. Because of the rising economic costs (EC) deriving from the growing use of expensive technology and population aging, and the need to invest in other basic health areas or global issues such as combating the effects of climate change, expenditure on health must achieve maximum efficiency, incorporating other health benefits and improving the quality of all.

Postoperative complications (PC) are a basic health outcome. Their presence has a negative effect on patients' quality of life in the short and/or long term and increases the consumption of human, technical and economic resources. An accurate appraisal of the effects of PC would allow us to determine which specific areas are need of investment in order to reduce their incidence, improve surgical results, raise patients' quality of life, and bring down EC.

## CURRENT SITUATION

At present no surgery service anywhere in the world systematically records all the morbidity associated with the surgical procedures it performs<sup>[2]</sup> or submits records of this kind to objective and impartial audit<sup>[3]</sup>. What is more, the analyses of the EC of PC lack consistency and transparency in terms of the presentation of reports on costs and resource use<sup>[4]</sup>. Without a complete and impartial registry of PC, the associated EC cannot be determined.

A prospective study by our group evaluating the PC of all surgical procedures at a surgery service found a morbidity rate of 27.7%. Depending on the complexity of the surgery, the PC ranged from 10.7% to 71.4%. In addition, among patients with complications, 51.5% presented two events or more<sup>[5]</sup>.

Even patient death has a negative economic effect. One cost analysis of 1200 patients undergoing major surgery showed that hospital costs incurred by a death were significantly higher than the costs of Clavien-Dindo classification (CDC) grade I to IIIb complications (although they were significantly lower than those associated with grade IV complications in all surgeries)<sup>[6]</sup>. In pancreatic surgery the costs in patients rescued from PC were 64% lower than those incurred in patients who died due to the complication<sup>[7]</sup>. In studies validating the association of scales with EC, deceased patients should not be included in the comparative statistical analysis due to possible distortions caused by non-survivor bias<sup>[5,6]</sup>. In addition to EC, the loss of patient productivity should be taken into account.

Obviously, PC must be monitored after discharge. For example, surgical site infections are a frequent PC<sup>[7]</sup>; an incidence of 15% has been reported<sup>[8,9]</sup> and of these as many as 58% occur after hospital discharge<sup>[9]</sup>.

From the hospital perspective, the minimum follow-up period of PC and their EC must be at least 90 d and readmissions must be considered. One prospective study of all interventions carried out at a general surgery service found an overall readmission rate after 90 d of 5.5%<sup>[10]</sup>.

Patel *et al*<sup>[4]</sup> conducted a systematic literature review to assess the relationship between EC and the severity of complications in 38 studies that included pancreatic, hepatic, gynecological, urological and thoracic surgeries, and also analyzed the methodologies used. They concluded that EC depend on the type and severity of the complication and are due mainly to prolonged hospitalization. Many of the studies included used general or national databases rather than databases from the hospitals themselves, and all 38 studies included only payer/hospital (*i.e.*, not societal) costs. It was also observed that the studies did not tend to use classifications of complication severity<sup>[4]</sup>.

The association between PC severity and increased EC has been demonstrated in major abdominal surgery<sup>[6,11-14]</sup>, and in another study of all procedures performed at a surgery service<sup>[15]</sup>. In the latter study for example, even the presence of mild CDC

grade 1 complications was found to double postoperative costs from the hospital perspective after cholecystectomy<sup>[15]</sup>.

In some cases it has been observed that complications are profitable for hospitals<sup>[16,17]</sup>. This is because the payment system rewards the volume of services provided to patients, rather than the quality of the patient's clinical outcome<sup>[18]</sup>.

## ISSUES TO CONSIDER IN THE ANALYSIS OF EC ASSOCIATED WITH PC

To determine the EC related to PC, we must: (1) Quantify and register all PC and the patients affected; (2) Identify and quantify all postoperative EC; and (3) Assess the difference in costs between complicated and non-complicated patients.

Let us now look at these three points in more detail.

### **Quantification, registration and classification of all PC**

The first step is to determine what we mean by PC. We agree with the definition of the CDC, which considers a complication to be any negative event occurring during hospitalization<sup>[5,19-21]</sup>, even if it is asymptomatic or only remotely related to the initial surgical procedure<sup>[5,15]</sup>. This classification is intended to avoid subjectivity and to ensure that all observers apply the same criteria. This fact entails a series of important consequences: (1) Postoperative patients who have had complications may be classified as uncomplicated: (A) In this case PC-related EC may be attributed to the mean or median costs of patients without complications. This overestimates the costs of patients without complications and brings down the mean EC of patients with complications included in the non-complicated group; and (B) Patients with complications who are wrongly classified as non-complicated do not appear in the PC group (these complications are usually minor). This misclassification pushes up the mean or median costs estimated for particular procedures associated with PC, and erroneously underestimates the total cost of patients with complications; and (2) This leads to errors of classification of diagnosis-related groups (DRG). DRG classify patients who have similar clinical characteristics and similar treatment costs. The purpose of their use is to relate a hospital's case load to the demand for resources and the costs incurred. Therefore, if a surgery service has a more complex case load according to DRG (as is the case when complications are recorded), this means that the hospital is treating patients who need more hospital resources and, by extension, represent a higher economic outlay. Failure to record all PC would have grave economic consequences for the management unit responsible for patient care because it would be assigned fewer resources (*i.e.*, the amount corresponding to non-complex DRG).

Secondly, PC must be recorded thoroughly. To achieve this, physicians must be formally obliged to prospectively document all complications affecting the patient during or after surgery. But this does not usually occur. In order to record all PC, researchers must evaluate the medical and (above all) the nursing logs and, if possible, specific complications forms in the electronic medical record<sup>[5]</sup>. An external audit is mandatory: The health authorities have an essential role to play here.

Thirdly, PC must be classified according to a severity or complexity system. We recommend the CDC, published in 2004<sup>[19]</sup>. This classification system is the most used worldwide, and the article in which it was described currently has 12318 citations<sup>[22]</sup>. The problem with the CDC is that it categorizes the entire postoperative period according to the most serious complication. This means that patients with two or more adverse events are underrepresented, and it has been demonstrated that between 44 and 51.5% of patients with morbidity at general surgery services have two or more complications<sup>[5,23]</sup>. To overcome this problem, in 2013 Slankamenac *et al*<sup>[24]</sup> developed a score that takes into account all the PC classified according to the CDC and summarizes them on a scale between 0 (without complications) and 100 (patient death). This scale, called the comprehensive complication index (CCI), has been validated both clinically<sup>[5]</sup> and from an economic perspective<sup>[14,15]</sup>.

It would be reasonable to include intraoperative complications in the calculation of EC. In a systematic review of abdominal surgery, Garbens *et al*<sup>[25]</sup> observed that intraoperative adverse events significantly increased the total EC, although the studies were of poor quality. However, despite the fact that a clinical classification of these complications has been published<sup>[26]</sup>, these events can only be reported by the surgical team; the surgeons may be fearful of the damage to their reputation of an audit (or its legal consequences) and may be tempted to conceal the complication. This means that there may be major differences and biases in the evaluation of clinical and economic results between different surgery services. In addition, even if they are



not taken into account, most of the intraoperative complications that affect EC would be detected during the postoperative period. In any case sensitivity studies might shed further light on this matter.

### **Identification and quantification of all postoperative EC**

The economic evaluation of PC may be performed from a variety of perspectives, depending on who is responsible for paying for them and the intended use of the results of the analysis. Different perspectives will consider different costs and time periods. Examples of these perspectives are the patient, the institution (*e.g.*, the hospital), the target group for specific services (*e.g.*, rehabilitation), the public health service, the public sector, or the sum of all these perspectives (*i.e.*, the social perspective)<sup>[27]</sup>.

ECs are not distributed equally across the hospital stay. Taheri *et al*<sup>[28]</sup> concluded that approximately 40% of variable costs are incurred during the first three days of admission. A reduction in length of stay of up to one full day at the end of admission reduces the total cost of care on average by 3% or less. Obviously, the occurrence of PC would change this trend.

It seems reasonable to assess EC until a specific time after patient discharge. Frequently, part of the costs of the care is transferred to the outpatient setting, in particular in patients who are discharged early. So, how long after the operation should EC be assessed? Ideally, all PC should be considered until the patient recovers from the consequences: Imagine a patient with a brain injury that leads to a definite tetraplegia. So, in the best-case scenario, all EC should be considered throughout the patient's life, although this may be impossible to achieve.

As noted by Drummond *et al*<sup>[27]</sup> in other economic evaluations, the follow-up time depends on the perspective applied. From the hospital's perspective, we think that the time period should be a minimum of 90 d, or preferably when the patient is able to return to work or their normal activities. However, in the case of certain PC such as a reconstruction of the intestine after an anastomosis dehiscence, this period could be lengthened. If the calculation is made from the perspective of other health agencies, the follow-up period might be for example one year, while from the perspective of the patient and/or family it might be considerably longer, even the entire lifetime<sup>[27]</sup>. So, the study should indicate the follow-up time used and why.

**Table 1** summarizes the different EC included in the literature which we believe should be taken into account in the economic evaluation of PC.

Given that most evaluations of PC-related EC have been carried out from the perspective of the hospital<sup>[4]</sup>, we would like to make some recommendations. It has been argued that taking micro costs into account (*i.e.*, each component of the resources used per patient)<sup>[27]</sup> is expensive. We do not share this view; with the electronic medical record, the investment for determining these costs is minimal but these costs are still assigned equally, independently of the individual cost. Some examples are: (1) The pharmacy costs associated with each patient are recorded electronically at almost all hospitals; (2) The cost of consumables used during surgical interventions (sutures, gauze, endostaplers, meshes) is very easy to calculate using a barcode reader and to incorporate into the medical record. In advanced surgery this EC is very high. However, operating room EC are usually assessed according to the time taken, regardless of the intervention, and not according to the material consumed; and (3) The cost of diagnostic tests, if they are assigned a unit cost. Consumables should also be individualized.

Specific measurement of each patient's use of these resources is essential to distinguish between the costs of patients with and without PC. Not taking them into account creates an obvious bias<sup>[15]</sup>. In addition, we believe that the practice of adjusted the number of stays by DRG is inappropriate<sup>[29]</sup>.

Since the economic evaluation of PC involves the comparison of two clinical evolutions, any costs that are common to both do not need to be quantified; we are interested only in differential costs and results, rather than totals. Any preoperative EC, including the EC of the initial or index surgery or PC caused by postoperative cancer treatments, should be excluded from the analysis.

To determine the cost of PC related to a specific procedure, the operations compared should be as similar as possible in terms of complexity and the patients compared should also be similar in terms of severity. The use of the Charlson morbidity scale can be particularly useful<sup>[30]</sup>.

**Table 2** describes the recommendations to be followed in the assessment of EC associated with PC and the data we think should be described in the economic study.

### **Assessment of the differences in costs between complicated and non-complicated patients**

Finally, to determine the EC of PC, the postoperative EC of patients with PC should

**Table 1** Types of costs to consider in the economic evaluation of postoperative complications**Direct costs**

## 1 Health-related

## 1.1 Postoperative resources used/consumed by the patient during hospitalization:

- (1) Shared or overhead costs. Financing of administrative infrastructure and fixed installations. Resources used by different services: Administration, records, electricity, heating, laundry, *etc.*
- (2) Central services: Documentation, computer services, ...
- (3) Installations and catering.
- (4) Health and non-health staff: Nurses, doctors, ancillary staff from the surgery service ...
- (5) Use of other surgical and medical services at the hospital.
- (6) Diagnostic procedures: Laboratory, radiological and endoscopic examinations, pathology studies, ...
- (7) Pharmacy.
- (8) Surgical, endoscopic and radiological procedures. Ideally, recording of consumables for each procedure.
- (9) Dressing materials.

## 1.2 Future postoperative costs related to the surgery performed and the postoperative complications arising:

- (1) Primary care and hospital visits.
- (2) Rehabilitation.
- (3) Diagnostic procedures: Analytical, radiological and endoscopic examinations, ...
- (4) Pharmacy.
- (5) Readmissions associated with the surgery, however remote the relation.
- (6) Care centres (for example, rehabilitation, convalescence or other long-term care centres)
- (7) Health transport: Ambulances.
- (8) Prostheses, wheelchairs, ...

## 2 Non-health-related

## 2.1 Monetary:

- (1) Social services: Home care, notification systems, ...
- (2) Time taken to receive medical care.
- (3) Transport costs incurred by patients and families.
- (4) Care for patient's dependents.
- (5) Adaptation of the home, vehicles, ...

## 2.2 Non-monetary (opportunity cost):

- (1) Time taken by the patient, families and friends in care of the illness.

**Indirect costs**

## 1.1 Productivity lost by patients (those in employment):

- (1) Temporary or definitive incapacity for work.
- (2) Readaptation in the workplace.
- (3) Death.

## 1.2 Productivity lost by family members (those in employment):

- (1) Paid time off work.
- (2) Non-paid time off work.

## 1.3 Associated with the legal issues that may occur.

be subtracted the postoperative EC of patients without PC. That is, costs in patients with and without complications should be compared. If the PC are classified according to the CDC and/or the CCI we can calculate the costs associated with each grade of the CDC or score on the CCI in any patient<sup>[15]</sup>.

## DISCUSSION

We believe that studies of EC associated with PC should ideally take into account the costs described in **Table 1** and the recommendations and clarifications in **Table 2**. Researchers should specify the ones they use. Some principles have been previously published in the CHEERS statement<sup>[31]</sup>.

In the best-case scenario, it would be possible to compare PC and their associated EC in different surgical procedures and in different hospitals at regional or global

**Table 2 Recommendations for the economic evaluations of costs associated with postoperative complications of surgical procedures**

No.	Data to be described in the economic study
1	Type of study
2	Center: Particular characteristics
3	Period of inclusion of cases
4	Population and subgroups analyzed; clarify why they were chosen
5	Surgical interventions considered
6	Methods for managing the heterogeneity of the population and the surgical techniques used; clarify the possible influence of this heterogeneity on the results
7	If possible, describe the characteristics of the severity or complexity of patients (ASA, Charlson, Frailty ...)
8	Patients excluded and why
9	Patients and procedures lost for analysis. Economic data lost. Clarify whether these can compromise the validity of the results
10	Definition of complication
11	How complications are classified (better, Clavien-Dindo classification and comprehensive complication index)
12	Sources used to obtain data on the complications (specific forms, medical and nursing evolution comments)
13	Perspective from which the economic evaluation is carried out: the patient, the specific institution (e.g., the hospital), the target group for specific services e.g., rehabilitation), the Public Health Service, the Public Sector in general or from all perspectives (social perspective)
14	Health-related costs (direct and indirect), non-health-related costs (table 1). Sources used to obtain data. Specify the costs obtained <i>en bloc</i> (for example, general costs) those obtained in microcosts (radiology, pharmacy, ...) In the estimation of operating room costs, specify whether the cost of the consumable material used in each patient is individualized
15	Describe the postoperative follow-up time in which complications and costs will be evaluated for each perspective and procedure. From the hospital perspective, consider 90 d minimum
16	Methods for converting costs into a common monetary base and the exchange rate. If it includes more than one calendar year, specify corrections made for inflation
17	Biases and limitations of the study, and measures used to reduce them
18	Conflict of interests of researchers. Linking researchers to the surgical service and hospital
19	Source of study financing

level. This would indicate the specific problems of each service and procedure, allow the introduction of improvements, and determine quality and efficiency benchmark services. But for this to be possible, care must be taken to measure the same phenomenon with the same tools: That is, assessing the complexity of the procedure and the severity of the patient, the concept of complication, the classification system, the evaluation perspective, the level of the hospital, and so on (Table 2).

Once the PC associated with a surgical procedure at a particular service have been identified, the introduction of a protocol aimed to allow early diagnosis and treatment of complications can help bring down morbidity and mortality rates and can also prove cost-effective<sup>[11]</sup>.

Obviously, without access to results expressed as real and audited PC of the various surgical interventions, it is impossible to consider the centralization of complex surgical procedures or the accreditation of surgical units or of training courses<sup>[3]</sup>. When these results are available, efficiency studies can be performed.

The training of surgeons is essential for reducing PC. Higher costs have been associated with surgeons who have performed fewer interventions<sup>[32,33]</sup> and a higher number of intraoperative complications<sup>[32]</sup>.

Outside the public health structure, the payment system in place may reward the volume of services provided to the patient rather than the patient's clinical outcome. Despite its obvious flaws, many hospitals have used this system for a long time<sup>[18]</sup>. Unfortunately, today there are no economic or social incentives to reduce PC, and the reporting of the results of surgery services all over the world continues to lack transparency. In fact, it is astonishing that in the year 2019 so little should be known



about a basic quality outcome of surgical services such as PC. It is an insult to science, patients and society as a whole.

We believe that PC at all surgery services should be audited in an objective and impartial manner by an external assessor. The health authorities would be able to carry out this process quickly and cheaply; using the electronic medical record, the assessment can be carried out in an average of 5 to 10 min per patient<sup>[2]</sup>. The audit should be maintained over a period of time and should be performed in all patients, not random samples. The audit of the results of the services is also likely to improve surgeons' practices due to the Hawthorne effect, though the mechanisms of this effect and its magnitudes need to be elucidated<sup>[34]</sup>.

The audit can enumerate the complications associated with each surgical procedure and measure their severity, thus identifying the areas where investment in interventions and improvements is most necessary to raise quality and reduce EC. Patients must be informed of the possible outcomes of the operation they are to undergo. The results available at present are unreliable, since they are based on evaluations made by surgeons at the service. This practice underestimates the number of PC, for many reasons: the fact that the better the recording system, the worse the results; the surgeon's sensation of personal failure; the fear of comparison with other surgeons and/or services; the fear that complex processes will be centralized, and so on. The responsibility for the fact that this audit of all surgical procedures has not been carried out so far in any surgery service in the world lies with the surgeons and the health authorities. Thorough and accurate data on the occurrence of PC will help to bring down their incidence and obviously the EC as well. In addition, it is a mandatory measure to ensure transparency.

## CONCLUSION

First of all, all the PC that are associated with a particular surgical procedure must be objectively determined. This is the responsibility of the surgical community and forms part of its commitment towards its patients, society, and science as a whole. Simply recording all PC is likely to reduce their incidence, improve quality and significantly lower EC. The information obtained would help to identify the surgical techniques and clinical management practices with the best postoperative results, to implement preventive measures in order to reduce PC and select the benchmarking services that should take charge of training. Currently, surgical training is not led by the services with the best clinical results. The EC saved can be reinvested in health and/or social areas. Complications should be audited externally and impartially by the health authorities, and in a universal, permanent manner in all surgical patients. Hospitals with the best results should be rewarded economically and not according to a payment system that may favour poor outcomes. This payment would only be justified in centres of proven quality.

ECs associated with PC should also be reported in a standardized way<sup>[31]</sup>. Appropriate and accurate methods should be used to track the use of resources and to estimate EC. Accounting must be accurate and detailed. Although the most frequently used perspective is that of the hospital, health authorities and health managers should bear in mind that EC associated with PC are much higher than the published figures, if the estimation includes future direct EC, non-health-related EC and indirect EC which have major repercussions for society and for patients.

Specifically, we think that recording and auditing of PC is likely will become the measure of the greatest impact on healthcare transparency, quality of care and the reduction of ECs in the coming decades.

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## Tailored classification of portal vein thrombosis for liver transplantation: Focus on strategies for portal vein inflow reconstruction

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### Abstract

Portal vein thrombosis (PVT) is currently not considered a contraindication for liver transplantation (LT), but diffuse or complicated PVT remains a major surgical challenge. Here, we review the prevalence, natural course and current grading systems of PVT and propose a tailored classification of PVT in the setting of LT. PVT in liver transplant recipients is classified into three types, corresponding to three portal reconstruction strategies: Anatomical, physiological and non-physiological. Type I PVT can be removed *via* low dissection of the portal vein (PV) or thrombectomy; porto-portal anastomosis is then performed with or without an interposed vascular graft. Physiological reconstruction used for type II PVT includes vascular interposition between mesenteric veins and PV, collateral-PV and splenic vein-PV anastomosis. Non-physiological reconstruction used for type III PVT includes cavoportal hemitransposition, renoportal anastomosis, portal vein arterialization and multivisceral transplantation. All portal reconstruction techniques were reviewed. This tailored classification system stratifies PVT patients by surgical complexity, risk of postoperative complications and long-term survival. We advocate using the tailored classification for PVT grading before LT, which will urge transplant surgeons to make a better preoperative planning and pay more attention to all potential strategies for portal reconstruction. Further verification in a large-sample cohort study is needed.

**Key words:** Portal vein thrombosis; Liver transplantation; Portal reconstruction; Grading; Anatomical; Physiological; Non-physiological

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**Core tip:** In the tailored classification for liver transplantation, portal vein thrombosis (PVT) is divided into types I, II and III according to the vascular sources used for portal reconstruction. The proposed algorithm for the tailored PVT classification and PV

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reconstruction strategy contributes to stratification of PVT patients by surgical complexity, risk of postoperative complications and long-term survival.

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## INTRODUCTION

Portal vein thrombosis (PVT) generally refers to complete or partial obstruction of blood flow in the portal vein and/or its main branches due to a non-neoplastic thrombus. A malignant embolus is termed tumorous invasion of the portal vein, constituting another clinical entity with different pathogenesis, treatment and prognosis<sup>[1]</sup>. PVT has long been considered a contraindication for liver transplantation (LT), as adequate portal inflow cannot always be ensured. However, when PVT is removed *en bloc* with the liver or through thrombectomy, a routine porto-portal anastomosis can still be performed. For some complex PVTs, alternative approaches can redirect the portal venous flow into the graft to achieve physiological reconstruction. Under these circumstances, PVT has no significant impact on post-LT outcomes<sup>[2]</sup>. Therefore, the portal vein inflow reconstruction pattern, and not the PVT *per se*, determines the efficacy of LT in patients with PVT. Current PVT grading systems, which are mainly based on the location and extent of the thrombus and the degree of occlusion in the vascular lumen, correlate weakly with the adequacy of portal vein inflow after thrombectomy or compensatory collaterals due to the PVT, both of which are crucial for portal vein inflow reconstruction during LT. We aimed in this opinion review to propose a tailored classification of PVT specific for LT, with the primary consideration of strategies for PV inflow reconstruction.

## PREVALENCE OF PVT IN LIVER TRANSPLANT CANDIDATES AND RECIPIENTS

A large epidemiologic study in northwest Italy identified 3535 new PVT cases from a total population of 13 million over an 11-year period, with an overall incidence of PVT of 3.78 and 1.7 per 100000 inhabitants in males and females, respectively<sup>[3]</sup>. However, the prevalence of PVT in liver transplant candidates and recipients is much higher. Analysis of the Organ Procurement and Transplant Network database between 2002 and 2013 showed that PVT was reported in 2819 (3.3%) patients listed for LT and in 3321 (6.8%) patients intraoperatively<sup>[4]</sup>. A Swedish study based on 23796 consecutive autopsies found that 33.1% of 254 PVT cases were associated with cirrhosis and hepatic carcinoma<sup>[5]</sup>, the main indications for LT. Specific for LT candidates and recipients, independent risk factors for preoperative PVT include older age, male sex, ethnicity, higher body mass index, longer waitlist time, autoimmune hepatitis, non-alcoholic steatohepatitis, diabetes mellitus, and transjugular intrahepatic portosystemic shunt<sup>[5-8]</sup>. With reference to ethnicity, African Americans had the lowest prevalence of PVT: 2.3% at registration and 4.9% at transplant, whereas Hispanic patients had a significantly higher prevalence: 4.6% at registration and 9.1% at transplant<sup>[6]</sup>.

## NATURAL COURSE OF PVT IN CIRRHOSIS

As a majority of liver transplant candidates and recipients have cirrhosis, it is crucial to obtain insight into the natural course of PVT in cirrhosis. All thromboembolic events can be traced pathophysiologically to the three fundamental components of Virchow's triads - alterations of normal blood flow, hypercoagulability, and vascular endothelial injury. For PVT in cirrhosis, the first two components are more decisive. Cirrhosis is a chronic process complicated by remodelling of the intrahepatic architecture and portal hemodynamics and by rebalancing of pro- and anticoagulant



activities<sup>[9]</sup>. Maruyam *et al*<sup>[10]</sup> performed an excellent study on the natural course of PVT in virus-related cirrhosis, with a rigorous design to exclude almost all interfering factors. None of the enrolled patients received any treatments related to PVT, including anticoagulants, vasoactive drugs, transjugular intrahepatic portosystemic shunt, surgery or even antiviral therapy. Over an 11-year period, *de novo* PVT developed in 28% of 150 patients with virus-related cirrhosis but without PVT at baseline. Moreover, the prevalence of PVT increased along the course of cirrhosis, with cumulative incidences of 12.8%, 20% and 38.7% at 1, 5 and 8-10 years, respectively. The baseline flow volume of the largest collateral vessel was the only independent risk factor for PVT, though collateral vessels were comparably common in the PVT and non-PVT groups, with baseline incidences of 93% and 96%, respectively. Follow-up of the 42 patients with PVT revealed PVT improvement in 47.6%, no change in 45.2%, and worsening in only 7.2%. These findings were consistent with the Organ Procurement and Transplant Network database analysis, which showed that 40% of the listed 1603 patients with PVT did not report PVT at LT<sup>[4]</sup>. Overall, these results are very interesting, indicating that PVT is a multifactor-induced event during the course of cirrhosis but tends to be stable or to resolve.

Portal pressure is a suitable surrogate reflecting remodelling of the intrahepatic architecture and portal hemodynamics. Portal pressure usually increases with the progression of cirrhosis, with a tendency to decrease from its peak value when portal blood flow is partly diverted by collateral vessels. Rebalancing of pro- and anticoagulant activities can be embodied as the intensity of thrombophilia, which increases during the early stage of cirrhosis and decreases during the decompensation period. We believe that the relationship between portal pressure and thrombophilia elucidates the three types of natural courses of PVT: Never occurring, occurring but stable or improved, and worsening (Figure 1).

## CURRENT GRADING SYSTEMS FOR PVT

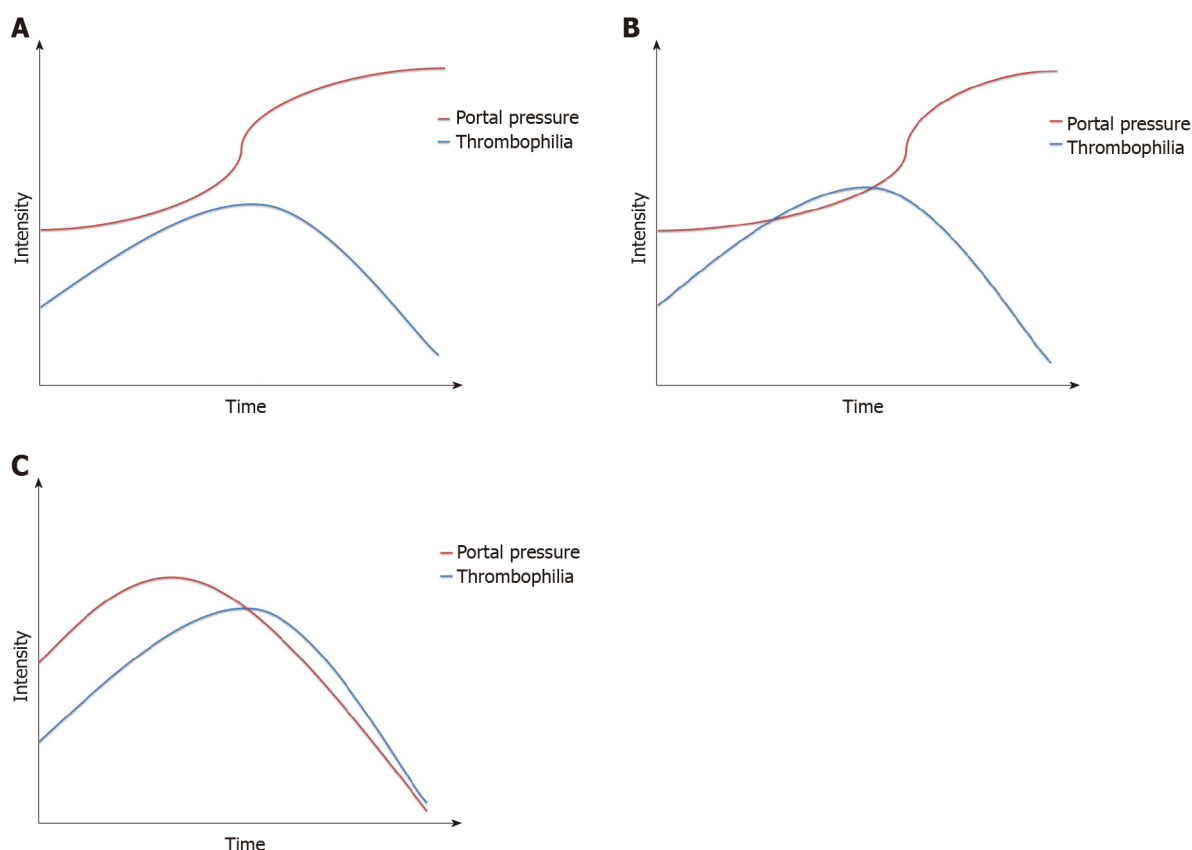
There are approximately ten grading systems for PVT, as reviewed in two major articles<sup>[1,11]</sup>. Essentially, these grading systems can be grouped into three categories. The early grading systems only considered the PVT's location, obstruction and extension, with the Yerdel grading system being the most representative and well recognized<sup>[12]</sup>. The collaterals and cavernous transformation were added in the later grading systems, among which the Jamieson grading system has been the most instructive<sup>[13]</sup>. The third category of PVT classification involved additional indicators, including duration, presentation and underlying liver disease, and was more complicated<sup>[1,14]</sup>. Nonetheless, in the setting of LT, these grading systems have limited value because a PVT located in the branches and distal trunk can be resected together with the liver but a more advanced PVT can also be removed through thrombectomy. Cases with insufficient portal blood inflow after thrombectomy and how to use alternative vessels for portal reconstruction are major challenges. Bhangu *et al*<sup>[11]</sup> proposed a dichotomy for PVT, which included complex PVT and noncomplex PVT, grouping Yerdel grade 4 or Jamieson grades 3 and 4 into the former and others into the latter. This classification was used as a guide for portal flow reconstruction during LT, which was also defined by a dichotomy of non-physiological and physiological reconstruction. However, a complex PVT has not always been assigned to a non-physiological reconstruction, and the relationship between a non-complex PVT and physiological reconstruction appears in a same manner.

## TAILORED CLASSIFICATION OF PVT WITH REFERENCE TO PV INFLOW RECONSTRUCTION

Portal vein inflow reconstruction in LT is a considerable challenge for advanced PVT complicated with structural and hemodynamic abnormalities of the portal venous system, not only with regard to surgical techniques but also with regard to postoperative complications and patient survival. Therefore, PV inflow reconstruction patterns can stratify patients with PVT undergoing liver transplantation. We propose a tailored classification of PVT with reference to three patterns of PV inflow reconstruction, mainly based on the vascular sources providing the portal inflow for the liver graft.

### **Type I PVT: Anatomical porto-portal anastomosis with or without devascularization of the collaterals**

Type I PVT is defined as a PVT located in the branches and distal trunk that can



**Figure 1 Relationship between portal pressure and thrombophilia in the natural course of portal vein thrombosis.** A: Portal pressure increases gradually, while the intensity of thrombophilia increases and then decreases. No crossover of the two curves means that portal vein thrombosis (PVT) never occurs; B: The two curves cross each other, but portal pressure continues to increase, and its curve is separate from the curve of thrombophilia; thus, PVT occurs but stabilizes or improves; C: Portal pressure increases at first and then decreases due to diversion of the blood flow by the collaterals. The two curves cross and remain close to each other, meaning that PVT occurs and worsens.

definitively be resected with the liver or removed through thrombectomy to recover adequate portal vein blood flow. This type is the most common in LT recipients with PVT. According to a systematic review of the surgical resolution of 1957 patients with PVT, PVT was resected through low dissection of the PV in 5% of the patients and removed through thrombectomy in 75% during LT<sup>[15]</sup>; both conditions would be assigned to type I PVT in our tailored classification, as an end-to-end donor-recipient portal anastomosis was performed thereafter.

Thrombectomy should always be maintained as the first option for any PVT, regardless of location, obstruction and extension; even for Yerdel grade 3 or 4 PVT, thrombectomy can still be performed successfully with appropriate approaches in experienced centres. The key points for thrombectomy include the following: (1) Low dissection of the PV as proximal as possible to reach the superior border of the pancreas; (2) Right operating spaces between the thrombus and the vascular wall; and (3) Appropriate Pringle manipulation with fingers to control bleeding and guide handling. Pan *et al*<sup>[16]</sup> introduced an improved eversion thrombectomy without cutting off the thrombus to obtain persistent traction from the diseased liver through the PVT. Using this technique and simple or eversion thrombectomy, all the Yerdel grade 1 or 2 PVTs in 218 cases were removed successfully, and the success rates for grades 3 and 4 PVT were 79.3% (23/29) and 50% (3/6), respectively. Kasahara *et al*<sup>[17]</sup> and Mizuno *et al*<sup>[18]</sup> also independently reported a pull-out technique for thrombectomy in 6 PVT patients. All inflow branches to the PV above the confluence of the superior mesenteric vein (SMV) and splenic vein (SV) were ligated and transected. The PV trunk was dissected posterior to the pancreas, pulled out inferior to the pancreas, and then transected at the confluence of the SMV and SV. After thrombectomy, the donor PV was placed posterior to the pancreas where the PV was used, and portal reconstruction was performed with or without an interposed vascular graft.

Occasionally, adequate portal vein flow or pressure is not regained even when the PVT is removed completely, probably due to diversion of blood flow by the collaterals. Under these circumstances, devascularization of the collaterals should be considered after restoration of blood inflow to the graft. The dominant collaterals

were compensatory enlarged gastric coronary veins and splenorenal shunts, as other collaterals, such as the pericholedochal vascular plexus and umbilical vein, were usually ligated and cut off during resection of the diseased liver, and mesentericocaval shunts in rare cases. The gastric coronary veins are usually easy to deal with in the operative field, though spontaneous or surgical splenorenal shunts are more complicated and require careful evaluation of preoperative images<sup>[19]</sup>. For splenorenal shunts that are difficult to dissect, ligation of the left renal vein might be an alternative strategy that does not affect renal function or recipient survival<sup>[20,21]</sup>. Diffuse and fine collaterals communicating portal and vena cava systems in the retroperitoneal region, which are common in pediatric recipients, can be thermally devascularized using bipolar electrocoagulators.

### **Type II PVT: Veins belonging to the recipient portal system used for portal inflow reconstruction**

Type II PVT is defined as PVT that cannot be removed successfully through thrombectomy to achieve adequate portal vein flow; thus, a substituted inflow vessel is used instead of or in addition to porto-portal anastomosis. The substituted vein belongs to the recipient portal system. Therefore, the reconstruction of portal inflow is still physiological, albeit not anatomical. The main substituted vessels used for patients with type II PVT are mesenteric veins and compensatory enlarged collaterals.

**Recipient mesenteric veins as portal influx:** Vascular graft interposition between recipient mesenteric veins and the donor portal vein was found to be the second most commonly used technique after thrombectomy, accounting for 8.4% of all PVT cases<sup>[15]</sup>. An interposed vascular graft can be an autologous vein such as the external iliac vein, ovarian vein or internal jugular vein, a donor vessel, a cadaveric cryopreserved vessel, or an artificial vessel. The SMV is the main portal influx source because it collects most of the hepatopetal blood from the gut and is closer to the portal vein. The vascular graft interposition has been performed using the jump method, by which the vascular graft is brought to the portahepatis through a transmesocolic route, anterior to the pancreas and posterior to the pyloric antrum. When the SMV distal to its confluence with the splenic vein is not available due to thrombus, hypoplasia or any other reasons, an enlarged inferior mesenteric vein can be used as a portal influx source with a jump vascular graft interposition<sup>[22,23]</sup>.

**Enlarged collaterals as portal influx:** Enlarged collaterals have been used as portal influx in 2.4% of all PVT cases<sup>[15]</sup>. For example, spontaneous splenorenal shunts were the most common collaterals in decompensated cirrhosis patients who underwent evaluation for liver transplantation, with an incidence of 23%<sup>[24]</sup>. Nevertheless, use of the left renal vein to graft PV anastomosis was preferred due to its technical simplification, which is discussed below for the type III PVT. A splenorenal shunt was directly used as portal influx when its confluence to the left renal vein was identified and sectioned, and then the splenorenal shunt was cautiously dissected and brought behind the stomach<sup>[25]</sup>. Among the collaterals, the enlarged left gastric vein or gastric coronary vein was the most commonly used portal influx, as it was superficial, close to the porta hepatis and easy to dissect. Pooled analysis for the left gastric vein or gastric coronary vein as portal influx showed that 92% of 24 patients were alive with a patent portal reconstruction at the last follow-up visit<sup>[11]</sup>. In some cases, a large pericholedochalvarix was the only collateral available for portal reconstruction, but a subsequent Roux-en-Y hepaticojejunostomy was reasonable for biliary reconstruction, as the dissection of recipient bile duct was abandoned beforehand to avoid injury to the pericholedochalvarix<sup>[26,27]</sup>. Nine of 10 (90%) patients with a pericholedochalvarix as portal influx were reported to be in good condition with a patent portal reconstruction. In case reports, other enlarged mesenteric vein tributaries, such as the right gastroepiploic vein and right and middle colic vein, have been used to reconstruct the portal flow to the graft<sup>[28-32]</sup>.

**The splenic vein as portal influx:** Heterotopic liver transplantation in splenic fossa after splenectomy has been proven to be feasible in technique and long-term results<sup>[33,34]</sup>, therefore providing an option for portal reconstruction in PVT patients using the splenic vein. For patients receiving heterotopic liver transplantation in the splenic fossa, the conditions are usually complicated not only by PVT but also by other problems, such as failure to remove the diseased liver. In addition to portal reconstruction, reconstructions for graft outflow, hepatic artery and bile duct are different from orthotopic liver transplantation. Three cases of PVT were reported using heterotopic liver transplantation in the splenic fossa, and two of the patients were in good condition with normal liver function and patent vessels at 60 months and 18 months after the operation<sup>[35,36]</sup>.



### **Type III PVT: Vessels that did not belong to the recipient portal system used for portal inflow reconstruction**

In patients with type III PVT, recovered portal inflow to the graft is partly or completely from vessels that do not belong to the recipient portal system, making the portal reconstruction a non-physiological pattern. The strategies for type III PVT include cavoportal hemitransposition (CPHT), renoportal anastomosis, portal vein arterialization and multivisceral transplantation.

**CPHT:** Cavoportal transposition was performed in early animal experiments to determine the effects of portal blood on liver regeneration, as well as the diversion of systemic blood to the liver, and was then used to treat glycogen storage disease clinically in the 1960s<sup>[37]</sup>. CPHT, in which only one anastomosis between the donor PV and recipient inferior vena cava was performed either in an end-to-end or end-to-side way, was first described in liver transplant recipients with diffuse PVT in 1998<sup>[38]</sup>. As a novel and simplified resolution for diffuse PVT, CPHT has been performed in many transplant centres, mainly in the 2000s. However, the results of CPHT were suboptimal and seemed unpredictable. Pooled analysis showed that 63% of 86 patients who underwent CPHT were alive at the last follow-up, but ascites were almost inevitable, with an incidence of portal or cavomesenteric thrombosis of 28% and an incidence of intra-abdominal bleeding of 30%-50%<sup>[11]</sup>. An eventless course after CPHT largely depends on two aspects, namely, proper perfusion to the graft and potential cavoportal shunts. The caval flow directed into the graft should be quick enough to avoid re-thrombosis, but not be too quick to avoid hyperperfusion. Potential cavoportal shunts guarantee the gradual correction of portal hypertension, reducing the risk of intra-abdominal bleeding and refractory ascites. Nonetheless, there is uncertainty in both aspects. Conversely, better results under the same circumstances have been achieved with renoportal anastomosis and multivisceral transplantation. Thus, CPHT has rarely been reported in diffuse PVT in the last decade.

**Renportal anastomosis (RPA):** RPA was first described in 1997 in a liver transplant recipient with PVT and a surgical splenorenal shunt<sup>[39]</sup>. A total of 64 cases of RPA among PVT patients have been reported<sup>[11,40-44]</sup>. Among 57 patients with available long-term results, 46 (80.7%) were alive with a patent portal reconstruction at the last follow-up visit; complications related to RPA included ascites (40.4%), renal dysfunction (28.1%), portal thrombosis or stenosis (5.3%) and variceal bleeding (3.5%). The left renal vein was observed to be comparable to the native PV in reference to the vessel size and blood flow. In addition, the proximal left renal vein close to the inferior vena cava was easy to dissect, which ensured the operability of RPA. This technique is particularly reasonable for PVT patients with a pre-existing splenorenal shunt, spontaneous or surgical. RPA drained the splanchnic blood through the splenorenal shunt, effectively decompressing portal hypertension and delivering portal trophic factors to the graft. A major concern for RPA lies in whether it is practicable for diffuse PVT without a splenorenal shunt. To date, 5 cases of RPA in the absence of a splenorenal shunt have been reported; 3 of the patients died, though none of the deaths were directly related to the procedure or complications of RPA. Performing a surgical splenorenal shunt followed by RPA appears to be a solution when RPA is the only option<sup>[11]</sup>.

**Portal vein arterialization (PVA):** PVA is more commonly used as a salvage technique when hepatic artery reconstruction is deemed impossible in liver transplantation or hepatopancreatobiliary surgery to increase the oxygen supply to the liver and alleviate ischaemic biliary necrosis<sup>[45]</sup>. This technique was first reported as a solution for portal reconstruction in pre- and post-LT-confirmed PVT in 1995<sup>[46]</sup>. A total of 18 cases of PVA for PVT in liver transplantation have been reported, mainly in the 1990s and 2000s<sup>[46-56]</sup>. In a few of them, PVA was performed to augment portal vein flow after native porto-portal anastomosis but contributed predominantly to portal inflow to the graft over the long term<sup>[52,55]</sup>. Among the 18 patients, 6 (33.3%) died postoperatively, and 2 underwent embolization of the diverting arteries due to aggravating portal hypertension with related complications. The main drawback of PVA is the impossibility of relieving portal hypertension, which usually leads to an eventful postoperative course. Another disadvantage is overarterialization, which may result in liver fibrosis. Therefore, the arterial flow should be calibrated, by using either medium-sized arterial vessels or partial ligation around the arterial side.

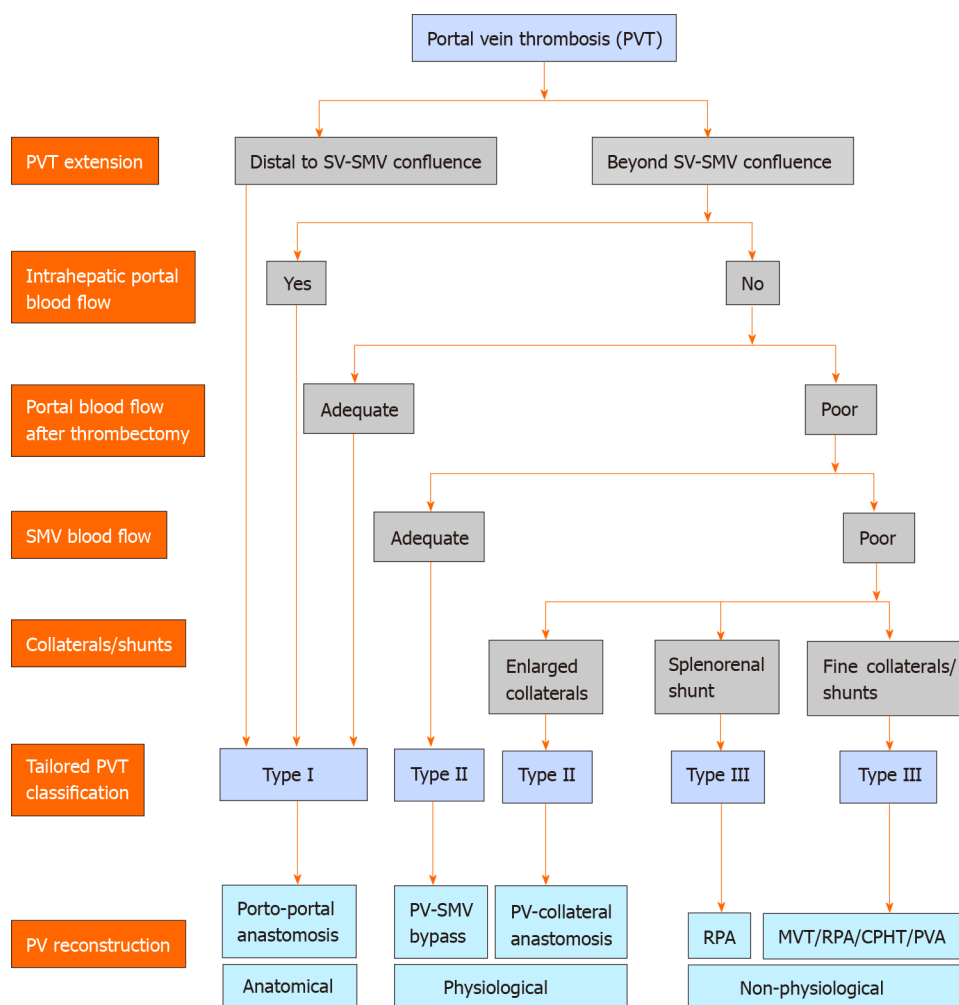
**Multivisceral transplantation (MVT):** MVT, which *en bloc* engrafts the liver, small intestine, stomach, pancreaticoduodenal complex and sometimes a segment of the colon, represents the last surgical option for diffuse PVT, replacing the entire

splanchnic venous system of the recipient. Due to the major challenges in this technique, immunosuppression and postoperative management, most MVTs have been performed by a few experienced teams, though there has been tremendous progress over the past decades<sup>[57-60]</sup>. The first case of successful MVT for diffuse portomesenteric thrombosis, which resulted from protein C deficiency, was reported in 2002<sup>[61]</sup>. The Indiana group reported 34 cases, the largest single-centre series of MVT for diffuse PVT, and patient survival was 80% at 1 year and 72% at 3 and 5 years postoperatively, with a median follow-up of 2.78 years<sup>[58,62]</sup>. However, surgical complications occurred in 56% of the patients. The steep learning curve and additional risks of the intestinal component of MVT, such as rejection, sepsis, malnourishment and post-transplant lymphoproliferative disorder, remain major obstacles for the routine adoption of MVT in diffuse PVT.

## ROLES OF THE TAILORED PVT CLASSIFICATION IN THE SETTING OF LIVER TRANSPLANTATION

Type I, II, and III PVTs strictly correspond to three patterns of PV inflow reconstruction in LT: Anatomical, physiological, and non-physiological reconstruction. This tailored classification can stratify PVT patients by surgical complexity and risk of postoperative complications, as well as long-term survival. Although it is not a preoperative classification, the PVT type can be predicted before liver transplantation through rigorous evaluations via abdominal CT angiography and symptoms of portal hypertension. A proposed algorithm for the tailored PVT classification and PV reconstruction strategy is illustrated in [Figure 2](#).

We advocate using this tailored classification for PVT grading before LT, even though the determined type may change during the operation. The tailored PVT classification allows for better preoperative planning, urging transplant surgeons to pay more attention to all potential strategies for portal reconstruction. In addition to the conditions of the recipients, the procurement of donor organs and interposed vascular grafts, as well as the technical capacity of MVT, should be fully considered. Only in this way can portal reconstruction be performed as planned rather than passively. Furthermore, a large-sample retrospective study should be performed with regard to the development of a model to accurately predict the tailored PVT type before LT, which should be verified in an independent cohort.



**Figure 2** A proposed algorithm for the tailored portal vein thrombosis classification and portal vein reconstruction strategy. CPHT: Cavoportal hemitransposition; MVT: Multivisceral transplantation; PV: Portal vein; PVA: Portal vein arterialization; RPA: Renoportal anastomosis; SMV: Superior mesenteric vein; SV: Splenic vein.

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## Role of regenerating islet-derived proteins in inflammatory bowel disease

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### Abstract

Inflammatory bowel disease (IBD) is an inflammatory disorder of the gastrointestinal tract that affects millions of patients worldwide. It has a complex and multifactorial etiology leading to excessive exposure of intestinal epithelium to microbial antigens, inappropriate activation of the immune system and ultimately to the damage of intestinal tissues. Although numerous efforts have been made to improve the disease management, IBD remains persistently recurring and beyond cure. This is due largely to the gaps in our understanding of the pathogenesis of IBD that hamper the development of timely diagnoses and effective treatment. However, some recent discoveries, including the beneficial effects of interleukin-22 (IL-22) on the inflamed intestine, have shed light on a self-protective mechanism in IBD. Regenerating islet-derived (REG/Reg) proteins are small secretory proteins which function as IL-22's downstream effectors. Mounting studies have demonstrated that IBD patients have significantly increased REG expressions in the injured intestine, but with undefined mechanisms and roles. The reported functions of REG/Reg proteins in intestinal homeostasis, such as those of antibacterial, anti-inflammatory and tissue repair, lead us to discuss their potential mechanisms and clinical relevance in IBD in order to advance IBD research and management.

**Key words:** Regenerating islet-derived proteins; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Interleukin-22; Intestinal bacteria

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**Core tip:** The clinical management of inflammatory bowel disease (IBD) remains a significant challenge due to the knowledge gap in its pathogenesis. In this review paper, we have discussed the literature regarding increased expressions of regenerating islet-derived proteins in IBD and proposed the potential clinical relevance of these proteins based on their known protective activities in the inflamed intestine. We therefore provide insight from a new perspective in order to advance IBD research and clinical management.

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## INTRODUCTION

Regenerating islet-derived (REG/Reg) proteins are small secretory C-type-like lectins. In 1979, De Caro *et al*<sup>[1]</sup> found that pancreatic stones in patients with chronic calcifying pancreatitis were made largely of a type of proteins. This pancreatic stone protein was later independently reported as pancreatic thread protein by Gross *et al*<sup>[2]</sup> in 1985, and as regenerating islet-derived protein by Terazono *et al*<sup>[3]</sup> in 1988. After the recognition of its different isoforms and the corresponding homologs across species in mammals, it was given the current name REG1 $\alpha$  in a sequence and structure-based classification of the family proteins<sup>[4,5]</sup>. The tissue distributions and physiological activities of this protein family are studied primarily in humans and mice. The human REG family consists of REG1 $\alpha$ , REG1 $\beta$ , REG3 $\alpha$ , REG3 $\gamma$  and REG4, and these family members share between 30 to 80 percent homologous sequences. In mice, the family members of Reg1, Reg2, Reg3 $\alpha$ , Reg3 $\beta$ , Reg3 $\gamma$ , Reg3 $\delta$  and Reg4 share more than 50 percent homologous sequences, and are structurally and functionally conserved with their human counterparts<sup>[5]</sup>. Studies of REG/Reg proteins have focused on their functions in the pancreas and intestine since REG/Reg proteins are predominantly expressed in these two digestive organs. We and others have shown that in acute pancreatitis, the production of REG/Reg proteins is significantly induced in pancreatic acinar cells to inhibit inflammatory cell infiltration and to promote tissue repair<sup>[6-11]</sup>. In the inflamed intestine, REG/Reg expression is highly upregulated in different crypt cells including Paneth cells, deep secreting cells and enteroendocrine cells, and extend to other intestinal epithelial cells depending on the isoform<sup>[12-16]</sup>. Although the precise roles of REG proteins in human inflammatory bowel disease (IBD) have not been defined, mouse studies have confirmed that Reg proteins are required to maintain intestinal homeostasis in both physiological and pathological conditions.

Ose *et al*<sup>[13]</sup> reported that Reg1 deficient mice had reduced stem cell proliferation in the crypts and impaired epithelial migration along the villi in the small intestine, revealing the role of Reg1 in the preservation and renewal of intestinal villous structure. In line with these findings, Sun *et al*<sup>[17]</sup> showed that Reg1 protein protected against intestinal damage caused by indomethacin, a nonsteroidal anti-inflammatory drug. In the same disease model, Kitayama *et al*<sup>[18]</sup> found that Reg1 deficiency severely attenuated the expression of tight junction proteins including claudins 3 and 4. Therefore, in addition to promoting the recovery of villous structure, Reg1 may enhance the integrity of the epithelial barrier during intestinal injury.

Like Reg1 protein, Reg3 proteins also have a protective role in injured intestine. Studies showed that Reg3 $\beta$  and Reg3 $\gamma$  could promote the growth of cultured colonic epithelial cells<sup>[19]</sup>, and their intestinal expression was highly upregulated in dextran sulfate sodium (DSS)- or pathogenic bacteria-induced mouse colitis<sup>[12,20,21]</sup>. Reg3 $\beta$  deficiency consistently exacerbated symptoms of colitis in DSS-treated mice<sup>[22]</sup>. Similarly, in a mouse model of graft-*vs*-host disease, intestinal expressions of Reg3 $\alpha$  and Reg3 $\gamma$  were upregulated in response to IL-22 signaling to enhance the survival of intestinal stem cells and Paneth cells<sup>[23,24]</sup>. Furthermore, compensation of Reg3 $\gamma$  deficiencies in IL-22 deficient mice by intraperitoneal injections of REG3 $\gamma$  or Reg3 $\gamma$  blocked the intestinal epithelial invasion of orally introduced *C. rodentium* and saved the animals' lives<sup>[25]</sup>.



Notably, in addition to Reg3 proteins' trophic effect on the epithelial barrier, the contribution of their direct inhibitory effect on bacterial invasiveness cannot be excluded<sup>[26,27]</sup>. Cash *et al*<sup>[28]</sup> showed that Reg3 $\gamma$  and its human counterpart REG3 $\alpha$  are antimicrobial proteins that bind to the peptidoglycan carbohydrate on Gram-positive bacteria. Further studies showed that the interaction was mediated by a Glu-Pro-Asn motif in the long loop region of REG3 $\alpha$ <sup>[29]</sup>. Upon interacting with peptidoglycan, REG3 $\alpha$  oligomerizes to form hexameric transmembrane pores that kill bacteria by increasing their membrane permeability<sup>[30]</sup>. Consistently, Reg3 $\gamma$  deficient mice have more Gram-positive bacteria in the intestinal mucosa<sup>[31]</sup>. In contrast to REG3 $\alpha$ /Reg3 $\gamma$ , Reg3 $\beta$  may attach to lipopolysaccharide to cause osmotic rupture in Gram-negative bacteria<sup>[26,27,32-34]</sup>, in line with the increased proportion of intestinal Gram-negative bacteria in Reg3 $\beta$  deficient mice<sup>[35]</sup>.

Unlike the expressions of Reg1/3 proteins in both the small and large intestines, Reg4 proteins are distributed predominantly in the colon. It has been shown that Reg4 is produced by deep crypt secretory cells and intestinal enteroendocrine cells, and expands to epithelial cells of the upper colonic crypts during inflammation<sup>[15,16]</sup>.

## INCREASED EXPRESSIONS OF REG PROTEINS IN IBD PATIENTS

IBD comprises two major disorders: Crohn's disease (CD) and ulcerative colitis (UC). CD and UC share the features of chronic and destructive mucosal inflammation, but they are pathogenically and clinically distinct. CD patients have abdominal cramps, pain, diarrhea and weight loss, and their intestinal injury is characterized by transmural and segmental lesions that may occur at any level of the gastrointestinal tract "from mouth to anus". UC patients frequently present with bloody stools and diarrhea, and their intestinal inflammation is localized to colonic mucosa. However, the mechanisms underlying the different pathogenesis in CD and UC remain poorly understood<sup>[36,37]</sup>.

Over the past decade, increased REG expression has been detected by various techniques in both CD and UC patients<sup>[12,14,38-52]</sup> (Table 1). Interestingly, the increased levels of different REG isoforms vary significantly in CD and UC, and in active and remissive phases of each disease, which could be attributed to the different transcriptional regulations of REG isoforms by inflammatory cytokines such as IL22 and TGF $\beta$  illustrated in Figure 1. CD and UC are characterized by different cytokine responses. In CD, the T-helper type 1 (Th1) cytokine interferon- $\gamma$  and the Th17 cytokines IL-17/IL-22 are the main inflammation regulators. In contrast, UC has a Th2-like cytokine response that activates IL-13/IL-5, producing natural killer T cells<sup>[53]</sup>. These distinct cytokine patterns are associated with relatively high IL-22 levels in patients with CD compared to those with UC<sup>[54-56]</sup>. Animal studies have shown comparable results. For example, in the Th17 response-mediated mouse model of CD generated by the transfer of CD45RB<sup>high</sup> T cells, IL-22 levels are high; but in the Th2 response-regulated UC model of T cell receptor  $\alpha$ -chain deficient mice, IL-22 levels are low<sup>[56]</sup>. A recent study by Leung *et al*<sup>[57]</sup> provided an explanation. They showed that in active UC, a Th22 subset of helper T cells that produces IL-22, but not IL-17, was depleted by increased TGF $\beta$  in patients' colonic tissues<sup>[57]</sup>.

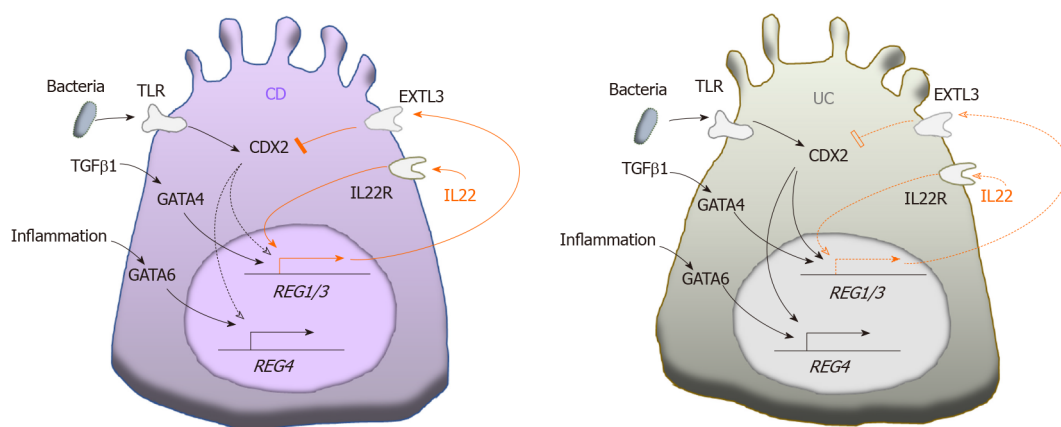
Tsuchida *et al*<sup>[52]</sup> showed that REG1 $\alpha$ , REG1 $\beta$ , and REG4 were all highly overexpressed in CD, while only REG4 was significantly overexpressed in UC. This could be caused by the limited IL-22 production in the colon because IL-22 signaling is essential for transcriptional activation of REG1/3 genes<sup>[25,46,52,58]</sup>, while REG4 transcription could be driven by the mechanisms other than IL-22 signaling<sup>[52]</sup>. For example, REG4 transcription is known to be activated by caudal type homeobox 2 (CDX2), a transcription factor that regulates multiple genes for maintaining intestinal homeostasis in response to the Toll-like receptor signaling<sup>[59-62]</sup>. Interestingly in this regard, CDX2 expression has been found to be inhibited by phosphoinositide 3-kinase<sup>[63]</sup>, which can be activated by exostosin-like glycosyltransferase 3 (EXTL3), a cell surface enzyme expressed in multiple organs including the intestine<sup>[64,65]</sup>. Since EXTL3 has been identified as a receptor for REG1/Reg1 and REG3/Reg3 proteins<sup>[64-67]</sup>, it is possible that as illustrated in Figure 1, CDX2-activated colonic REG4 transcription is inhibited in CD, but activated in UC, due to higher REG1/3 levels in CD than UC. In addition, GATA binding proteins (GATAs) could possibly regulate REG genes transcription in an IL-22 independent manner. Studies have shown that Reg1/3 transcriptions can be activated by GATA4, which is normally expressed in the proximal small intestine<sup>[68,69]</sup>, but abnormally expressed in the inflammatory lesions of the distal small intestine and colon<sup>[70]</sup>. On the other hand, REG4 transcription is specifically activated by GATA6<sup>[52,71]</sup>, which is expressed in both the small and large

**Table 1** Reported REG expressions in inflammatory bowel disease patients

Ref.	REGs	Samples (size)	Relevant findings
Lawrance <i>et al</i> <sup>[38]</sup> , 2001	REG1 $\alpha$ /1 $\beta$ /3 $\alpha$	CD (6) UC (6) control (6)	Increased intestinal REG1 $\alpha$ /1 $\beta$ /3 $\alpha$ in IBD detected by microarray
Shinozaki <i>et al</i> <sup>[39]</sup> , 2001	REG1 $\alpha$	CD (9) UC (21) control (5 non-IBD, 6 normal)	Increased intestinal REG1 $\alpha$ in IBD detected by RT-PCR and ISH
Desjeux <i>et al</i> <sup>[40]</sup> , 2002	REG3 $\alpha$	CD (124) normal control (54)	Increased serum REG3 $\alpha$ in active CD detected by ELISA
Dieckgraefe <i>et al</i> <sup>[41]</sup> , 2002	REG1 $\alpha$ /1 $\beta$ /3 $\gamma$	CD (3) UC (5) control (4)	Increased intestinal REG1 $\alpha$ /1 $\beta$ /3 $\gamma$ in IBD detected by microarray and IHC
Ogawa <i>et al</i> <sup>[42]</sup> , 2003	REG3 $\alpha$	CD (20) UC (23) control (18)	Increased intestinal REG3 $\alpha$ in IBD detected by ISH and Northern blot
Kämäräinen <i>et al</i> <sup>[42]</sup> , 2003	REG4	CD (N/A) UC (N/A)	By ISH and IHC, REG4 constitutively expressed in neuroendocrine cells, and upregulated in inflamed epithelial cells
Gironella <i>et al</i> <sup>[43]</sup> , 2005	REG3 $\alpha$	IBD (171) control (14 non-IBD, 29 normal)	Increased serum REG3 $\alpha$ correlated with IBD severity detected by ELISA. Higher REG3 $\alpha$ in CD than UC. REG3 $\alpha$ localized to colonic Paneth cells
Wu <i>et al</i> <sup>[44]</sup> , 2007	REG1 $\beta$ /REG3 $\alpha$	CD (9) UC (5) control (4)	Increased intestinal REG1 $\beta$ in CD and REG3 $\alpha$ in CD and UC detected by microarray
Nanakin <i>et al</i> <sup>[45]</sup> , 2007	REG4	UC (22) normal control (5)	Increased intestinal REG4 in UC detected by RT-PCR, ISH and IHC
Sekikawa <i>et al</i> <sup>[46]</sup> , 2010	REG1 $\alpha$	UC (60) control (10)	Increased intestinal REG1 $\alpha$ in UC detected by RT-PCR and IHC
Tanaka <i>et al</i> <sup>[47]</sup> , 2011	REG1 $\alpha$	UC (31) control (5)	Increased intestinal REG1 $\alpha$ in UC detected by IHC
Granlund <i>et al</i> <sup>[14]</sup> , 2011	REG1 $\alpha$ /1 $\beta$ /3 $\alpha$ /4	CD/control (12/21) UC/control (32/34)	Increased intestinal REG1 $\alpha$ /1 $\beta$ /3 $\alpha$ /4 in IBD detected by microarray. Different cellular localizations of REG1 $\alpha$ and REG4 detected by IHC
van Beelen Granlund <i>et al</i> <sup>[48]</sup> , 2013	REG1 $\alpha$ /1 $\beta$ /3 $\alpha$ /4	CD (N/A) UC (N/A)	By ISH, REG1 $\alpha$ /1 $\beta$ /3 $\alpha$ localized to Paneth cells in the crypt base, REG4 localized to enteroendocrine cells towards the luminal face
Planell <i>et al</i> <sup>[49]</sup> , 2013	REG1 $\alpha$ /4	Microarray: UC (15 active/8 remissive), Non-IBD (13); RT-PCR: UC (8 active/12 remissive), non-IBD (10)	Comparably increased intestinal REG4 in active and remissive UC, and significantly increased REG1 $\alpha$ in active UC but not in remissive UC, detected by microarray and RT-PCR
Marafini <i>et al</i> <sup>[50]</sup> , 2014	REG3 $\alpha$	CD (72) UC (22)	Infliximab treatment decreased the high serum REG3 $\alpha$ in CD and UC
Nunes <i>et al</i> <sup>[51]</sup> , 2014	REG3 $\alpha$	CD (66) UC (74)	Increased serum REG3 $\alpha$ serum in active CD but not UC detected by ELISA
Tsuchida <i>et al</i> <sup>[52]</sup> , 2017	REG1 $\alpha$ /1 $\beta$ /3 $\alpha$ /4	CD (49) UC (39) control (44)	Increased intestinal REG1 $\alpha$ /1 $\beta$ /4 in CD, and REG4 in UC detected by RT-PCR

IHC: Immunohistochemistry; ISH: *In situ* hybridization; RT-PCR: Reverse transcription-polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay. N/A: Not available.

intestines<sup>[68]</sup>. Deficiency of GATA4 or GATA6 causes abnormal alterations in intestinal cells including Paneth cells, enteroendocrine and goblet cells<sup>[72,73]</sup>. Both GATA4- and GATA6-regulated *REG* transcriptions could be mediated by inflammation in IBD. Haveri *et al*<sup>[70]</sup> suggested the upregulation of GATA4 in inflamed intestine by activated signaling of TGF $\beta$ , whose expression is elevated in active but not remissive CD and UC<sup>[74,75]</sup>. This explains the significant increase of intestinal REG1 $\alpha$  in active phase but not remissive phase of UC, which is in contrast to the disease status-independent increase of REG4 in UC<sup>[49]</sup>. As discussed before, *REG4* transcription could be activated by CDX2 in UC when IL-22 is diminished. In addition, the activation of GATA6 by inflammation also possibly contributes to REG4 expression in UC (Figure 1). In support of this view, Mustfa *et al*<sup>[76]</sup> showed that in IBD, inflammation globally



**Figure 1** A model of the differential transcription of *REG* genes in colon crypt cells in Crohn's disease and ulcerative colitis. Increased interleukin-22 induced transcriptions of *REG1/3* in Crohn's disease (orange solid arrows) are relatively attenuated in ulcerative colitis (orange dotted arrows), leading to CDX2-activated *REG* transcriptions in ulcerative colitis (black solid arrows), but not in Crohn's disease (black dotted arrows). IL: Interleukin; TGF: Transforming growth factor; TLR: Toll-like receptor.

decreased SUMOylation, a post-translational modification that inhibits *GATA6* transcription, in colonic cells<sup>[77]</sup>.

Of note, other factors, such as exclusive enteral nutrition diet commonly recommended for CD patients, influence the bacteria population, inflammation and mucosal healing in IBD<sup>[78]</sup>. Therefore, they could also have a direct and/or indirect regulatory effect on intestinal *REG* expressions, which should be investigated in future studies.

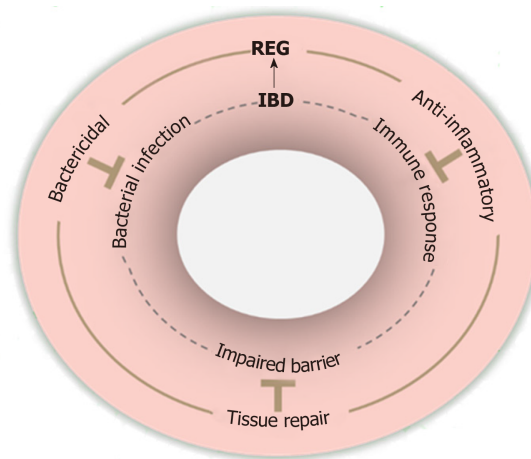
## REG PROTEINS ARE POTENTIALLY PROTECTIVE IN IBD

Studies have supported that IBD progression is driven by defective bacterial clearance, aberrant immune responses, and impaired epithelial barrier<sup>[37]</sup>. Notably, *REG* proteins appear to have the corresponding protective effects that can counteract these defects in IBD as illustrated in **Figure 2**.

In line with *REG/Reg* proteins' bactericidal activities, mice carrying genetically modified *REG/Reg* genes have altered compositions of intestinal bacterial microbiota<sup>[16,31,35,79,80]</sup> (**Table 2**), indicating the regulatory roles played by *REG/Reg* proteins in the gut microbiome. Notable among these are that mice with *REG3α* overexpression and mice with *Reg4* deficiency were resistant to DSS-induced colitis, suggesting the importance of *REG/Reg*-regulated intestinal microbiota in IBD pathogenesis<sup>[16,79]</sup>. Furthermore, mice with *Reg3γ* overexpression had an enriched fraction of beneficial *Lactobacilli* in the gut microbiome, suggesting the positive selection of "good bacteria" by *Reg3γ*<sup>[80]</sup>. In support of the importance of an altered intestinal microbiome in IBD pathogenesis, a systematic review and meta-analysis of CD and UC patients showed different intestinal microbiome compositions in patients with active disease as compared to those in remission<sup>[81]</sup>. Given the fact that patients with active IBD have higher levels of *REG* proteins, further studies are needed to define the significance between increased *REG* expression and altered microbiome composition observed in these patients.

In addition to their bactericidal activities, *REG3/Reg3* proteins are anti-inflammatory since they can inhibit proinflammatory cytokine secretion, inflammatory cell activation and infiltration in inflammatory diseases including IBD<sup>[8-10,12,82-84]</sup>. *REG3α* incubation inhibited the proinflammatory cytokine secretion in intestinal mucosa harvested from patients with active CD in a dose dependent manner, and decreased the adhesive molecules, such as E-selectin, ICAM-1 and VCAM-1, which were found to be upregulated on endothelial cells to promote inflammatory infiltration<sup>[12]</sup>. Additionally, *REG3/Reg3* proteins regulate the activities of macrophages, which regulate inflammatory injury in IBD<sup>[84-87]</sup>. It is thus possible that *REG3/Reg3* proteins may also alleviate IBD inflammation via macrophages.

As previously mentioned, *REG/Reg* proteins have trophic effects on intestinal epithelium in both physiological and pathological conditions. These trophic effects have been attributed to the activation of pro-survival and pro-proliferative signaling pathways, such as MEK1/2, ERK1/2, phosphoinositide 3-kinase-Akt and JAK2-



**Figure 2** A mechanistic model of REG proteins' protective activities in inflammatory bowel disease. IBD: Inflammatory bowel disease.

STAT3<sup>[5]</sup>. Therefore, the direct tissue protection and repair of mucosa in IBD by REG/Reg proteins cannot be excluded. Indeed, transgenic overexpression of REG3 $\alpha$  or intrarectal administration of REG3 $\alpha$  alleviated the epithelial damage in 2,4,6-trinitrobenzene sulphonic acid-induced mouse colitis<sup>[79]</sup>, while Reg3 $\beta$  deficiency worsen DSS colitis in mice<sup>[22]</sup>. Similarly, administration of REG3 $\gamma$  /Reg3 $\gamma$  improved epithelial integrity in *C. rodentium*-induced mouse colitis<sup>[25]</sup>. Additionally, Reg4<sup>+</sup> deep crypt secretory cells promoted the formation of organoids derived from Lgr5<sup>+</sup> colonic stem cells, and Reg4 stimulated the growth of colonic organoids isolated from mice with DSS-induced colitis<sup>[15,16]</sup>.

## REG PROTEINS' PROSPECTIVE CLINICAL RELEVANCE IN IBD

Despite the recent advancements, there are unmet needs in current IBD management, particularly including disease activity detection and treatment<sup>[88,89]</sup>. REG proteins have been considered as potential diagnostic markers and/or therapeutic targets for immune-mediated diseases<sup>[90]</sup>. The recognized upregulation of REG proteins in IBD and REG proteins' beneficial activities provide unique opportunities to address some of these unmet needs for improving IBD management, such as serving as biomarkers for disease activity, shifting the composition of bacterial microbiota, and enhancing the repair of intestinal epithelium.

The clinical diagnosis of IBD remission/relapse depends on the endoscopic biopsy, which has its own limitations including invasiveness, financial burden and inter-user variability. Non-invasive imaging such as ultrasound, and CT and MR enterography are useful modalities but also have drawbacks such as inter-operator variability, radiation exposure, and financial burden<sup>[91,92]</sup>. Therefore, efforts have been made to study potential IBD biomarkers in serum and feces including C-reactive protein (CRP), anti-Saccharomyces cerevisiae antibody, which is chiefly linked with CD, and perinuclear antineutrophil cytoplasmic antibody, which is linked with UC<sup>[93-96]</sup>. However, these biomarkers have high specificities but limited sensitivities. Of note, inhibition of tumor necrosis factor alpha by Infliximab reduced serum REG3 $\alpha$  levels in CD and UC patients<sup>[50]</sup>, supporting the potential use of REG3 $\alpha$  for evaluating the response of treatment. Furthermore, a multicenter prospective study showed increased serum REG3 $\alpha$  with 94% specificity and 60% sensitivity for active CD<sup>[38]</sup>. Similarly, a recent study showed increased serum REG3 $\alpha$  in CD patients 3 mo prior to relapse, but with only 73% specificity and 50% sensitivity<sup>[51]</sup>. The authors explained that the lower specificity and sensitivity in their study compared with those from the previously mentioned report was likely due to the patients' mild disease activity and low relapse rate. Given the functional redundancy among REG family members and confirmed upregulated REG1 $\alpha/\beta$  in CD patients (Table 1), it is possible that CD patients with lower REG3 $\alpha$  levels may have higher serum REG1 $\alpha/\beta$  levels. If so, combining REG3 $\alpha$  and REG1 $\alpha/\beta$  measurements in each CD patient could collectively improve the sensitivity for evaluating the relapse. On the other hand, this study showed that serum REG3 $\alpha$  levels were not correlative with UC activity<sup>[51]</sup>. This could be due to the relatively attenuated upregulation of REG3 in UC (Figure 1). Therefore,

**Table 2** Effects of genetically modified *REG/Reg* genes on the composition of intestinal bacterial microbiota in mice

Class	Order	Family	Genera
Actinobacteria	Bifidobacteriales	<i>Bifidobacteriaceae</i>	<i>Bifidobacterium</i> ↑ <sup>2</sup>
		<i>Coriobacteriaceae</i> ↓ <sup>1</sup> ↓ <sup>3</sup>	
		<i>Eggerthellaceae</i>	<i>Enterorhabdus</i> ↓ <sup>3</sup>
Alphaproteobacteria	Caulobacteriales	<i>Caulobacteraceae</i>	<i>Brevundimonas</i> ↓ <sup>3</sup>
		<i>Bartonellaceae</i>	<i>Bartonella</i> ↑ <sup>3</sup>
Bacilli	Bacillales	<i>Bacillaceae</i>	<i>Oceanobacillus</i> ↑ <sup>3</sup>
		<i>Staphylococcaceae</i>	<i>Staphylococcus</i> ↓ <sup>2</sup>
			<i>Lactococcus</i> ↑ <sup>3</sup>
		N/A	<i>Gemella</i> ↑ <sup>3</sup>
		<i>Lactobacillaceae</i> − <sup>1</sup>	<i>Lactobacillus</i> ↓ <sup>2</sup> ↑ <sup>3</sup> − <sup>4</sup> ↓ <sup>5</sup>
		<i>Enterococcaceae</i>	<i>Enterococcus</i> ↑ <sup>2</sup> ↑ <sup>3</sup>
		<i>Aerococcaceae</i>	<i>Facklamia</i> ↓ <sup>3</sup>
		<i>Carnobacteriaceae</i>	<i>Carnobacterium</i> ↑ <sup>3</sup>
		<i>Prevotellaceae</i> ↓ <sup>1</sup> ↑ <sup>3</sup>	<i>Prevotella</i> ↓ <sup>1</sup> ↑ <sup>2</sup> ↑ <sup>3</sup>
		<i>Rikenellaceae</i> − <sup>1</sup>	
Bacteroidia	Bacteroidales	<i>Porphyromonadaceae</i> ↓ <sup>1</sup> ↓ <sup>5</sup>	<i>Parabacteroides</i> ↓ <sup>1</sup> ↑ <sup>3</sup> ↑ <sup>5</sup>
			<i>Barnesiella</i> ↓ <sup>1</sup>
		<i>Bacteroidaceae</i> ↓ <sup>1</sup>	<i>Bacteroides</i> ↓ <sup>1</sup> − <sup>2</sup> − <sup>3</sup> − <sup>4</sup> ↑ <sup>5</sup>
		<i>Sutterellaceae</i> ↓ <sup>1</sup>	<i>Parasutterella</i> ↑ <sup>1</sup> ↓ <sup>3</sup>
		<i>Lachnospiraceae</i> ↑ <sup>1</sup> ↑ <sup>3</sup> − <sup>5</sup>	<i>Roseburia</i> ↑ <sup>3</sup>
		<i>Ruminococcaceae</i> − <sup>2</sup> ↑ <sup>3</sup> − <sup>5</sup>	<i>Faecalibacterium</i> ↑ <sup>3</sup>
		<i>Clostridiaceae</i>	<i>Clostridium</i> ↑ <sup>2</sup> ↑ <sup>3</sup> (XI Cluster) ↓ <sup>5</sup> (XIVa Cluster) ↑ <sup>5</sup>
			<i>Candidatus Arthromitus</i> ↑ <sup>3</sup>
			<i>Candidatus Savagella</i> ↑ <sup>4</sup>
		<i>Eubacteriaceae</i>	<i>Eubacterium (rectale)</i> ↑ <sup>4</sup>
Betaproteobacteria	Burkholderiales	<i>Oscillospiraceae</i>	<i>Oscillibacter</i> ↑ <sup>1</sup>
		<i>Peptococcaceae</i> ↑ <sup>3</sup>	
		<i>Desulfovibrionaceae</i> − <sup>5</sup>	<i>Lawsonia</i> − <sup>5</sup>
			<i>Desulfovibrio</i> − <sup>5</sup>
Epsilonproteobacteria	Campylobacteriales	<i>Helicobacteraceae</i>	<i>Helicobacter</i> ↑ <sup>2</sup> ↑ <sup>3</sup>
Erysipelotrichia	Erysipelotrichales	<i>Erysipelotrichaceae</i> ↑ <sup>3</sup> ↑ <sup>5</sup>	<i>Turicibacter</i> ↑ <sup>3</sup>
			<i>Allobaculum</i> − <sup>3</sup> ↓ <sup>5</sup>
Gammaproteobacteria	Enterobacteriales	<i>Enterobacteriaceae</i> ↑ <sup>3</sup>	<i>Escherichia</i> ↓ <sup>2</sup>
			<i>Enterobacter</i> ↓ <sup>1</sup>
		<i>Morganellaceae</i>	<i>Proteus</i> ↓ <sup>2</sup>
		<i>Moraxellaceae</i>	<i>Psychrobacter</i> ↑ <sup>3</sup>
	Pseudomonadales		<i>Acinetobacter</i> ↑ <sup>3</sup>
		<i>Xanthomonadaceae</i>	<i>Stenotrophomonas</i> ↑ <sup>3</sup>
		<i>Acidaminococcaceae</i>	<i>Phascolarctobacterium</i> ↑ <sup>3</sup>
		<i>Veillonellaceae</i> ↑ <sup>3</sup>	
Negativicutes	Acidaminococcales		
Verrucomicrobiae	Verrucomicrobiales	<i>Akkermansiaceae</i>	<i>Akkermansia</i> ↓ <sup>1</sup> ↑ <sup>3</sup> ↑ <sup>5</sup>
		<i>Verrucomicrobiaceae</i> ↓ <sup>1</sup>	

Increased (↑), decreased (↓) and unaltered (−) intestinal bacterial proportions are indicated in:

<sup>1</sup>hepatocyte specific *REG3a* transgenic mice<sup>[79]</sup>.

<sup>2</sup>intestinal cell specific *Reg4* knockout mice<sup>[16]</sup>.

<sup>3</sup>intestinal cell specific *REG3γ* transgenic mice<sup>[80]</sup>.

<sup>4</sup>the intestinal mucosa of *Reg3γ* knockout mice<sup>[31]</sup>.

<sup>5</sup>the intestinal mucosa of *Reg3β* knockout mice<sup>[35]</sup>.

it would be more informative to assess the use of REG4 as a biomarker for UC, since it is more specific and highly upregulated in UC<sup>[45,49,52]</sup>.

Multiple clinical trials have shown that fecal microbiota transplantation is a promising treatment to induce remission in active UC<sup>[97-100]</sup>. However, the specific



bacteria that protect against UC have not been identified. Studying altered intestinal bacteria populations in DSS colitis resistant *REG3a* transgenic mice and *Reg4* deficient mice therefore could help to identify the protective bacteria and associated REG/Reg regulation in UC [16,79].

Bowel resection is considered for the patients that are refractory to medical therapy or with serious complications of the medications<sup>[101-103]</sup>. However, in addition to the risk of short bowel syndrome, septic complications are commonly associated with anastomotic leaks<sup>[103]</sup>. The early diagnosis of anastomotic leaks enables a timelier intervention essential for a better outcome. It has been shown that a low CRP on postoperative day 4 is a reliable biomarker for excluding postoperative infectious complications in abdominal surgery, while high CRP levels should prompt aggressive imaging for possible anastomotic failure<sup>[104]</sup>. Like CRP, REG/Reg proteins have been considered to be acute phase proteins that could be used as markers of septic complications in patients including those undergoing abdominal surgery<sup>[105-110]</sup>. Therefore, determining whether REG proteins can serve as a more sensitive and specific sensor of postoperative anastomotic leak could have significant clinical potential.

It is also worthy of note that Reg proteins do not lead to immune suppression or immunogenicity, the side-effects that are associated with risks of infection and immune dysregulation in some agents used in IBD treatment<sup>[88,89]</sup>. Given that REG proteins have bactericidal, anti-inflammatory and tissue repair functions in the inflamed intestine (Figure 2), the use of REG proteins as an adjunct to reduce the doses of current medications for IBD could potentially minimize complications. Despite these benefits, however, concerns of long-term application of REG proteins remain. For example, REG/Reg proteins may potentially overactivate the oncogenic STAT3 signaling pathway<sup>[5,27]</sup>, even though the gastrointestinal tract administration may decrease the oncogenic risk in other organs. Additionally, CD patients are prone to bowel stricturing/stenosis formation<sup>[111]</sup>. Based on our finding of Reg1 as an activator of stellate cells, the predominant producer of collagen in pancreatitis<sup>[67]</sup>, the potential of REG1 for bowel stricturing/stenosis should be clarified, which could argue against its use in patients with CD.

## CONCLUSION

IBD remains a significantly challenging disorder due to as yet unresolved issues in its pathogenesis, diagnosis and clinical management. In this review, by discussing the expressions and activities of REG proteins in the inflamed intestine, we have attempted to illuminate potential applications of these REG proteins that may help to improve detection and treatment of the disease, but further comprehensive studies are necessary to clarify and confirm these benefits in IBD.

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## Alternative uses of lumen apposing metal stents

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### Abstract

The advent of lumen apposing metal stents (LAMS) has revolutionized the management of many complex gastroenterological conditions that previously required surgical or radiological interventions. These procedures have garnered popularity due to their minimally invasive nature, higher technical and clinical success rate and lower rate of adverse events. By virtue of their unique design, LAMS provide more efficient drainage, serve as conduit for endoscopic access, are associated with lower rates of leakage and are easy to be removed. Initially used for drainage of pancreatic fluid collections, the use of LAMS has been extended to gallbladder and biliary drainage, treatment of luminal strictures, creation of gastrointestinal fistulae, pancreaticobiliary drainage, improved access for surgically altered anatomy, and drainage of intra-abdominal and pelvic abscesses as well as post-surgical fluid collections. As new indications of endosonographic techniques and LAMS continue to evolve, this review summarizes the current role of LAMS in the management of these various complex conditions and also highlights clinical pearls to guide successful placement of LAMS.

**Key words:** Lumen apposing metal stents; Walled off necrosis; Gallbladder drainage; Biliary drainage; Gastric access temporary for endoscopy; Gastric outlet obstruction; Therapeutic endoscopy

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**Core tip:** Lumen apposing metal stents have become widely adopted as a preferred modality in the treatment of pancreatic walled-off necrosis, with even broader applications for multiple alternative conditions. Current literature suggests that the use of these novel stents may significantly improve treatment response and provide a much needed minimally invasive therapeutic option to patients in need.

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## INTRODUCTION

The introduction of novel lumen apposing metal stents (LAMS) over the past decade has ushered in a new era of therapeutic gastrointestinal endoscopy. Originally approved by the United States food and drug administration (FDA) in 2013, with a primary goal of managing pancreatic fluid collections, LAMS have become widely adopted as a preferred modality in the treatment of walled-off necrosis (WON), with even broader applications for multiple alternative conditions. Alternative uses that have not yet become FDA approved include treatment of luminal strictures, creation of gastrointestinal fistulae, achievement of pancreaticobiliary drainage, improved access for surgically altered anatomy, and drainage of intra-abdominal and pelvic abscesses as well as post-surgical fluid collections. In this review, we highlight both FDA approved as well as non-FDA approved uses of LAMS and provide a detailed summary of the current evidence to support the alternative uses for a variety of conditions.




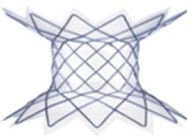

## DESIGN AND FUNCTIONALITY OF LUMEN APPOSING METAL STENTS

The advent of LAMS has radically changed the landscape of therapeutic endoscopy, allowing for multiple minimally invasive treatments for conditions previously thought to require surgical management. Among the various types of stents available, LAMS have revolutionized the field of therapeutic endoscopy given their unique advantages over traditional plastic stents. While variability in design exists between individual stent types, LAMS possess a barbell or saddle shape that allows for the ability to hold two luminal structures in apposition-leading to a lower risk of leakage. Additional benefits of LAMS include a large intraluminal diameter which is able to accomplish more efficient drainage. Furthermore, by virtue of this wider lumen, LAMS may serve as a conduit providing endoscopic access for interventions to various structures abutting the gastrointestinal tract. These bi-flange stents are covered by a silicone layer which helps prevent tissue ingrowth and thus facilitates easy removal. At present, different types of LAMS have been developed and used for transluminal interventions. While there are various similarities between the types, there are subtle differences in terms of their features and delivery mechanisms as summarized on [Figure 1](#). The most commonly utilized LAMS, and the only stent approved in the United States, is the AXIOS stent (Boston Scientific, Natick, MA). Although four additional stents are commercially available, the majority of research and literature is based upon use with the AXIOS stent. However, we have included all available stents within this review to appeal to a more global audience who may be more familiar with alternative LAMS.

## PANCREATIC FLUID COLLECTIONS AND PANCREATIC WALLED-OFF NECROSIS

Pancreatic fluid collections typically develop as sequelae of acute pancreatitis or may arise from chronic injury to the pancreas. While these collections resolve spontaneously in most cases, patients with collections that persist beyond four weeks



	<b>Axios</b> (Boston Scientific, Natick, MA, United States)	<b>Spaxus</b> (Taewoong Medical, Gimposi, South Korea)	<b>Nagi</b> (Taewoong Medical, Gimposi, South Korea)	<b>Aixstent</b> (Leufen Medical, Berlin, Germany)	<b>Hanarostent Plumber</b> (M.I Tech, Pyeongtaek-si, South Korea)
Stents					
Availability in United States	Yes	No	No	No	No
Flange diameter (mm)	21, 24, 29	23, 25, 31	20	25	22 to 28
Length (mm)	10	20	10, 20, 30	30	10, 30
Lumen diameter (mm)	10, 15, 20	8, 10, 16	10, 12, 14, 16	10, 15	10, 12, 14, 16
Delivery catheter (Fr)	10.8	10	9, 10	10	10.5
Material	Silicone-covered Nitinol-braided stent	Silicone-covered Nitinol stent	Silicone-covered Nitinol stent	Silicone-covered Nitinol stent	Silicone-covered Nitinol stent
Deployment through-the-scope	Yes	Yes	Yes	Yes	Yes
Delivery in single step	Yes	No	No	No	No

**Figure 1** Types of lumen-apposing metal stents. (All stent images available on manufacturer website).

and develop a mature wall (*i.e.*, pancreatic pseudocyst or pancreatic WON) may develop obstructive symptoms such as abdominal pain, early satiety, malnutrition, or infection and warrant drainage<sup>[1]</sup>. Traditionally, these collections have been drained *via* a percutaneous and surgical approach; however, these strategies may be associated with significant morbidity and high rates of adverse events. With the evolution and common adoption of endoscopic ultrasound (EUS)-guided drainage, the morbidity of drainage has decreased as have the associated adverse events—thereby becoming the preferred approach due to its superiority or comparability in terms of efficacy, cost-effectiveness, and safety<sup>[2-5]</sup>.

Early EUS-guided drainage previously relied upon double pigtail plastic stents in an effort to create a communication between the bowel lumen and the cyst or fluid collection cavity. Plastic stents, however, have various limitations that include longer length and narrow luminal diameter predisposing to occlusion, ineffective drainage of solid debris, long procedure times, and frequent need for repeat intervention. Therefore, development of large diameter, bi-flanged LAMS were able to overcome the shortcomings of plastic stents with subsequent FDA approval in 2013 for treatment of WON with less than 30% solid necrosis. Appropriate patient selection and location of WON is very important to achieve successful LAMS placement as proximity to the gastric wall is ideal with the collection within approximately 1 to 1.5 cm of the bowel wall (Table 1). For this procedure, a transgastric approach is typically recommended; transduodenal route is also possible though may be associated with a longer course<sup>[6,7]</sup>. From experience, these authors also have noted that large collections extending into the paracolic gutters may not be ideal for endoscopic drainage though this approach may be preferred for patients with a history of gastric varices given EUS-guided visualization.

Treatment of pancreatic pseudocysts and WON were first described by Itoi *et al*<sup>[9]</sup> in 2012 and Gornals *et al*<sup>[8]</sup> in 2013, respectively. The first study reported outcomes of 15 patients with resolution of all pseudocysts after a single drainage procedure with a technical success rate of 100%<sup>[9]</sup>. In this study by Itoi *et al*<sup>[9]</sup>, one stent migration was reported with a median time to removal of 35 d. In the Gornals *et al*<sup>[8]</sup> study evaluating pancreatic fluid collections, 9 patients were enrolled and technical success rate of 89% was reported. Two stent migrations occurred with 2 patients developing recurrence.



**Table 1 Clinical pearls when performing procedures with lumen apposing metal stent**

Type of procedure	Summary of keys to success
Pancreatic fluid collection and walled-off necrosis	Transgastric approach is typically recommended Ensure collection is within one cm of the gastric wall May be less effective for large collections extending into the paracolic gutters
EUS-guided gallbladder drainage	Ensure the echoendoscope is advanced into the gastric antrum or duodenal bulb Transgastric or transduodenal approach is recommended (transgastric preferred) Freehand placement or over a wire after fine needle injection and dilation of tract
EUS-guided choledochoduodenostomy	Use of a pigtail stent through LAMS to decrease risk of sump syndrome Reserve LAMS use for optimal candidates for traditional metal stent placement
Gastric access temporary for endoscopy	Avoid penetration of the diaphragm to minimize patient discomfort Avoidance of gastric staple line to reduce risk of persistent gastro-gastric fistula Consider gastro-gastric fistula to decrease risk of LAMS dislodgement
EUS-guided gastroenterostomy	Prone/ swimmer's positioning prior to beginning procedure Distention of the bowel with dilute contrast and sterile water Use of glucagon to decrease motility of the bowel Placement of a wire may push small bowel away from the stomach
Benign gastrointestinal strictures	First traverse entire length of stricture (if possible) Use of a guidewire is also important to prevent trauma
Post-surgical fluid collections	Favorable collection locations include adjacent to stomach, duodenum, or rectum

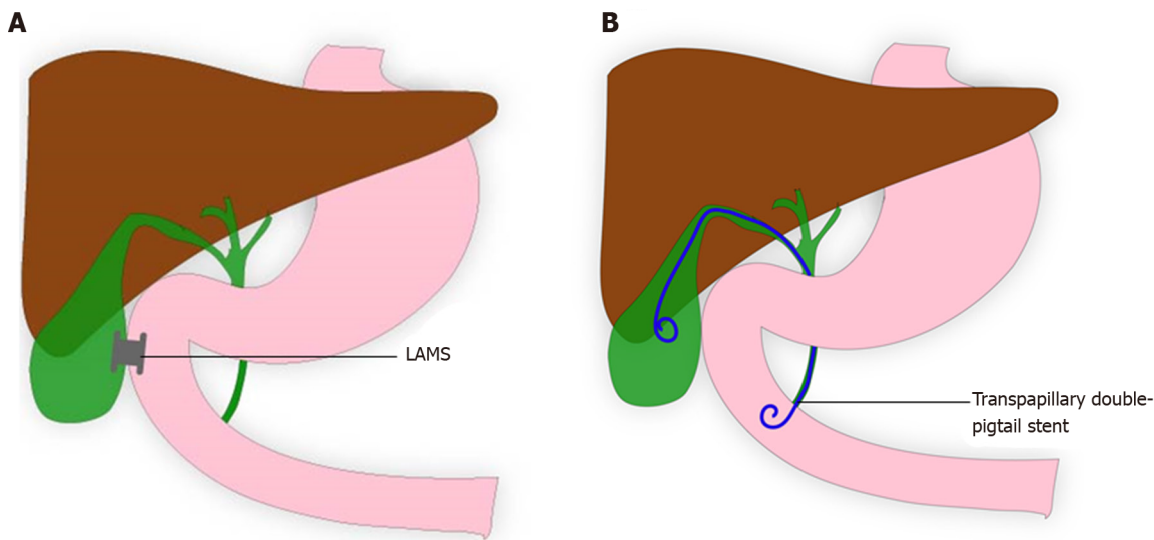
EUS: Endoscopic ultrasound; LAMS: Lumen apposing metal stent.

Additional literature evaluating the efficacy and safety of LAMS for pancreatic fluid collections has shown impressive results<sup>[10]</sup>. A landmark prospective multi-center study was performed by Shah and colleagues in 2015, evaluating stent placement in 33 patients with symptomatic pancreatic pseudocysts or WON<sup>[11]</sup>. The mean size of these collections was  $9.0 \pm 3.3$  cm with a technical success (defined as the ability to place LAMS successfully) of 91% and resolution of the pancreatic fluid collections in 27 of the 29 patients (93.1%). Additionally, these stents allowed for endoscopic debridement in 11 patients with mild-to-moderate adverse events reported in 15% of cases. Multiple systematic reviews and meta-analyses have since demonstrated similar efficacy and safety results for LAMS and support the notion that metal stents are advantageous compared to plastic stents for pancreatic fluid collections<sup>[12-14]</sup>. Given these impressive results for pancreatic fluid collections, it is no surprise that the use of LAMS is being increasingly adopted for the treatment of alternative conditions.

## GALLBLADDER DRAINAGE FOR HIGH-RISK SURGICAL PATIENTS

Although laparoscopic cholecystectomy remains the mainstay of treatment for patients with acute cholecystitis, percutaneous transhepatic gallbladder drainage (termed cholecystostomy) performed by interventional radiology has been traditionally utilized for poor surgical candidates or in the setting of acute illness when surgical removal is contraindicated. Percutaneous drainage has been a preferred treatment strategy for symptomatic gallbladder disease among high-risk surgical candidates; however, it is limited by significant risk of inadvertent self-removal of the catheter and risk of serious adverse events such as pneumoperitoneum, pneumothorax, and catheter leakage associated with this technique<sup>[15,16]</sup>. To avoid or reduce some of the complications, endoscopic drainage *via* a transpapillary or transmural approach has been devised (Figure 2).

While transpapillary drainage is a well-established endoscopic technique for gallbladder drainage, achieved through an endoscopic retrograde cholangiography (ERC) approach, traversing the cystic duct can be challenging due to anatomy. A

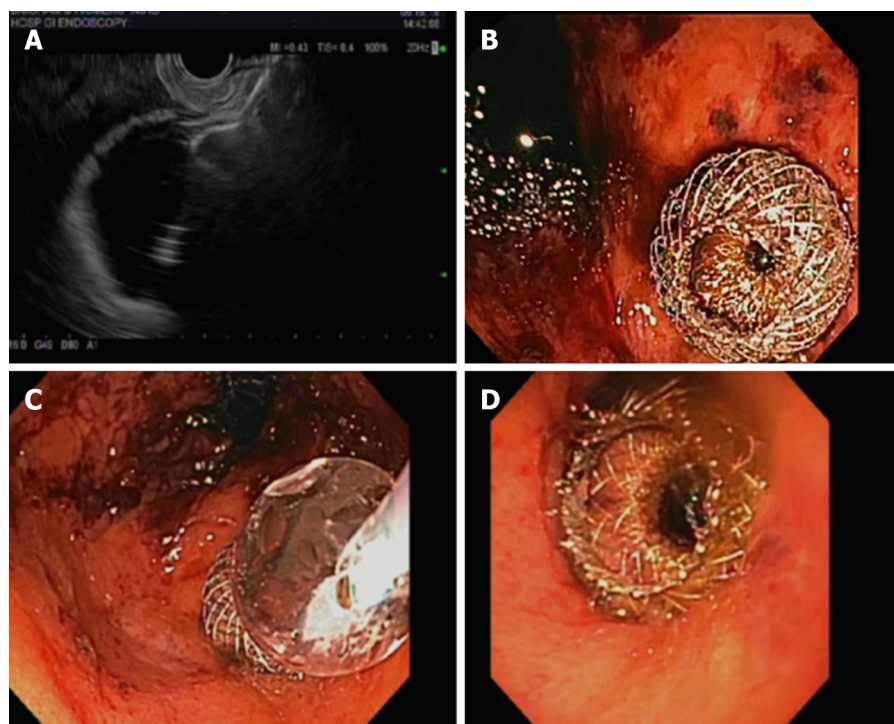


**Figure 2** Endoscopic drainage via a transmural or transpapillary approach. A: Endoscopic ultrasound-guided transmural gallbladder drainage using lumen apposing metal stent; B: Endoscopic ultrasound-guided transpapillary drainage of gallbladder. LAMS: Lumen apposing metal stent.

more novel approach, utilizing EUS-guided stent placement *via* a transgastric or transduodenal approach, is also able to achieve drainage of the gallbladder. In the transmural approach, a cholecystogastric or cholecystoduodenal fistula is created using EUS-guided placement of LAMS (Figure 3). To accomplish this, the echoendoscope is advanced into the gastric antrum or duodenal bulb. Next, the decision should be made on how to create a fistulous tract from the stomach or duodenum to the gallbladder lumen. Once the route of access (*i.e.*, transgastric *vs* transduodenal) is determined, two options are typically recommended: (1) performing freehand placement of an electrocautery-enhanced LAMS; or (2) placement of non-cautery enhanced LAMS over a wire after fine-needle injection and dilation of the tract (Table 1). The decision regarding these strategies is based primarily on the type of LAMS as well as individual provider expertise.

This technique with LAMS drainage, was first successfully described by Itoi *et al*<sup>[9]</sup> in 2012 with 5 patients with acute cholecystitis who underwent four cholecystoduodenostomies and one cholecystogastrostomy. Technical and clinical success was 100% with resolution of acute cholecystitis observed immediately after stent implantation at a follow-up period of 5 mo. Another pilot study by de la Serna-Higuera and colleagues utilized a transgastric approach in 12 patients and transduodenal in one patient<sup>[17]</sup>. Technical success in this initial series was 84.6% with two failures reported. Overall, clinical success was 100% for patients that underwent drainage with LAMS, and the stents were left in place in 10 of 11 patients without further symptom recurrence at a median follow-up of 100 d. In another patient with acute cholecystitis who had previously failed percutaneous cholecystostomy drainage, Teoh *et al*<sup>[18]</sup>, demonstrated the feasibility of a single-step EUS-guided puncture and delivery of a LAMS for gallbladder drainage using a novel cautery-tipped stent delivery system. While use is limited to centers with expertise, Walter and others performed the first multi-center, prospective study of LAMS for EUS-guided gallbladder drainage among high-risk surgical patients with acute cholecystitis<sup>[19]</sup>. Thirty patients were included in this study and demonstrated a technical success rate of 90% and clinical success of 96%; however, there were 4 LAMS-related adverse events reported. Despite this high adverse event rate, subsequent studies have not confirmed this data, with only one adverse event of a post-operative fever reported among 15 patients in a study by Irani *et al*<sup>[20]</sup>. A meta-analysis of LAMS use in EUS-guided gallbladder drainage revealed a procedure-related adverse event rate of 10.6% with a majority (5.7%) developing delayed events at a median follow-up of 6 mo<sup>[21]</sup>. These conflicting results underscore the importance of proper patient selection prior to EUS-guided LAMS placement for gallbladder drainage.

More recently, a meta-analysis was performed by Luk *et al*<sup>[22]</sup> comparing EUS-guided versus percutaneous transhepatic gallbladder drainage. On subgroup analyses of only studies utilizing LAMS, three studies were found and demonstrated no difference in pooled technical success [OR 0.21 (95%CI, 0.04 to 1.10)], clinical success [OR 1.43 (95%CI, 0.42 to 4.81)], or rate of adverse events [OR 0.42 (95%CI 0.14 to 1.28)]. However, all three studies found that hospital length of stay was shorter (mean



**Figure 3 Transmural approach.** A: Endoscopic ultrasound-guided gallbladder drainage with proximal lumen apposing metal stent (LAMS) deployment in gallbladder; B: Endoscopic view status post LAMS placement; C: Dilation of the LAMS with through-the-scope balloon; D: Successful endoscopic ultrasound-guided cholecystogastric fistula formation using LAMS.

difference of 2.76 d;  $P = 0.03$ ) with fewer readmissions [OR 0.14 (95% CI, 0.03 to 0.70)] and fewer reinterventions [OR 0.15 (95% CI, 0.02 to 0.98)] in the LAMS treatment arm compared to percutaneous cholecystostomy. Additional authors have concluded, that for patients with terminal diagnoses, EUS-guided gallbladder drainage may provide a better quality of life than other non-surgical techniques such as percutaneous cholecystostomy due to need for repeated intervention<sup>[23,24]</sup>.

## BILIARY DRAINAGE FOR MALIGNANT DISTAL BILIARY OBSTRUCTION

Along with EUS-guided gallbladder drainage, drainage of the biliary system *via* EUS-guided hepaticogastrostomy, choledochoduodenostomy, and cholecystostomy are feasible treatments with LAMS when or if ERCP is not feasible<sup>[25,26]</sup>. Although other non-lumen apposing metal stents may achieve choledochoduodenostomy, the bi-flanged design may improve drainage and decrease the risk of stent migration. From the same authors that originally described EUS-guided gallbladder drainage, Itoi and Binmoeller also first reported EUS-guided choledochoduodenostomy in a patient with pancreatic cancer who failed traditional transpapillary access *via* ERCP<sup>[27]</sup>. In this study, LAMS was utilized to prevent leakage with the proximal and distal anchor flanges designed to hold the bile duct and the duodenal wall in apposition. The procedure was successful with subsequent studies demonstrating similar successful results. A multi-center study in Europe included 57 patients with malignant distal biliary obstruction<sup>[28]</sup>. In this study, cautery-enhanced and non-cautery LAMS were used with a technical success of 98.2% with a range of sizes employed. Clinical success was 94.7% with a low adverse event rate of 7.0%. A prospective multi-center study by Tsuchiya *et al*<sup>[29]</sup> was recently performed to evaluate the long-term effectiveness of EUS-guided choledochoduodenostomy with LAMS placement. This study included 19 patients (all of which had failed prior ERCP) and found a technical success rate of 100% with no immediate adverse events reported. Moreover, 18 of the 19 stents remained in place at 6 mo follow-up period although there were four reported episodes of stent occlusion.

While promising, LAMS may not, in fact be required to achieve EUS-guided choledochoduodenostomy as traditional metal stents have shown similarly impressive results. Furthermore, use of LAMS typically requires a larger diameter bile

duct and may increase the risk for sump syndrome after placement. We recommend the use of a pigtail stent through the LAMS to decrease this risk of sump syndrome post-choledochoduodenostomy (Table 1). Therefore, these authors typically reserve the use of LAMS for specific individuals that may be less optimal candidates for traditional metal stent placement.

## ACCESS TO THE REMNANT STOMACH AND POST-BARIATRIC SURGICAL ANATOMY

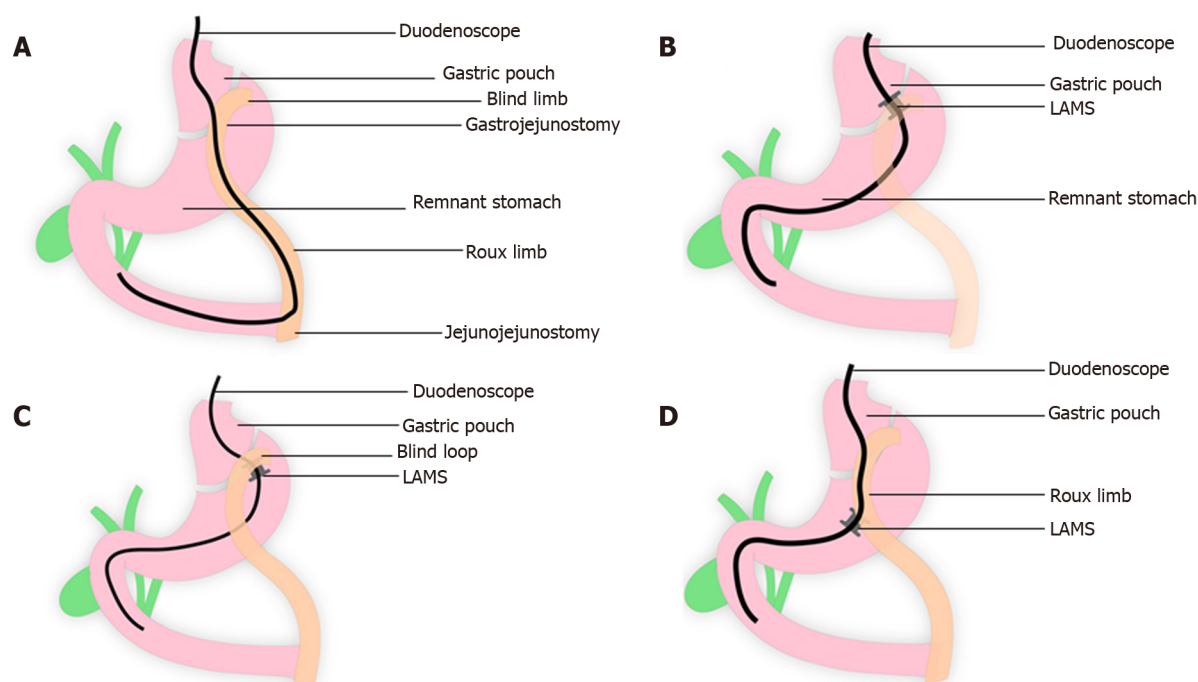
Given the prevalence of obesity and subsequent bariatric surgery in the United States and worldwide, gastroenterologists may increasingly encounter difficult pancreaticobiliary conditions in patients with surgically altered anatomy. Rapid weight loss following bariatric surgery is associated with a higher incidence of cholelithiasis and risk for choledocholithiasis among patients with their gallbladders in situ. In patients with Roux-en-Y gastric bypass (RYGB) anatomy, access to the biliary and pancreatic tract *via* ERCP is not easily achieved with need to advance the endoscope distally from the gastric pouch to the jejunojejunostomy and then retrograde through the biliopancreatic limb to access the biliary system. While enteroscopy- and laparoscopy-assisted ERCP may provide alternative treatment options to access the pancreaticobiliary system, gastric access temporary for endoscopy (GATE) is a minimally invasive alternative. This procedure utilizes the unique LAMS design with large intraluminal diameter to create a fistulous tract and a working channel between the gastric pouch and remnant stomach.

Although multiple other descriptions or terminology have been proposed to describe this procedure [endoscopic ultrasound-guided transgastric fistula (EUS-TG), endoscopic ultrasound-guided gastro-gastric-endoscopic cholangiopancreatography (EUS-GG-ERCP), EUS-directed transgastric ERCP (EGDE), or EUS-directed transgastric intervention (EDGI)], these authors will use the common term GATE as this can be applied to procedures other than ERCP. The procedure entails EUS-guided access to the excluded stomach using the large diameter of the LAMS as a working channel-allowing access for not just ERCP but other procedures such as EUS, endoscopic mucosal resection, and endoscopic submucosal dissection of the foregut<sup>[30]</sup>.

Using an echoendoscope, the excluded stomach is located to create a gastro-gastric fistula. We suggest accessing the remnant or excluded stomach from the proximal pouch to create a favorable angle and minimize chances of stent dislodgement when advancing the endoscope through the LAMS and pylorus. The LAMS is deployed under fluoroscopic and endosonographic guidance with the distal flange in the excluded stomach and the proximal flange in the gastric pouch. The lumen of the stent is then dilated up to the diameter of the stent lumen, thereby allowing for easy passage of any wider endoscope to access the remnant stomach to complete the desired procedure (Figure 4). It is important to avoid penetration of the diaphragm to minimize patient discomfort as well as avoidance of the gastric staple line to reduce the risk of a persistent gastro-gastric fistula (Table 1). Early LAMS removal with placement of a double pigtail stent to maintain the tract may also help to minimize subsequent gastro-gastric fistula. It may also be possible to create a fistulous tract from the jejunum to the remnant stomach; however, creation of a gastro-gastric connection is generally recommended when possible as a transjejunal approach may carry a higher risk of LAMS dislodgement.

Kedia and colleagues first performed this procedure in a single stage using a LAMS to create a gastro-gastric fistula in a patient with a history of RYGB<sup>[31]</sup>. Since the first successful report of this technique, there have not yet been large scale studies to date. However, a few case series have been published which have demonstrated the efficacy of the GATE procedure. In a multi-center case series of 13 patients using LAMS to create an EUS-guided gastro-gastric fistula to facilitate per-oral ERCP, technical success was reported to be 100% with LAMS dislodgement noted in 2 patients<sup>[32]</sup>. One retrospective study by these authors reported duodenal endoscopic submucosal dissection and sutured defect closure after performing GATE in a patient with RYGB<sup>[33]</sup>. In another study by authors of this review, 10 patients underwent the GATE procedure using a novel algorithmic approach using gastric and jejunal access points for LAMS deployment<sup>[30]</sup>. This study demonstrated a clinical and technical success rate of 100% with GATE and concluded that it was a safe and effective procedure to be considered as the preferred approach to ERCP in patients with RYGB anatomy at centers with LAMS experience. A comparator study was also performed by Bukhari *et al*<sup>[34]</sup> to compare GATE with ERCP versus enteroscopy-assisted ERCP. Technical success was significantly higher in the GATE group versus enteroscopy-assisted group (100% *vs* 60%;  $P < 0.001$ ) with decreased total procedure time (49.8 min





**Figure 4** The lumen of the stent is dilated up to the diameter of the stent lumen, thereby allowing for easy passage of any wider endoscope to access the remnant stomach to complete the desired procedure. A: Normal Roux-en-Y gastric bypass (RYGB) anatomy showing long endoscopic route to be traversed to access the biliary system; B: Lumen apposing metal stent (LAMS) placement between gastric pouch and remnant stomach in RYGB anatomy; C: LAMS placement between blind limb and remnant stomach in RYGB anatomy; D: LAMS placement between Roux Limb and remnant stomach in RYGB anatomy. LAMS: Lumen apposing metal stent.

*vs* 90.7 min;  $P < 0.001$ ) and length of hospital stay (1 d *vs* 10.5 d;  $P = 0.02$ ) and no difference in adverse events (10% *vs* 6.7%  $P = 1.0$ ). A more recent study by Kedia and colleagues, sought to compare outcomes of GATE versus laparoscopy-assisted ERCP and found no difference in technical or clinical success, as well as adverse events; however, noted that GATE was associated with significantly shorter procedure times and length of hospital stay<sup>[35]</sup>. In 2018, a case report of successful drainage of a large, isolated fluid collection in the gastric remnant was described by Schulman and Thompson<sup>[36]</sup>. In this case, a GATE procedure was first performed followed by placement of a second LAMS to reconstitute dependent flow from the remnant stomach to the jejunum.

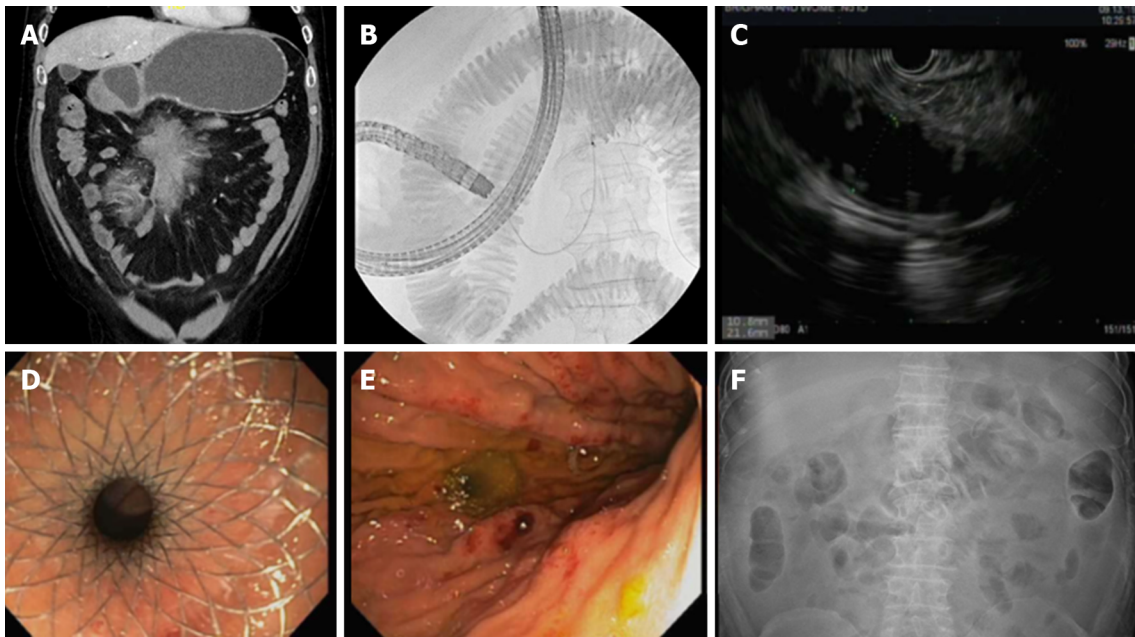
These results are no doubt promising; however, similar to EUS-guided gallbladder drainage, LAMS use for GATE is limited to centers with expertise. Furthermore, it is important to understand that the two potential ramifications of this procedure are the potential for weight regain due to creation of a gastro-gastric fistula, as well as the potential for stent migration. However, despite these concerns, this procedure improves access to the remnant stomach, provides the ability to perform the procedure in a single session, and is a minimally invasive alternative to traditional laparoscopic-assisted techniques.

## MANAGEMENT OF GASTRIC OUTLET OBSTRUCTION

Traditionally, surgical gastrojejunostomy has been the primary treatment for both benign and malignant gastric outlet obstruction despite the procedure being associated with a high complication rate approaching nearly 40%<sup>[37,38]</sup>. Given this significant adverse event profile, enteral stenting has been widely utilized, though stent occlusion and migration have also resulted in an increased need for repeat intervention<sup>[39]</sup>. As such, EUS-guided gastroenterostomy (EUS-GE) has emerged as an attractive procedure to treat patients with gastric outlet obstruction as an alternative to surgery (Figure 5)<sup>[40,41]</sup>.

This EUS-GE procedure first requires precise location of the small bowel distal to the gastric outlet obstruction (either distal duodenum or jejunum) endosonographically from the gastric antrum or body. Typically, a guidewire is passed beyond the area of obstruction under direct fluoroscopic guidance as well as the use of a





**Figure 5** An attractive procedure to treat patients with gastric outlet obstruction as an alternative to surgery. A: Initial computed tomography demonstrating gastric outlet obstruction; B: Fluoroscopy with duodenal stenosis and distal filling with contrast diluted in sterile water; C: Endoscopic ultrasound-guided gastroenterostomy demonstrating filling of distal bowel; D: Successful placement of lumen apposing metal stent (LAMS); E: Endoscopic image of gastroenterostomy placement with LAMS; F: Follow-up radiograph demonstrating successful LAMS placement to achieve gastroenterostomy.

catheter to fill the distal small bowel with a mixture of contrast and sterile water. Then using fluoroscopy and EUS-guidance, the bi-flanged LAMS is placed from the stomach to the small bowel – thus creating a newly formed fistulous tract and thereby bypassing the point of gastric outlet obstruction. The positioning of the patient in a prone/swimmer's position and use of fluoroscopy is essential. Distention of the bowel with dilute contrast with or without methylene blue and use of water (not saline), and use of glucagon to decrease motility of the bowel is also key. A freehand technique may also be adopted as placement of a wire may push small bowel away from the stomach (Table 1).

Technical feasibility of EUS-GE was first demonstrated by Binmoeller and colleagues using 5 porcine models in 2012<sup>[42]</sup>. Since that initial animal study, translation to humans has been achieved with more widespread adoption of the EUS-GE procedure. The first report of LAMS associated gastroenterostomy was performed by Ikeuchi *et al*<sup>[43]</sup> in 2015. Subsequent literature with variable techniques has suggested EUS-GE is safe and effective for the treatment of gastric outlet obstruction. Kashab *et al*<sup>[44]</sup> reported outcomes for 10 patients with gastric outlet obstruction. This study showed technical success rate of 90% with no associated adverse events. A similarly high technical and clinical success rate was observed in a multi-center study of 26 patients by Tyberg *et al*<sup>[45]</sup> at 92% and 85% respectively. Itoi and colleagues also demonstrated similarly impressive results and demonstrated the efficacy of EUS-GE predominantly as palliative treatment of malignant gastric outlet obstruction<sup>[46]</sup>. In a patient with both biliary and duodenal obstruction, a case report by Abidi and Thompson described successful choledochoduodenostomy and gastrojejunostomy with LAMS as well. In this case, initial ERCP and EUS-guided rendezvous were unsuccessful, prompting placement of a LAMS to create a choledochoduodenostomy and subsequent electrocautery-enhanced LAMS without an initial needle or wire access to create an endoscopic gastrojejunostomy to relieve biliary and duodenal obstruction in an 84-year-old gentleman<sup>[47]</sup>.

A recent meta-analysis including benign and malignant gastric outlet obstructions by the authors of this review showed a technical success rate of 92.90% and clinical success of 90.11%<sup>[41]</sup>. More importantly, serious adverse events occurred in 5.61% of cases with a reintervention rate of 11.43%. In a study by Manuel Perez-Miranda *et al*<sup>[48]</sup> comparing surgical and EUS-guided strategies, technical success was not different between an EUS-GE cohort and patients undergoing laparoscopic gastrojejunostomy (88% *vs* 100%,  $P = 0.11$ ); however, EUS-GE was associated with a significantly lower rate of adverse events (12% *vs* 41%,  $P = 0.0386$ ). Another study by the authors of this review examined EUS-GE versus enteral stenting and found EUS-GE was associated higher rate of initial clinical success (95.8% *vs* 76.3%;  $P = 0.042$ ) and a lower rate of

stent failure requiring repeat intervention (8.3% *vs* 32.0%;  $P = 0.021$ )<sup>[49]</sup>. Not only does the current literature support the feasibility, efficacy, and safety of EUS-GE as a treatment for gastric outlet obstruction, but it also appears to suggest EUS-GE may be a preferred treatment strategy compared to surgical gastrojejunostomy and enteral stenting.

## TREATMENT OF BENIGN GASTROINTESTINAL STRICTURES

Current management options for benign gastrointestinal strictures include endoscopic balloon dilation, incisional treatment, steroid injection, and self-expandable metal stents (SEMS)<sup>[50-54]</sup>. These endoscopic management techniques are effective but limited by recurrence rates requiring repeated interventions<sup>[55]</sup>. Additionally, due to the bi-flanged design, LAMS have a lower risk of stent migration compared to fully-covered SEMS<sup>[56,57]</sup>. When performing the procedure, it is recommended to first traverse the entire length of the stricture with a smaller diameter endoscope (if possible) to ensure that the one cm length of the LAMS is adequate. Use of a guidewire is also important to prevent trauma and reduce the risk of perforation as the LAMS deployment catheter is relatively rigid and may navigate tortuous downstream bowel without wire guidance (Table 1).

Current literature has demonstrated LAMS use for the management of multiple types of strictures including refractory esophageal strictures, pyloric stenoses, and gastrojejunostomy stricturing after RYGB<sup>[58]</sup>. Successful placement of LAMS for esophageal or gastric strictures as well as small bowel and colonic stenoses have been reported in numerous case series to date<sup>[59-66]</sup>. A retrospective study by the authors of this review demonstrated that non-electrocautery enhanced LAMS could be an effective treatment for RYGB patients with persistent gastrojejunal anastomosis stenosis<sup>[67]</sup>. This study examined 18 patients with a technical success rate of 100% and clinic success reported in 94% of patients with 6 patients developing adverse events. In another multi-center study, a total of 49 patients underwent 56 LAMS procedures with a technical success rate of 100% and clinical success rate of 96.4%<sup>[68]</sup>. Despite these impressive results, stent migration occurred in 17.9% of procedures, notably more likely to occur for strictures in the lower gastrointestinal tract. Although LAMS were well-tolerated by patients in this study, symptom recurrence at the time of removal was also common. In a systematic review of 8 studies ( $n = 192$  patients) evaluating LAMS for benign gastrointestinal strictures, LAMS demonstrated statistically better outcomes in regards to stent migration and post-procedure pain when compared with fully-covered SEMS and biodegradable stents. In our practice, we typically recommend removal of LAMS after approximately 3 mo with determination at that time whether a second LAMS placement is needed based upon response.

## DRAINAGE OF POST-SURGICAL FLUID COLLECTIONS AND PELVIC ABSCESES

Post-surgical fluid collections are a common cause of morbidity and mortality in post-operative patients<sup>[69-71]</sup>. Common causes of collections may include peripancreatic fluid collections after pancreatic surgery, bile leaks post-cholecystectomy, and pelvic fluid collections or abscesses after low anterior resection, colectomy, appendectomy, and gynecologic surgeries<sup>[72]</sup>. Though these collections can be drained surgically, for over a decade the mainstay of management has been percutaneous drainage under radiologic guidance. A percutaneous approach has classically been preferred due to lower mortality and morbidity compared to surgical drainage and has a lower associated procedure cost. However, percutaneous drainage requires external percutaneous drain placement that may remain *in situ* for weeks to months and may be associated with an increased risk of fluid and electrolyte loss, catheter or drain dislodgement, as well as formation of a cutaneous fistulae<sup>[69,70]</sup>.

More recently, with the advent of EUS-guided drainage and the innovation of LAMS, there has been a transition in the management of post-surgical fluid collections from a percutaneous to endoscopic-guided approach. The advantage of EUS-guided transmural drainage lies in the ability to internally drain the fluid – minimizing the risk of infection as well as fluid and electrolyte derangement associated with percutaneous drainage. This has been further associated with higher clinical success rates, lower costs, and an overall improvement in the quality of life<sup>[73,74]</sup>. Favorable

locations for endoscopic drainage with LAMS and avoidance of percutaneous or surgical drainage include collections that are located adjacent to the stomach, duodenum or rectum (Table 1). Prior case series have also demonstrated successful drainage of distal pancreatectomy-related collections as well as pelvic abscesses using EUS-guided placement of plastic stents<sup>[71,75,76]</sup>. However, the literature on the use of LAMS for drainage of post-surgical fluid collection was lacking until 2017 when Mudireddy *et al*<sup>[72]</sup> published the first study on the role of LAMS for this purpose. In this retrospective study, they reported technical and clinical success rates of 93.6% and 89.3%, respectively. Further studies on the use of LAMS for this purpose are still scarce. In a recent multi-center study evaluating the safety and efficacy of the use of LAMS in the management of postsurgical fluid collections, technical success rate was 96.8% and clinical success rate was 91.9% with no procedure-related mortality but intraoperative adverse events of 1.6% and postoperative adverse events of 11.3%<sup>[77]</sup>. To date, there is a paucity of literature available regarding EUS-guided LAMS drainage of post-surgical fluid collections and pelvic abscesses, though available literature suggests a promising role for LAMS.

## CONCLUSION

In conclusion, LAMS have revolutionized the role of interventional endoscopists in the management of a variety of complex conditions. These expanded indications include not only treatment of pancreatic fluid collections, but also broader applications to treat acute cholecystitis, distal malignant biliary obstruction, post-bariatric surgery complications, benign gastrointestinal strictures, gastric outlet obstruction, and intra-abdominal, pelvic, and post-surgical fluid collections. Current literature suggests the use of these novel stents may significantly improve treatment response and provide a much needed minimally invasive therapeutic option to patients in need. We have provided a comprehensive literature review of current LAMS indications as well as clinical pearls to achieve a successful placement. We anticipate that the next decade will see expanded applications for LAMS providing minimally invasive treatment options for more patients.

## ACKNOWLEDGEMENTS

All authors approved the final version of the manuscript.

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## Importance of investigating high-risk human papillomavirus in lymph node metastasis of esophageal adenocarcinoma

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### Abstract

High-risk human papillomavirus has been suggested as a risk factor for esophageal adenocarcinoma. Tumor human papillomavirus status has been reported to confer a favorable prognosis in esophageal adenocarcinoma. The size of the primary tumor and degree of lymphatic spread determines the prognosis of esophageal carcinomas. Lymph node status has been found to be a predictor of recurrent disease as well as 5-year survival in esophageal malignancies. In human papillomavirus driven cancers, e.g. cervical, anogenital, head and neck cancers, associated lymph nodes with a high viral load suggest metastatic lymph node involvement. Thus, human papillomavirus could potentially be useful as a marker of micro-metastases. To date, there have been no reported studies regarding human papillomavirus involvement in lymph nodes of metastatic esophageal adenocarcinoma. This review highlights the importance of investigating human papillomavirus in lymph node metastasis of esophageal adenocarcinoma based on data derived from other human papillomavirus driven cancers.

**Key words:** Esophageal cancer; Esophageal adenocarcinoma; Metastasis; Lymph nodes; Human papillomavirus

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**Core tip:** Esophageal adenocarcinoma (EAC) is one of the fastest growing cancers in the western world. EAC has been associated with high-risk human papillomavirus and has

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been shown to grant a positive prognosis in EAC. In some human papillomavirus (HPV) driven malignancies (*e.g.*, cervical and head and neck tumors), associated lymph nodes with a high viral load suggest metastatic lymph node involvement. Therefore, HPV is a potential marker of micro-metastases. This review highlights the importance of investigating HPV in lymph node metastasis of EAC based on data derived from other HPV driven malignancies.

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## INTRODUCTION

The incidence rates for esophageal adenocarcinoma (EAC) has rapidly risen in the western world<sup>[1-7]</sup>. EAC has a high mortality rate with a 5-year survival of less than 15%<sup>[8,9]</sup>. Even patients (< 50%) considered fit for surgery (with neo-adjuvant therapy) and a curative intent, the outcome remains poor with a 5-year survival rate of < 45%<sup>[10,11]</sup>. This is predominantly because esophageal cancer often proliferates through the lymphatic system. Lymph node metastasis (LNM) is the most crucial prognostic factor in esophageal carcinoma, whereby the number of lymph nodes (LN) is directly correlated with disease progression<sup>[12-21]</sup>. The extent of the primary tumor and lymphatic spread determines the prognosis of esophageal carcinoma. In particular, it has been found that LN status is a predictor of recurrent disease as well as 5-year survival<sup>[19,20,22]</sup>.

Over 200 years ago, LeDran *et al*<sup>[23]</sup> first observed the difference in prognosis in breast cancer patients with metastatic spread of tumor cells to regional lymph nodes (RLNs) and patients without spread. Ever since, several studies have focused on the mechanisms involved in this phenomenon. Tumor cells migrate to regional lymph nodes through systematic circulation. Nearly all types of cancers involve circulating tumor cells<sup>[24]</sup>. Few studies supporting the intravasation of these tumor cells into blood vessels have been reported. LNM is a well-established prognostic factor in breast cancer, head and neck cancer, colorectal cancer and prostate cancer. However, the methods of vascular invasions (lymphatic invasion, hematogenous dissemination, *etc.*) vary in different cancers<sup>[25-29]</sup>. Hosch *et al*<sup>[30]</sup> classified esophageal carcinoma as an immunogenic tumor. Mechanisms reported include immune evasion due to functional suppression of cytotoxic T-lymphocytes by DNA methylation in MHC class I phenotype esophageal cancers and hypercoagulation<sup>[30-33]</sup>.

Human papillomavirus (HPV) has been associated with tumorigenesis of several cancers including cervical, anal, head and neck, oral and oropharyngeal squamous cell carcinomas<sup>[34-37]</sup>. The presence of HPV DNA in the RLNs has been associated with disease recurrence and early signs of metastasis<sup>[20,38-43]</sup>.

HPV is a non-enveloped, double-stranded DNA virus from the papillomavirus family. It is approximately 8kB in size with its genome encoding for at least 8 proteins of which 2 oncoproteins E6 and E7 are of importance. The E6 oncogene causes disruption to the p53 pathway with ultimate degradation of the cell. The E7 oncogene binds and inactivates the retinoblastoma protein (pRb) which is responsible for the major G1 checkpoint that blocks the S-phase entry and cell growth phase transition, thereby upregulating p16 expression<sup>[44,45]</sup>.

HPV can be classified into high risk type *i.e.* HPV type 16 and 18 that causes cervical malignancy and head and neck cancers and low-risk type *e.g.* HPV type 6 and 11 which are linked to genital warts and benign changes in the cervix. The causal link between high-risk human papillomavirus (hr-HPV) infection and cervical malignancy, anogenital, and a subset of oropharyngeal squamous cell carcinoma has been well established by molecular and epidemiological investigations<sup>[46-50]</sup>.

HPV detection in these cancers has been undertaken using a combination of techniques: Polymerase chain reaction (PCR), in-situ hybridization (ISH) and p16<sup>INK4A</sup> expression. Detection of both high-risk and low-risk HPV types in tumor samples has been performed by nested PCR. The PCR amplifies the L1 gene of HPV using MY09 and MY11 and GP5+ and GP6+ primers. PCR products were then separated on 2% agarose gel and sequencing performed to determine the genotypes. Viral load has

been calculated by real-time PCR assay method. It measures E6 and E7 copy numbers using genotype-specific HPV-16 and HPV-18 primers. Hr-HPV16 and 18 E6/E7 mRNA has been determined by RT-PCR and/or RNA ISH, which are considered the gold standard for detection of transcriptionally active hr-HPV. p16<sup>INK4A</sup> expression has been assessed by immunohistochemical staining on FFPE (formalin fixed paraffin-embedded) tumor biopsies and controls. These techniques have been widely used in all the HPV driven cancers<sup>[50-53]</sup>.

Currently, Barrett's esophagus (BE) is the only recognized visible precursor lesion for EAC<sup>[54,55]</sup>. It has been proposed that a metaplasia – dysplasia – cancer sequence exists<sup>[56,57]</sup>, whereby patients with BE (no dysplasia) undergo dysplastic transformation (low- and high-grade dysplasia) before cancer development. Although, the cancer risk in BE gets progressively downgraded, the exponential rise in EAC continues unabated<sup>[58,59]</sup>.

Syrjanen reported the association of HPV with esophageal squamous cancer for the first time in 1982<sup>[60,61]</sup>. In 2010, Rajendra *et al*<sup>[62]</sup> hypothesized that HPV could be a common denominator in the pathogenesis of EAC due to similar immunogenetics of BE and cervical neoplasia. Later in 2013, they provided world first evidence for a strong association of transcriptionally active hr-HPV with EAC<sup>[52,63]</sup>. Hr-HPV has now been proposed as one of the risk factors for esophageal adenocarcinoma<sup>[64]</sup>.

Despite the suggested association of HPV with Barrett's dysplasia and EAC, a few studies found contradictory results. A study by Antonsson *et al*<sup>[65]</sup> found no evidence of HPV in 233 formalin-fixed EAC tissue specimens > 10 years old. In another case-control study by Serag *et al*<sup>[66]</sup>, no viral presence was detected in 127 patients with BE in a US cohort, as the virus is not associated with metaplastic tissue. Rai *et al*<sup>[67]</sup> again confirmed the virtual absence of HPV infection in Barrett's metaplasia in the UK population. Iyer *et al*<sup>[68]</sup> found no statistical difference in the HPV prevalence between BE (27.4%), EAC (31%) and controls (20.7%) and thus concluded that it was of no etiologic significance. Cross-contamination may explain their results. Plausible reasons for negative studies in EAC include limited sample size, geographical location of patient cohorts, racial disparity, methods of HPV detection especially as the viral load in esophageal tissue is low, sample collection, storage, age of specimens and poor tissue classification. HPV detection by PCR is an extremely sensitive assay. Thus, quality of tissue samples, storage conditions and contamination can all adversely affect the assay. For example, the PCR primers MY09/MY11 might not amplify the 450 bp target area on the biopsy tissues collected over 10 years ago. Also, HPV detection is significantly superior in fresh-frozen samples than in the paraffin embedded tissues<sup>[34,69]</sup>.

A systematic review has reported HPV prevalence rates of 35% in EAC ( $n = 174$ ), which is not much different to our findings. Another systematic review which included 19 studies concluded that the pooled prevalence of HPV in EAC was 13%. The authors suggested that the low prevalence rate may have been caused by small sample sizes and compromised detection methods<sup>[34,69]</sup>. Increasing hr-HPV viral load and integration status has been linked with more severe disease along the Barrett metaplasia-dysplasia-adenocarcinoma sequence. In situ-hybridization (ISH) demonstrated for the first time that the hr-HPV genome is present in BE, BD and EAC cells but not the squamous epithelium (Figure 1)<sup>[70]</sup>. The presence of HPV oncogene E6/7 mRNA was also observed in the BD and EAC (Figure 2)<sup>[51]</sup>.

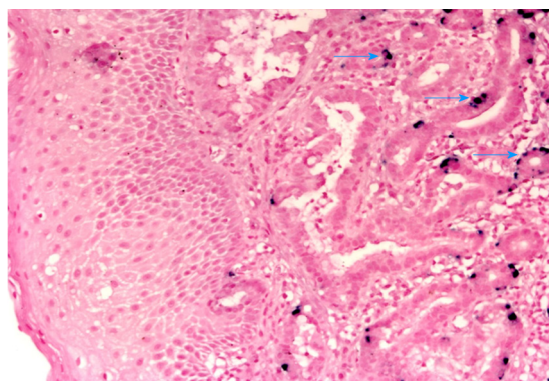
Moreover, treatment failure after endoscopic ablation of Barrett's dysplasia (BD) or EAC is predicted by persistent hr-HPV infection and overexpression of the p53 gene (TP53)<sup>[71]</sup>. Epidemiological data indicate various other risk factors for EAC including Caucasian race, gender, age, obesity and smoking<sup>[72-74]</sup>.

HPV-16 DNA has been detected in the cervical lymph nodes of esophageal squamous cell carcinoma (ESCC)<sup>[42]</sup>. Currently no studies have been reported regarding HPV involvement with LNM in EAC. This review will therefore emphasize the significance of conducting such an investigation.

## ASSOCIATION OF HPV INFECTION AND LYMPH NODE METASTASIS IN VARIOUS CANCERS

Cervical cancer is the archetypal HPV driven malignancy. This has led investigators to examine HPV involvement in other less common cancers which involve the transformation zone and/or the genital area. Examples are head and neck tumors which have a physiological transformation zone rather an anatomical one, esophageal adenocarcinoma (anatomical transformation zone), penile, vulvar and anal carcinomas. Summarized below, we review LNM in the HPV driven cancers.





**Figure 1** DNA *in-situ* hybridization showing high-risk human papillomavirus DNA in Barrett's dysplasia /esophageal adenocarcinoma tissue only. (Adapted from: Al-Haddad *et al*<sup>[70]</sup>, 2014)

### Anogenital cancer

**Cervical cancer:** More than 90% of cervical cancer cases are caused by untreated HPV infection<sup>[75-77]</sup>. The presence of HPV DNA in lymph nodes ranges between 40%-99.7% and has been linked to early signs of metastasis with poor prognosis. The disparity in results can be due to technical differences and variations in patient cohorts<sup>[20,38,43,78-84]</sup>. Although, there is a more apparent correlation of HPV-16 and LNM, both hr-HPV types 16 and 18 have been reported to be predictive of LNM in cervical cancer thus affecting prognosis<sup>[82,85,86]</sup>.

Early detection of epithelial cells containing transcriptionally active HPV in lymph nodes has been proposed as a marker for metastases<sup>[87,88]</sup>. In 2012, Zhang *et al*<sup>[89]</sup> reported hr-HPV positivity in 95.8% metastatic lymph nodes with same HPV type as in the primary lesion. Thus, testing for HPV DNA in pathologically negative lymph nodes could be a crucial marker of micro-metastases<sup>[89]</sup>. About 15% of cervical cancer cases have non-metastatic lymph nodes and detection of HPV in negative lymph nodes has been indicated as a marker of micro-metastasis<sup>[87,90]</sup>. HPV positive DNA in pelvic lymph nodes has been found in 25%-80% of women in early stages cervical cancer<sup>[83,91]</sup>. In contrast to the above findings, few studies reported no correlation between HPV positivity and LNM<sup>[92-94]</sup>.

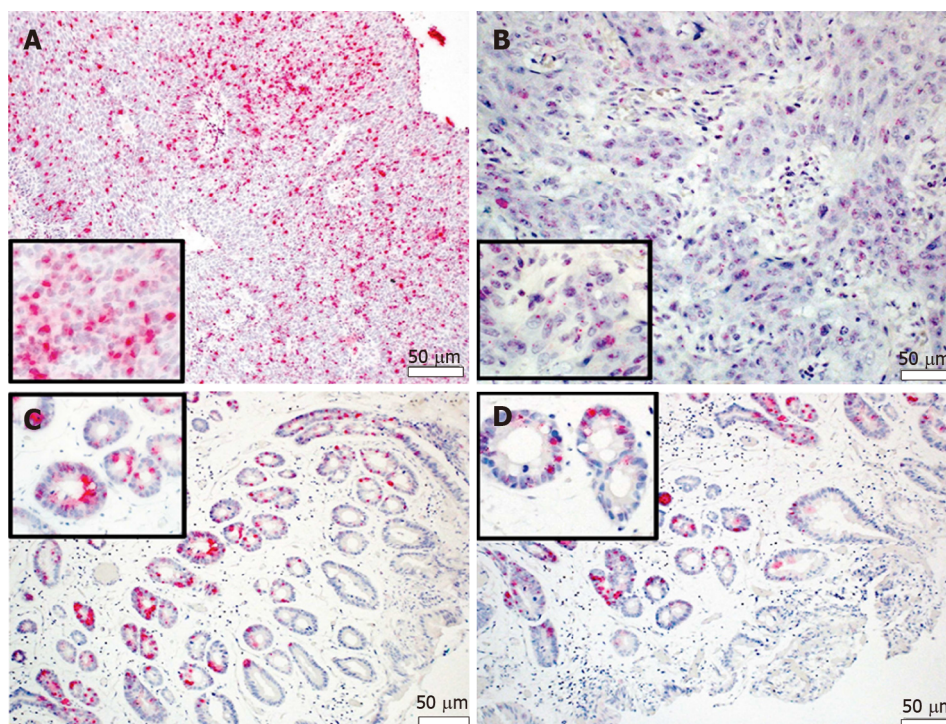
While, some studies associate HPV-positive lymph nodes with higher risk of disease recurrence and lower overall survival<sup>[88,81]</sup>, others find no notable differences in these parameters between HPV positive and negative lymph node status<sup>[93,94]</sup>. Coutant *et al*<sup>[91]</sup> reported no correlation between the HPV status in pelvic lymph nodes and overall prognosis. These disparities could be due to limited sample size and DNA detection methods. A study by Lukaszuk *et al*<sup>[41]</sup> concluded that LNM detection is significantly more accurate by ascertaining HPV status than normal histopathological testing.

**Anal, penile and vulvar cancers:** The incidence of penile and vulvar cancers is highest in some developing countries. HPV has been linked with the pathogenesis of a subset of these cancers<sup>[95-98]</sup>. Studies have reported the overall prevalence of HPV DNA in other anogenital cancers as: Penile cancer (15%-71%); anal (80%) and vulvar (40.1%) cancers have been reported to be HPV-associated<sup>[95,99]</sup>. These prevalence rates are obviously dependent on the methods of DNA detection, patient cohort selection as well as sample handling and processing.

Hr-HPV 16 and 18 has been detected in penile and vulvar cancer tissues using polymerase chain reaction, Southern blot and in-situ hybridization techniques<sup>[100,101]</sup>. The prevalence of HPV DNA has been reported higher (> 55%) in basaloid and warty penile cancers as compared to verrucous and keratinizing cancers (< 10%)<sup>[95]</sup>. Involvement of inguinal lymph node in penile cancer is an unfavorable prognostic indicator<sup>[102]</sup>. In a study by Picconi *et al*<sup>[103]</sup>, 71% of cases were positive for HPV DNA. The positivity was consistent in both the primary tumor and lymph node metastasis. Also, HPV positivity was found in histologically normal lymph nodes which could indicate early metastasis and disease recurrence<sup>[103]</sup>.

In regard to the prevalence of HPV and location in the transformation zone, anal cancer mimics cervical malignancy better than other HPV associated anogenital cancers. Using the PCR detection technique, HPV positivity has been found in up to 89% of the squamous cell anal carcinoma cases. HPV 16 is the most prevalent high-risk type<sup>[104,105]</sup>.





**Figure 2** *In-situ* hybridization detection of transcriptional activity of high-risk human papillomavirus types 16 and 18 E6/E7 mRNA. A: HPV16 cervical squamous cell carcinoma; B: HPV16 head and neck squamous cell carcinoma; C: HPV16/18 positive EAC; D: Barrett's esophagus with HGD. (Adapted from: Rajendra *et al*<sup>[71]</sup>, 2015).

### Head and neck cancer

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer worldwide<sup>[106]</sup>. Most of the primary tumors detected in HNSCC are of unknown primary origin and are found in the oropharynx. Cervical LNM in oropharyngeal squamous cell carcinoma (OSCC) has been linked to regional recurrence and decreased overall survival<sup>[106-109]</sup>. Lymph node metastasis has been described as an independent prognostic factor in head and neck cancers<sup>[108,110,111]</sup>.

Around 80-90% oropharyngeal tumors are associated with hr- HPV<sup>[112-115]</sup>. Of all high-risk types, HPV-16 is the most frequent genotype. HPV driven HNSCCs have considerably superior prognosis and superior chemo- and radio-sensitivity than the typical non-HPV related HNSCC. Normally, HNSCC is keratinizing and is usually caused by genetic alterations; whereas, HPV-associated HNSCC is non-keratinizing and is triggered by inactivation of tumor suppressor genes (*e.g.* TP53 and RB) by viral oncogenes E6 and E7<sup>[116]</sup>. However, a subset of patients with HPV related HNSCC develop metastasis similar to non-HPV related HNSCC<sup>[117]</sup>.

Furthermore, transcriptionally active HPV has been detected in metastatic lymph nodes in these cancers<sup>[118,119]</sup>. Takes *et al*<sup>[120]</sup> proposed that hr-HPV positive DNA in metastatic cervical lymph nodes, could identify the primary origin of head and neck cancers. 70% of OSCC patients were diagnosed with LNM at early stages and 20-30% have occult metastases. Cancer free lymph nodes with high viral load could indicate occult metastasis<sup>[42]</sup>.

HPV driven head and neck cancer especially in oropharyngeal and tonsillar regions have an increased overall survival. Schwartz *et al*<sup>[121]</sup> and Nicolas *et al*<sup>[122]</sup> found that patients with HPV-positive tumors have an increased disease free, overall survival and reduced disease specific mortality rates compared to viral negative head and neck cancers. Fujita *et al*<sup>[121]</sup> concluded that LNM was more prevalent in HPV linked HNSCC and HPV negative tumors were associated with disease recurrence.

### Gastric cancer

Gastric cancers (GC) have been caused by various microbial infections. Major pathogens linked to GC are *Helicobacter pylori* (*H. pylori*) and Epstein Barr virus (EBV)<sup>[122-124]</sup>. EBV infection raises the risk of gastric cancer. It is present in 9% of gastric carcinomas<sup>[125]</sup>. Very few studies have been reported on the prevalence of EBV in lymph nodes in GC. These studies found that EBV positive gastric cancer was negatively associated with lymph node metastasis, resulting in better prognosis<sup>[126-128]</sup>.

The association of HPV and gastric cancers is still poorly understood. de Souza *et*

*al*<sup>[128]</sup> and Fakhraei *et al*<sup>[129]</sup> observed only 3% and 5% of samples infected with HPV. Although, hr-HPV 16 and 18 were found in some tumors, E6 and E7 expression was negative implying the absence of biologically active virus. These studies therefore concluded that HPV had no role in initiation or progression of GC. Where some studies reported higher frequencies (37.5%-52%), other publications documented complete absence of the virus in GC tumor samples<sup>[130-135]</sup>. This discrepancy can be attributed to DNA detection and tumor samples processing methods. In a meta-analysis of 1917 patients, Zeng *et al*<sup>[136]</sup> reported that the HPV positivity was greater in the Chinese population (31%) than in non-Chinese regions (9%). Also, higher rates of HPV infection were obtained using the ISH (36%) method than PCR technique (26%).

## CONCLUSION

HPV associated LNM has been well established as a major prognostic factor in oropharyngeal, head and neck squamous cell carcinoma and other anogenital cancers. Studies have demonstrated an association of transcriptionally active HPV with esophageal adenocarcinoma. The viral involvement in LNM in EAC is still unexplored and unidentified. Through this review, we propose that HPV detection should be undertaken in LNM of EAC tissue samples which test positive for HPV infection in the primary tumor, using PCR, DNA ISH, RT-PCR, E6-E7 mRNA ISH and p16<sup>INK4A</sup>. We shall correlate HPV status of the primary tumor and lymph nodes with clinical outcomes including overall survival, disease-free survival micro-metastasis and diseases-specific death. The results of these studies we hope, shall be keenly anticipated in the coming years.

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## Post-transplant diabetes mellitus and preexisting liver disease - a bidirectional relationship affecting treatment and management

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### Abstract

Liver cirrhosis and diabetes mellitus (DM) are both common conditions with significant socioeconomic burden and impact on morbidity and mortality. A bidirectional relationship exists between DM and liver cirrhosis regarding both etiology and disease-related complications. Type 2 DM (T2DM) is a well-recognized risk factor for chronic liver disease and vice-versa, DM may develop as a complication of cirrhosis, irrespective of its etiology. Liver transplantation (LT) represents an important treatment option for patients with end-stage liver disease due to non-alcoholic fatty liver disease (NAFLD), which represents a hepatic manifestation of metabolic syndrome and a common complication of T2DM. The metabolic risk factors including immunosuppressive drugs, can contribute to persistent or *de novo* development of DM and NAFLD after LT. T2DM, obesity, cardiovascular morbidities and renal impairment, frequently associated with metabolic syndrome and NAFLD, may have negative impact on short and long-term outcomes following LT. The treatment of DM in the context of chronic liver disease and post-transplant is challenging, but new emerging therapies such as glucagon-like peptide-1 receptor agonists (GLP-1RAs) and sodium-glucose cotransporter 2 inhibitors (SGLT2i) targeting multiple mechanisms in the shared pathophysiology of disorders such as oxidative stress and chronic inflammation are a promising tool in future patient management.

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**Key words:** Diabetes mellitus; Liver transplantation; Non-alcoholic fatty liver disease; Metabolic syndrome; Insulin-resistance; Glucagon-like peptide-1 receptor agonists; Sodium-glucose cotransporter 2 inhibitors

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**Core tip:** This review explores complex relationships and mechanisms involved in the interplay between diabetes mellitus and liver disease before and after liver transplantation, especially in the term of non-alcoholic fatty liver disease, which then relate to management issues, newer treatment options and patient outcomes.

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## INTRODUCTION

Diabetes mellitus (DM) represents a group of heterogeneous diseases caused by an impaired insulin secretion and/or action. It is characterized by hyperglycemia and derangements in carbohydrate, lipid and protein metabolism, causing the development of numerous complications resulting in increased morbidity and mortality of patients<sup>[1]</sup>. According to the World Health Organization data, 422 million adults live with DM worldwide and global prevalence is estimated to rise to 592 million by 2035<sup>[2,3]</sup>.

The liver has a key role in glucose metabolism. It is responsible for the maintenance of total carbohydrate stores and for gluconeogenesis. Regulation of the complex and interdependent glycolytic-gluconeogenic pathways is dependent on multiple factors, including hormonal status and the relative availability of nutrient substrates<sup>[4]</sup>.

The association between DM and liver disease is well established and complex in nature. Insulin resistance (IR) and type 2 DM (T2DM) within the metabolic syndrome are important risk factors for the development of non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC)<sup>[5]</sup>. On the other hand, liver cirrhosis of any etiology can cause impaired glucose regulation due to diminished liver function, and the term hepatogenous diabetes has been proposed for this entity<sup>[6,7]</sup>. However, it is sometimes difficult to establish this diagnosis, due to the aforementioned bidirectional relationship between impaired glucose metabolism and chronic liver disease.

Liver transplantation (LT) is a well-established treatment option for end-stage liver disease of any etiology. Following LT, glucose metabolism can improve and DM resolve, especially in patients with diabetes following liver cirrhosis development. However, this may not be true for patients with pre-existing diabetes, and to further complicate this relationship, DM may develop post-transplant, due to genetic factors and lifestyle changes of the patient, immunosuppressive treatment, donor-dependent and procedure-related factors (Figure 1).

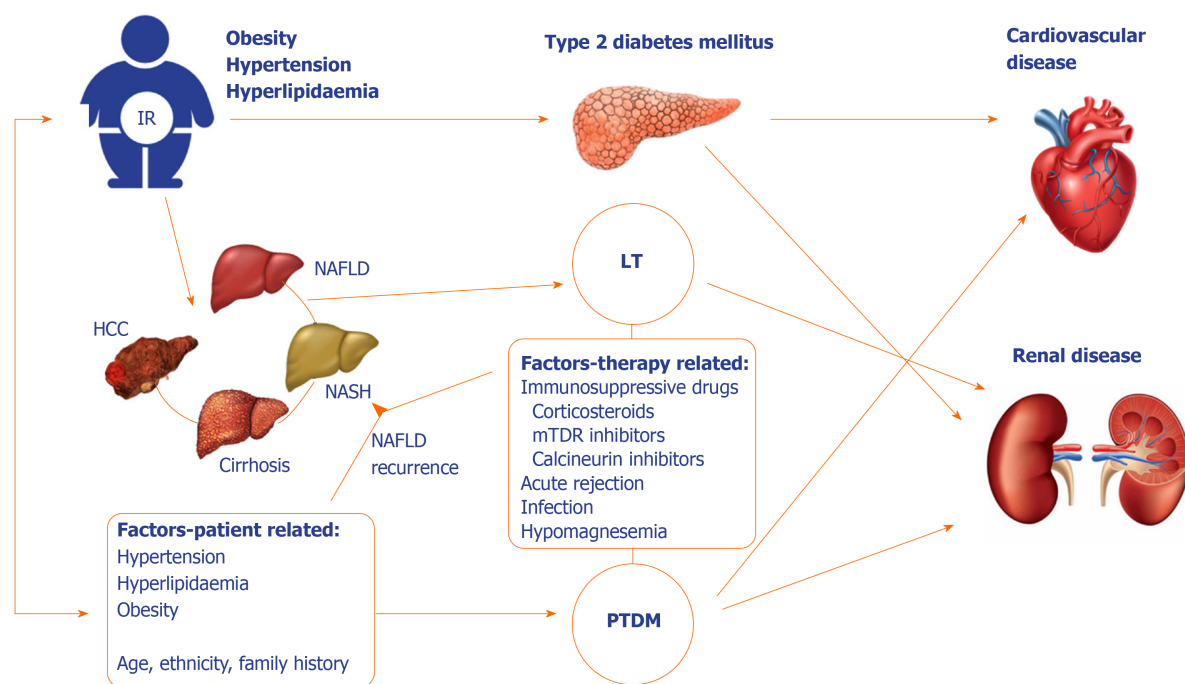
The complex relationship and mechanisms involved in the interplay between DM and liver disease before and after LT will be explored, and related to management issues, newer treatment options and patient outcomes.

## NAFLD

The global prevalence of metabolic syndrome characterized by the morbidity cluster of obesity, T2DM, hypertension and dyslipidemia is rapidly rising, reflecting the changes in eating habits and inclination towards sedentary lifestyle. In parallel, its liver manifestation NAFLD is becoming the most common cause of chronic liver disease and the leading cause of liver-related mortality<sup>[8]</sup>. The global prevalence of NAFLD is now estimated around 25%, the highest being in South America and in the Middle East (30%-31%) and the lowest in Africa (13%)<sup>[9]</sup>.

NAFLD encompasses a wide spectrum of histological changes in the liver, ranging





**Figure 1 A complex relationship between liver disease and diabetes mellitus.** Although in case of diabetes mellitus following liver cirrhosis glycemia might improve after liver transplantation (LT), this may not be the case in the preexisting type 2 diabetes mellitus. Moreover, diabetes can develop following LT (post-transplant diabetes mellitus) due to different patient and procedure-related factors. Both diabetes and liver disease after transplant increase the cardiovascular risk, which is the main cause of mortality in the long-term follow-up. LT: Liver transplantation; HCC: Hepatocellular carcinoma; PTDM: Post-transplant diabetes mellitus; IR: Insulin resistance; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

from simple steatosis or non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis, fibrosis and finally cirrhosis<sup>[10]</sup>. Approximately 25% of NAFLD patients develop NASH, and 5% progress to cirrhosis. Severe liver-related events, such as death or need for LT are relatively uncommon, and occur in 1-2% of patients with NAFLD. However, given its high global prevalence, NAFLD has become the fastest growing indication for LT<sup>[11]</sup>.

The pathogenesis of NAFLD is multifactorial and complex, with multiple mechanisms involved in the development and progression of the disease<sup>[12,13]</sup>. Genetic factors, dietary habits and other environmental factors lead to obesity and insulin resistance (IR), which is the key mechanism responsible for the NAFLD forming cascade of events. Impaired inhibition of adipose tissue lipolysis with consequent increased influx of free fatty acids into the liver together with increased hepatic *de novo* lipogenesis outweigh fatty acid oxidation and triglyceride secretion, resulting in the accumulation of triglycerides in hepatocytes<sup>[12,13]</sup>. Increased lipotoxicity causes mitochondrial dysfunction, oxidative stress and production of reactive oxygen species; these changes result in a more severe form of NASH<sup>[14]</sup>. Obesity and insulin resistance are associated with alterations in gut microbiota and changes in intestinal permeability with consequent activation of inflammatory pathways and release of proinflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin 1 and 6<sup>[12,13]</sup>. Changes in the secretion of adipokines, hormones derived from adipose tissue, such as adiponectin, leptin, resistin or ghrelin, contribute to chronic inflammatory state which consequently leads to further liver injury, where long-standing liver damage and repair responses result in activation of hepatic stellate cells and deposition of fibrous matrix, leading to cirrhosis and development of HCC<sup>[15,16]</sup>. However, although steatosis in NAFLD usually precedes inflammation, it has been recognized that hepatic inflammation and fibrosis can exist without steatosis<sup>[12,17]</sup>. Numerous and diverse processes which contribute to the development of steatosis and liver inflammation occur in parallel, and it is possible that different risk of progression of simple steatosis or NASH to more advanced liver disease may actually allude that these two are separate entities in terms of pathogenesis<sup>[12,17]</sup>.

Given the high burden of NAFLD and its consequences, end-stage liver disease and HCC, NAFLD represents a growing indication for LT both in Europe and the United States. In the near future, the prevalence of NAFLD as indication for LT is expected to rise due to increasing prevalence of obesity, T2DM and metabolic syndrome, but also because of lack of symptoms and reliable non-invasive tests for the timely and



accurate diagnosis, as well as lack of effective treatment options that could significantly alter the course of the disease. The problem is augmented by the reportedly high rates of NAFLD recurrence after LT, ranging between 30%-100% of NAFLD liver transplant recipients, and *de novo* occurrence of NAFLD in 20%-30% of patients undergoing LT for other indications<sup>[18-20]</sup>.

## DM AND NAFLD - CARDIOVASCULAR DISEASE EQUIVALENTS?

T2DM is an important risk factor for the development of NAFLD, and vice versa. NAFLD is associated with up to five-fold increased risk of T2DM<sup>[21]</sup>. The prevalence of DM is higher (48.4%) in NASH than in other indications waitlisted for LT. Moreover, NASH patients are more likely to be white, female, older, and with higher body mass index<sup>[22]</sup>. Concomitant NAFLD in patients with diabetes often compromises achievement of desired glycemic targets and moreover aggravates complications such as chronic kidney disease by almost 2-fold; these factors later on contribute to overall poor treatment outcomes<sup>[23,24]</sup>.

Available evidence suggests an intimate relationship between NAFLD/NASH, T2DM, cardiovascular disease (CVD) and chronic kidney disease<sup>[25]</sup>. This is not a surprise since NAFLD/NASH and T2DM share many metabolic risk factors with cardiovascular and chronic kidney disease, such as insulin resistance, atherogenic dyslipidaemia, hypertension and obesity. Different pathophysiological mechanisms have been described that contribute to CVD and chronic kidney disease development in patients with NAFLD/NASH and T2DM and this topic has been extensively reviewed elsewhere<sup>[25-27]</sup>. Although epidemiological evidence from many primarily cross-sectional, case-control and retrospective studies involving NAFLD patients with and without diabetes have shown an increased prevalence of CVD and chronic kidney disease, the complex interplay between the traditional cardiometabolic risk factors makes a causal relationship between NAFLD, T2DM, and cardiovascular and chronic kidney disease sometimes difficult to establish<sup>[28,29]</sup>. In the Multi-Ethnic Study of Atherosclerosis (MESA), NAFLD proved as independent risk factor for latent atherosclerosis, chronic inflammation and coronary artery calcium scores<sup>[30]</sup>. Similarly in the Kuopio Ischaemic Heart Disease Risk Factor Study (KIHD), the fatty liver index was a predictor of incident CVD in the middle-aged men over a median of 17-year follow-up<sup>[31]</sup>. In addition, data coming from observational studies showed the association between NAFLD and significant increase in the risk of incident chronic kidney disease<sup>[32]</sup>.

## DM AND LIVER CIRRHOSIS

Liver cirrhosis represents the end-stage liver disease caused by various etiologies and mechanisms of liver injury that lead to inflammation, necrosis, and consequent fibrogenesis. It is an important cause of morbidity and mortality worldwide, with significant regional differences in both the prevalence and etiology of the disease. Cirrhosis represents the 14th most common cause of death in adults and results in 1.03 million deaths per year<sup>[33]</sup>.

T2DM and IR are important risk factors for the development of NAFLD and NASH, which can progress to liver cirrhosis. On the other hand, IR and hyperinsulinemia are common features in cirrhosis<sup>[6,34]</sup>. Recent studies using current criteria for DM diagnosis have shown that, based on fasting plasma glucose levels, alone or associated with measurements of hemoglobin (Hb) A1c, prevalence of DM in cirrhotic individuals is approximately 30%-40%, but if individuals were subjected to an oral glucose tolerance test, the prevalence of abnormal glucose tolerance and DM would rise up to 96%<sup>[35]</sup>.

Although it has been recognized that liver cirrhosis of any etiology represents a risk factor for the development of DM, the mechanisms responsible for the development of cirrhosis-associated DM are multiple and not completely understood<sup>[6,7]</sup>. A body of evidence suggests that liver dysfunction in cirrhosis directly affects insulin secretion, clearance and insulin sensitivity, but certain etiological agents may cause IR in the earlier, pre-cirrhotic stages, through various mechanisms<sup>[6,36-39]</sup>.

Numerous epidemiological, clinical and experimental studies have reported hepatitis C virus to be strongly associated with hepatic steatosis, IR and T2DM, even before cirrhosis development<sup>[40]</sup>. HCV induces IR in the liver and peripheral tissues through multiple mechanisms. A large meta-analysis of 34 studies confirmed a positive correlation between HCV infection and increased risk of T2DM in

comparison to the general population<sup>[41,42]</sup>. The prevalence of T2DM was also reported to be higher in patients with chronic hepatitis caused by HCV compared to subjects with chronic liver diseases from other etiologies<sup>[6,43]</sup>. However, prevalence of T2DM was higher in HCV infected patients following cirrhosis development, compared to earlier stages of fibrosis, and was similar to patients with cirrhosis of other etiologies, thus suggesting that diabetogenic potential of liver dysfunction was even higher than that of hepatitis C virus itself<sup>[6]</sup>. HCV related IR and steatosis appear to be associated with progression of fibrosis and development of hepatocellular carcinoma<sup>[40]</sup>.

Alcohol is known to reduce insulin-mediated glucose uptake upon acute ingestion, although the effects of chronic alcohol intake are less well understood<sup>[44]</sup>. Patients with chronic consumption of alcohol frequently suffer from chronic pancreatitis and damage to pancreatic islet  $\beta$ -cells can result in diabetes development<sup>[44]</sup>. The risk of developing DM seems to be related to the amount of alcohol ingested and increased twofold in persons with high alcohol intake compared to those with moderate intake<sup>[45]</sup>. Prevalence of diabetes among patients with alcohol-related liver cirrhosis according to studies ranges between 10% and 40%, with an average prevalence of around 27%<sup>[46]</sup>.

Hereditary haemochromatosis is a metabolic disorder characterized by iron accumulation and deposition in several organs. Haemochromatosis as well as alcohol consumption induce iron deposition in the liver which could interfere with the ability of insulin to suppress hepatic gluconeogenesis. However, the pathogenesis of diabetes due to haemochromatosis has also been related to concomitant injury of pancreatic  $\beta$ -cells caused by iron deposits<sup>[6]</sup>. For this reason DM represents a clinical manifestation of haemochromatosis, and can be found in 40% to 85% of patients in advanced stages<sup>[44,47,48]</sup>.

Unlike chronic HCV infection, the relationship between hepatitis B virus infection (HBV) and insulin resistance has not been completely understood as the studies produced conflicting results<sup>[49]</sup>. However, most studies concur that the incidence of DM in patients with HBV infection is not increased compared to patients without infection indicating that HBV may not influence the development of DM<sup>[49]</sup>. However, a meta-analysis comprising results of 15 studies found that the prevalence of T2DM among HBV infected patients was 1.3 times higher and concluded that chronic HBV infection does increase the risk of T2DM development<sup>[50]</sup>. The prevalence of DM is higher among patients with HBV who developed cirrhosis (22.2%), compared to those without cirrhosis (12.8%)<sup>[51]</sup>. The pooled prevalence of DM in patients with HBV-related cirrhosis was around 22.2% (ranging from 9.4% to 35.0% in various studies)<sup>[46]</sup>. This was significantly lower than in patients with cirrhosis related to NAFLD which had the highest prevalence of DM (a pooled estimate of 56.1%), or alcohol-related, HCV or cryptogenic cirrhosis (estimated prevalence ranging from 27.3% to 50.8%). The lowest prevalence of DM was found in patients with cirrhosis secondary to cholestatic liver disease (overall 7.1%, ranging from 1.3% to 15.2%)<sup>[46]</sup>.

Regardless of the etiology of cirrhosis, and irrespective of which developed first, when cirrhosis and DM coexist, they interfere with each other with reflection on course of the disease. In a population-based Italian study, the risk of death from cirrhosis was significantly higher among patients with DM<sup>[52]</sup>. A number of prospective and retrospective studies indicated that complications of liver disease (spontaneous bacterial peritonitis and other bacterial infections, ascites, hepatic encephalopathy, and gastrointestinal hemorrhage) as well as death rates are higher in cirrhotic patients with DM compared to non-diabetic patients with advanced liver disease<sup>[6]</sup>. Moreover, DM associated with poor glycemic control ( $\text{HbA1c} > 9\%$ ) is an independent risk factor for development of HCC in patients with chronic liver disease<sup>[53]</sup> although poor glycemic control in cirrhotic patients is not necessarily reflected through high  $\text{HbA1c}$ , but rather higher glucovariability<sup>[54]</sup>. Therefore, both uncontrolled and controlled T2DM as well as glucose intolerance can negatively impact survival of patients with liver cirrhosis<sup>[52,55]</sup>. The mechanisms involved are still not clear. Plausible explanation is that insulin resistance alone, even without the element of hyperglycemia increases adipokine production through chronic inflammation, such as leptin and TNF alpha leading to activation of TGF beta1, a profibrotic cytokine in turn activating hepatic stellate cells to produce high quantities of collagen and extracellular matrix proteins. Of course, all these unfavorable effects of chronic inflammation mediated through IR are then largely potentiated by chronic hyperglycemia<sup>[56-61]</sup>.

## POST-TRANSPLANT DM

Post-transplant DM (PTDM) has been recognized as a major complication after LT

with the potential for serious consequences and ultimately worse patient and graft survival<sup>[62]</sup>. It seems that in case of LT, the onset of PTDM is rapid; 50% of cases occurring within first 6 months and 75% after 12 mo following procedure<sup>[63]</sup>. Although IR and DM related to liver dysfunction and cirrhosis can improve following LT, the presence of metabolic syndrome and extrahepatic comorbidities, as well as immunosuppressive drugs can contribute to persistent or *de novo* DM following LT. It occurs in 7%-45% of liver transplant recipients and is associated with increased morbidity, health care costs and impairs both patient and graft survival<sup>[62-64]</sup>. So far, parameters such as advanced age, ethnicity, family history, body mass index, hepatitis C virus infection and immunosuppressive drugs (mainly corticosteroids and tacrolimus) have all been reported as risk factors for PTDM after LT<sup>[65,66]</sup>. Moreover, obesity, either predating or gained after transplantation, is associated with an increased risk for PTDM but also post-transplant NAFLD<sup>[67]</sup>. According to 2014 International Consensus Meeting diagnosis of PTDM can be established in patients after achieving stable doses of maintenance immunosuppressants, with stable allograft function, once they have been discharged from the hospital to avoid misdiagnosis of transient hyperglycemia<sup>[64]</sup>. Preferred criteria to diagnose PTDM (either fasting plasma glucose > 7 mmol/L, random plasma glucose > 11.1 mmol/L accompanied by symptoms or 2-hour post 75g oral glucose test plasma glucose > 11.1 mmol/L) are those of American Diabetes Association/World Health Organization criteria for the diagnosis of diabetes, and HbA1c > 6.5% should not be used alone to screen for PTDM within the first year after transplant.

As we enter an era where recurrent hepatitis C after LT becomes a rare entity and *de novo* or recurrent NAFLD increases in incidence, we are likely to see changes in the patterns of incidence and impact of PTDM. Current data suggest that PTDM increases the risk of infection, chronic renal failure, biliary complications and decreases survival<sup>[68,69]</sup>. Poor LT outcomes seem not only to be related to PTDM, but also to much earlier derangements in the pathophysiology of hyperglycemia, during IR stage<sup>[70]</sup>. Still, nearly 30% of deaths in LT patients are caused by cardiovascular or cerebrovascular diseases<sup>[71]</sup>.

## NAFLD AFTER LT

After LT, NAFLD/NASH may re-occur or develop *de novo*. Histological features of recurrent or *de novo* NAFLD/NASH are the same as in native livers, and there are no features to differentiate recurrent from *de novo* NAFLD/NASH in the graft. The diagnosis is based on correctly identifying preexisting liver disease – NASH-related or in some cases cryptogenic cirrhosis<sup>[72]</sup>. The studies assessing NAFLD recurrence after LT report a wide range from 10 to 100%<sup>[19,20,73-75]</sup>. However, regardless of indication for LT, risk factors for metabolic syndrome and NAFLD increase over time in the post-transplant setting<sup>[76,77]</sup>. Side-effects of immunosuppressive medications and rapid weight gain in the post-transplant period can lead to impaired glucose control, dyslipidemia, development of metabolic syndrome and *de novo* NAFLD. In fact, it has been reported in 20%-30% of patients following LT for non-NAFLD/NASH etiologies<sup>[18,19]</sup>. Weight gain is common in patients following LT and 30%-60% of patients become overweight or obese, with a mean weight gain of 2-9 kg usually within the first year after LT. After one year post-LT, weight gain typically slows down<sup>[18,78-81]</sup>. Obesity appears to be one of the strongest risk factors for the development of NAFLD in the post-transplant period<sup>[18,20]</sup>. Moreover obese patients after LT have a 2-fold higher risk of mortality compared to normal weight LT recipients<sup>[82]</sup>. The mechanisms leading to a post-transplant weight gain are driven by a complex interaction of genetic, physiological, behavioral, and environmental factors<sup>[83,84]</sup>. Dyslipidemia and hypertension occur in 40%-70% of patients, usually related to use of mammalian target of rapamycin (mTOR) inhibitors and calcineurin inhibitors. Metabolic syndrome has been reported in 40%-50% of patients after LT, both in patients with previous NAFLD as well as other etiologies of chronic liver disease. In a recent report from Mayo Clinic, 84.6% of patients had *de novo* allograft steatosis and 15.4% recurrent NAFLD after 2 months post-liver transplant. After 10 years, 48% of liver transplant recipients had evidence of allograft steatosis, including 77.6% of patients transplanted for NASH and 44.7% of individuals transplanted for other indications<sup>[20]</sup>. Interestingly, in this study allograft steatosis was not associated with worse outcomes regarding graft and patient survival. However, the incidence of cardiovascular events 5 years post-transplant was approximately 40% in patients transplanted for NASH compared to 5%-10% incidence in LT recipients transplanted for non-NASH etiologies, regardless of graft steatosis<sup>[20]</sup>. This could suggest that impaired metabolic profile before LT could have more significant impact on CV

events and outcomes than the post-transplant NAFLD itself. However, the long-term outcomes and consequences of complex interplay between T2DM, obesity, metabolic syndrome, pre- and post-liver transplant NAFLD with CV events require further investigation and explanation.

## T2DM TREATMENT IN THE CONTEXT OF NAFLD

Treating type 2 diabetes, although now with available wide range of armamentarium, still remains challenging, especially in the presence of coexisting NAFLD and obesity. Acknowledging the fact that NAFLD and T2DM act synergistically in driving adverse outcomes, in the way that the NAFLD accelerates the development of diabetic chronic micro- and macrovascular complications, while the diabetes increases the likelihood of more severe forms of NAFLD such as steatohepatitis, cirrhosis, and HCC could be an important issue in choosing the treatment modality and pharmacological agent<sup>[24,85-89]</sup>. Therefore, when taking into account pathophysiology, treating NAFLD/NASH could prevent T2DM development and/or progression, but, also the other way around<sup>[90]</sup>.

As there is no specific pharmacotherapy yet available for treating NAFLD, anti-diabetic drugs with pleiotropic effects, targeting multiple metabolic disorders, seem promising. Among them, sodium-glucose cotransporter 2 inhibitors (SGLT2i) and glucagon-like peptide-1 receptor agonists (GLP-1RAs) are recognized by American Diabetes Association (ADA) and European Association for Study of Diabetes (EASD) as the best treatment option for patients with T2DM and cardiovascular disease, heart failure and/or chronic kidney disease. These two classes of drugs both exert weight losing effect, making them especially interesting for patients with associated obesity, and according to results of both randomized controlled trials (RCTs) and open-label studies, offer promising effects in reducing liver fat content<sup>[91]</sup>.

Previously used agents such as metformin and thiazolidinediones (TZDs), affecting insulin resistance, did show benefit on biochemical and metabolic features of NAFLD, but improvement of patients' histological response or fibrosis was modest and studies were usually short-term therefore lacking information on liver-related long-term outcomes. Moreover, side-effects usually override their wider use in NAFLD patients<sup>[92-95]</sup>. Especially interesting results came from studies with NASH patients receiving TZDs. Mentioned agents activate the nuclear receptor of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) located in adipose, liver and muscle tissue and exert insulin-sensitizing activity. Additionally, TZDs increase adiponectin, reduce liver fat content and modulate intra hepatic inflammation<sup>[94]</sup>. Recently published large cohort study in Taiwan using propensity score matching suggested patients with newly diagnosed T2DM receiving either rosiglitazone (not widely used anymore due to associated increased CV risk) or pioglitazone for mean of 3.84 years, compared to newly diagnosed T2DM patients that were non-TZD users, had lower risk of hepatic cirrhosis (incidence rate: 0.77 *vs* 1.95 per 1000 person-y)<sup>[96]</sup>.

One of the newer therapeutic modalities that needs to be addressed is certainly pre-transplant bariatric surgery in the obese patients with or without diabetes. So far, available data for this particular subset of patients are scarce and data from randomized clinical trials are lacking. However two major points should be considered regarding bariatric procedure in liver transplant (LT) – timing and type of procedure. In a study including 37 patients who underwent LT, and 7 who underwent LT combined with sleeve gastrectomy (SG), majority of patients with LT alone had weight gain to BMI > 35, post-LT diabetes and steatosis with 3 deaths and 3 grafts losses as compared to patients undergoing the combined procedure, who had no graft losses and there were no developed post-LT DM or steatosis, with significant weight loss (mean BMI = 29) after 12 months of follow up. One patient developed a leak from the gastric staple line, and one had excess weight loss. Average time from SG to LT was 17 months<sup>[97]</sup>. This was confirmed in other study performed in liver and kidney transplant patients who underwent SG 16 months before transplant<sup>[98]</sup>. There are also some small studies available investigating SG after solid organ transplant showing favorable outcomes regarding weight loss and PTDM incidence<sup>[99-101]</sup>.

In a large cohort study comparing in-hospital mortality and length of stay for patients with no cirrhosis, compensated cirrhosis, and decompensated cirrhosis, who underwent bariatric surgery, patients with decompensated cirrhosis had highest postoperative mortality rate compared to those with compensated and non-cirrhosis patients (16.3% *vs* 0.9% and 0.3%, respectively,  $P = 0.0002$ )<sup>[102]</sup>.

Therefore, we can conclude that sleeve gastrectomy is procedure of choice in liver transplant patients with compensated liver cirrhosis since it does not affect absorption compared to other operative modalities and provides beneficial effects in terms of



weight reduction, development of PTDM and liver steatosis.

## GLP-1RA IN THE TREATMENT OF T2DM

Glucagon-like peptide-1 receptor agonists (GLP-1RAs) are a newer class of antidiabetic medications with a spectrum of beneficial metabolic effects. Besides improving glycaemia by promoting glucose-dependent insulin secretion and inhibition of glucagon secretion, these agents promote weight loss by increasing satiety through central nervous system, delaying gastric emptying and potentially increasing thermogenesis in brown fat tissue. Moreover, GLP-1RAs have proven cardiovascular safety, while some representatives of the class have shown additional cardiovascular benefits/protection in large cardiovascular outcome trials<sup>[103-106]</sup>.

Although receptors for the GLP-1 on the hepatocytes are still a matter of scientific dispute, they appear to have a direct effect on the lipid metabolism of hepatocytes, which leads to reduction of hepatic steatosis and their potential in NAFLD treatment<sup>[107-110]</sup>. Of additional interest in NAFLD treatment would be their lipid-lowering and anti-inflammatory potential<sup>[111,112]</sup>.

## GLP-1RA IN THE TREATMENT OF NAFLD

In patients with NAFLD intrahepatic triglyceride accumulates, while hepatic as well as muscle insulin sensitivity declines, which correlates with the IR at the adipose tissue level and its dysfunction. This was a scientific context for assessing the potential of GLP-1Ras in the treatment of NAFLD/NASH from which optimistic results showing reduction in liver enzymes and intrahepatic triglycerides content in patients with T2DM first came<sup>[113-116]</sup>. It seems that GLP-1RA inhibit dysfunctional endoplasmic reticulum stress response to fatty-acids in hepatocytes and therefore provide protective effects on hepatocytes<sup>[110]</sup>. In human studies including exenatide (5-10 µg/d) improvements in weight, body mass index, glycemic control, liver enzymes, hsCRP (high-sensitivity C-reactive protein) and adiponectin were seen in patients with ultrasound diagnosed NAFLD treated with GLP-1RA compared to metformin<sup>[117]</sup>, **Table 1**.

Moreover, smaller studies suggested beneficial effects of exenatide and liraglutide on liver histology<sup>[118-120]</sup>. Regarding short acting GLP-1RA lixisenatide, studies in obese or overweight T2DM patients reported increase in the proportion of patients achieving normalization of ALT<sup>[121]</sup>, but there is no data on lixisenatide effects on hepatic steatosis or fibrosis.

The first RCT confirming the efficacy of GLP-1RA in the resolution of NASH in patients with biopsy-proven diagnosis was the LEAN trial (Liraglutide Efficacy and Action in NASH) which followed patients for 48 weeks while taking liraglutide 1.8 mg daily<sup>[122]</sup>. Resolution was achieved in 39% of patients in the active arm compared with 2/22 (9%) participants in the placebo group. It seems that the improvements in liver fat were greater with prolonged duration of liraglutide treatment<sup>[119,123-126]</sup>.

Similar results were suggested in the recently published large scale RCT with semaglutide, a once weekly GLP-1RA including patients with biopsy-proven NASH<sup>[127]</sup>. There is currently no published data available on oral form of semaglutide regarding the effect on NAFLD/NASH, although it shows superiority over liraglutide in terms of weight loss<sup>[128,129]</sup>.

Another long acting GLP-1RA with a once weekly dosing, dulaglutide, according to results of a Japanese retrospective case series study, and results from the AWARD (Assessment of Weekly Administration of LY2189265 (dulaglutide) in Diabetes) development program seems promising for NASH patients in terms of reduction of liver enzymes and liver stiffness (measured by transient elastography)<sup>[130,131]</sup>. So far, no data is available for albiglutide (another once weekly administered GLP-1 RA) on NAFLD/NASH features.

## SGLT2i IN THE TREATMENT OF T2DM

Sodium-glucose cotransporter-2 (SGLT2) inhibitors are widely used oral anti diabetic agents targeting hyperglycemia in an untraditional way, by enhancing glycosuria (with the daily glucose loss ranging between 60 and 80 g). In addition to blood glucose lowering effect, glycosuria also enhances calorie loss (240–320 kcal) and thus gliflozins exert a clinically significant weight-lowering effect. Moreover, their osmotic diuretic and natriuretic effects contribute to plasma volume contraction, and decrease



**Table 1 Proven and possible effects of glucagon-like peptide-1 receptor agonists and sodium-glucose cotransporter 2 inhibitors in diabetes and metabolism-associated fatty liver disease before and after liver transplantation**

Therapy effect	NAFLD	T2DM	PTDM	Post-LT liver disease
GLP-1RA proven	Improvement in liver enzymes and intrahepatic triglycerides content <sup>[113-116,130,131]</sup> Improvements in weight, body mass index, glycemic control, liver enzymes, hsCRP <sup>[117]</sup> Improvement in liver histology <sup>[118-120]</sup> Resolution of NASH <sup>[122,127-129]</sup>	Improvement of glycemia; increase insulin secretion in a glucose-dependent manner; inhibit glucagon secretion; weight losing effect; cardiovascular protection <sup>[103-106]</sup>	Improvement of insulin and normalization of glucagon concentrations <sup>[150]</sup> Resistance against toxicity of IS drugs <i>in vitro</i> <sup>[154]</sup> Prevention of steroid diabetes <sup>[155]</sup>	None metabolized by liver, no dose adjustments needed <sup>[156]</sup> Weight loss during first weeks after LT <sup>[158]</sup>
GLP-1RA possible	Reduction of hepatic steatosis; anti-inflammatory effect <sup>[107-112]</sup>		Improvement of cardiovascular outcomes <sup>[103-106]</sup> , GI side effects potentiating GI disturbances caused by IS drugs <sup>[157]</sup>	
SGLT-2i proven	Reduction of liver enzymes <sup>[65,135-142]</sup> Reduction of body weight and body fat <sup>[143-147]</sup>	Improvement of glycemia; weight losing effect; decrease in systolic and diastolic blood pressures; cardiovascular benefit <sup>[132-134]</sup>	Reduction of weight and blood pressure <sup>[162]</sup> Improved glycemia <sup>[163]</sup>	
SGLT2i possible	Reduction of oxydative stress and inflammation <sup>[143-147]</sup>		Genitourinary infections <sup>[164]</sup>	Reduction in fat mass and visceral adipose tissue <sup>[165]</sup>

GLP-1RA: Glucagon-like peptide-1 receptor agonists; IS: Immunosuppressive; LT: Liver transplant; NAFLD: Non-alcoholic fatty liver disease; T2DM: Type 2 diabetes mellitus; PTDM: Post-transplant diabetes mellitus; SGLT2i: Sodium-glucose cotransporter 2 inhibitors.

in both systolic and diastolic blood pressures. In the large, recently published cardiovascular outcome trials (EMPA-REG OUTCOME trial with empagliflozin, CANVAS Program with canagliflozin, DECLARE-TIMI 58 with dapagliflozin) SGLT2i proved cardiovascular benefits, primarily seen through the reduction of heart failure risk and studies assessing their renal protective effects are on their way<sup>[132-134]</sup>.

## SGLT2i IN THE TREATMENT OF NAFLD

SGLT2 inhibitors as a class have shown promising results in open-label studies and RTCs in reducing liver fat<sup>[92,135,136]</sup>. Majority of mentioned studies used biochemical markers, but some also employed different imaging techniques for assessing fatty liver content and fibrosis, while one small pilot study conducted liver biopsy to determine the hepatic effects of SGLT2 use<sup>[137-140]</sup>. Gliflozins were effective in reducing liver enzymes in diabetic patients, and more pronounced results were seen in those with accompanying NAFLD<sup>[141]</sup>. The mechanisms underlying the improvement of NAFLD with SGLT2 inhibitors remain unknown and can only be speculated, [Table 1](#).

It seems that SGLT2 inhibitors, compared to other oral antidiabetic agents with comparable glycemic control, offer more pronounced reductions in serum liver enzymes<sup>[142]</sup>. Mentioned effect, exceeding glucose-lowering activity, relies on other mechanisms including reduction of body weight and body fat, reduction of serum uric acid, oxidative stress and inflammation which might prove important in NAFLD treatment<sup>[143-147]</sup>.

## PTDM IN LT PATIENTS AND THE ROLE OF GLP-1RA

There are no specific guidelines for treatment of PTDM thus general recommendations for diabetes management are followed due to paucity of data regarding usage of antihyperglycemic drugs in transplanted patients.

The main challenge in treatment of post-transplant diabetes is increased cardiovascular risk observed in patients after solid organ transplantation<sup>[148]</sup>. Due to severe metabolic disturbances caused by weight gain after transplantation and immunosuppressant drugs incidence of cardiovascular events is higher than in non-

transplant patients and are the leading cause of mortality<sup>[149]</sup>. Given that recent data from large cardiovascular outcome trials demonstrated cardiovascular benefits for majority of long acting GLP-1 RA (liraglutide, semaglutide, albiglutide and dulaglutide<sup>[103-106]</sup>) in terms of reducing 3 point major adverse cardiovascular endpoints comprised of non-fatal myocardial infarction, non-fatal stroke and cardiovascular mortality this therapeutic option has its merits in treatment of PTDM.

Presently, there are no clinical trials published regarding GLP 1RA therapy in PTDM. The only studies available are conducted in case series of patients after kidney and pancreas transplant providing enough scientific rationale for this treatment concept in liver PTDM<sup>[150-153]</sup>. In a study performed by Halden *et al*<sup>[150]</sup> in patients with and without PTDM after kidney transplant GLP infusion in hyperglycemic clamp led to improvement of insulin and normalization of glucagon concentrations. Furthermore, GLP 1RAs also exhibit resistance against toxicity of immunosuppressive drugs *in vitro*<sup>[154]</sup> and seem to prevent steroid diabetes by diminishing glucocorticoid-induced glucose metabolism impairment and beta-cell dysfunction in healthy humans<sup>[155]</sup>.

Since long acting GLP 1RA agonists are eliminated through enzymatic degradation and renally cleared, drug-drug interactions are avoided which is extremely important in terms of immunosuppressant drug metabolism<sup>[156]</sup>. However, by delaying gastric emptying, absorption of other drugs could be affected. In a two studies of case series of kidney transplant recipients with PTDM safe co-administration of liraglutide and tacrolimus was reported mitigating, at least partially, concerns regarding absorption interference<sup>[152,153]</sup>.

One of the major downsides of GLP 1RA therapy are gastrointestinal (GI) side effects which could potentiate a GI disturbances caused by immunosuppressant drugs leading to limited usage of GLP 1 RA in spite of their numerous beneficial effects<sup>[157]</sup>, [Table 1](#).

## USE OF GLP-1RA IN TREATING NAFLD AND POST-TRANSPLANT NAFLD IN LT PATIENTS

Pharmacokinetic and pharmacodynamic properties of GLP 1 RA seems to be of extreme convenience in LT since none are metabolized by the liver and no dose adjustment is needed<sup>[156]</sup>. As already mentioned, there is growing body of evidence suggesting beneficial effects of GLP 1RA therapy in patients with NAFLD/NASH affecting not only liver enzymes normalization but also reducing inflammation and progression of fibrosis<sup>[122,127,130,131]</sup>. There are two different aspects of potential mechanisms of action involving GLP 1RA in post-transplant NAFLD. For one, improvement of metabolic disturbances through induction of weight loss, especially seen during first few weeks after transplantation<sup>[158]</sup>. Thus, lipogenesis is decreased leading to a reduction in free fatty acid and triglyceride toxic metabolites positively affecting insulin resistance, oxidative stress and inflammation, while increasing insulin secretion initiating changes in gut liver axis which are not completely understood yet<sup>[4]</sup>. The second relates to direct anti-fibrotic effects independent of metabolic changes recently shown in animal and human studies<sup>[159]</sup>. Unfortunately there are no clinical studies performed in post-transplant NAFLD/NASH patients, although based on present knowledge this therapeutic approach would be completely justified, [Table 1](#).

## PTDM IN LT PATIENTS AND THE ROLE OF SGLT2i

SGLT2 inhibitors have recently been recommended as a treatment of choice for patients with diabetes and high cardiovascular risk, which is also a characteristic of patients with PTDM<sup>[160]</sup>. The evidence of the use of gliflozins in PTDM in LT patients is scarce, but results from animal studies are promising. Rats with tacrolimus-induced diabetes that received empagliflozin had lower glycemia and increased plasma insulin levels<sup>[161]</sup>. In a case series of patients with post heart transplant diabetes mellitus, empagliflozin reduced weight, and blood pressure<sup>[162]</sup>. In a small group of kidney recipients, canagliflozin treatment improved glycemia, reduced weight and blood pressure<sup>[163]</sup>. Recently published study using empagliflozin in a larger population of PTDM patients after kidney transplantation showed improvement of glycemic control compared to placebo, and a concomitant reduction of body weight<sup>[164]</sup>. Major potential problem with SGLT2 inhibitors in PTDM might be genitourinary infections, considering concurrent immunosuppressive therapy and glycosuria. Up to date collected data, mainly in the kidney transplant patients with PTDM suggests

incidence of the GU infections is not higher than that seen in T2DM patients and resolves with usual antibiotics/antifungal treatment<sup>[164]</sup>, **Table 1**.

## USE OF SGLT2i IN TREATING NAFLD AND POST-TRANSPLANT NAFLD IN LT PATIENTS

Currently, SGLT2 inhibitors have not the indication of improving NAFLD and dedicated research is mandatory in this evolving field. In several RCTs SGLT2 inhibitors have shown positive effects on NAFLD in patients with T2DM, but the exact mechanisms are still speculative, as is their role in treating post-transplant NAFLD in patients with and without diabetes<sup>[165]</sup>. SGLT2 inhibitors are cleared by the liver, but pharmacokinetic trials involving patients with mild to moderate hepatic impairment demonstrated their safety and no need for dose adjustments in the setting of liver disease<sup>[166]</sup>. Besides safety, by reducing liver fat content and hepatocyte injury biomarkers which was shown in several gliflozin RCTs they might prove liver protective and improve NAFLD-associated endoplasmic reticulum stress and mitochondrial function<sup>[91]</sup>. Additionally, SGLT2 inhibitors are associated with significant weight loss, especially reduction in fat mass and visceral adipose tissue, which makes them interesting agents in treating post-transplant NAFLD in LT patients. Additionally, they attenuate the renin-angiotensin-aldosterone pathway by inhibition of sodium reabsorption which in theory makes the SGLT2 inhibitors ideal for patients with cirrhosis<sup>[165]</sup>, **Table 1**.

## CONCLUSION

In conclusion, pharmacological management of PTDM is further complicated given there are no published randomized clinical trials regarding effectiveness and safety of antihyperglycemic agents. Particular characteristics of PTDM include prodiabetogenic potential of immunosuppressant drugs as well as increased cardiovascular risk due to higher incidence of metabolic disturbances present in post-transplant period both of which could be successfully resolved with GLP 1RA and SGLT2i therapy. Further prospective studies including larger number of patients are needed.

Considering that currently NASH is one of the main causes of LT and it is associated with metabolic disorders mainly IR, which is a particular problem in transplanted patients, GLP 1 RA and SGLT2i treatment certainly has the potential to influence precisely this bidirectional relationship between PTDM and preexisting liver disease.

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## Vaccine therapy for dysbiosis-related diseases

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### Abstract

Progress in genomic analysis has resulted in the proposal that the intestinal microbiota is a crucial environmental factor in the development of multifactorial diseases, such as obesity, diabetes, rheumatoid arthritis, and inflammatory bowel diseases represented by Crohn's disease and ulcerative colitis. Dysregulated gut microbiome contributes to the pathogenesis of such disorders; however, there are few effective treatments for controlling only disease-mediating bacteria. Here, we review current knowledge about the intestinal microbiome in health and disease, and discuss a regulatory strategy using a parenteral vaccine with emulsified curdlan and CpG oligodeoxynucleotides, which we have recently developed. Unlike other conventional injectable immunizations, our vaccine contributes to the induction of antigen-specific systemic and mucosal immunity. This vaccine strategy can prevent infectious diseases such as *Streptococcus pneumoniae* infection, and control metabolic symptoms mediated by intestinal bacteria (e.g. *Clostridium ramosum*) by induction of high titers of antigen-specific IgA at target mucosal sites. In the future, our vaccination approach could be an effective therapy for common infectious diseases and dysbiosis-related disorders that have been difficult to control so far.

**Key words:** Dysbiosis; IgA; Microbiome; Mucosal immunity; Pathobiont; Vaccine

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**Core tip:** How to control intestinal pathogenic bacteria that mediate multifactorial diseases is a major concern worldwide. There are few methods for controlling only

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intestinal pathogenic bacteria; therefore, we have developed a prime–boost type, next-generation mucosal vaccine, and have used it for control of bacterial intestinal diseases. This vaccine can contribute to prevention of *Clostridium ramosum*-mediated obesity. Thus, this approach might be useful for protecting against microbe-associated disorders of the intestine.

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## INTRODUCTION

With the rapid progress of next-generation sequencing and genome analysis technology, human genome analysis has ended, and the focus has shifted to research on commensal microbiomes<sup>[1-8]</sup>. Body sites that are exposed to a wide variety of external antigens through mucosal sites, such as the respiratory organs and gastrointestinal tract, are constantly colonized with microorganisms, resulting in a symbiotic relationship. If this relationship is broken, the host immune response to microorganisms is distorted, sometimes causing disease. Dysbiosis, which is defined as an imbalance in the repertoire of the intestinal microbiota, is associated with many disorders in humans<sup>[9-11]</sup>. Therefore, novel strategies to control dysbiosis-associated diseases by attenuating the function of related microorganisms are necessary.

Antibiotics, which were first deployed in 1910, have drastically changed our lives<sup>[12]</sup>. In particular, penicillin discovered in 1928 contributed to the discovery of naturally occurring antibiotics. Antibiotics have extended our lifespans by > 20 years. However, a rapid increase in multidrug-resistant bacteria has arisen because of overuse and inappropriate consumption and application of antibiotics, which reveals that antibiotics are not a panacea for infectious diseases<sup>[13,14]</sup>. In addition, antibiotics sometimes cause dysbiosis and can lead to diseases such as *Clostridioides difficile* (*C. difficile*) infection<sup>[15]</sup>. Thus, although antibiotics are available for killing disease-specific commensal bacteria, they are not suitable for eliminating only pathogens.

Fecal microbiota transplantation (FMT), an effective therapy for dysbiosis-related diseases such as *C. difficile* infection, has been shown to improve aberrant intestinal microbiota<sup>[16,17]</sup>. Feces from healthy individuals, which are considered relatively safe, are usually used for FMT. However, it was recently reported that antibiotic-resistant bacteria from donor feces were transferred to recipients and induced bacteremia<sup>[18]</sup>. This is an emergency issue and FMT is not now a recommended regimen. In fact, elimination of only pathobionts through the intestinal mucosa is difficult; therefore, development of novel methods to control dysbiosis-related diseases by attenuating the function of pathobionts is strongly desired.

In this review, we present current knowledge about the intestinal microbiome in health and disease, and discuss a prime–boost type, next-generation mucosal vaccine that we have recently developed and reported for control of disease mediated by intestinal bacteria.

## INTESTINAL MICROBIOME IN HEALTH AND DISEASE

Intestinal commensal microbes have primarily been analyzed through single bacterial species isolation. Since most enteric bacteria do not like aerobic conditions, it has been difficult to culture them. However, advances in culture-independent technologies such as next-generation sequencing have shown the dynamics of the human intestinal microbiota<sup>[9,19]</sup>. For example, trillions of intestinal microbes reside in the gastrointestinal tract and dysbiosis is correlated with diseases such as obesity<sup>[20-22]</sup>, diabetes<sup>[23-25]</sup>, rheumatoid arthritis (RA)<sup>[26-31]</sup>, and inflammatory bowel diseases (IBDs) including Crohn's disease and ulcerative colitis<sup>[32]</sup>. Therefore, in addition to the current best treatment, it is suggested that controlling dysbiosis may improve these diseases.

It is widely accepted that metabolic diseases, such as obesity and diabetes, are intimately correlated with diet and dysbiosis<sup>[22,33]</sup>. Germ-free (GF) mice do not develop western-diet-induced obesity<sup>[34-36]</sup>. It was also shown in 2006 that colonization of GF

mice with intestinal microbiota from obese mice led to a significantly greater increase in total body fat than colonization with microbiota from lean mice<sup>[21]</sup>. This suggests a strong association between the intestinal microbiota and host metabolism. The intestinal microbiome from obese mice and humans has a significantly higher ratio of Firmicutes to Bacteroidetes (F/B ratio) than that from their lean counterparts<sup>[21,37-40]</sup>. In addition, the bacterial diversity is lower in the microbiota from obese than lean individuals<sup>[39,41]</sup>. However, other studies have shown no difference in the F/B ratio between obese and lean individuals<sup>[42-46]</sup>. Therefore, although the diversity in obese individuals is low compared with that in lean individuals, the correlation between obesity and the F/B ratio is unclear.

There is an increased risk of developing type 2 diabetes in obesity; therefore, dysbiosis might also influence type 2 diabetes. Previous reports have shown that disorder of intestinal carbohydrate metabolism and low-grade gut inflammation cause insulin resistance<sup>[47-49]</sup>. A reduced abundance of short chain fatty acids such as butyrate is associated with type 2 diabetes<sup>[50]</sup>. Vrieze *et al*<sup>[51]</sup> showed that FMT improved insulin resistance in individuals with metabolic syndrome by altered levels of butyrate-producing intestinal bacteria, indicating that gut microorganisms might be developed as therapeutic tools in the future.

RA is a systemic inflammatory disorder including in polyarthritis that leads to joint destruction. Although both genetic and environmental factors are involved in the pathogenesis of RA, intestinal microbiota analysis has recently attracted much attention, along with single nucleotide polymorphism analysis. When mice are reared in GF conditions, arthritis does not develop, indicating that intestinal microbiota is related to onset of arthritis<sup>[28,52-54]</sup>. Abdollahi-Roodsaz *et al*<sup>[53]</sup> showed that interleukin-1 receptor antagonist knockout mice do not spontaneously develop T-cell-mediated arthritis under GF conditions. However, they do develop arthritis under specific-pathogen-free conditions, and monocolonization of the mice with *Lactobacillus bifidus* induces arthritis<sup>[53]</sup>. Matsumoto *et al*<sup>[55]</sup> also showed that K/BxN T-cell receptor transgenic mice develop arthritis under specific-pathogen-free conditions, but not GF conditions, and monocolonization of the mice with segmented filamentous bacteria induces arthritis. Previous studies have shown that composition of the microbiota is altered in early RA<sup>[26,28,56]</sup>. In the preclinical stages of RA, *Prevotella* species such as *Prevotella copri* (*P. copri*) are dominant in the intestine. Maeda *et al*<sup>[28]</sup> showed that microbiota isolated from RA patients whose fecal bacteria contained high levels of *P. copri* contributes to the development of Th17-dependent arthritis, and monocolonization of SKG mice with *P. copri* is sufficient to induce arthritis. Thus, although more precise investigations are needed to determine which bacterium is a target for RA treatment, it is strongly suggested that there are intestinal pathogens that are related to the pathogenesis of human RA.

IBDs are increasing in incidence worldwide<sup>[57]</sup>. Also in Japan, the numbers of IBD patients have rapidly increased over the past 30 years, suggesting that in addition to genetic predisposition, environmental factors such as dysbiosis are more involved in the development of IBDs<sup>[58]</sup>. Various changes in the intestinal microbiota have been reported in IBD patients<sup>[59-61]</sup>. The advent of next-generation sequencing has revealed a range of altered microbiota in the intestine. However, a common problem is that it is unclear whether the dysbiosis observed in IBD patients is a cause or a consequence of intestinal inflammation. Given the complicated relationships between the intestinal immune system and gut microbiota, further studies are needed to elucidate the pathogenesis of IBDs and develop more effective treatments.

## PRIME-BOOST TYPE MUCOSAL VACCINE

Conventional injectable vaccines, including subcutaneous vaccines, have the ability to induce antigen-specific IgG, maintain antigen-specific immune memory, and contribute to prevention of severe infection<sup>[62-64]</sup>. Pediatric vaccination is a key factor in protection against many life-threatening infections<sup>[64]</sup>. However, despite progress in vaccine technology, many infections remain incompletely controlled in both humans and animals worldwide.

Mucosal immune responses are thought to be effective for prevention of infection because foreign antigens, such as microorganisms and food antigens, enter the host through mucosal surfaces<sup>[65-69]</sup>. In the mucosal sites, secretory IgA (SIgA) plays an important role in regulating intestinal health and disease prevention<sup>[70-78]</sup>. The major functions of IgA are (1) prevention of adherence, colonization, and invasion of pathogenic microorganisms that invade the mucosal surface; (2) neutralizing effect on toxins and enzymes produced by pathogenic microorganisms; (3) capturing pathogenic microorganisms in the mucus layer; and (4) antimicrobial activity. Only

limited numbers of mucosal vaccines are available to date; therefore, a new mucosal vaccine strategy is strongly desired for induction of beneficial systemic immune responses.

IgA is the most abundant antibody in mucosal secretory components. In the intestinal mucosa, there are two types of IgA production mechanisms, represented by T-cell-dependent and T-cell-independent immune responses<sup>[79-82]</sup>. In the gut, T-cell-dependent antibody responses are involved in activation of B cells by antigen in the organized lymphoid tissue of Peyer's patches, mesenteric lymph nodes and isolated lymphoid follicles<sup>[82-84]</sup>. It has been shown that both CD40L and transforming growth factor- $\beta$ 1 are essential for the induction of T-cell-dependent IgA class switching<sup>[85]</sup>. In contrast, T-cell-independent IgA class switch recombination occurs in B1 cells of the gut-associated lymphoid tissue (GALT), where IgA is constitutively induced by stimulation with commensal bacteria<sup>[82]</sup>.

GALT, such as Peyer's patches and isolated lymphoid follicles, is the primary site for IgA induction<sup>[86,87]</sup>. It has been reported that antigen-specific IgA-producing B cells develop in GALTs with the aid of GALT-dendritic cells (DCs). It is notable that retinoic acid synthesized by GALT-DCs can contribute to IgA synthesis<sup>[87-89]</sup>. GALT-DCs are also able to imprint gut-homing chemokine receptors such as  $\alpha$ 4 $\beta$ 7 integrin and C-C chemokine receptor type 9 on B and T cells, which is an essential process for lymphocyte migration to the intestines<sup>[90]</sup>.

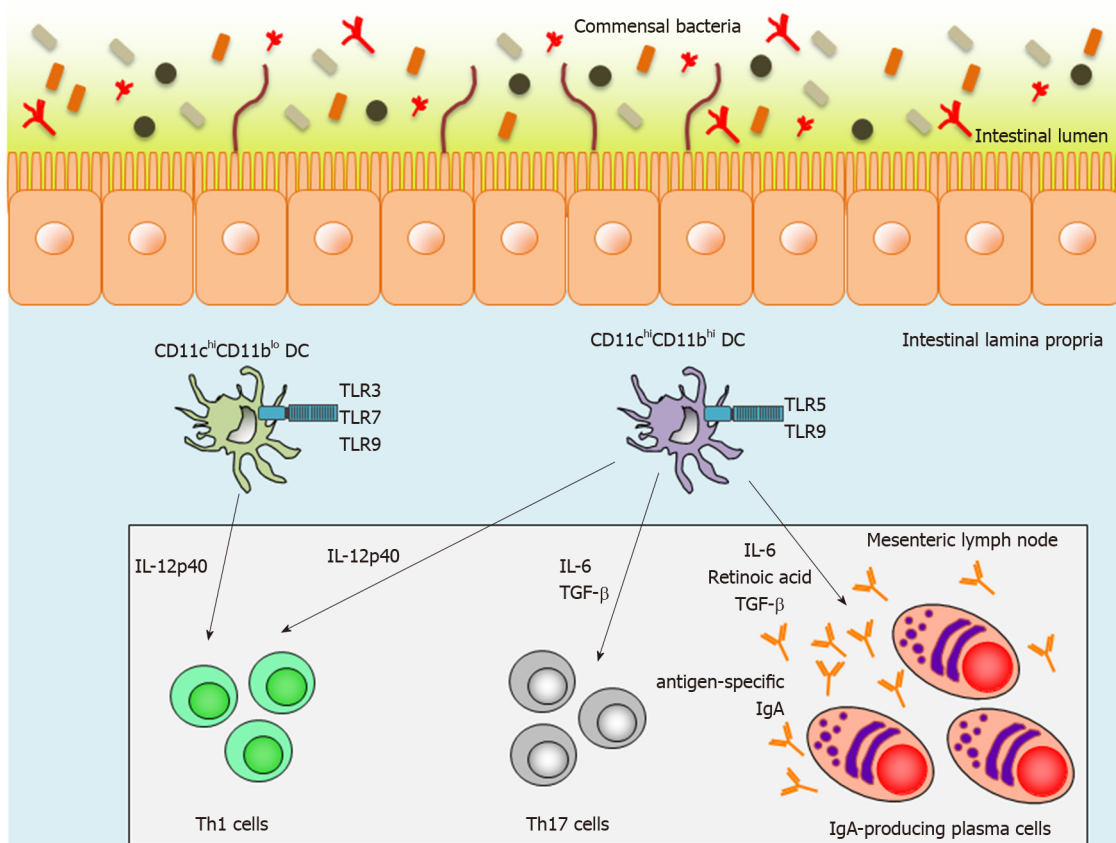
Intestinal lamina propria DCs (LPDCs) are also crucial inducers of SIgA-producing B cells in a T-cell-independent manner. We have previously reported two subsets of small-intestinal LPDCs based on their differential CD11c and CD11b expression patterns: CD11c<sup>hi</sup>CD11b<sup>lo</sup> LPDCs and CD11c<sup>hi</sup>CD11b<sup>hi</sup> LPDCs<sup>[91-93]</sup> (Figure 1). CD11c<sup>hi</sup>CD11b<sup>hi</sup> intestinal LPDCs express the gene encoding the retinoic-acid-converting enzyme, *Raldh2*, and are able to induce antigen-specific SIgA as well as systemic immunity mediated by Toll-like receptor (TLR) 5 or 9 stimulation<sup>[91]</sup> (Figure 1). In contrast to CD11c<sup>hi</sup>CD11b<sup>hi</sup> LPDCs, CD11c<sup>hi</sup>CD11b<sup>lo</sup> LPDCs express TLR3, TLR7 and TLR9, which recognize dsRNA, ssRNA, and CpG oligodeoxynucleotides (ODNs), respectively<sup>[93]</sup> (Figure 1). They do not express *Raldh2* and are not involved in IgA synthesis in the small-intestinal lamina propria<sup>[93]</sup>. In addition, high titers of antigen-specific IgA were detected in fecal extracts from antigen-loaded CD11c<sup>hi</sup>CD11b<sup>hi</sup> LPDC-immunized mice<sup>[93]</sup>. Accordingly, CD11c<sup>hi</sup>CD11b<sup>hi</sup> LPDCs are considered to be an ideal target for a mucosal vaccine, but it has thus far been technically difficult to induce antigen-specific mucosal immunity using conventional injectable vaccines.

We have recently reported that splenic DCs stimulated with both curdlan, dextrin-1 ligand, and CpG-ODN, TLR9 ligand, successfully induced antigen-specific fecal IgA as well as antigen-specific serum IgG and splenic Th1 and Th17 responses in mice<sup>[94]</sup>. This indicates that combination of curdlan and CpG-ODN is available as an adjuvant of parenteral vaccination to induce broad functional immunity against mucosal antigens. We found that intramuscular immunization with the combination of curdlan and CpG-ODN emulsified with incomplete Freund's adjuvant induced antigen-specific fecal IgA as well as serum IgG and splenic Th1 and Th17 responses<sup>[94]</sup> (Figure 2). However, although antigen-specific IgG in serum was continuously detected after prime injection, antigen-specific IgA production in feces was only transiently detected by parenteral immunization with curdlan + CpG-ODN<sup>[94]</sup>. Therefore, additional immunization, for example, boosting, to induce more durable mucosal immunity at targeted mucosal sites is thought to be necessary. We have demonstrated that after oral, nasal or vaginal antigen administration, high titers of long-lasting antigen-specific intestinal, lung or vaginal IgA are inducible<sup>[94]</sup> (Figure 2). Also, this prime-boost vaccine is effective against cholera-toxin-induced diarrhea and *Streptococcus pneumoniae* (*S. pneumoniae*) infection<sup>[94]</sup>. Thus, we established intramuscular antigen injection adjuvanted with curdlan + CpG-ODN and subsequent antigen administration on target mucosal sites (prime-boost vaccination) as a new vaccine strategy capable of inducing strong and durable systemic and mucosal immunity.

## FUTURE REGULATION OF DYSBIOSIS-ASSOCIATED DISORDERS

Intestinal dysfunction has been correlated with multifactorial diseases<sup>[9]</sup>, suggesting that the mucosal immune responses provide a solid causal link between pathological symptoms in the host and disease-associated dysbiosis. Several studies have identified some pathobionts, such as *Clostridium ramosum* (*C. ramosum*)<sup>[95]</sup>, *P. copri*<sup>[26,28]</sup>, *Helicobacter pylori*<sup>[96]</sup>, adherent invasive *Escherichia coli*<sup>[97]</sup>, *Clostridium scindens*<sup>[98]</sup>, and *Enterococcus gallinarum*<sup>[99]</sup>. Therefore, regulating the function of disease-associated



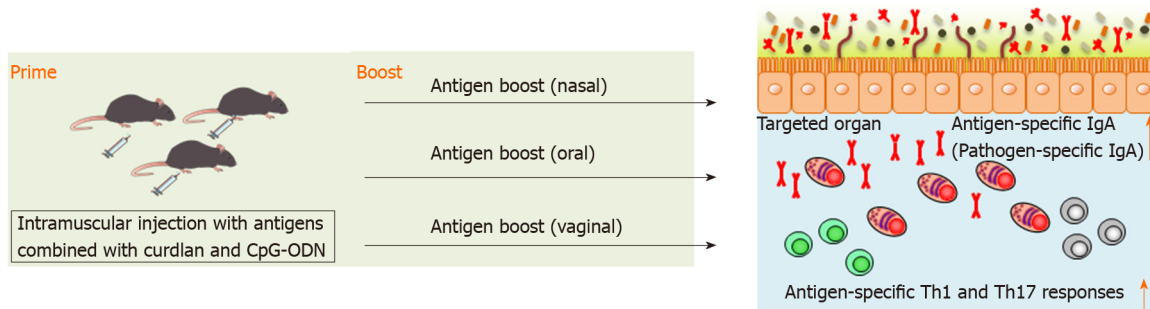


**Figure 1 Function of two distinct lamina propria dendritic cells in the small intestine.** Mouse small-intestinal lamina propria dendritic cells (LPDCs) are divided into two subsets on the basis of CD11c and CD11b expression. CD11c<sup>hi</sup>CD11b<sup>lo</sup> LPDCs express Toll-like receptor (TLR) 3, TLR7 and TLR9, whereas CD11c<sup>hi</sup>CD11b<sup>hi</sup> LPDCs express TLR5 and TLR9. After TLR stimulation, activated CD11c<sup>hi</sup>CD11b<sup>lo</sup> LPDCs can produce interleukin (IL)-12p40, IL-6, transforming growth factor-β and retinoic acid, and subsequently induce antigen-specific Th1 and Th17 responses and antigen-specific IgA-producing plasma cells. In contrast to CD11c<sup>hi</sup>CD11b<sup>hi</sup> LPDCs, activated CD11c<sup>hi</sup>CD11b<sup>lo</sup> LPDCs can induce antigen-specific Th1 responses, but not antigen-specific Th17 responses and antigen-specific-IgA-producing plasma cells. TLR: Toll-like receptor; TGF: Transforming growth factor; IL: Interleukin; DC: Dendritic cell.

pathobionts can lead to prevention or treatment of dysbiosis-related disorders. However, antibiotics are not suitable for eliminating only pathogens because they have the possibility to induce dysbiosis or multidrug-resistant bacteria<sup>[100]</sup>.

*C. ramosum* is an obligate anaerobic bacterium first identified in an appendicitis patient in 1898 and widely inhabits the human gastrointestinal tract. Increased levels of *C. ramosum* are associated with human obesity and diabetes<sup>[20,23]</sup>. *C. ramosum* is also associated with clinical symptoms of metabolic disorders in gnotobiotic mice colonized with *C. ramosum* alone and a simplified human intestinal microbiome containing *C. ramosum*. Furthermore, it has been shown that the numbers of *C. ramosum* are higher in mice fed a high-fat compared with normal-fat diet, and this results in increased expression of *Slc2a2* in the small-intestinal mucosa<sup>[95]</sup>. Therefore, we recently applied our prime-boost vaccination to control *C. ramosum*-mediated diseases. Our vaccine for *C. ramosum* significantly inhibited body weight gain and the increased levels of *C. ramosum* in the intestinal mucosa under a high-fat diet<sup>[94]</sup>. It also resulted in decreased expression of *Slc2a2* and subsequently ameliorated glucose intolerance<sup>[94]</sup>. It is notable that this immunization strategy did not induce dysbiosis<sup>[94]</sup>. Thus, it might be effective for preventing *C. ramosum*-associated obesity and diabetes.

Until now, there have been few methods that can induce high titers of antigen-specific IgA at target mucosal sites using an injection-type mucosal vaccine. It is noteworthy that we have developed a next-generation prime-boost mucosal vaccine using curdlan and CpG-ODN, and used it for control of diseases such as *S. pneumoniae* infection, and other diseases mediated by intestinal bacteria<sup>[94]</sup>. With the advent of gnotobiotic technology, function of the intestinal microbiome has been revealed. However, since there are few methods for specifically attenuating the function of intestinal bacteria, many diseases mediated by intestinal bacteria are still not fully elucidated. Our vaccination is the world's first immunization strategy, and has the potential to be an excellent technique for functional analysis of intestinal bacteria.



**Figure 2 Scheme of antigen-specific immune responses by prime-boost vaccination.** Parenteral immunization with antigen emulsified in curdlan and CpG-oligodeoxynucleotides induces antigen-specific fecal IgA as well as serum IgG and splenic Th1 and Th17 responses. Once primed, high titers of long-lasting antigen-specific lung, intestinal, or vaginal IgA are induced after nasal, oral, or vaginal antigen administration, respectively. Also, antigen-specific Th1 and Th17 responses are induced at the targeted organs. CpG-ODN: CpG oligodeoxynucleotides.

## CONCLUSION

As the link between various diseases and aberrant intestinal microbiota becomes apparent, there is an urgent need to develop and disseminate control strategies for dysbiosis in addition to existing effective treatments. Antibiotics are not specific to pathobionts and may induce dysbiosis that can lead to disease. Attempts have also been made to control diseases mediated by intestinal bacteria using FMT or probiotic treatments, but these are established and effective treatments. An important treatment for diseases mediated by intestinal bacteria is to improve the underlying disease without inducing new dysbiosis. Vaccination with curdlan + CpG-ODN and antigens and subsequent antigen administration can effectively induce antigen-specific systemic and mucosal immunity. This prime-boost vaccine method has been patented in Japan and prime-boost vaccines targeting various infectious diseases are being developed for future human prescription. There is no doubt that the vaccine technology discussed in this review will become a new treatment in the next generation of antimicrobial strategies. Further analysis of the gut microbiota is necessary, but we are eagerly looking forward to developing pathobiont-specific treatments for human diseases in the future.

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## Gut microbiome in primary sclerosing cholangitis: A review

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### Abstract

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by biliary inflammation and stricturing. Exploration of the pathogenesis of PSC in light of its association with inflammatory bowel disease (IBD) and the “gut-liver” axis is an emerging area of interest. A growing number of studies have begun to elucidate the role of the gut microbiota, its metabolites and its influence on host immune responses in the development of PSC and PSC-IBD. Studies of the fecal microbiota have highlighted enriched levels of certain species, including *Veillonella*, *Streptococcus* and *Enterococcus*, among others. A heightened immune response to enteric dysbiosis and bacterial translocation have also been implicated. For example, *Klebsiella pneumoniae* strains derived from gnotobiotic mice transplanted with PSC-IBD microbiota were found to induce pore formation in human intestinal epithelial cells and enhanced Th17 responses. Gut microbes have additionally been hypothesized to be implicated in PSC pathogenesis through their role in the synthesis of various metabolites, including bile acids (BAs), which function as signaling molecules with important gut and hepatic effects. An expanded knowledge of the gut microbiome as it relates to PSC offers critical insight into the development of microbe-altering therapeutic interventions, such as antibiotics, nutritional interventions and fecal microbial transplantation. Some of these have already shown some preliminary evidence of benefit. Despite exciting progress in the field, much work remains to be done; areas that are particularly lacking include functional characterization of the microbiome and examination of pediatric populations. In this review, we summarize studies that have investigated the microbiome in PSC and PSC-IBD as well as putative mechanisms, including the potential role of metabolites, such as BAs. We then briefly review the evidence for interventions with microbe-altering properties for treating PSC.

**Key words:** Bile acids; Colitis; Inflammatory bowel disease; Microbiome; Microbiota; Primary sclerosing cholangitis

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**Core tip:** The frequent coexistence of primary sclerosing cholangitis (PSC) and inflammatory bowel disease (IBD) points to the gut-liver axis as central to pathogenesis. The gut microbiome is hypothesized to be involved. A growing body of literature supports that PSC and PSC-IBD are associated with a distinct gut microbiome and more recent animal studies suggest a potential causal relationship. Microbial metabolites, such as bile acids, may mediate the effects of the gut microbiota in PSC. A sound understanding of the PSC microbiome has the potential to inform the development of microbe-altering therapeutic interventions.

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## INTRODUCTION

Primary sclerosing cholangitis (PSC), a cholestatic liver disease that causes inflammation and fibrosis of the biliary tree, represents a pressing unmet need in the field of Hepatology<sup>[1]</sup>. Although a rare disease in the general population (prevalence 0-16 per 100000 adults<sup>[1]</sup>, and 1.5 per 100000 children)<sup>[2]</sup>, a striking association exists between PSC and inflammatory bowel disease (IBD); approximately 75% of adults and children with PSC have IBD<sup>[3,4]</sup>, and conversely, up to 8% of IBD patients are found to have large duct PSC when screening magnetic resonance cholangiopancreatography is applied<sup>[5]</sup>. The natural history of PSC is progression to end-stage biliary cirrhosis requiring liver transplantation (LT); in adults, the median LT-free survival after diagnosis is 14.5 years<sup>[6]</sup>, and 30% of children require a LT by 10 years after diagnosis<sup>[4]</sup>. Furthermore, PSC is associated with a markedly increased risk of colorectal malignancy (20%-30% lifetime risk, four times higher than colitis without PSC) and hepatobiliary malignancy, particularly cholangiocarcinoma, which has a 20% lifetime risk and poor prognosis<sup>[7]</sup>. To date, no medical therapy has been shown to alter the natural history of PSC. The significance of PSC is underscored by the fact that it has been identified as the only predictor of premature mortality in IBD populations<sup>[8]</sup>. Even in the pediatric age range, it is a significant risk factor for cancer-related mortality<sup>[9]</sup>.

## PSC-IBD: A UNIQUE IBD PHENOTYPE

There are now abundant data to support that IBD occurring in association with PSC ("PSC-IBD") is distinct from conventional IBD not associated with PSC. A robust body of adult literature supports a distinct PSC-IBD clinical phenotype, characterized by a higher frequency of pancolitis (typically worse in the right colon, contrary to chronic ulcerative colitis (UC), which is most severe distally in the left colon), rectal sparing and backwash ileitis<sup>[3,10,11]</sup>. In addition, subclinical inflammation (mucosal disease in the presence of no or minimal symptoms) has been described and is hypothesized to contribute to the heightened neoplastic potential<sup>[12]</sup>. In a large retrospective review, we recently demonstrated that a similar phenotype is also observed in children<sup>[13]</sup>. In addition to the unique clinical phenotype of PSC-IBD, there is relatively little genetic overlap between PSC-IBD and conventional IBD, further supporting that PSC-IBD is a distinct entity<sup>[14]</sup>. This growing body of literature is important for several reasons; it highlights the need to conceptualize (and investigate) PSC-IBD as separate from IBD and the characteristic gut phenotype may provide insight into underlying pathogenesis.

## GUT-LIVER AXIS IN PSC

Although the pathogenesis of PSC remains obscure, the striking association between PSC and IBD points to the gut-liver axis as integrally involved. While strong HLA



associations and greater than 23 non-HLA susceptibility loci have been identified, known genetic risk factors currently account for less than 10% of PSC liability, highlighting the likely critical role of environmental factors<sup>[15]</sup>, chief among them the gut microbiota. The microbiota can be considered an environmental factor on its own, but is also influenced by other environmental factors, such as diet, medications, hygiene and even mood<sup>[16]</sup>. A theory of PSC pathogenesis invoking the gut microbiota has long been postulated. The basic hypothesis is that gut microbes (or their products) translocate across a leaky gut barrier (due to inflammation) and access the liver *via* the portal circulation, where they trigger inflammatory and ultimately fibrotic processes. In the 1990s, Lichtman *et al*<sup>[17,18]</sup> provided the first compelling support for this hypothesis, showing that small bowel bacterial overgrowth, achieved using a blind jejunal loop, induced cholangiographic changes resembling PSC in rats. Moreover, these changes were reversible with antibiotics and peptidoglycan-degrading enzymes, but not prednisone or ursodiol. Since then, numerous additional findings have reinforced the role of the gut microbiota and its interactions with innate immune responses in PSC, including the identification of FUT2 (involved in handling translocated bacteria) as a PSC risk locus<sup>[14]</sup>, and the observation that lipo-polysaccharide levels correlate with LT-free survival in PSC<sup>[19]</sup>.

## GUT MICROBIOTA IN PSC AND PSC-IBD

In the past five years, there has been a flurry of publications aimed at characterizing the gut microbiota in PSC/PSC-IBD compared to healthy controls (HCs) and IBD populations. There have been seven publications (four full-length papers<sup>[20-23]</sup>, three letters<sup>[24-26]</sup>) on the fecal microbiota, and five (four full-length papers<sup>[27-30]</sup>, one letter<sup>[31]</sup>) on the mucosal microbiota in PSC. To date, only one study, a letter investigating the fecal microbiota, examined a pediatric population (in Japan)<sup>[26]</sup>. All used 16S rRNA methods to establish microbial composition. These studies are summarized in Table 1. The findings are further summarized in Figure 1, which illustrates microbial taxa that are enriched and depleted in PSC compared to HCs and IBD patients.

In interpreting studies of the microbiota, it is important to appreciate that microbial populations differ substantially between the gut lumen and mucosal surfaces. For example, in a large study of new-onset pediatric Crohn's disease (CD), mucosal-associated dysbiosis was only weakly reflected in stool, and mucosal samples were superior to fecal samples for distinguishing CD from healthy controls<sup>[32]</sup>. It is also important to note that studies of the mucosal microbiota (compared to the fecal microbiota) may be susceptible to greater variation stemming from differences in sampling methods (biopsies, brushings, luminal washes, *etc.*) and other procedural factors.

Indeed, studies of the fecal microbiome in PSC are more consistent (than those of the mucosal microbiome) and they reveal a few themes. The first is that the gut bacterial alpha diversity is lower in PSC than HCs (in contrast, studies comparing alpha diversity between PSC and IBD cohorts have yielded variable findings). The second is that, although studies are not entirely consistent in terms of the specific microbes altered in PSC, they are consistent in demonstrating that the overall bacterial community is different in PSC/PSC-IBD *vs* HCs and IBD. The third is that, despite this heterogeneity, a few bacterial taxa are fairly consistently altered in the stool of PSC patients compared to HCs. *Veillonella*, in particular, was higher in the stool of PSC patients than HCs in all the studies in Table 1. *Enterococcus*, *Streptococcus* and *Lactobacillus* are also frequently enriched, as are members of the Proteobacteria phylum, such as *E. coli*. Several studies also support a relative depletion of short chain fatty acid (SCFA)-producing Firmicutes, such as *Faecalibacterium* and *Coproccoccus*, in PSC.

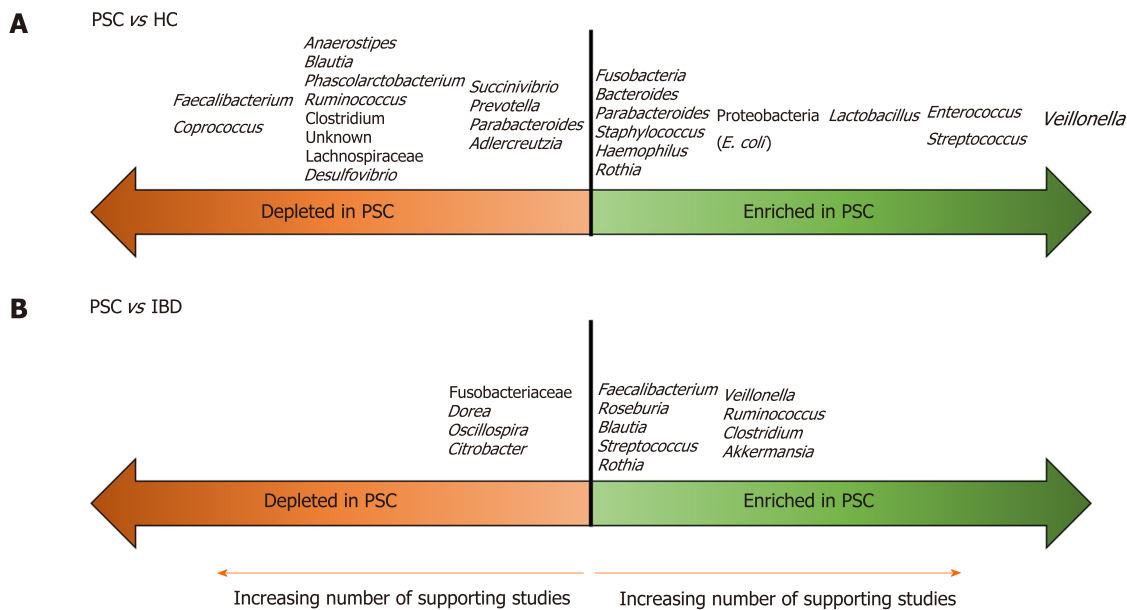
In one study, *Veillonella* was 4.8-fold higher in individuals with PSC compared to HCs and displayed a positive correlation with the Mayo Risk Score (a PSC prognostic index). Moreover, *Veillonella* discriminated PSC from HCs with an area under the receiver operator characteristic curve (AUC) of 0.64<sup>[21]</sup>. This improved to 0.78 when other PSC-associated genera were added. Interestingly, *Veillonella* has been associated with pulmonary fibrotic conditions and CD recurrence post resection<sup>[33,34]</sup>. It is tempting to speculate that *Veillonella* directly contributes to fibrogenesis in PSC, but such evidence is lacking. *Veillonella* is not specific to PSC either; it is observed in a broad range of liver diseases, such as primary biliary cirrhosis, hepatitis B cirrhosis and hepatic encephalopathy, and may therefore reflect the common end-stage of multiple hepatic pathologies<sup>[35-37]</sup>. In another study by Sabino and colleagues, *Enterococcus*, *Lactobacillus* and *Fusobacteria* were overrepresented in PSC patients and a model including these three microbes correctly classified PSC 95% and 71% of the

Table 1 Studies of the fecal or mucosal microbiota in primary sclerosing cholangitis

Ref.	Population	Sample source	Methods	Taxa depleted in PSC	Taxa enriched in PSC	Diversity in PSC
Adult						
Mucosal						
Quraishi <i>et al</i> <sup>[30]</sup> , 2020	10 PSC-IBD 10 UC; 10 HC	Mucosal biopsies, sigmoid	16S rRNA; V4 amplified Illumina MiSeq	<i>vs</i> HC: Lachnospiraceae  <i>vs</i> UC: 26 taxa, including Lentisphaerae, Gammaproteobacteria, Enterobacteriaceae, Prevotellaceae, Paraprevotellaceae, Coriobacteriaceae, Erysipelotrichaceae, Desulfovibrionaceae, Myxococcales, Streptococcus, Blautia	<i>vs</i> HC: Bacilli, Pseudomonas, Streptococcus, Haemophilus parainfluenzae  <i>vs</i> UC: 24 taxa, including Bacilli, Staphylococcus, Parvimonas sp., Bacteroides fragilis, Roseburia spp., Shewanella spp., Clostridium ramosum, Sphingomonas sp., Actinomyces, Rothia	Alpha: No difference; Beta: All 3 groups significantly different from each other
Quraishi <i>et al</i> <sup>[31]</sup> , 2017 (Letter)	11 PSC-IBD 10 IBD 9 HC	Mucosal biopsies, ascending, transverse, descending colon (results not different by site)	16S rRNA; V3-V4 amplified; Illumina MiSeq	<i>vs</i> HC: Prevotella, Roseburia, Bacteroides	<i>vs</i> HC: Escherichia, Lachnospiraceae, Megaspheara	Beta: Different from HC and IBD
Torres <i>et al</i> <sup>[27]</sup> , 2016	13 PSC-UC; 6 PSC-CD; 1 PSC only; 13 UC; 2 CD; 9 HC	Mucosal biopsies from terminal ileum, right and left colon (results not different by site)	16S rRNA; V3-V4 amplified; Illumina MiSeq	<i>vs</i> IBD	<i>vs</i> IBD: Barnesiellaceae, Blautia, Ruminococcus obeum	Alpha: Similar to HC and IBD; Beta: Similar to HC and IBD
Kevans <i>et al</i> <sup>[28]</sup> , 2016	31 PSC-UC; 56 UC; 0 HC	Mucosal biopsies, left colon	16S rRNA; V4 amplified; Illumina MiSeq	Nil after multiple testing adjustment	Nil after multiple testing adjustment	Alpha: ↓ <i>vs</i> UC (in 1 cohort only); Beta: No difference
Rossen <i>et al</i> <sup>[29]</sup> , 2015	8 PSC-UC; 4 PSC-CD; 11 UC; 9 HC	Mucosal biopsies from ileocecum	16S rRNA using HITChip	<i>vs</i> HC: Uncultured Clostridiales II  <i>vs</i> UC: Uncultured Clostridiales II	<i>vs</i> HC  <i>vs</i> UC	Alpha: ↓ <i>vs</i> HC; Beta: Similar to HC and IBD
Fecal						
Lemoinne <i>et al</i> <sup>[38]</sup> , 2019	16 PSC-UC/IBD-U; 11 PSC-CD; 22 PSC only; 33 IBD; 30 HC	Stool	16S rRNA; V3-V4 amplified; Illumina MiSeq	<i>vs</i> HC: Ruminococcus, Faecalibacterium, Lachnospiraceae, Blautia	<i>vs</i> HC: Veillonella, Sphingomonadaceae, Alphaproteobacteria, Rhizobiales	Alpha: ↓ <i>vs</i> HC and IBD; Beta: PSC-IBD different from HC and IBD, PSC-IBD similar to PSC only
Rühlemann <i>et al</i> <sup>[20]</sup> , 2019	75 PSC-IBD; 62 PSC only; 118 UC; 133 HC	Stool	16S rRNA; V1-V2 amplified MiSeq	<i>vs</i> HC: CoprococcusHoldemania, Desulfovibrio, Faecalibacterium, Clostridium IV  <i>vs</i> UC  PSC-IBD <i>vs</i> PSC only: Bilophila and Bacteroides OTU ↓	<i>vs</i> HC: Veillonella, Streptococcus, Lactobacillus, Enterococcus, Proteobacteria (Gammaproteobacteria), Lactobacillales (Bacilli), Parabacteroides, Bacteroides spp.  <i>vs</i> UC: Firmicutes  PSC-IBD <i>vs</i> PSC only: Nil	Norwegian cohort; Alpha: ↓ <i>vs</i> HC, similar to UC; Beta: Different from HC and IBD; German cohort; Alpha: Similar to HC, ↑ <i>vs</i> UC; Beta: Different from HC and IBD, PSC-IBD similar to PSC only
Rühlemann <i>et al</i> <sup>[25]</sup> , 2017 (Letter)	38 PSC-UC; 35 PSC only; 88 UC; 98 HC	Stool	16S rRNA; V1-V2		<i>vs</i> HC: Veillonella (no difference compared to UC)	Beta: Different from HC and IBD, PSC-IBD similar to PSC only

Kummen <i>et al</i> <sup>[21]</sup> , 2017	44 PSC-UC; 11 PSC-CD; 30 PSC only; 36 UC; 263 HC	Stool	16S rRNA; V3-V4 amplified; Illumina MiSeq	<p><i>vs</i> HC: ML615J-28 unknown genus, <i>Succinivibrio</i>, <i>Desulfovibrio</i>, RF32 unknown genus, <i>Phascolarctobacterium</i>, <i>Coproccoccus</i>, Lachnospiraceae unknown genus, Christensenellaceae unknown genus, Clostridiales unknown genus, YS2 unknown genus, S24.7 unknown genus</p> <p><i>vs</i> UC: <i>Dorea</i>, <i>Oscillospira</i>, <i>Citrobacter</i></p> <p>PSC-IBD <i>vs</i> PSC only: Nil</p>	<p><i>vs</i> HC: <i>Veillonella</i> (<i>V. dispar</i>, <i>V. parvula</i>)</p> <p><i>vs</i> UC: <i>Veillonella</i> (<i>V. dispar</i>, <i>V. parvula</i>); <i>Akkermansia</i>, <i>Clostridium</i>, <i>Ruminococcaceae</i></p> <p>PSC-IBD <i>vs</i> PSC only: Nil</p>	Alpha: ↓ <i>vs</i> HC, similar to IBD; Beta: Different from HC and IBD, PSC-IBD similar to PSC only
Bajer <i>et al</i> <sup>[22]</sup> , 2017	32 PSC-IBD; 11 PSC only; 32 UC 31 HC	Stool	16S rRNA; V4 amplified Illumina MiSeq	<p><i>vs</i> HC: <i>Coproccoccus</i> (<i>C. catus</i>), unidentified Lachnospiraceae genera, <i>Faecalibacterium prausnitzii</i>, <i>Ruminococcus gnavus</i>, <i>Prevotella copri</i> (PSC only and PSC-IBD), <i>Phascolarctobacterium</i> (PSC-IBD); <i>Adlercreutzia equolifaciens</i> (PSC only)</p> <p><i>vs</i> UC: Fusobacteriaceae</p>	<p><i>vs</i> HC: <i>Rothia</i>, <i>Enterococcus</i>, <i>Streptococcus</i>, <i>Clostridium</i>, <i>Veillonella</i>, <i>Haemophilus</i> (PSC only and PSC-IBD); <i>Staphylococcus</i>, <i>Coprobacillus</i>, <i>Escherichia</i>, <i>Corynebacterium</i>, <i>Lactobacillus</i> (PSC-IBD)</p> <p><i>vs</i> UC: <i>Rothia</i>, <i>Streptococcus</i>, <i>Veillonella</i>, <i>Blautia</i>; <i>Akkermansia muciniphila</i>, <i>Clostridium colinum</i></p>	Alpha: Similar to HC and UC; Beta: Different from HC, PSC-IBD different from IBD, PSC-IBD similar to PSC only
Sabino <i>et al</i> <sup>[23]</sup> , 2016	18 PSC only; 27 PSC-UC; 21 PSC-CD; 13 UC; 30 CD; 66 HC	Stool	16S rRNA; V4 amplified; Illumina MiSeq	<p><i>vs</i> HC: Firmicutes <i>Anaerostipes</i></p> <p>PSC-IBD <i>vs</i> PSC only: Nil</p>	<p><i>vs</i> HC: Bacteroidetes, Fusobacteria, <i>Streptococcus</i>, <i>Enterococcus</i>, <i>Lactobacillus</i>, <i>Fusobacterium</i>, <i>Veillonella</i><sup>1</sup></p> <p>PSC-IBD <i>vs</i> PSC only: Nil</p>	Alpha: ↓ <i>vs</i> HC; Beta: Different from HC, PSC-UC different from UC, PSC-IBD similar to PSC only
In a re-analysis by Vieira-Silva <i>et al</i> <sup>[24]</sup> in 2019, <i>Fusobacterium</i> was associated with intestinal inflammation and <i>Enterococcus</i> with cholangitis/biliary obstruction.						
Pediatric						
Iwasawa <i>et al</i> <sup>[26]</sup> , 2017 (Letter)	27 PSC; 16 UC; 23 HC	Stool	16S rRNA; 1-V2 amplified	<p><i>vs</i> HC: <i>Parabacteroides</i> (<i>P. distasonis</i>), <i>Anaerostipes hadrus</i>, <i>Blautia obeum</i></p> <p><i>vs</i> UC</p>	<p><i>vs</i> HC: <i>Enterococcus faecium</i>, <i>Enterococcus</i> sp. NBRC 107345, <i>Streptococcus parasanguinis</i>, <i>Veillonella</i> sp. 3_1_44</p> <p><i>vs</i> UC: <i>Faecalibacterium</i>, <i>Ruminococcus</i>, <i>Roseburia</i></p>	Alpha: ↓ <i>vs</i> HC, ↑ <i>vs</i> IBD; Beta: Different from HC and IBD

<sup>1</sup>Significant only when patients with cirrhosis included. CD: Crohn's disease; HC: Healthy control; IBD: Inflammatory bowel disease; IBD-U: Inflammatory bowel disease-unclassified; PSC: Primary sclerosing cholangitis; UC: Ulcerative colitis.



**Figure 1** Enriched and depleted taxa in primary sclerosing cholangitis compared to healthy controls and inflammatory bowel disease. A: Primary sclerosing cholangitis (PSC) vs healthy controls; B: PSC vs inflammatory bowel disease. Taxa farther from the midline are supported by a larger number of studies. PSC: Primary sclerosing cholangitis; HC: Healthy controls; IBD: Inflammatory bowel disease.

time in training and validation cohorts<sup>[23]</sup>. An *Enterococcus* operational taxonomic unit (OTU) correlated with alkaline phosphatase (ALP), a marker of PSC severity. More recently, this dataset was reanalyzed while accounting for stool moisture variation; *Fusobacterium* was found to be associated with intestinal inflammation severity, while *Enterococcus* was associated with biliary pathology<sup>[24]</sup>. High-*Veillonella* stool was observed across all disease groups in this study, whereas high-*Fusobacteria* stool was specific to CD or PSC-CD. In a recent study by Rühlemann *et al*<sup>[20]</sup>, which investigated two geographically distinct cohorts (Germany and Norway), the fecal microbial composition identified PSC with AUC 0.88. These data, coupled with those presented above, highlight the possible utility of microbial signatures as prognostic and/or diagnostic markers in PSC. Rather than simply characterize the gut microbiota, Lemoine and colleagues examined alterations in the network of correlations between bacteria in PSC patients; they found that compared to HCs and IBD patients, individuals with PSC had a decreased density of bacterial network correlations<sup>[38]</sup>.

The 2019 study by Rühlemann *et al*<sup>[20]</sup> is important because it showed that the microbial alterations in PSC are independent from associated colitis; there were no significant differences between patients with PSC with or without concomitant IBD in beta diversity and taxonomic differences were few, limited to decreased *Bilophila* and a *Bacteroides* OTU in PSC-IBD. Similarly, several other studies from Table 1 support that the microbial findings in PSC are independent of IBD<sup>[21-23]</sup>. All of this ties in to an additional important theme, which is that the presence of IBD appears to have little effect on the composition of the gut microbiota in PSC patients, suggesting that it is PSC that drives the primary changes in microbial composition. Therefore, in addition to a distinct clinical phenotype and genetic architecture, PSC-IBD is characterized by a distinct gut microbiome compared to IBD without associated PSC.

Studies of the mucosal microbiome in PSC have been fewer in number and less consistent. Two studies found little to no changes between PSC patients and HCs<sup>[28,29]</sup>, while others showed some features congruent with the fecal studies, including increased Bacilli, such as *Streptococcus*, and *Proteobacteria* (*E. coli*)<sup>[30,31]</sup>. A very recently published pilot study by Quraishi *et al*<sup>[30]</sup> warrants further discussion as it is the first to apply an integrative biology approach to the joint analysis of the gut microbiome, intestinal gene expression and immune cell signatures in PSC-IBD compared to UC and HCs. In this study, the microbial alterations in PSC-IBD *vs* UC (and inferred metagenomics) and the differentially expressed genes between these two groups implicated dysregulation of bile acid (BA) metabolism in PSC-IBD. Moreover, multi-omics integration revealed networks involved in bile acid homeostasis and cancer regulation in PSC-IBD. Other upregulated pathways were related to glucuronidation. Interestingly, amine oxidase-expressing bacteria, such as *Sphingomonas sp.*, were also increased in PSC-IBD *vs* UC; this enzyme is associated with aberrant homing of gut



lymphocytes to the liver, which is postulated to be involved in PSC pathogenesis.

While the focus has undoubtedly been on bacteria to date, investigators are beginning to examine the gut mycobiome in PSC as well. In the first such study, which examined fecal fungal profiles in 112 individuals, PSC patients were found to have fungal gut dysbiosis, characterized by altered composition and increased biodiversity (alpha diversity), compared to patients with IBD only and HCs<sup>[38]</sup>. In particular, an increase in the abundance of *Exophiala* genus and decrease of *Saccharomyces cerevisiae* were noted. In addition, the fungal microbiota of PSC patients displayed a disrupted correlation network with bacteria; a disrupted correlation network between bacteria and fungi has also been reported in patients with cirrhosis<sup>[39]</sup>.

While the above studies represent an important first step along the path toward elucidating the role of the gut microbiome in PSC, they have important limitations. These include their cross-sectional nature and lack of functional data (through metagenomics, proteomics, metatranscriptomics and metabolomics investigations). Furthermore, there is a paucity of pediatric data. Children, particularly if sampled close to diagnosis, offer the important advantage of temporal proximity to disease origin and relatively fewer comorbidities, which may help to disentangle cause from consequence and minimize confounding variables.

## EMERGING EVIDENCE SUPPORTING A CAUSAL ROLE FOR THE MICROBIOME

Until recently, PSC microbiota studies have been largely associational. In the past few years, however, a handful of elegant animal experiments have yielded more persuasive support for a causal link, while simultaneously shedding light on the mechanisms by which pathobionts might interface with host immune responses to cause hepatobiliary injury in PSC<sup>[40]</sup>. Three of these recent studies have been very informative<sup>[41-43]</sup>. All three support the concepts of dysbiosis, bacterial translocation across the gut barrier, and heightened immune responses (adaptive or innate) in PSC pathogenesis. While the term “dysbiosis” is difficult to specifically define, in these studies, it generally denotes differences in the composition of the gut microbiota, as it relates to diversity and relative abundance of specific taxa, compared to a control group<sup>[44]</sup>. Importantly, two of these studies showed transferability of the PSC phenotype with fecal microbiota transplant (FMT)<sup>[41,42]</sup>. Specifically, Nakamoto *et al*<sup>[41]</sup> demonstrated that inoculation of germ-free (GF) mice with feces from patients with PSC-UC was associated with Th17 priming in the liver and increased susceptibility to hepatobiliary injury by diethyldithiocarbamate. Investigators isolated *Klebsiella pneumoniae*, *Proteus mirabilis* and *Enterococcus gallinarum* from the mesenteric lymph nodes of these animals. Specific *K. pneumoniae* strains were found to induce pore formation on human intestinal epithelial organoids, providing a putative mechanism for bacterial translocation, as further supported by the observation that transferring non-pore-inducing strains resulted in lower serum endotoxin levels and decreased Th17 responses. An imbalance between Th17 and T regulatory (Treg) responses has previously been implicated in PSC<sup>[45,46]</sup>. Tedesco *et al*<sup>[43]</sup> and Liao *et al*<sup>[42]</sup> both investigated MDR2<sup>-/-</sup> mice, an animal model of human PSC. Both groups confirmed dysbiosis and increased gut permeability in MDR2<sup>-/-</sup> mice. Tedesco *et al*<sup>[43]</sup> documented increased interleukin (IL)-17A levels, further supporting the role of Th17 responses, but extended previous findings by showing that this was mediated, at least in part, by expansion of unconventional T cells ( $\gamma\delta$  T cells). *Lactobacillus gasseri*, which was pH- and bile-resistant (suggesting selection of specific bacteria under PSC-induced conditions) was isolated from MDR2<sup>-/-</sup> livers and found to stimulate IL-17 production from MDR2<sup>-/-</sup>-derived  $\gamma\delta$  T cells. Interestingly,  $\gamma\delta$  T cells from PSC patients, but not patients with other liver diseases like hepatitis C, produced IL-17 when stimulated. The gut dysbiosis in MDR2<sup>-/-</sup> mice reported by Liao *et al*<sup>[42]</sup> was characterized by significant alterations in *Lachnospiraceae*. In addition, *Enterococcus* was isolated from MDR2<sup>-/-</sup> livers. MDR2<sup>-/-</sup> mice displayed increased NLRP3 inflammasome activation in the liver and gut. Transferability of phenotype (NLRP3 activation, increased gut permeability, including suppressed zonulin-1 tight junction expression, elevated serum transaminases and an inflammatory infiltrate dominated by monocyte-derived macrophages) was achieved with FMT of MDR2<sup>-/-</sup> fecal samples into GF mice. In all three studies, inhibition of the postulated immunologic mechanism using (1) a small molecule antagonist of retinoid-related receptor- $\gamma$ t (a selective inhibitor of Th17 differentiation; (2) anti- $\gamma\delta$  T-cell receptor; and (3) a pan-caspase inhibitor led to improvement of the phenotype.

While this initial support for a potential causal relationship between gut microbes

and PSC is exciting and represents critical progress, it is important to recall that one cannot directly extrapolate the findings from these animal experiments to human PSC populations. Moreover, although these studies have begun to shed light on important implicated processes, none thus far has delineated a complete and coherent causal pathway between the microbiota and the clinical manifestations of PSC.

## POTENTIAL ROLE OF MICROBIAL PRODUCTS: EMPHASIS ON BILE ACIDS

The gut microbiota may also contribute to PSC/PSC-IBD pathogenesis through synthesis of compounds or co-metabolism of molecules produced by the host. Bile acids (BAs) are one such example. The primary human BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesized from cholesterol in the liver and secreted into the gut. The majority (95%) are actively reabsorbed in their conjugated form in the terminal ileum *via* the apical sodium-dependent BA transporter (ASBT). This constitutes the BA enterohepatic circulation (EHC). Microbial deconjugation generates unconjugated BAs that escape active reuptake and reach the colon. Deconjugation of primary BAs is mediated by bile salt hydrolases, which are widely distributed among the gut microbiota, including members of the Firmicutes, Bacteroidetes and Actinobacteria phyla, such as *Clostridium*, *Bacteroides*, *Lactobacillus*, *Bifidobacterium* and *Enterococcus*<sup>[47-50]</sup>. About 5% of unabsorbed BAs enter the distal ileum and colon, where they undergo chemical diversification by the gut microbiota. The predominant secondary BAs are lithocholic acid (LCA) and deoxycholic acid (DCA), but over 50 different types of microbially derived secondary BAs can be detected in human feces<sup>[51]</sup>. Only a small fraction of gut microbes possess the functional ability to carry out the 7 $\alpha$ -dehydroxylation step involved in generating secondary BAs, such as *Clostridium spp*<sup>[48,52-57]</sup>. DCA and LCA generated in this way can then be further microbially altered to produce other secondary BAs.

BAs function as critical signaling molecules *via* their interactions with several receptors, including the nuclear receptor Farnesoid X receptor (FXR) and the membrane-bound G protein-coupled bile acid receptor-1, also known as TGR5. These receptors have multiple effects on the liver and gut. The secondary BAs DCA and LCA are the most potent TGR5 ligands. TGR5 agonism is involved in cholangioprotective and anti-inflammatory effects; on cholangiocytes, TGR5 stimulates chloride secretion *via* CFTR to maintain the bicarbonate umbrella; on macrophages, TGR5 activation leads to decreased NF- $\kappa$ B transcriptional activity, thereby decreasing inflammatory cytokine production<sup>[58]</sup>. Stimulation of the TGR5-cAMP-protein kinase A axis by secondary BAs also inhibits NLRP3 inflammasome activation<sup>[59]</sup>, which as described above, may be a mechanism involved in PSC. Tabibian *et al*<sup>[60]</sup> demonstrated that the GF state in MDR2<sup>-/-</sup> mice leads to more severe hepatobiliary injury, an observation that is hypothesized to be due to the absence of microbially derived secondary BAs. It is interesting to note that colessevelam administration has been shown to improve liver injury in MDR2<sup>-/-</sup> mice and that this benefit occurred in conjunction with a shift in the BA pool toward secondary BAs and enhanced TGR5 signaling<sup>[61,62]</sup>. TGR5 is relevant for the gut as well. TGR5 deficiency exacerbates injury-induced colitis in mice, while treatment of wild-type mice with oleanolic acid (which resembles LCA in shape and functions as a TGR5 agonist) attenuates colitis and monocyte infiltration<sup>[63]</sup>. FXR is another critical BA receptor. Activation in lamina propria monocytes and macrophages results in NF- $\kappa$ B repression and downregulation of proinflammatory cytokines<sup>[64]</sup>. BA-dependent FXR activation also prevents gut barrier dysfunction and bacterial translocation in cholestatic rats<sup>[65]</sup>. Temporal fluctuations in DCA at the site of bowel injury, mediated by FXR and driven by regional shifts in the microbiota, have been shown to be integral for colonic repair processes<sup>[66]</sup>. In addition to the effects described above mediated by TGR5 and FXR, LCA derivatives have recently been demonstrated to control host immune responses by directly modulating Th17/Treg balance<sup>[67]</sup>.

In summary, BAs, including secondary BAs, shaped by the gut microbiota, offer a potential link between the gut and liver in PSC-IBD. To date, there have been only two studies to report on fecal BA profiles in PSC-IBD (15 PSC-IBD/15 IBD patients in one<sup>[68]</sup>, and 7 PSC-IBD/8 IBD/8 HCs in the other)<sup>[69]</sup>. Both were small and in several ways inconsistent, but some observations are noteworthy. In the first study, the total BA pool was significantly reduced in the PSC-IBD group and there was a trend toward more conjugated BAs in this group as well. The overall combination of fecal BAs allowed excellent separation of PSC-IBD from IBD, and the relative abundance of bacterial genera that correlated with BAs was 12% in PSC-IBD compared to only 0.4% in IBD. A negative correlation was seen between secondary BAs and endoscopic

activity. In the second study, there were less secondary BAs in the PSC-IBD group with a lower DCA/CA ratio. Notably, there were two patients with colonic dysplasia, and they both had minimal fecal DCA. This line of investigation requires further exploration; pediatric data and mucosal BA characterization are lacking. Importantly, further understanding of the specific roles of BAs in PSC could lead to novel therapies, as discussed below.

SCFAs (acetate, butyrate and propionate), derived from microbial fermentation of host-indigestible complex carbohydrates, represent another category of bacterial metabolites. As demonstrated in Table 1 and Figure 1, SCFA-producing Firmicutes are depleted in PSC/PSC-IBD relative to HCs. The potential role of SCFAs in PSC has been little explored. SCFAs influence both adaptive and innate immune responses, including the differentiation of regulatory T cells and production of proinflammatory cytokines<sup>[70]</sup>, which may bear relevance to PSC disease processes.

## MICROBIOME AS A THERAPEUTIC TARGET OR MEDIATOR IN PSC

Additional support for the role of the microbiota in PSC/PSC-IBD comes from preliminary interventional studies targeting or manipulating the gut microbiome. While large definitive randomized controlled trials (RCTs) are awaited, a handful of small RCTs and case series have shown biochemical improvement with antibiotic treatment (primarily vancomycin and metronidazole) of PSC patients<sup>[71]</sup>. More recently, in a pilot study of FMT in 10 PSC-IBD patients, 30% displayed an ALP reduction of at least 50%, without any adverse events<sup>[72]</sup>. An increase in bacterial diversity and engraftment correlated with a reduction in ALP<sup>[50]</sup>. Future study considerations include assessment of the efficacy of maintenance therapy and FMT *via* other, more practical routes, such as oral capsule.

Given the potential role of BAs in PSC, pharmacotherapies targeting BA pathways represent an active area of research for the treatment of PSC. Ursodeoxycholic acid (UDCA) is widely used. Its administration has been associated with biochemical benefits but not improvements in long-term clinical outcomes such as liver transplant or death. In fact, at higher doses of 28-30 mg/kg per day, UDA has been linked with an increased risk of serious adverse events<sup>[73]</sup>. In a small study of 15 adults with PSC that combined UDCA with all-trans retinoic acid, ALP levels fell but the change was not statistically significant<sup>[74]</sup>. As a result of the emerging evidence of potential harm at higher doses (without convincing evidence of clinically meaningful benefit), the most recent PSC guideline, put forth by the British Society of Gastroenterology in 2019, recommends against the routine use of UDCA for adults newly diagnosed with PSC<sup>[75]</sup>. A derivative of UDCA, termed *nor*ursodeoxycholic acid (*nor*UDCA), has recently garnered attention. It showed biochemical benefit in a phase II RCT including 161 adult PSC patients. Twelve weeks of treatment with *nor*UDCA resulted in significant reductions of serum ALP in a dose-dependent manner, with a similar safety profile to placebo<sup>[76]</sup>. Pharmacologic FXR agonists represent another category of BA-related therapies that are being explored in PSC. Obeticholic acid (OCA), an endogenous FXR ligand, was investigated in a phase II double-blind, placebo controlled RCT of 76 adult PSC patients. At week 24, OCA was associated with a significant decrease in ALP, satisfying the study's primary outcome. There was, however, a higher rate of pruritus with OCA than placebo<sup>[77]</sup>. Cilofexor is a nonsteroidal FXR agonist. It too was recently tested in a phase II RCT and led to lower ALP levels at week 12. This improvement was seen regardless of UDCA treatment status and, importantly, cilofexor use was well tolerated<sup>[78]</sup>. Disruption of the EHC with inhibitors of ASBT (to prevent ileal BA reuptake) represents yet another potential therapeutic strategy for PSC. Studies of ASBT inhibitors are ongoing<sup>[73]</sup>.

Further investigation is needed to definitively establish the utility of the above strategies and agents. Key limitations to existing data include their short-term (12 - 24 week) nature and the absence of good surrogate endpoints that have been shown to correlate with clinically meaningful outcomes. This latter limitation is a significant hindrance to the study of PSC in general.

## CONCLUSION

In summary, PSC represents an urgent unmet need in the fields of IBD and Hepatology alike. The frequent coexistence of PSC and IBD points to the important role of the gut-liver axis and there is mounting evidence to suggest that the gut microbiota is implicated. Numerous studies demonstrate that the gut microbiome in

individuals with PSC and PSC-IBD is distinct from that in healthy controls and individuals with IBD without liver disease, and a handful of animal experiments provide more compelling evidence of a causal role. Microbial metabolites, such as BAs and SCFAs, may represent an important intermediary between gut microbes and PSC disease processes. Despite tremendous progress in this field of study over the past several years, much work remains to be done including functional characterization of the gut microbiota and more careful examination of pediatric populations. The relationship between the microbiota and malignancy in PSC-IBD has largely been overlooked as well. The distinct PSC-IBD phenotype may offer important insight into pathophysiologic mechanisms. A better understanding of the gut microbiota in PSC and PSC-IBD may inform the development of useful microbe-altering interventions, with already some preliminary evidence of benefit, though longer-term and larger studies are needed.

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## Innate immune recognition and modulation in hepatitis D virus infection

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### Abstract

Hepatitis D virus (HDV) is a global health threat with more than 15 million humans affected. Current treatment options are largely unsatisfactory leaving chronically infected humans at high risk to develop liver cirrhosis and hepatocellular carcinoma. HDV is the only human satellite virus known. It encodes only two proteins, and requires Hepatitis B virus (HBV) envelope protein expression for productive virion release and spread of the infection. How HDV could evolve and why HBV was selected as a helper virus remains unknown. Since the discovery of Na<sup>+</sup>-taurocholate co-transporting polypeptide as the essential uptake receptor for HBV and HDV, we are beginning to understand the interactions of HDV and the immune system. While HBV is mostly regarded a stealth virus, that escapes innate immune recognition, HBV-HDV coinfection is characterized by a strong innate immune response. Cytoplasmic RNA sensor melanoma differentiation antigen 5 has been reported to recognize HDV RNA replication and activate innate immunity. Innate immunity, however, seems not to impair HDV replication while it inhibits HBV. In this review, we describe what is known up-to-date about the interplay between HBV as a helper and HDV's immune evasion strategy and identify where additional research is required.

**Key words:** Hepatitis D virus; Hepatitis B virus; Innate immunity; Pathogen-associated molecular pattern molecules; Immune evasion; Immunosuppression

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**Core tip:** Hepatitis D virus (HDV) is the only known human satellite virus requiring hepatitis B virus (HBV) coinfection for productive viral release. However, it was recently shown that HDV can be disseminated by viruses other than HBV in experimental setups, so it remains unexplained why HDV chose HBV as a helper virus.



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As HDV might possibly profit from HBV mediated immunosuppression, we first focus on recent findings on HDV recognition by the innate immune system. Later on, we summarize partially controversial data on immunomodulatory mechanisms of both, HBV and HDV.

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## INTRODUCTION

First identified in 1977 by Rizzetto *et al*<sup>[1]</sup>, Hepatitis D virus (HDV) represents a unique pathogen that defines its stand-alone genus Deltavirus. Eight genotypes of HDV varying in their RNA-genome sequences have been described. As a satellite RNA virus, HDV does not encode its own envelope proteins for packaging of its ribonucleoprotein (RNP) and therefore depends on the envelop glycoproteins of the hepatitis B virus (HBV) for virion assembly, envelopment and transmission. HDV has a broad cell and host tropism and, theoretically, several virus genera can provide help by enveloping the HDV RNP<sup>[3]</sup>. Clinically, however, HDV infection has so far only been described as coinfection with HBV or as a superinfection of chronic HBV carriers. Both, co- and superinfection may lead to HDV persistence and inflammatory liver disease, called hepatitis D. Currently, World Health Organization estimates that 15-20 million people are infected with HDV worldwide, while others predict up to 70 million carriers.

The pathogenesis of hepatitis D has been recently summarized by Koh *et al*<sup>[6]</sup>. Coinfection with HBV and HDV tends to result in both, acute hepatitis B and D at the same time, leading to most severe disease. In a superinfection scenario, HDV profits from pre-existing hepatitis B surface antigen (HBsAg) expression for progeny virus production but at the same time decreases HBV replication rates<sup>[7,8]</sup>. Chronic hepatitis D is the viral hepatitis form that is most likely to lead to liver cirrhosis and it is associated with a significant risk of hepatocellular carcinoma development and high mortality rates<sup>[6]</sup>. The reasons for more severe disease progression in HBV-HDV infection compared to HBV monoinfection have not been ultimately resolved. Chimpanzee studies indicate that liver damage by HBV is immune mediated whereas in HDV infection it is mainly cytopathic<sup>[9,10]</sup>. Direct cytopathic effects and induction of liver fibrosis by HDV antigen (HDAg) were also indicated in *in vitro* studies<sup>[11-14]</sup>.

Current treatment options rely on interferon alpha and are largely unsatisfactory leaving chronically infected at high risk to develop liver cirrhosis and hepatocellular carcinoma. New treatment options include interferon lambda, a farnesyl transferase inhibitor (Lonafarnib), the entry inhibitor peptide Mycludex B (Bulevirtide), nucleic acid polymers (*e.g.* REP 2139-Ca) that are applied alone or in combination with interferon and show promising results in phase II clinical trials<sup>[6]</sup>.

## HDV

The viral genome of HDV is a single-stranded, circular, negative sense RNA with a length of approximately 1680 nucleotides. Due to broad base pairing within the RNA molecule, the genome appears as a double stranded, rod-like structure resembling a plant viroid. During HDV replication, exclusively taking place in the nucleus, three distinct RNAs, which includes the genome, the positive-stranded antigenome, and viral mRNA, are generated by host RNA polymerases. RNA-Pol I drives the transcription of genome to antigenome in the nucleolus, whereas RNA-Pol II is responsible for genome replication using the antigenome as template on the one hand and for transcription of mRNA in the nucleoplasm on the other hand. Genome replication functions *via* a double rolling-circle mechanism. RNA oligomers of genomic and antigenomic orientation are generated, followed by self-cleavage into monomers through genome and antigenome intrinsic ribozyme activity, respectively.

Although there are several open reading frames within the HDV genome, only a single one is actively transcribed leading to the expression of two isoforms of HDAg. The small HDAg (S-HDAg) is composed of 195 amino acids, and the large HDAg (L-

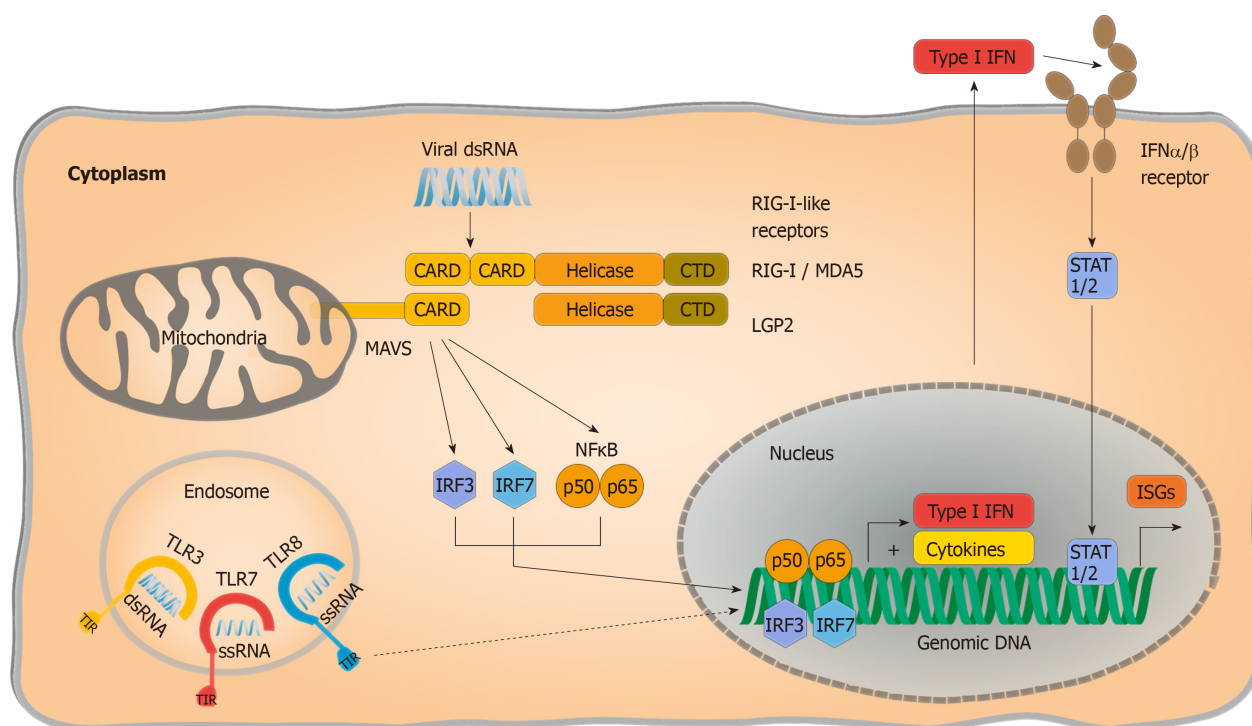
HDAG) is comprised of 214 amino acids. Initially, only S-HDAG is expressed because a termination codon prevents protein translation of L-HDAG. In order to produce the large isoform, the stop-codon (UAG) within the antigenome is mutated into a tryptophan codon (UGG) by the cellular enzyme Adenosine Deaminase Acting on RNA (ADAR1). ADAR1 is an “RNA editor” induced by interferon. Transcription of this modified genome into mRNA extends the open reading frame until the next stop codon is reached, resulting in the translation of L-HDAG harbouring an additional 19 C-terminal amino acids<sup>[23,24]</sup>.

For both isoforms, post-translational modifications play an important role. For S-HDAG it has been shown that phosphorylation of a serine residue enables interactions with the cellular RNA-Pol II, which is essential for HDV replication. Due to its C-terminal elongation, L-HDAG incorporates a nuclear export signal and a prenylation site, which allows farnesylation. The farnesylated form of L-HDAG inhibits HDV replication by masking a conformational epitope present in S-HDAG that is essential for trans-activating HDV RNA replication. The farnesylated L-HDAG is also crucial for virion assembly through its promotion of the interaction of the viral genome with a tryptophan-rich domain in the cytosolic loop of HBsAg. Common arginine rich motifs within S- and L-HDAG allow their mutual binding to RNA, leading to the formation of the so-called RNP complex, which consists of HDV genomic RNA and both HDAG isoforms. The RNP is subsequently exported into the cytoplasm, where virion assembly takes place. Export is likely mediated by nuclear export factor 1 and the cellular RNA export factor REF/Aly. During these different steps of viral replication, HDV induces a pronounced cytokine response and activates a broad range of host defence mechanisms<sup>[31-33]</sup>. This review focuses on the mode of HDV-detection by cellular pattern recognition receptors (PRRs) and selective modulatory properties of the HDV antigens. Additionally, immune-evasive and immunosuppressive strategies of HDV, and its coexisting host virus HBV, are discussed.

## PATTERN RECOGNITION OF VIRUSES

The immune system of vertebrates acts as protective mechanism against damage on cellular and organism level, and is subdivided into two branches, the innate and the adaptive immune systems<sup>[34]</sup>. Innate immunity, as the evolutionary older system, is the frontline of host defence which upon infection with a pathogen initiates and fine-tunes pathogen-specific adaptive immunity. For this purpose, innate immunity possesses the capacity to distinguish between self and non-self, as well as different classes of pathogens by recognizing certain structural patterns. This function is enabled by the expression of PRRs that detect distinct pathogen associated molecular patterns, also referred to as “PAMPs”, such as unusually structured or located nucleic acids or characteristic bacterial proteins which are not found in a given cellular compartment under physiological conditions. In the case of viral infections, innate immune sensing mostly depends on characteristic modifications of viral genomes or genome-replication intermediates and mRNA as well as special RNA structures which are normally absent in eukaryotic cells.

Extensive studies have narrowed viral RNA detection down to two families of PRRs: Endosomal Toll like receptors (TLRs) and cytosolic RIG I like receptors (RLRs) (Figure 1). The latter consists of two activating PRRs, retinoic acid inducible gene 1 (RIG I) and melanoma differentiation associated gene 5 (MDA5), as well as a third signalling-incompetent accessory molecule termed laboratory of genetics and physiology 2. Double-stranded RNA regions are both required for RIG I and MDA5 activation, although MDA5 was reported to bind longer double-stranded RNA whereas RIG I activation is mostly thought to be triggered by shorter double-stranded RNA or hairpin structures with a 5' phosphorylation. Interaction of RLRs with their specific RNA patterns results in intramolecular conformational changes, exposing their “Caspase activation and recruitment domain” site for interaction with the mitochondrial antiviral signalling (MAVS) protein<sup>[37]</sup>. Subsequently, MAVS functions as a scaffold and initiates two divergent immune signalling pathways: (1) Proinflammatory cytokine release is provoked in a nuclear factor “kappa-light-chain-enhancer” of activated B-cells (NF-κB) dependent manner; and (2) Phosphorylation and nuclear translocation of “Signal transducer and activator of transcription” (STAT 1/2) induces production of interferon (IFN). IFN signalling activates the expression of interferon-stimulated gene (ISGs) by modulating cellular homeostasis in both autocrine and paracrine manners, resulting in an antiviral state that protects both infected and noninfected cells and suppresses viral replication and progeny virus production.



**Figure 1 RNA-sensing by pattern recognition receptors.** Intracellular pathogenic RNA is sensed by endosomal Toll-like receptors (TLRs) and retinoic acid inducible gene I (RIG I) like receptors. TLR3 detects double-stranded RNA (dsRNA), whereas TLR7 and TLR8 detect single-stranded RNA in a sequence-specific manner and signal via their Toll/interleukin-1 receptor homology domains. RIG I and melanoma differentiation antigen 5 bind cytoplasmic dsRNA structures and activate conformational changes leading to the exposure of Caspase activation and recruitment domains (CARDs). Signalling-deficient laboratory of genetics and physiology 2 only consists of a helicase and a C-terminal domain and functions as an accessory receptor. This enables interaction of CARDs with mitochondrial antiviral signalling protein (MAVS), resulting in subsequent signalling cascades that release nuclear factor “kappa-light-chain-enhancer” of activated B-cells inducing a proinflammatory cytokine response. MAVS also activates interferon regulatory factor 3/7 signalling and signal transducers and activators of transcription 1/2-dependent type I interferon production and antiviral state with upregulation of interferon-stimulated genes in the host cell. TLRs: Toll-like receptors; RIG I: Retinoic acid inducible gene I; LGP2: Laboratory of genetics and physiology 2; ssRNA: Single-stranded RNA; CTD: C-terminal domain; RLRs: RIG I like receptors; NF-κB: Nuclear factor “kappa-light-chain-enhancer” of activated B-cells; MAVS: Mitochondrial antiviral signalling protein; TIR: Toll/interleukin-1 receptor; dsRNA: Double-stranded RNA; MDA5: Melanoma differentiation antigen 5; CARDs: Caspase activation and recruitment domains; IRF: Interferon regulatory factor; STAT1/2: Signal transducers and activators of transcription 1/2; Type I IFN: Type I interferon; ISGs: Interferon-stimulated genes.

## PATTERN RECOGNITION OF HDV

Partial dependence on RLR signalling has been reported by Suárez-Amarán *et al*<sup>[38]</sup> in immune pattern recognition of HDV. The authors used adenovirus-associated virus (AAV) to deliver HBV and HDV genomes (AAV-HBV and AAV-HDV) into murine liver cells to circumvent species-specific limitations of viral entry. Both wildtype (wt) and MAVS-knockout (MAVS-ko) mice showed HDV gene expression and replication, but the innate immune response to HDV infection was diminished in MAVS-ko cells. HDV-induced immune activation resulting in type I and type III IFN production was later found to be dependent on MDA5 in both primary human hepatocytes and hepatoma-cell lines<sup>[32]</sup>. While pattern recognition of HDV RNA is considered the primary source of immune activation, direct induction of IFN-signalling by L-HDAg has also been reported<sup>[39]</sup>. Nevertheless, pattern recognition of HDV infection has not been conclusively resolved. As residual IFN-responses are still detectable in the absence of RLR-signalling<sup>[38]</sup>, the impact of synergic immune activating pathways require further investigation. Additionally, the nature of HDV-specific molecular patterns that activate PRRs and changes in HBV-induced cellular immunoregulatory pathways are still poorly characterized. Considering that HDV only occurs as a satellite virus and chose HBV as a helper under natural conditions although theoretically a broad variety of viruses could provide their envelopes<sup>[3]</sup>, the detailed characterization of pattern recognition should regard potential confounding effects of HBV coinfection.

## IMPACT OF HBV COINFECTION

The impact of HBV infection on innate immunity has been subject to numerous

studies and discussions. Numerous studies proved that HBV is sensitive to interferons and other antiviral cytokines *in vivo* in the liver<sup>[40,41]</sup>, in primary hepatocytes or in HepaRG cells that have maintained their sensitivity to innate immune stimulation<sup>[42,43]</sup>. Cytokines can block HBV replication at transcriptional and posttranscriptional steps (Summarized in: Xia *et al* 2017) and affect cccDNA stability by inducing the enzymes that edit and subsequently digest it<sup>[45,46]</sup>. Up to date, the discussion is ongoing whether HBV can actively interfere with or suppress innate immunity, and thus support HDV persistence.

HBV is primarily regarded a stealth virus, neither activating nor inhibiting an innate immune response during virus replication<sup>[47-49]</sup>. Macrophages may, however, recognize virus particles early during infection<sup>[50,51]</sup>. This may be responsible for suppression of HBV replication shortly after infection and allow to prevent early activation of adaptive immunity. Several HBV proteins have been reported to have distinct features resulting in active interference with immune recognition or immune suppression. A number of these studies were done in settings where HBV proteins were overexpressed resulting in controversial discussions about the physiological relevance of the results<sup>[42,52-55]</sup>. An inhibition of interferon responses by HBV, however, has also been described in mice with humanized livers<sup>[56]</sup>. An inhibition of interferon responses by HBV, however, has also been described in mice with humanized livers. One would expect these livers to be close to the human physiological situation although HBV replication levels may be higher due to the lack of adaptive immunity and a cross-talk between human hepatocytes and murine non-hepatocytes.

A recent publication showed that HDV can be efficiently disseminated by helper viruses other than HBV from different genera, including flavivirus, vesicular stomatitis virus and the hepatitis C virus *in vitro* and in mice<sup>[3]</sup>. HDV particles packaged within a vesicular stomatitis virus envelope were able to overcome liver specificity conferred by the HBV envelope proteins and efficiently infect human embryonic kidney cell derived 293 cells<sup>[3]</sup>. In the context of tissue-specific pattern recognition, liver tropism may only confer a minor advantage for HDV since dsRNA-detecting TLR3, RIG I and MDA5 functions have been verified despite low protein expression levels *in vitro*<sup>[42,57,58]</sup> and *in vivo*<sup>[59]</sup>.

From an evolutionary standpoint, one would argue that coinfection with HBV must be favourable for HDV, leading to the question of what benefit this confers. One possible explanation could be that HBV does indeed prevent or block innate immunity and that HDV profits from this. Regarding HDV recognition by MDA5, downregulation of MAVS-induced signalling by HBV-encoded X-protein has been proposed<sup>[60-64]</sup>. Interference has also been reported by the HBV polymerase<sup>[65]</sup> or by HBV induced microRNA146a<sup>[66]</sup> – all of which could support the survival of HDV infected cells. Proving this, however, requires additional studies using infection models because there are potential confounding effects from overexpression of HBV proteins in these experiments. The impact of HBV infection on downstream immune pathways also remains controversial. Direct blocking of interferon-signalling by HBV polymerase<sup>[52,54,67]</sup>, HBV envelope protein<sup>[68]</sup>, X-protein<sup>[69,70]</sup> or microRNA 146a<sup>[71]</sup> has been reported, which could also benefit HDV infection. These effects may well be subtle as HDV envelopment requires a certain level of interferon activity to allow induction of expression of the interferon-stimulated ADAR1 that is essential for L-HDAg expression. Regardless of the exact mode of HBV-induced immunosuppression, dependency of HDV on help to survive innate immunity seems likely, given that it only encodes for a single protein.

## SENSITIVITY OF HDV TO ANTIVIRAL CYTOKINES

In addition to exploiting immunosuppressive and immune-evasive mechanisms, HDV possesses some resistance to interferon-mediated antiviral effects. In contrast to HBV, HDV induces interferon signalling in both cell lines and mice without viral replication being affected<sup>[31,32,38]</sup>. The most prominent interferon-induced protein in HDV infection is ADAR1, which both inhibits viral replication and promotes RNA packaging. ADAR1 exists in two isoforms, constitutively expressed short ADAR1p110 present in the nucleus and IFN-inducible long ADAR1p150 which is both present in the cytoplasm and nucleus. In untreated Huh7-cells, non-inducible ADAR1p110 is mainly responsible for L-HDAg expression, whereas ADAR1p150 can enhance HDV-RNA editing rates up to one-third upon IFN-treatment<sup>[24,74,75]</sup>. Though ADAR1p150 editing was hypothesized to be partially responsible for the antiviral effects of interferon- $\alpha$  therapy, this effect seems to be limited due to additional regulatory mechanisms.

HDV production appears to remain unaffected or even promoted during



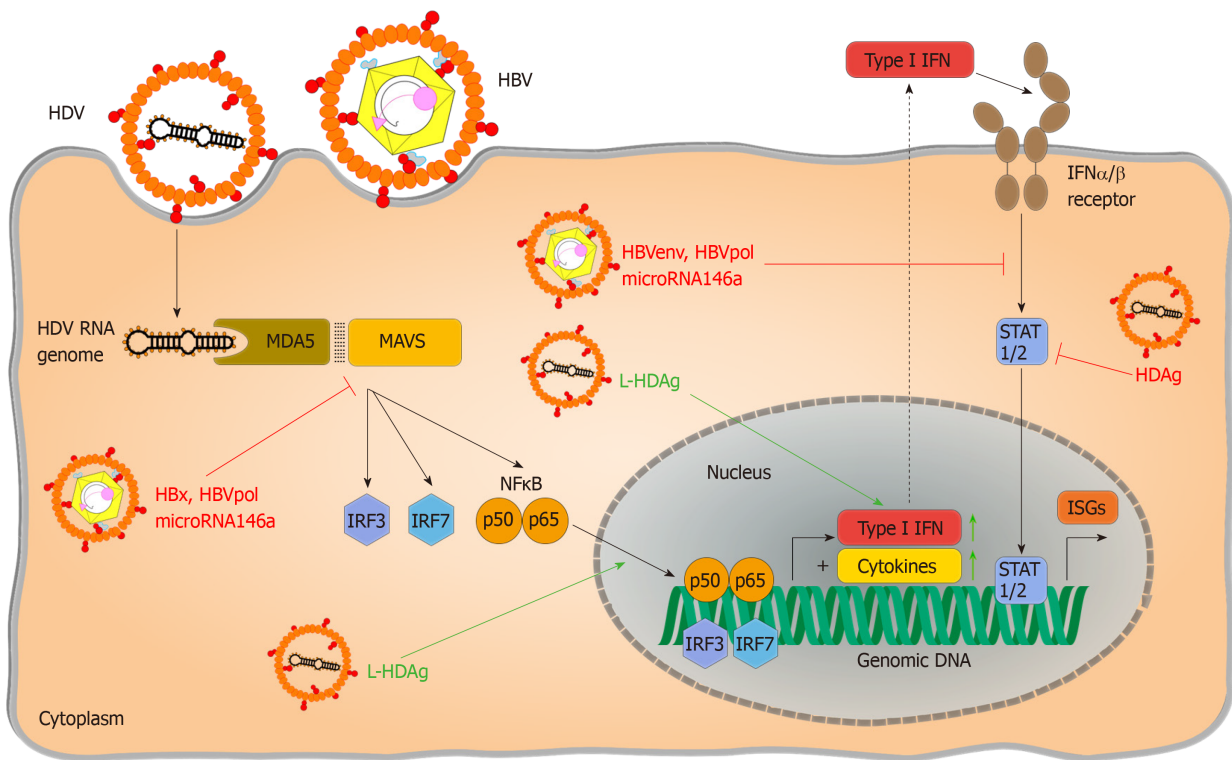
proinflammatory cytokine responses. Various groups have reported L-HDAg enhanced NF- $\kappa$ B translocation to the nucleus and the upregulation of proinflammatory genes in response to transfection of HDV-encoding plasmid<sup>[76-78]</sup>. This might be necessary for viral assembly as L-HDAg translocation from nucleus to cytoplasm was reported to be induced by NF- $\kappa$ B activation. However, all these experiments were performed as transient HDAg overexpressions, which could also induce unfolded protein response in the endoplasmic reticulum, leading to NF- $\kappa$ B activation<sup>[80,81]</sup>. These circumstances were caused by unavailability of HDV-susceptible cell lines until the identification of Na<sup>+</sup>-taurocholate co-transporting polypeptide (NTCP) as an essential factor for HBV/HDV infection<sup>[82]</sup>. Newly developed infection systems utilizing AAV-HDV, as well as NTCP-expressing cell lines and mouse models, should be used to strengthen previously published results on antiviral activity of cytokines against HDV.

## IMMUNE EVASION BY HDV

Despite the very limited coding capacity of its genome, HDV has evolved mechanisms to escape immunity (Figure 2). First of all, the HDV genome avoids direct contact with cytoplasmic or endosomal PRRs by replicating in the nucleus, taking advantage of cellular compartmentalization. Furthermore, it forms a circular RNA genome without “open” 5’ or 3’ ends to prevent PRR binding, as circular RNA has been reported not to activate RIG I<sup>[83]</sup>, and an RNP complex reducing the binding of PRRs to virus-characteristic structures. Transfection of HDV-cDNA and HDV-encoding plasmid pSVL(D3) in Huh7 cells reduced STAT-signalling and expression of ISGs in response to IFN- $\alpha$  treatment and inhibited phosphorylation and nuclear translocation of STAT-proteins. This direct inhibition of interferon signalling was hypothesized to account for poor responsiveness to IFN treatment in infected patients. However, these results have not been reproduced in HDV-infection so far and, contradictory to the complete blocking of IFN signalling observed in this study, HDV triggers immune activation *via* MDA-5<sup>[32]</sup>. Whether HDV immune recognition by alternate PRR plays a role and which HDV-RNA structures trigger HDV-immune recognition still needs to be identified. It also remains ambiguous if HDV initiates IFN production in infected patients, since as to the authors’ knowledge no data exist on this so far. HDV also offers little for adaptive immunity to attack since there is only two proteins expressed, and S-HDAg and L-HDAg largely overlap in their protein sequence. Thus, few HDV-derived peptides can be presented on infected cells and recognized by T cells. In a systematic screen to define CD8 epitopes, the overall number of epitopes identified was very low compared to other hepatotropic viruses<sup>[85]</sup>. When sequences of HDV RNA and HLA class I alleles that present epitope peptides to CD8<sup>+</sup> T cells in patients with persistent HDV infection were analyzed, HDV variants were identified that can escape T cell-mediated immunity<sup>[85,86]</sup>. As an RNA virus, HDV genomes are mutated during virus replication allowing immune escape variants to emerge. Hereby, HDV escape from the immune response was associated with uncommon HLA class I alleles, indicating that HDV has evolved, at the population level, to evade recognition by common HLA class I alleles<sup>[86,87]</sup>. T cell exhaustion doesn’t seem to be a major reason for failure to clear HDV. Activated HDV-specific CD8<sup>+</sup> T cells target conserved epitopes and seem to contribute to disease progression. Even memory-like HDV-specific CD8<sup>+</sup> T cells remain functional but are unable to clear HDV because of the presence of escape variants<sup>[86,87]</sup>. Thus, HDV mainly escapes adaptive immunity because there are so few epitopes that may be presented by human HLA haplotype repertoire and recognized by T cells.

## CONCLUSION

As the only known satellite virus known in humans, HDV has chosen HBV as a helper virus although HDV per se is promiscuous. HDV seems to profit from the co-existence of HBV. Whether the advantage conferred is the strict liver tropism of HBV where pattern recognition is tuned down due to the constant exposure to bacterial components, or whether active HBV-induced immunosuppression contributes remains open. HDV shows some capabilities to escape immune responses and also a certain degree of resistance to interferon activity. Detailed studies on the mode of HDV-induced immune regulation and immune activation could contribute valuable key information to target this virus and develop new therapies against this fatal disease.



**Figure 2 Immune evasion and immunomodulation in hepatitis D virus infection.** Pattern recognition of hepatitis D virus RNA was reported to be both inhibited by hepatitis B virus (HBV) specific proteins like HBV X protein, HBV envelope proteins, HBV polymerase as well as the hepatitis delta antigen and in particular its large variant. Inhibitions of major pathways are indicated with red flat arrows, activation of cytokine response is indicated in green pointed arrows. HBV: Hepatitis B virus; HDV: Hepatitis D virus; HBx: Hepatitis B virus X protein; HBV env: Hepatitis B virus envelope proteins; HBV pol: Hepatitis B virus polymerase; HDAg: Hepatitis delta antigen; L-HDAg: Large hepatitis delta antigen; TLRs: Toll-like receptors; RIG I: Retinoic acid inducible gene I; LGP2: Laboratory of genetics and physiology 2; ssRNA: Single-stranded RNA; CTD: C-terminal domain; RLRs: RIG I like receptors; NF-κB: Nuclear factor "kappa-light-chain-enhancer" of activated B-cells; MAVS: Mitochondrial antiviral signalling protein; TIR: Toll/interleukin-1 receptor; dsRNA: Double-stranded RNA; MDA5: Melanoma differentiation antigen 5; CARDs: Caspase activation and recruitment domains; IRF: Interferon regulatory factor; STAT1/2: Signal transducers and activators of transcription 1/2; Type I IFN: Type I interferon; ISGs: Interferon-stimulated genes.

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## Basic Study

# Use of zebrafish embryos as avatar of patients with pancreatic cancer: A new xenotransplantation model towards personalized medicine

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## Abstract

### BACKGROUND

The response to chemotherapy treatment of patients with pancreatic ductal adenocarcinoma (PDAC) is difficult to predict and the identification of patients who most likely will benefit from aggressive chemotherapy approaches is crucial. The concept of personalized medicine has emerged in the last years with the objective to tailor the medical treatment to the individual characteristics of each patient, and particularly to the tumor biology of each patient. The need for in-vivo xenotransplantation models for cancer patients has increased exponentially, and for this reason zebrafish avatars have gained popularity. Preliminary studies were conducted also with PDAC tissue.

### AIM

To develop a simple, not expensive, diffusible zebrafish embryo model as avatar for patients affected by PDAC.

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#### Institutional review board

**statement:** The study was approved by Ethics committee of "Area Vasta Nord Ovest (CEAVNO)" (protocol number 70213).

#### Institutional animal care and use

**committee statement:** Zebrafish (Danio Rerio) were handled in compliance with local animal welfare regulations (authorization n. 99/2012-A, 19.04.2012) and standard protocols approved by Italian Ministry of Public Health, in conformity with the Directive 2010/63/EU.

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## METHODS

Tumor tissue was taken from the surgical specimen by the histopathologist. After its fragmentation into small pieces, they are stained with CM-Dil. Small pieces of stained tissue were transplanted into the yolk of wt AB zebrafish embryos with a glass capillary needle. Embryos were incubated at 35 °C in E3 medium supplemented with 1% Pen/Strep in the presence or absence of drugs for the following days in respect of the treatment plan (Gemcitabine; Gemcitabine and Oxaliplatin; Gemcitabine and nab-Paclitaxel; 5-Fluorouracil and Folinic acid and Oxaliplatin and Irinotecan). The response of zebrafish xenografts to the chemotherapy options has been analyzed by monitoring the fluorescent stained area at 2 h post injection (hpi), 1 d and 2 d post injection (dpi). In each time point, the mean size of the stained area was measured by ImageJ and it was normalized with respect to the 1 dpi time point mean relative tumor area (RTA). We evaluated the effect of the chemotherapy exposition comparing the mean RTA of each treated subgroup and the control group and evaluating the percentage reduction of the mean RTA by comparing each treated subgroup with the control group.

## RESULTS

Between July 2018 and October 2019, a total of 15 patients with pancreatic cancer were prospectively enrolled. In all cases, it was possible to take a fragment of the tumor from the surgical specimen for the xenotransplantation in the zebrafish embryos. The histological examination confirmed the presence of a PDAC in all cases. In absence of chemotherapy (control group), over time the Dil-stained area showed a statistically significant increase in all cases. A statistically significant reduction of the mean RTA in the treated subgroups for at least one chemotherapy scheme was reported in 6/15 (40%) cases. The analysis of the percentage reduction of the RTA in treated subgroups in comparison to the control group revealed the presence of a linear relationship in each subgroup between the percentage reduction of the RTA and the number of cases reporting each percentage threshold considered for the analysis.

## CONCLUSION

Our model seems to be effective for the xenotransplantation of PDAC tissue and evaluation of the effect of each chemotherapy scheme on the xenotransplanted tumor tissue.

**Key words:** Pancreatic ductal adenocarcinoma; Zebrafish embryos; Personalized medicine; Xenotransplantation; Chemotherapy efficacy; Avatar of oncological patients

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**Core tip:** Patient-derived xenograft model has emerged as an important tool for personalized medicine. Zebrafish embryos offer several advantages: the short generation time, the large number of offspring, the transparency, and the small size therefore making zebrafish a more practical and less expensive laboratory system than others *in vivo* cancer models. We developed a model to use zebrafish embryos as avatar of patients with pancreatic ductal adenocarcinoma, standardizing the protocol for the xenotransplantation of pancreatic tumor tissue, for the exposition of the xenotransplanted zebrafish embryos to the chemotherapy drugs, and for the evaluation of the effects of chemotherapy on the xenotransplanted tumor tissue.

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## INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive malignancies worldwide, with a median survival time of less than 18 mo and with a 5-year survival rate for pancreatic cancer increased from 6% to 9% from 2014 to 2018<sup>[1,2]</sup>. The annual death rate for PDAC is almost equal to the incidence rate<sup>[3]</sup>, suggesting the lack of an effective screening method for targeted drug therapy. A radical surgical resection for a curative treatment option can be performed in less than 20% of cases because most patients have an advanced stage of the disease at the diagnosis. Chemotherapy plays a fundamental role for the treatment of patients affected by PDAC, as neoadjuvant, adjuvant or as unique treatment in non resectable PDAC. However, the response to chemotherapy treatment is difficult to predict and today different chemotherapy schemes are disposable for the treatment of PDAC. Recent trials showed significant improvement in over-all survival with the use of combined chemotherapy modalities compared to the gemcitabine monotherapy<sup>[4,5]</sup>. Different regimens have been developed such as gemcitabine plus nab-paclitaxel and FOLFIRINOX and direct comparisons of these regimens are not available<sup>[6,7]</sup>. Moreover, the incidence of adverse events increases with the use of combination regimens compared to gemcitabine: The proportion of patients who experienced at least one grade 3 or higher treatment-related, treatment-emergent adverse event was 77% with nab-paclitaxel plus gemcitabine and 51% with gemcitabine-alone in the MPACT study<sup>[6]</sup>. FOLFIRINOX produced higher rate of both hematological and non-hematological grade 3 or higher toxicities such as diarrhea and sensory neuropathy<sup>[7]</sup>. An appropriate patient selection is crucial to identify those that are most likely to benefit from aggressive chemotherapy approaches and those who are more likely to have only little benefit due to increased rates of severe side effects. Some clinical parameters can help in the choice of the most effective scheme. However, the concept of precision medicine has emerged in the last years with the objective to tailor the medical treatment to the individual characteristics of each patient, and particularly to the tumor biology of each patient. In this context, precision oncology seeks to identify the most effective therapy for an individual patient, based on the characterization of his cancer. "Mouse Avatar" is an emerging approach of precision medicine in oncology that has recently grown in popularity<sup>[8]</sup>. It implicates the xenotransplantation of cancer cells from patient tumor sample in mouse models testing drug efficacy to run the so called "co-clinical trials". The advantage of this approach is that each patient has his/her own tumor growing in an *in vivo* system, thereby allowing the identification of a personalized therapeutic approach. However, the use of mice as avatars has some limits such as high costs, the time-consuming process and the requirement of immunosuppressed strains. Recently, the use of zebrafish as avatar for oncological patients has gained popularity. After the first experiment reported in 2005<sup>[9]</sup>, nowadays the use of the zebrafish model of xenotransplantation is one option for implementing strategies of personalized medicine, together with other models such as mouse patients-derived xenografts, patient-derived organoids<sup>[10,11]</sup> or the whole tumor genome sequencing<sup>[12,13]</sup>. Several human cancer cells *e.g.*, melanoma, glioma, breast and prostate cancer cells as well as fragments of human cancer tissues have been tested to date<sup>[14]</sup>. Preliminary studies were conducted also with patient-derived pancreatic cancer cells or tissue<sup>[15-17]</sup>. The aim of this study is to propose a model that is possibly simple, not expensive and diffusible to use the zebrafish embryos as avatars for patients affected by PDAC to predict the efficacy of the different chemotherapy schemes and the clinical response to the treatment.

## MATERIALS AND METHODS

Patients affected by PDAC that had undergone pancreatic resection were enrolled after written informed consent. After the surgical operation, the specimen was analyzed by the pathologist and a fragment of the tumor was taken for the xenotransplantation in zebrafish embryos.

Preoperative data included diagnosis, age, gender, body mass index (BMI), value of tumor marker CA 19.9, and neoadjuvant chemotherapy and/or radiotherapy for neoplastic disease. Operative data included type of surgical procedure, if an associated vascular resection was performed, and if there were problems in taking a fragment of the tumor for the xenotransplantation. Histological data included: Histological type of the tumor, grade of differentiation, tumor dimension, number of harvested lymph nodes, number of metastatic lymph nodes, presence of angioinvasion and perineural infiltration, presence of vascular infiltration in case of vascular resection. Patients were staged according to the T and N definitions

proposed for the American Joint Committee on Cancer 8<sup>th</sup> edition<sup>[18]</sup>. Proposed T-stage definitions are the following: T1  $\leq$  2 cm maximal diameter, 2 < T2  $\leq$  4 cm maximal diameter, T3 > 4 cm maximal diameter, T4 = locally unresectable. Extra-pancreatic extension was not included in these T-stage definitions. Proposed N-stage definitions included the following: N0 = node negative, N1 = 1-3 nodes positive for metastatic disease, N2  $\geq$  4 nodes positive for metastatic disease.

### **Animal care and use statement**

Zebrafish (*Danio rerio*) were handled in strict compliance with local animal welfare regulations (authorization n. 99/2012-A, 19.04.2012) and standard protocols approved by Italian Ministry of Public Health, in conformity with the Directive 2010/63/EU. Fish were housed at an average temperature of 28 °C in a recirculating system with a 14:10 h light to dark cycle. Zebrafish fertilized eggs were obtained by natural mating of wild-type AB strain at our facilities and the developing embryos were staged in incubator at 28 °C according to Kimmel *et al.*<sup>[19]</sup>. Before any procedure, embryos were anesthetized in 0.02% tricaine.

### **Zebrafish embryos xenotransplantation and chemotherapy test**

We used the protocol described in our article on validation of zebrafish embryos as Avatar to test chemotherapy drugs<sup>[20]</sup>. The tumor tissue taken from the surgical specimen by the histopathologist was washed three times with RPMI supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL Amphotericin B and cut into small pieces (1-3 mm) using a scalp blade. The pieces were then transferred to a 5 mL tube, and stained with 40 µg/mL CM-Dil in Dulbecco's phosphate buffered saline (D-PBS). The tissue pieces were incubated for 15 min at 37 °C and for 15 min in ice cubes. Tissue pieces were then washed and centrifuged three times by D-PBS and re-suspended in D-PBS supplemented with 10% fetal calf serum. For tissue transplantation, we used the manual method proposed by Marques *et al.*<sup>[15]</sup> 2009. Small pieces of stained tissue were further disaggregated using Dumont forceps (No.5) into a relative size of 1/4 to 1/2 the size of the yolk. Tissue pieces with the correct size were transferred to 1% agarose disks in which the 2-d post injection (dpi) embryos were laying, ready for transplantation. A glass transplantation needle was used to implant the tissue into the yolk. The tissue was picked up, put on the top of the yolk and then pushed inside. The yolk usually sealed itself and in the majority of embryos, the tumor remained in the yolk. After transplantation, embryos were incubated for 2 h at 35 °C, then embryos were checked for presence of tissue and incubated at 35 °C in E3 1% Pen/Strep medium with the presence or absence of drugs for the following days in respect of the treatment plan. The tumor tissue was xenotransplanted in  $n = 100$  zebrafish embryos and injected embryos were randomly allocated among 5 groups (4 therapeutic options and one control group). The treated groups were exposed to the four main chemotherapy options used for PDAC: Gemcitabine (GEM), Gemcitabine + Oxaliplatin (GEMOX), Gemcitabine + nab-Paclitaxel (GEM/nab-P), and 5-Fluorouracil + Folinic acid + Oxaliplatin + Irinotecan (FOLFOXIRI). The chemotherapies were dissolved in fish water, using the equivalent dose (ED = 5) calculated with a toxicity study on zebrafish embryos and validated by test with cellular lines or tumor tissue xenotransplanted in zebrafish embryos<sup>[20]</sup>. Two days post treatment the response of zebrafish xenografts to the chemotherapy options was analyzed by monitoring the stained area at 2 hpi, 1 dpi and 2 dpi using ImageJ (Figure 1). The mean size of the tumor mass area measured in each time point was normalized with respect to the 1 dpi time point mean relative tumor area (RTA). We evaluated the efficacy of the chemotherapy exposition using two modalities. The first one was the comparison of the mean RTA between each treated subgroup and the control group calculating the mean difference between the two groups. Moreover, we evaluated the possibility to use in the zebrafish model the "Response evaluation criteria in solid tumors (RECIST)" used in the common clinical practice to evaluate the response to the chemotherapy in oncological patients<sup>[21]</sup> as an objective parameter for evaluation of data obtained with zebrafish embryos tests. To do this, we evaluated the percentage reduction of the mean RTA in each treated subgroup taking as reference the mean RTA of the control group. After that, we calculated how many cases reported a reduction of at least a percentage value equivalent to a referent threshold included between 0% and -90% decreasing the threshold value of 10% each time. We evaluated for each treated subgroup if there was a linear relationship between the threshold values of the percentage reduction of the mean RTA and the number of cases reporting a percentage reduction of the mean RTA equal or greater to each threshold value and calculated the linear regression line equation. We researched in the literature data the percentage of partial response according to the RECIST criteria reported for each chemotherapy protocol tested in xenotransplanted zebrafish embryos. Then, using the linear regression line equation, we calculated for each

protocol the expected percentage RTA reduction (theoretically the percentage of RTA reduction reported in a percentage of cases in test with xenotransplanted zebrafish embryos equal to the percentage of partial response reported in literature) with the following formula: Expected percentage reduction of the mean RTA = (percentage of PR reported in literature - *q*linear regression line equation)/*m*linear regression line equation. Then, for each chemotherapy protocol we calculated the mean value of the expected percentage reduction of the mean RTA in treated xenotransplanted zebrafish embryos corresponding to a partial response in oncological patient and compared them.

### **Xenografts imaging and quantification**

All xenografts were imaged by fluorescence Nikon Eclipse E600 microscope to monitor the RTA and analyzed using ImageJ software. For the volumetric analysis of the nuclei the images were acquired by using a Nikon A1 confocal microscope with a 5  $\mu$ m z-stacks. The quantifications were performed using the 3D Objects Counter function of ImageJ software.

### **Formalin-Fixed Paraffin Embedded samples preparation of Xenografts**

**Histological analysis:** Two days post treatment zebrafish patient-derived xenografts (zPDX) were sampled for histopathological examination. Briefly, the embryos were fixed in 10% neutral buffered Formalin and dehydrated through increasing ethanol scale. Then the zPDX were embedded after immersion in liquid paraffin at 60 °C for 2 h. The Formalin-Fixed Paraffin Embedded (FFPE) were sectioned at 3  $\mu$ m-thick, then rehydrated using xylene and ethanol solutions. Finally, zPDX sections were stained with hematoxylin and eosin for morphological analysis.

**Immunohistochemistry:** The expression of Pan-Cytokeratin proteins (PanCKs) was examined using immunohistochemistry in the preclinical models. FFPE sections were hydrated. Antigen retrieval was performed through soaking 3 times for 3 min in 10 mmol/L citrate buffer pH 6 at 96 °C. The sections were treated for endogenous peroxidase quenching, by incubating the specimens in a 3% H<sub>2</sub>O<sub>2</sub> solution at room temperature in the dark for 15 min. Samples were incubated with monoclonal mouse anti-human PanCK antibody at 1:100 dilution for 1 h and stained with avidin-biotinperoxidase complex (Ventana system). The sections were counterstained with hematoxylin. Positive cells were identified through brown color visualization.

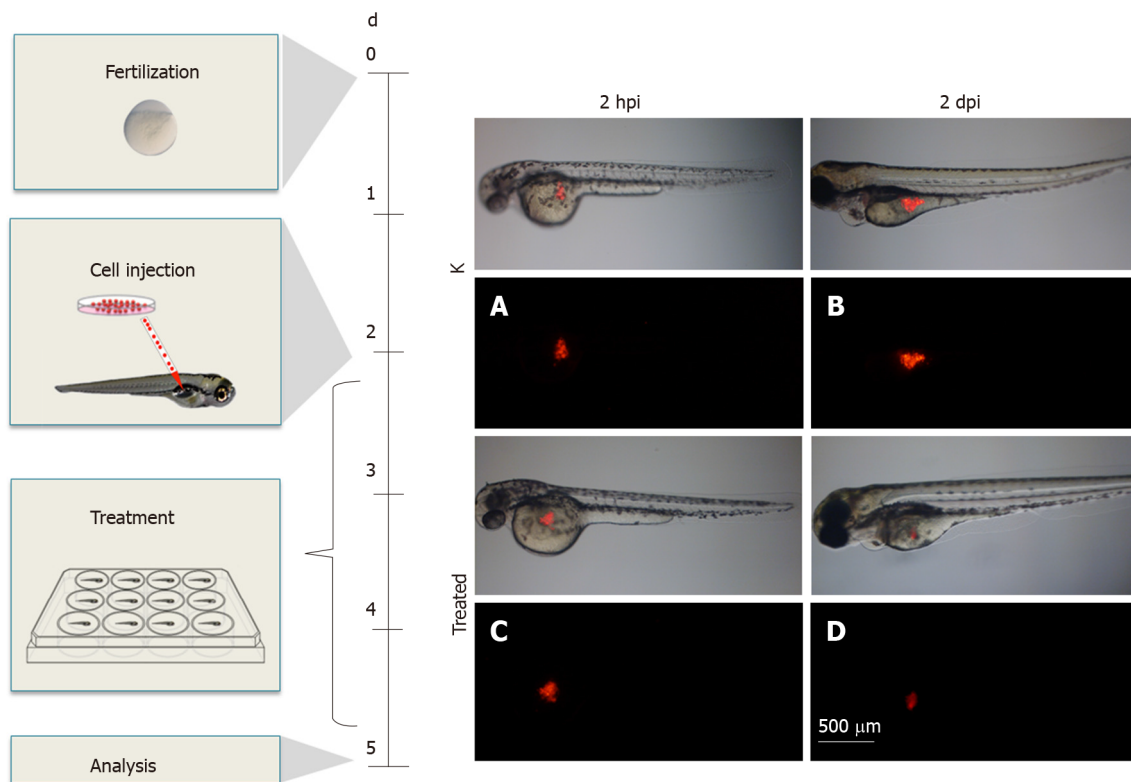
### **Statistical analysis**

We used GraphPad Prism 7 as statistical analysis software. Data analysis was performed by ANOVA, followed by Bonferroni correction or Dunnett's post-hoc test. Statistical significance was set to 5%.

Continuous variables with normal distribution are expressed as mean  $\pm$  SD and compared using *t* test. The statistical analysis was performed using SPSS (Statistical Production and Service Solution for Windows, SPSS Inc., Chicago, IL, United States), version 23.

## **RESULTS**

Between July 2018 to October 2019, a total of 15 patients with PDAC were enrolled. Patients characteristics are summarized in Table 1. Of these 15 patients, 8 (53.3%) were male. The mean age was 71.2  $\pm$  9.9 years (range 44.1-83.2) and the mean BMI was 26.4  $\pm$  5.1 kg/m<sup>2</sup> (range 17.6-40.4). The mean preoperative Ca 19.9 was 347.1  $\pm$  543.0 U/mL (range 1.7-2094) and it was increased in 12/15 (80%) patients. A pancreaticoduodenectomy was performed in 11/15 (73.3%) patients, a distal splenopancreatectomy in 3/15 patients (20%) and a total splenopancreatectomy in 1/15 (6.7%) patients. An associated vascular resection was performed in 4/15 (26.7%) patients: A venous resection in 3 cases and resection of the celiac trunk in 1 case. No intra-operative complications were reported, and, in all cases, it was possible to take a fragment of the tumor from the surgical specimen for the xenotransplantation in the zebrafish embryos. The histological examination confirmed the presence of a PDAC in all cases. A moderately differentiated adenocarcinoma (G2/3) was reported in 11/15 (73.3%) cases, while a poorly differentiated adenocarcinoma (G3/3) was reported in 4/15 (26.7%) cases. The mean diameter of the pancreatic neoplasia was 3.3  $\pm$  1.2 cm (range 1.5-5.0). The mean number of harvested lymph nodes was 40.6  $\pm$  18.0 (range 19-74), while the mean number of positive lymph nodes was 5.7  $\pm$  6.0 (range 0-22). The presence of positive lymph nodes was documented in 14/15 (93.3%) patients: a N1 status was reported in 7/15 (46.7%) patients, while a N2 status was reported in 7/15 (46.7%) patients. The presence of perineural infiltration was reported in 11/15 (73.3%)



**Figure 1 Protocol used for the evaluation of the chemotherapy drugs efficacy.** A piece of pancreatic ductal adenocarcinoma tumor tissue is injected into the yolk sac of zebrafish embryos 2 dpf. At 2 h post injection (hpi) the embryos are imaged and exposed to chemotherapy for 2 d. A and B: On the right panel a representative image of control embryos after 2 hpi and 2 dpi; C and D: On the right panel a representative image of treated embryos after 2 hpi and 2 dpi. Qualitatively images show the increased of fluorescent area in control group versus the regression in xenotransplanted embryos treated with chemotherapy.

patients, while the angioinvasion was reported in 2/15 (13.3%) patients. In 2/4 cases of vascular resection the histological examination confirmed the presence of vascular infiltration.

### Results of tests with zebrafish embryos

Tumor tissue was successfully engrafted in healthy zebrafish embryos in all cases (Figure 2). The volumetric analysis of the volume occupied by the nuclei of the patient's tumor fragment inoculated in the zebrafish embryos revealed a volume of  $6411 \pm 10.96 \mu\text{m}^3$  (mean  $\pm$  SEM) derived from two embryos xenotransplanted with different patient's tumors. Considering a diameter of 6  $\mu\text{m}$  for a typical mammalian nucleus, this value corresponds to about 60 cells per xenotransplant. We calculated the percentage of epithelial cells (mean PDAC counterpart) out of the total surface area ( $31.8\% \pm 4.9\%$ ,  $n = 3$ , Figure 2D). The mean survival rate of xenotransplanted zebrafish embryos was 71.5% at 1 dpi and 52.4% at 2 dpi (Table 2). In absence of chemotherapy (control group), the Dil-stained areas showed a statistically significant increase over time in all cases, while we observed a tendency to a reduction of the mean RTA in treated subgroups. In Figure 3 it is possible to observe the alteration due to the chemotherapy of xenotransplanted cells of some treated xenotransplanted zebrafish embryos. The mean RTA of each subgroup and the differences of cases RTA between the control group and the treated subgroups are reported in Table 3. The analysis of the mean RTA revealed a statistically significant reduction of the mean RTA in the treated subgroups for at least one chemotherapy scheme in 6/15 (40%) cases (Figure 4). The percentage reduction of the mean RTA in each treated subgroup, taking as reference the mean RTA of the control group, is reported in Table 4.

Table 5 shows how many cases in each subgroup presented a percentage reduction of the mean RTA equal or greater to each threshold value in comparison to the control group. The analysis of percentage reduction of the RTA in the treated subgroups, compared to the control group, revealed the presence of a linear relation in each subgroup between the percentage reduction of the RTA and the number of cases reporting at least each percentage threshold considered for the analysis (Figure 5). The linear regression line equations for each subgroup are reported in Figure 5. Using the linear regression line equations, we calculated the expected RTA reduction corresponding to the percentage of partial response reported in the literature for each



**Table 1 Patients characteristics (n = 15)**

Characteristics	
Mean age, years $\pm$ SD	71.2 $\pm$ 9.9 (44.1-83.2)
M:F, n (%)	8:7 (53.3:46.7)
Mean BMI, kg/m <sup>2</sup> $\pm$ SD	26.4 $\pm$ 5.1 (17.6-40.4)
Ca 19.9, U/mL $\pm$ SD	347.1 $\pm$ 543.0 (1.7-2094)
Type of surgical procedure, n (%)	
Pancreatoduodenectomy	11 (73.3)
Distal splenopancreatectomy	3 (20)
Total splenopancreatectomy	1 (6.7)
Vascular resection, n (%)	4 (26.7)
Grade of differentiation, n (%)	
G2/3	11 (73.3)
G3/3	4 (26.7)
Mean tumour dimension, cm	3.3 $\pm$ 1.2 (1.5-5.0)
Mean harvest lymph nodes, n	40.6 $\pm$ 18.0 (19-74)
Mean positive lymph nodes, n	5.7 $\pm$ 6.0 (0-22)
T status, n (%)	
T1	2 (13.3)
T2	7 (46.7)
T3	6 (40)
N status, n (%)	
N0	1 (6.6)
N1	7 (46.7)
N2	7 (46.7)
Stage, n (%)	
IB	1 (6.7)
IIB	6 (40)
III	8 (53.3)
Angioinvasion, n (%)	2 (13.3)
Perineural infiltration, n (%)	11 (73.4)
Vascular infiltration, n (%)	2 (13.3)

BMI: Body mass index; M: Male; F: Female.

chemotherapy scheme administered to each treated subgroup. Data are reported in Table 6. The mean conversion factor calculated was -60.4% for FOLFOXIRI, -59.3% for GEM-nab/P, -62.4% for GEMOX, and -63.7% for GEM ( $P = 0.626$ ).

## DISCUSSION

In the last decade, patient-derived xenograft model has emerged as an important tool for translational research, retaining much of the complexity of the tumor microenvironment and heterogeneity of the original tumor in an individual patient, and representing the first step towards personalized medicine<sup>[22]</sup>. Different patient-derived tumor models, both *in vitro* and *in vivo*, have been developed. Organoid cultures were a major breakthrough in the *in vitro* culture of tumor cells from patients, becoming the most attractive tool to be used as an *in vitro* screening platform. Recently, this technique was further developed to generate organoids from patient-derived cancer tissues<sup>[23]</sup>. However, only few retrospective studies correlated patient clinical outcomes with organoid drug response<sup>[24-26]</sup>. Moreover, the use of organoids has some limitations in comparison to the zebrafish model. First of all, the initial organoid generation requires 4-5 wk<sup>[23,27]</sup>, which is border line for the time frame needed to guide first clinical decisions. Moreover, for the lack of stromal components, these models lack many complex interactions observed in the tumor microenvironment or in a living organism and do not allow, for instance, the evaluation of the metastatic or angiogenic potential<sup>[23]</sup>. PDX into immunodeficient mice has been

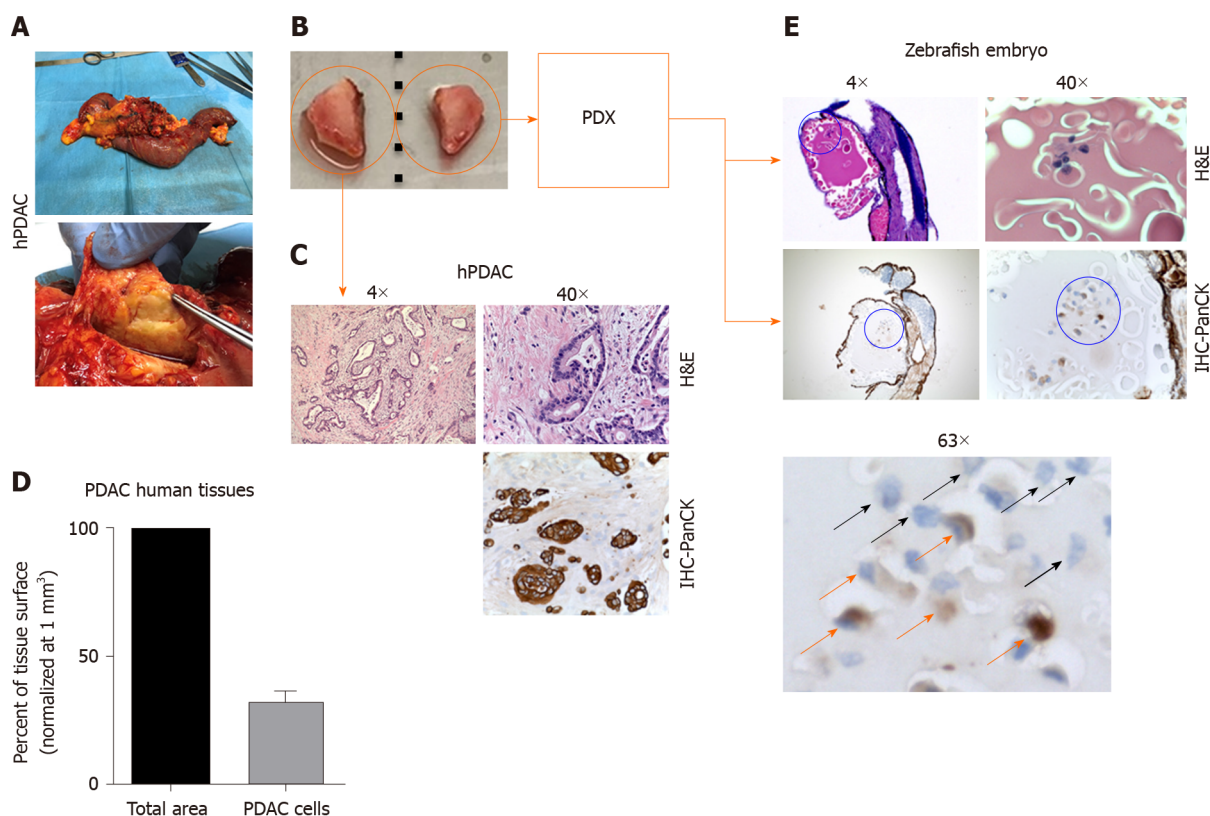
Table 2 Survival rate of the xenotransplanted zebrafish embryos

No. of case	1 d post injection					2 d post injection			
	Control group	GEM	GEMO-X	GEM/ nab-P	FOLF-FOXIRI	Control group	GEM	GEMOX	GEM/nab-P
1	0.909	0.833	0.917	0.917	0.900	0.818	0.667	0.833	0.583
2	0.417	0.800	0.917	0.667	0.833	0.333	0.583	0.667	0.500
3	0.833	1.000	0.636	0.917	0.833	0.417	0.818	0.182	0.75
4	0.682	0.438	0.7692	0.750	0.647	0.545	0.375	0.692	0.688
5	0.533	0.750	0.667	1.000	0.555	0.533	0.625	0.267	0.824
6	0.476	0.429	0.462	0.563	0.688	0.477	0.429	0.462	0.563
7	0.857	0.692	0.846	0.733	0.688	0.667	0.538	0.615	0.267
8	0.864	0.533	0.765	0.933	0.588	0.727	0.533	0.539	0.800
9	0.652	0.471	0.058	0.471	0.556	0.609	0.353	0.059	0.471
10	0.800	0.611	0.733	0.800	0.800	0.150	0.5	0.667	0.600
11	0.917	0.923	1.000	0.800	1.000	0.500	0.462	0.571	0.500
12	0.789	0.75	0.687	0.556	0.529	0.632	0.625	0.312	0.167
13	0.957	0.824	0.75	0.765	0.833	0.739	0.647	0.500	0.471
14	0.700	0.467	0.556	0.500	0.333	0.550	0.267	0.278	0.167
15	0.846	0.786	0.786	0.538	0.833	0.615	0.357	0.429	0.462
Mean survival rate	0.749	0.687	0.703	0.727	0.708	0.554	0.519	0.471	0.521

GEM: Gemcitabine; GEMOX: Gemcitabine + Oxaliplatin; GEM/nab-P: Gemcitabine + nab-Paclitaxel; FOLF-FOXIRI: 5-Fluorouracil + Folinic acid + Oxaliplatin + Irinotecan.

considered the gold standard model for assessing pre-clinical efficacy of cancer drugs. The methodology of initiation and propagation consists in sectioning the fresh surgical tissue into about 3 mm<sup>3</sup> pieces, followed by subcutaneous or orthotopic implantation into the flank of an immunodeficient mouse. During the engraftment phase, tumors are allowed to establish and grow and then are harvested upon reaching a size of 1500 mm<sup>3</sup>. Similar protocols are employed for subsequent expansion cohort and treatment cohort<sup>[28]</sup>. Despite this preclinical model more closely recapitulate the heterogeneity of human tumors, there are inherent limitations. For example, mice are expensive and require large vivarium space, limiting the scale of experimentation. Moreover, the tumor propagation required a long amount of time, resulting in several genetic, pathological, histological and micro-environmental niche changes, that may not mirror the patient's tumor accurately. Mice are also furred, and is not possible to image disseminated cancer cells throughout the whole animal<sup>[29]</sup>. To address these issues, have been developed zebrafish xenograft transplantation approaches to engraft human cancers into two days post fertilization zebrafish embryos at a stage prior to the development of the adaptive immune system<sup>[15]</sup>. Moreover, both in organoids and in mPDX, to amplify tumor tissue to obtain enough material for chemoresponse analysis, the cells are subjected to a strong selection pressure, diverging from the original tumor<sup>[30]</sup>. For our purpose, we do not need cell expansion, instead we directly observe the drug response of human tumor material xenotransplanted in zebrafish embryos. We believe that this is an appeal of zebrafish xenotransplantation and not a limitation.

When compared to other vertebrate model systems, zebrafish embryos offer several advantages. The short generation time, the large number of offspring, transparency (enabling noninvasive imaging), the external development of the embryos and the small size make zebrafish a more practical and less expensive laboratory system than other *in vivo* cancer models<sup>[31]</sup>. The appeal of zebrafish xenograft lies also in the possibility to overcome some drawbacks of murine xenograft, such as the larger number of tumor cells needed (about 1 million), the long time required (from several weeks to months) to have a visible tumor implant, the need of immunosuppressed animals to avoid transplant rejection and the high difficulties to generate mouse xenotransplant models able to metastasize<sup>[32]</sup>. The patients-derived zebrafish avatars do not require tumor cell expansion and the results of the chemosensitivity assay can be obtained in just few days. Previously study with different tumor type (patient derived gastric xenografts) demonstrate sensitivity to chemotherapy after 2 d post treatment<sup>[33]</sup>. The difference in time scale of the assay is not due to the fast zebrafish's biology, but rather because zebrafish larvae are 10000 times smaller than adult mice therefore they require reduced cells number to perform the injection (ranging from



**Figure 2 Tumor tissue was successfully engrafted in healthy zebrafish embryos in all cases.** A: Surgical specimen was obtained from patient with diagnosed pancreatic ductal adenocarcinoma (PDAC); B: Two fragments of the tumor were taken; C and D: One of them was used for histological evaluation (C) revealing that the percentage of epithelial cells (mean PDAC counterpart) out of the total surface area is  $31.8 \pm 4.9\%$  ( $n = 3$ ) (D); E: The second fragment of the tumor was used for xenotransplantation into the yolk of zebrafish embryos 2 dpf. After 2 d post xenotransplantation, the zebrafish patient-derived xenografts (zPDX) are Formalin-Fixed Paraffin Embedded. We performed the hematoxylin and eosin staining and immunohistochemistry using anti-Human Pan-Cytokeratin antibody on zPDX sections, highlighting the presence of epithelial PDAC cells (orange arrows) and the immune-negative counterpart (black arrows) that might be associated with the microenvironment side of PDAC human tissue. PDAC: Pancreatic ductal adenocarcinoma; PDX: Patient-derived xenografts; PanCK: Pan-Cytokeratin antibody.

500 to 5000 cells in each zebrafish embryo instead of  $1 \times 10^6$  in the mouse). A lower quantity of PDAC cells let to perform a higher number of xenograft that provide powerful statistical analysis<sup>[23,34]</sup>. All these aspects make possible to evaluate an evident effect of chemotherapy in just few days.

Zebrafish transplantation models offer the possibility to study many hallmarks of cancer and steps of cancer progression, such as self-renewal, tumor-induced angiogenesis, invasion and dissemination, interaction between tumor and host, and drug responses<sup>[4]</sup>. In fact, several studies have exemplified the potential of zebrafish models as transplantation metastatic models or to contribute more significantly and directly to precision oncology through identifying and testing drugs for targeted inhibition of specific pathways/alterations by utilizing zebrafish as an *in vivo* drug screening platform. In fact, zebrafish model has some advantages. Firstly, the aquatic environment of the zebrafish means that many drugs (depending on solubility) can be added directly to the embryo water and absorbed through the fish, avoiding the burden of administering drug to each individual animal<sup>[35]</sup>. However, it is absolutely essential to determine the toxicity curve for each compound before embarking on xenograft studies and following completion of toxicity curves and in this setting drug toxicity can be easily evaluated in zebrafish. Indeed, xenografted zebrafish can be exposed to the appropriate concentration of drug and observed over time for cancer progression, in terms of cell proliferation, migration and angiogenesis<sup>[32]</sup>. In this regard, we have performed a safety study with the calculation of the ED human to fish able to impair the increase of the mean RTA of xenotransplanted tumor tissue in zebrafish embryos<sup>[20]</sup>. As reported in our initial experience, an ED = 5 was effective both for cancer cell lines and for tumor tissue xenotransplanted in zebrafish embryos. Therefore, in this analysis we used this ED in the tests performed with pancreatic tissue directly xenotransplanted in the zebrafish embryos.

To date, very few authors have used zebrafish embryos to test new drugs after xenotransplantation of pancreatic cancer tissue. Weiss and colleagues used zebrafish embryos as a patient-derived transplantation model of metastasis for pancreatic

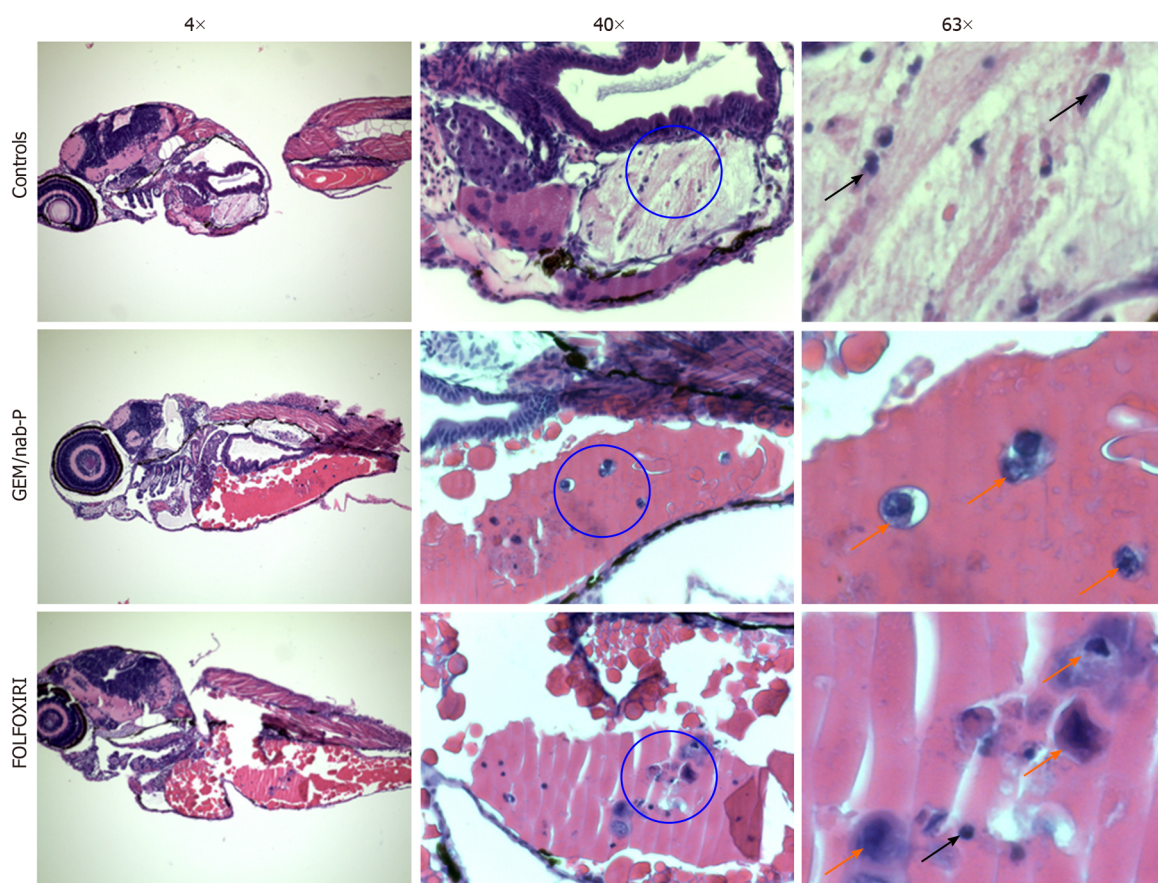
**Table 3 Results of exposure to chemotherapy in xenotransplanted zebrafish embryos**

Mean relative tumor area					Mean difference				95%CI				P value			
Con- trol group	FOLF- OXIRI	GEMO -X	GEM/ nab-P	GEM	k vs FOLF- OXIRI	k vs GEMO -X	k vs GEM/ nab-P	k vs Gemci -tabine	k vs FOLF- OXIRI	k vs GEMO -X	k vs GEM/ nab-P	k vs Gemci -tabine	k vs FOLF- OXIRI	k vs GEMO -X	k vs GEM/ nab-P	k vs Gemci -tabine
3.026	0.790	1.243	0.988	1.213	2.236	1.784	2.038	1.813	0.286 to 4.187	-0.167 to 3.734	-0.047 to 4.123	-0.198 to 3.823	<b>0.020</b>	0.081	0.057	0.087
1.964	0.755	1.188	0.529	1.674	1.209	0.776	1.435	0.290	-0.016 to 2.434	-0.522 to 2.073	0.099 to 2.771	-0.978 to 1.558	0.054	0.361	<b>0.032</b>	0.936
1.699	0.524	1.599	1.238	0.706	1.175	0.100	0.461	0.993	-0.026 to 2.376	-1.348 to 1.548	-0.645 to 1.567	-0.113 to 2.099	0.057	0.999	0.659	0.088
1.604	0.951	1.990	1.767	1.013	0.653	-0.385	-0.163	0.592	-0.452 to 1.759	-1.551 to 0.780	-1.269 to 0.942	-0.724 to 1.907	0.381	0.817	0.987	0.616
2.357	2.260	1.689	1.566	1.263	0.097	0.668	0.792	1.094	-2.112 to 0.261	-1.363 to 2.700	-0.679 to 2.262	-0.479 to 2.668	> 0.990	0.817	0.458	0.244
2.098	0.714	1.423	1.130	1.672	1.384	0.674	0.968	0.426	-2.112 to 0.261	-0.826 to 2.175	-0.411 to 2.346	-1.075 to 1.926	<b>0.034</b>	0.610	0.236	0.879
2.170	1.663	1.746	1.205	0.965	0.507	0.424	0.966	1.205	-2.112 to 0.261	-0.760 to 1.607	-0.478 to 2.409	0.022 to 2.388	0.560	0.770	0.270	<b>0.040</b>
1.224	0.489	1.802	1.189	1.221	0.735	-0.578	0.035	0.003	-2.112 to 0.261	-2.130 to 0.975	-1.311 to 1.382	-1.549 to 1.556	0.730	0.750	> 0.990	> 0.990
1.606	0.710	0.423	0.606	0.945	0.896	1.183	1.000	0.662	-2.112 to 0.261	-0.383 to 2.748	0.083 to 1.918	-0.417 to 1.740	0.057	0.183	<b>0.029</b>	0.342
1.046	0.575	1.037	0.468	0.514	0.471	0.009	0.579	0.533	-2.112 to 0.261	-1.094 to 1.113	-0.557 to 1.714	-0.586 to 1.650	0.643	> 0.990	0.506	0.563
2.669	1.203	1.207	1.428	1.371	1.466	1.462	1.241	1.298	-2.112 to 0.261	-1.407 to 4.331	-1.326 to 3.807	-1.268 to 3.864	0.338	0.492	0.537	0.499
1.371	0.852	0.705	1.383	1.077	0.520	0.666	-0.012	0.294	-0.667 to 1.706	-0.775 to 2.107	-1.452 to 1.429	-0.774 to 1.363	0.627	0.584	> 0.990	0.888
1.648	0.8481	0.8028	1.174	1.273	0.7996	0.8449	0.4737	0.3748	0.019 to 1.580	-0.030 to 1.720	-0.3717 to 1.319	-0.471 to 1.220	<b>0.043</b>	0.061	0.433	0.632
1.275	1.704	0.888	2.157	1.683	-0.429	0.387	-0.882	-0.408	-1.975 to 1.117	-0.957 to 1.731	-2.428 to 0.664	-1.954 to 1.138	0.879	0.864	0.394	0.896
1.041	1.966	1.167	0.789	1.091	-0.926	-0.126	0.252	-0.050	-2.112 to 0.261	-1.356 to 1.104	-1.035 to 1.539	-1.281 to 1.180	0.162	> 0.990	0.963	> 0.990

k: Control group; GEM: Gemcitabine; GEMOX: Gemcitabine + Oxaliplatin; GEM/nab-P: Gemcitabine + nab-Paclitaxel; FOLFOXIRI: 5-Fluorouracil + Folinic acid + Oxaliplatin + Irinotecan.

cancer<sup>[16]</sup>. They transplanted pancreatic carcinoma cells and resected specimens of human pancreatic carcinoma into zebrafish embryos and the model was used to demonstrate the *in vivo* anti-metastatic activity of retinoid acid receptor antagonists, following the identification of the retinoid acid target miR-10A as a key mediator of metastasis in pancreatic cancer. Also, Guo *et al*<sup>[17]</sup> used the pancreatic adenocarcinoma xenograft model in zebrafish embryos evaluating their possible use for the screening of new anti-cancer compounds. They found that a known small molecule inhibitor, U0126, targeting the KRAS signaling pathway, represses proliferation and migration of the transplanted of Mia PaCa-2 cells in zebrafish larvae<sup>[17]</sup>. Due to the permeability of zebrafish embryos to small molecules, a number of compounds can be added directly to the embryo water, and Guo *et al*<sup>[17]</sup> dissolved the drug treatment, U0126, in DMSO and added it directly to the water, as we performed in our study. The results reported by Guo *et al*<sup>[17]</sup> suggest that zebrafish larvae as xenotransplantation model of pancreatic cancer is useful for facilitating *in vivo* drug screening and identification of new anti-pancreatic cancer compounds. Instead, in our experience, we tested the different chemotherapy schemes already used for the treatment of pancreatic cancer in the common clinical practice in order to evaluate if the zebrafish model could be used for the definition of a personalized treatment plan for each patient with pancreatic cancer. The idea of precision oncology is that in the future primary specimens from patients diagnosed with cancer could be xenotransplanted in zebrafish embryos to test the responses of the patient cancer cells to various available drugs and the output of the test, obtainable in a few days, will dictate the most effective treatment for the individual patient. The application of the zebrafish model to precision oncology is still in its infancy, and there are not yet examples of direct use





**Figure 3 Representative hematoxylin and eosin stained sections of zebrafish patient-derived xenografts.** Visible morphologic alteration of the human pancreatic ductal adenocarcinoma nuclei (orange arrows) in zebrafish patient-derived xenografts exposed to Gemcitabine + nab-Paclitaxel and 5-Fluorouracil + Folinic acid + Oxaliplatin + Irinotecan could be associated with a chemotherapy damage. Control group shows normal nuclei (black arrows). GEM/nab-P: Gemcitabine + nab-Paclitaxel; FOLFOXIRI: 5-Fluorouracil + Folinic acid + Oxaliplatin + Irinotecan.

of zebrafish to guide patient-specific cancer treatments in clinical practice. However, this field has matured enough to move towards this aim in the near future. Modeling cancer in zebrafish has provided important insights that contribute to the development of precision oncology as well as straightforward examples of advantages and feasibility of direct clinical utilization<sup>[36]</sup>. As reported by different studies, it is possible to xenotransplant in zebrafish embryos tumor cells from cancer cell lines<sup>[32]</sup> and preliminary studies about the possibility of a direct, real-time application of zebrafish xenograft models in clinical practice suggests the possibility to use zebrafish embryos for a precision oncology<sup>[37]</sup>. However, in order to obtain a tool for precision medicine and personalized medicine, it is important to transplant primary cancer cells from biopsy or surgical specimen. In fact, studies with the use of commercially available cell lines are criticized because the results gleaned do not always correlate with those found in primary cancers<sup>[32]</sup>. In fact cell lines do not capture the heterogeneity that exists within a given cancer because clones with a higher proliferative rate than that of the primary tumor are selected during the process of adaptation, and thus they could not be really representative of the cancer cell population<sup>[38]</sup>. However, the experience with pancreatic tissue is limited. Marques *et al*<sup>[15]</sup> described the first use of primary patient material as the transplanted tissue using small samples from pancreas, colon and stomach adenocarcinomas. Pancreatic tumor fragments showed invasion and migration in the developing zebrafish and, comparing the non-invasive pancreatitis tissue xenografted with those from infiltrating pancreatic adenocarcinoma, only the latter invaded the embryos<sup>[15]</sup>. Guo *et al*<sup>[17]</sup> used cells acquired from culture dishes using a non-enzymatic cell-lifting solution and injected approximately 100-200 cancer cells labeled with CM-DiI into the perivitelline cavity of each zebrafish. Instead in our experience, in order to preserve the tumor micro-environment, we decided to xenotransplant fresh tissue fragments, by modifying the protocol published by Marques *et al*<sup>[15]</sup>. Our data confirmed the possibility to xenotransplant tumor cells taken directly from the tumor tissue of each patient, obtaining a model for testing the sensibility to the different chemotherapies associated with the specific alterations present in the tumor of each patient. In fact, in

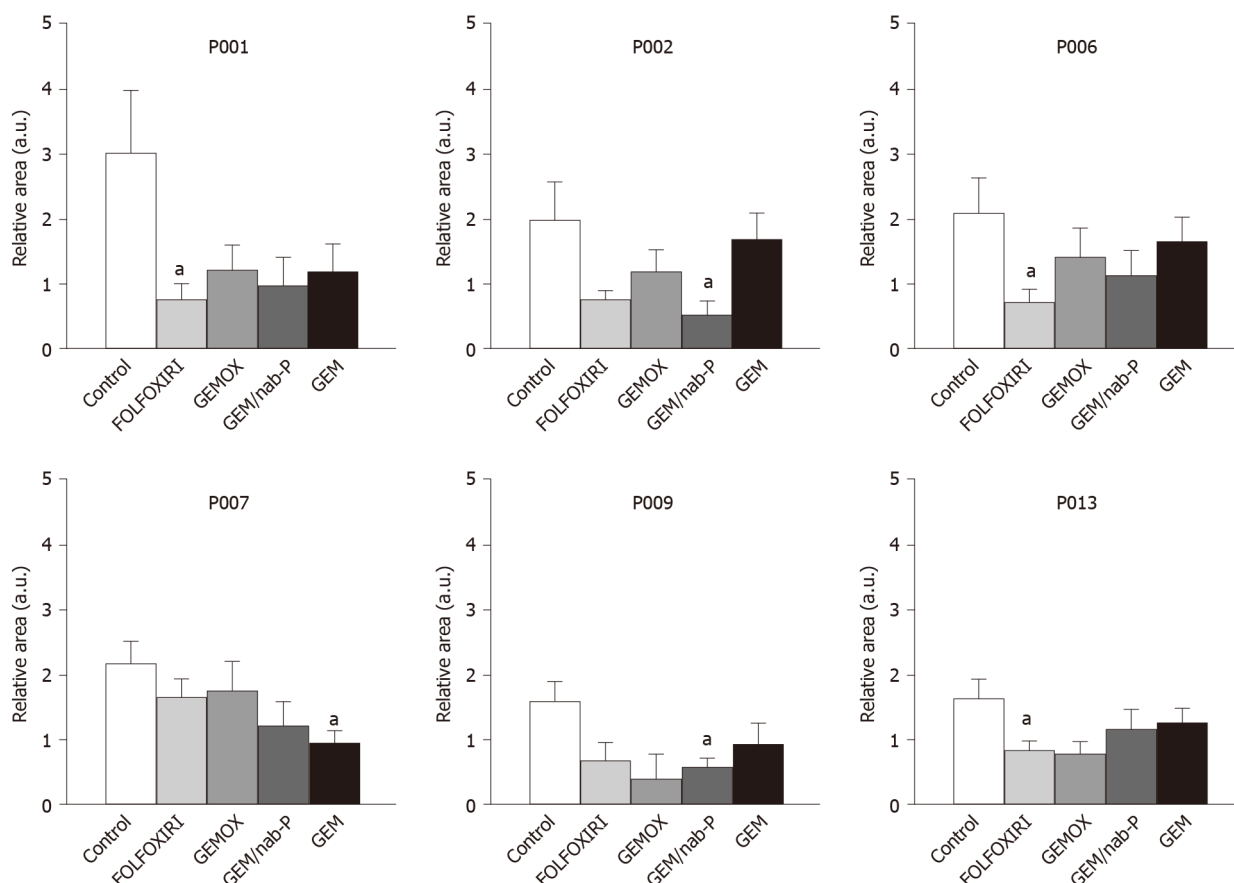
**Table 4** Percentage reduction of the mean relative tumor area

No. of case	k vs FOLFOXIRI (%)	k vs GEMOX (%)	k vs Gem/nab-P (%)	k vs Gemcitabine (%)
1	-74	-59	-67	-60
2	-62	-40	-73	-15
3	-69	-6	-27	-58
4	-41	24	10	-37
5	-4	-28	-34	-46
6	-66	-32	-46	-20
7	-23	-20	-44	-56
8	-60	47	-3	0
9	-56	-74	-62	-41
10	-45	-1	-55	-51
11	-55	-55	-46	-49
12	-38	-49	1	-21
13	-49	-51	-29	-23
14	34	-30	69	32
15	54	-8	-38	-14

k: Control group; GEM: Gemcitabine; GEMOX: Gemcitabine + Oxaliplatin; GEM/nab-P: Gemcitabine + nab-Paclitaxel; FOLFOXIRI: 5-Fluorouracil + Folinic acid + Oxaliplatin + Irinotecan.

all cases of the control group the tumor area increased at 1 dpi and 2 dpi.

Very few studies evaluate the use of zebrafish embryos for personalized medicine and passing to the best of our knowledge there are no results in literature of treatment correlation between zebrafish xenografts and matched PDAC patients. However a preclinical human cancer xenotransplantation platform has been recently developed in zebrafish to inform therapeutic decisions in patients with T-cell acute lymphoblastic leukemia<sup>[37]</sup>. Moreover, a retrospective study with zPDXs from multiple myeloma cells, demonstrate that zPDXs showed a response equivalent to patient's clinical outcome<sup>[39]</sup>. Similarly, Wu *et al*<sup>[33]</sup> showed a retrospective correlation with one gastric tumor patient clinical outcome. In 2017 Fior *et al*<sup>[34]</sup> performed a retrospective study, showing that colorectal patient's avatars are predictive of patient clinical outcome in 4 out 5 patients (80%). These results bode well for our co-clinical trial (NCT03668418). Our study is the first one that evaluate the use of zebrafish embryos as avatar for patients affected by pancreatic cancer. Preliminary results are encouraging. Indeed, in all cases the tumor cells successfully engrafted in the xenotransplanted zebrafish embryos and the mean xenotransplanted zebrafish embryos survival rate was 71.5% at 1 dpi but decreased at 52.4% at 2 dpi, probably due to the high invasiveness of the tumor xenografts and the everyday embryo anesthetization and manipulation to image the tumor. Comparing directly the mean RTA between the treated subgroups and the control group we obtained a statistically significant reduction of the mean RTA in the treated subgroups for at least one chemotherapy scheme in only 6/15 (40%) cases. Moreover, due to the too short follow-up of the enrolled patients and the consequent absence of clinical data of response to chemotherapy treatments, we tried to use the RECIST criteria in the experiment with the xenotransplanted zebrafish embryos, with the intent to use an objective parameter in the evaluation of the robustness of our model. In this way, we evaluate if the results of the tests with the different chemotherapy scheme could be in accordance with data reported in large number of series published in literature. We evaluated the percentage of the mean RTA reduction in the treated subgroups in comparison to the control group for each case, reporting interesting results. First of all, we reported for each chemotherapy scheme tested the presence of a linear relationship between each threshold of the percentage reduction of the mean RTA and the number of cases in which we reported the mean RTA reduction at least for a percentage equal or greater to the threshold value. Interestingly, the FOLFOXIRI scheme resulted the most efficacious in accordance with data reported in the literature in the common clinical practice for the treatment of patients with PDAC<sup>[7,40-45]</sup>. Moreover, using the linear regression line equation, we calculated a conversion factor for each chemotherapy scheme evaluating the percentage reduction of the mean RTA in each treated subgroup corresponding to the percentage of patients with a partial response after chemotherapy treatment reported in literature. Interestingly, the conversion factor value calculated for each chemotherapy scheme resulted similar to



**Figure 4** Cases with a statistically significant reduction of the mean relative tumor area for at least one chemotherapy scheme (the code below each diagram corresponds to the case number). Results are expressed as average  $\pm$  SEM, <sup>a</sup> $P < 0.05$ , 1-way ANOVA followed by Dunnett's multiple comparisons test. Control group shows normal nuclei (black arrows). GEM: Gemcitabine; GEMOX: Gemcitabine + Oxaliplatin; GEM/nab-P: Gemcitabine + nab-Paclitaxel; FOLFOXIRI: 5-Fluorouracil + Folinic acid + Oxaliplatin + Irinotecan.

the other ones with a value ranging between -63.7% and -59.3%. So, we can hypothesize that our model reflects the different efficacy of the various chemotherapy schemes used for the treatment of patients affected by PDAC. In fact, we obtained different percentage reductions of the mean RTA and different linear regression lines for each treated subgroup and these data are in accordance with the different efficacy reported in literature of the various chemotherapy schemes used in the common clinical practice. In fact, we reported the same conversion factor in all subgroups. Naturally these are preliminary data and we do not have confirming data from the chemotherapy treatment of the patients enrolled because of the too short follow-up. We know that this comparison has important limitations and could not replace the comparison with data obtainable with the follow-up of the enrolled patients. We performed this analysis only to give an initial assessment of the reliability of our model. However, taking in consideration the previous premises, these preliminary results are encouraging because first of all they confirmed the possibility to successfully xenotransplant directly the tumor tissue in the zebrafish embryos. Moreover, we observed the effect of the chemotherapy on the xenotransplanted tumor tissue with the possibility to use the zebrafish embryos as a model to predict the possible efficacy of the chemotherapy treatment. The correlation between these data and the real clinical response to adjuvant chemotherapy will be essential to determine the possible role of our model in predicting the efficacy of the chemotherapy scheme administered to patients with PDAC. A co-clinical trial (NCT00070213) is underway in our institution and will be object of further publication.

In conclusion, the described model appears to be an effective, usable and not expensive model for the xenotransplantation of pancreatic tumor tissue and for the evaluation of the efficacy of the different chemotherapy schemes available for the treatment of patients with PDAC. This could open a new frontier to personalized medicine because the results of the tests obtained in our model in the xenotransplanted zebrafish embryos could reflect the clinical course of the patients' medical history, and such an approach might improve the evaluation of the patient's

**Table 5 Analysis of the percentage reduction of the mean relative tumor area in the treated subgroups in comparison to the control group**

Threshold value	Number of cases, <i>n</i> (%)			
	k vs FOLFOXIRI	k vs GEMOX	k vs Gem/nab-P	k vs Gemcitabine
-10%	13 (87)	13 (87)	12 (80)	13 (87)
-20%	12 (80)	10 (67)	11 (73)	11 (73)
-30%	11 (73)	8 (53)	9 (60)	8 (53)
-40%	10 (67)	6 (40)	7 (47)	7 (47)
-50%	7 (47)	5 (33)	4 (27)	4 (27)
-60%	5 (33)	1 (7)	4 (27)	1 (7)
-70%	1 (7)	1 (7)	1 (7)	0 (0)
-80%	0 (0)	0 (0)	0 (0)	0 (0)

k: Control group; GEM: Gemcitabine; GEMOX: Gemcitabine + Oxaliplatin; GEM/nab-P: Gemcitabine + nab-Paclitaxel; FOLFOXIRI: 5-Fluorouracil + Folinic acid + Oxaliplatin + Irinotecan.

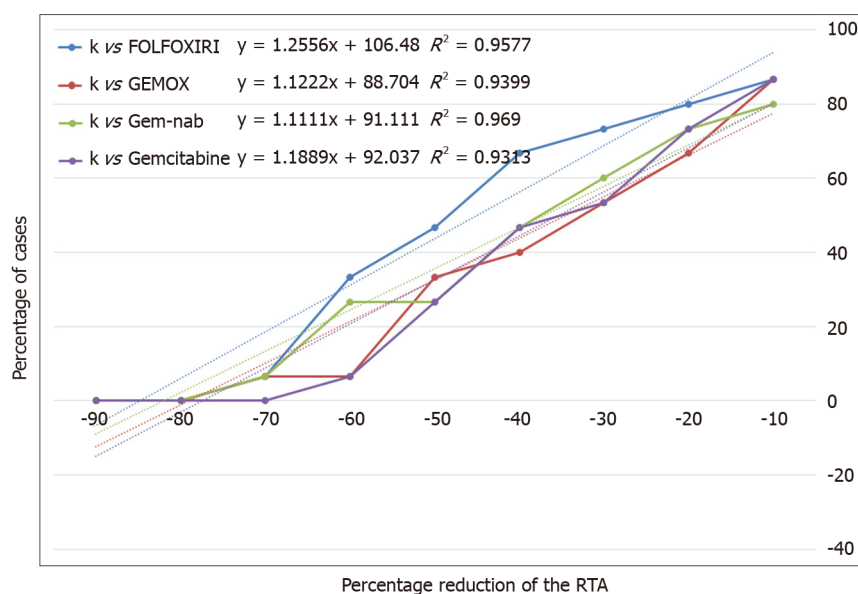
prognosis and the identification of the most appropriate individualized therapy.



**Table 6** Calculation of the conversion factor for the test with xenotransplanted zebrafish embryos

	Study	Percentage of partial response reported in literature	Conversion Factor for xenotransplanted zebrafish embryos	Mean conversion factor ( $P = 0.626$ )
k vs FOLFOXIRI	Conroy <i>et al</i> <sup>[7]</sup> , 2011	31	-60.1	-60.4
	Tong <i>et al</i> <sup>[40]</sup> , 2018	35.9	-56.2	
	Lakatos <i>et al</i> <sup>[41]</sup> , 2017	18.8	-69.8	
	Nitsche <i>et al</i> <sup>[42]</sup> , 2015	42.8	-50.7	
	Sadot <i>et al</i> <sup>[43]</sup> , 2015	29	-61.7	
	Wang <i>et al</i> <sup>[44]</sup> , 2019	32.3	-59.1	
k vs GEMOX	Shi <i>et al</i> <sup>[46]</sup> , 2007	26.8	-55.2	-59.3
	Li <i>et al</i> <sup>[47]</sup> , 2010	25	-56.8	
k vs Gem/nab-P	Von Hoff <i>et al</i> <sup>[48]</sup> , 2013	23	-61.3	-62.4
	Casanova-Martinez <i>et al</i> <sup>[49]</sup> , 2018	20	-64.0	
	Karasic <i>et al</i> <sup>[50]</sup> , 2019	21.1	-63.0	
	Montes <i>et al</i> <sup>[51]</sup> , 2017	23	-61.3	
k vs Gemcitabine	Conroy <i>et al</i> <sup>[7]</sup> , 2011	16	-63.9	-63.7
	Louvet <i>et al</i> <sup>[52]</sup> , 2005	17.3	-62.8	
	Ergun <i>et al</i> <sup>[53]</sup> , 2018	15.3	-64.5	

k: Control group; GEM: Gemcitabine; GEMOX: Gemcitabine + Oxaliplatin; GEM/nab-P: Gemcitabine + nab-Paclitaxel; FOLFOXIRI: 5-Fluorouracil + Folinic acid + Oxaliplatin + Irinotecan.



**Figure 5** Percentage of cases with a percentage reduction of mean relative tumor area equal or greater to each threshold value. RTA: relative tumor area; Control group shows normal nuclei (black arrows). k: Control group; GEM: Gemcitabine; GEMOX: Gemcitabine + Oxaliplatin; GEM/nab-P: Gemcitabine + nab-Paclitaxel; FOLFOXIRI: 5-Fluorouracil + Folinic acid + Oxaliplatin + Irinotecan.

## ARTICLE HIGHLIGHTS

### Research background

The response of patients with pancreatic ductal adenocarcinoma (PDAC) to the different chemotherapy schemes is difficult to predict. The concept of precision medicine has emerged recently with the objective to tailor the medical treatment to the individual characteristics of each patient, and particularly to the tumor biology of each patient.

### Research motivation

Recently, the use of zebrafish as avatar for oncological patients has gained popularity. However,

only preliminary studies were conducted with patient-derived pancreatic cancer cells or tissue.

### Research objectives

The aim of this study is to evaluate the usability of zebrafish embryos as a model possibly simple, not expensive and diffusible, that could be used as avatar for patients affected by PDAC, to predict the efficacy of the different chemotherapy schemes and the clinical response to the treatment.

### Research methods

A fragment of the tumor was taken from the surgical specimen and sectioned into about 3 mm<sup>3</sup> pieces for DiI staining. Small pieces of stained tissue were xenotransplanted into the yolk of  $n = 100$  zebrafish embryos 2 dpf. The zebrafish xenografts were incubated at 35°C with the presence or absence of drugs (GEM, GEMOX, GEM/nab-P, FOLFOXIRI) dissolved in fish water, using the equivalent dose (ED = 5). Firstly, we compared the mean relative tumor area (RTA) between each treated subgroup and the control group. Secondly, we evaluated the percentage reduction of the mean RTA (PRmRTA) in each treated subgroup in comparison to the control group, evaluating the presence of a linear relationship between each threshold value of the PRmRTA and the number of cases reporting a PRmRTA equal or greater to each threshold value. Using the linear regression line equation, we calculated for each protocol the expected percentage RTA reduction with the following formula:  $\text{Expected percentage reduction of the mean RTA} = (\text{percentage of PR reported in literature} - \text{qlinear regression line equation}) / \text{mlinear regression line equation}$ . For each chemotherapy protocol, we calculated the mean value of the expected PRmRTA and compared each other's.

### Research results

In the control group the DiI-stained areas showed a statistically significant increase over time in all cases, while a tendency to a reduction of the mean RTA was observed in treated subgroups, with a statistically significant reduction of the mean RTA for at least one chemotherapy scheme in 6/15 (40%) cases. The presence of a linear relation between the percentage reduction of the RTA and the number of cases reporting at least each percentage threshold was observed in each subgroup. The mean conversion factor was -60.4% for FOLFOXIRI, -59.3% for GEM-nab/P, -62.4% for GEMOX, and -63.7% for GEM ( $P = 0.626$ ).

### Research conclusions

This study provides a simple, reliant and not expensive PDAC patients-derived xenograft model for the rapid pre-clinical evaluation of the efficacy of different chemotherapy schemes available for the treatment of each individual PDAC patient's case.

### Research perspectives

Our model seems to reflect the clinical response rate reported in literature. However, to determinate the possible capability of our model in predicting the efficacy of the chemotherapy scheme administered to patients with PDAC, it is necessary to evaluate the correlation between these data and the real clinical response to adjuvant chemotherapy on patients. A co-clinical trial (NCT00070213) is underway in our institution and will be object of further publication.

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## Basic Study

# Gan Shen Fu Fang ameliorates liver fibrosis *in vitro* and *in vivo* by inhibiting the inflammatory response and extracellular signal-regulated kinase phosphorylation

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### Institutional review board

**statement:** The study was reviewed and approved by the Institutional Review Board at Beijing University of Chinese Medicine.

### Institutional animal care and use

**committee statement:** All experimental procedures were

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## Abstract

### BACKGROUND

Liver fibrosis is a common health problem worldwide and there is still a lack of effective medicines. The Chinese herbal medicine, Gan Shen Fu Fang (GSFF) is composed of salvianolic acid B and diammonium glycyrrhizinate. In this study, we observed the effects of GSFF on liver fibrosis *in vivo* and *in vitro* in an attempt to provide some hope for the treatment.

### AIM

To observe the effects of GSFF on liver fibrosis *in vivo* and *in vitro* and investigate the mechanism from the perspective of the inflammatory response and extracellular signal-regulated kinase (ERK) phosphorylation.

### METHODS

Common bile duct-ligated rats were used for *in vivo* experiments. Hepatic stellate cells-T6 (HSC-T6) cells were used for *in vitro* experiments. Hematoxylin and eosin staining and Masson staining, biochemical assays, hydroxyproline (Hyp) assays, enzyme-linked immunoassay and western blotting were performed to evaluate the degree of liver fibrosis, liver function, the inflammatory response and ERK phosphorylation. The CCK8 assay, immunofluorescence and western

conducted in accordance with the guidelines for the use of experimental animals and were approved by the Institutional Review Committee on Animal Care and Use at the Experimental Animal Centre of Beijing University of Chinese Medicine [certificate of conformity: SCXK (2012-0001)].

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blotting were applied to test the effect of GSFF on HSC-T6 cell activation and determine whether GSFF had an effect on ERK phosphorylation in HSC-T6 cells.

## RESULTS

GSFF improved liver function and inhibited liver fibrosis in common bile duct-ligated rats after 3 wk of treatment, as demonstrated by histological changes, hydroxyproline assays and collagen I concentrations. GSFF alleviated inflammatory cell infiltration and reduced the synthesis of pro-inflammatory cytokines [tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$ ] and NF- $\kappa$ B. In addition, GSFF decreased ERK phosphorylation. *In vitro*, GSFF inhibited the viability of HSC-T6 cells with and without transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) stimulation and decreased the synthesis of collagen I. GSFF had the greatest effect at a concentration of 0.5  $\mu$ mol/L. GSFF inhibited the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), a marker of HSC activation, in HSC-T6 cells. Consistent with the *in vivo* results, GSFF also inhibited the phosphorylation of ERK and downregulated the expression of NF- $\kappa$ B.

## CONCLUSION

GSFF inhibited liver fibrosis progression *in vivo* and HSC-T6 cell activation *in vitro*. These effects may be related to an alleviated inflammatory response and downregulated ERK phosphorylation.

**Key words:** Liver fibrosis; Gan Shen Fu Fang; Inflammatory response; Extracellular signal-regulated kinase phosphorylation; *In vivo*; *In vitro*

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**Core tip:** Liver fibrosis results from various kinds of chronic liver diseases and there is no specific treatment so far. Inflammatory response and extracellular signal-regulated kinase cascade play an important role in liver fibrosis development and progression. In this study, we observed the effects of herbal medicine, Gan Shen Fu Fang (GSFF) on liver fibrosis *in vivo* and *in vitro*. The results indicate that GSFF alleviates liver fibrosis progression *in vivo* and inhibits HSC-T6 activation *in vitro*, which may be related with inhibited inflammatory response and downregulated extracellular signal-regulated kinase phosphorylation. GSFF may provide hope for liver fibrosis treatment.

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## INTRODUCTION

Liver fibrosis, characterized by the excessive deposition of extracellular matrix (ECM), is the common result of various kinds of chronic liver diseases (chronic viral hepatitis, alcoholic hepatitis, nonalcoholic fatty liver disease, etc.). Liver fibrosis progresses and ultimately leads to liver cirrhosis. Cirrhosis is characterized by the replacement of normal liver with fibrous septa, which disrupts the normal liver architecture and causes the formation of numerous abnormal nodules. Liver cirrhosis can induce severe consequences, such as ascites, splenomegaly, collateral circulation formation, and even upper gastrointestinal bleeding that can lead to death<sup>[1]</sup>. Therefore, exploring the mechanism of liver fibrosis and developing effective and safe anti-fibrosis medicines remains a research focus.

Hepatic stellate cells (HSCs), which are located in the dissepimental space, are a kind of nonparenchymal cell. HSCs maintain a quiescent phenotype as fat-storing cells in the body<sup>[2]</sup>. Once the liver is injured, HSCs undergo dramatic phenotypic transformation. HSCs become activated and trans-differentiate into myofibroblasts, which are characterized by increased cell proliferation, survival,  $\alpha$ -SMA expression, and ECM production (including collagen I, collagen III and fibronectin)<sup>[3]</sup>. Thus, activation of

HSCs is a central event in liver fibrosis<sup>[4,5]</sup>. Inflammation is present in virtually all patients with liver fibrosis and correlated with fibrosis progression. The chronic inflammatory response is believed to sustain chronic liver disease progression<sup>[6,7]</sup>. Inflammation has been demonstrated to activate quiescent HSCs into myofibroblasts through enhanced TGF- $\beta$  signalling<sup>[8]</sup>. In addition, activated macrophages, which participate in the inflammatory response induced by aseptic or septic stimuli, release TGF- $\beta$  to promote ECM synthesis by activated HSCs<sup>[9]</sup>. Therefore, one strategy to alleviate liver fibrosis is regulation of the inflammatory response. NF- $\kappa$ B activation in macrophages and HSCs is a key precipitating factor that increases pro-inflammatory mediators, such as TNF- $\alpha$  and interleukin-1 $\beta$ . Such cytokines regulate inflammation, immune responses and cell survival in hepatic fibrosis<sup>[10,11]</sup>.

The extracellular signal-regulated kinase (ERK) cascade, which is a critical MAPK signalling pathway, plays a major role in liver fibrogenesis. The extracellular signals that stimulate the ERK cascade include platelet-derived growth factor (PDGF), TGF- $\beta$ , epidermal growth factor (EGF), and reactive oxygen species (ROS)<sup>[12]</sup>. Once activated, the signal is transmitted through the sequential phosphorylation and activation of sequential kinases. The phosphorylation of hundreds of substrates results in the induction of several extracellular signal-regulated kinase1/2(ERK1/2)-dependent processes. In liver fibrosis, the ERK cascade is strongly related to HSC activation and has been shown to lead to the increased proliferation and survival of HSCs<sup>[13,14]</sup>, synthesis of ECM<sup>[15]</sup>, and plays pro-inflammatory, immune-modulatory<sup>[7]</sup>, and pro-angiogenic roles<sup>[16]</sup>. Therefore, drugs and strategies designed to target the ERK1/2 signalling pathway provide hope for anti-fibrotic treatment.

Gan Shen Fu Fang (GSFF) (previously named Glytan), a Chinese herbal medicine, is composed of salvianolic acid B (SA-B) and diammonium glycyrrhizinate (DG). SA-B is extracted from *Salvia miltiorrhiza* and DG is from *liquorice*. *Salvia miltiorrhiza* and *liquorice* are commonly used herbal medicines in China. The molecular formulas of both SA-B and DG have been established. Previous studies on GSFF focused on its role in portal hypertension<sup>[17]</sup>. We have shown that GSFF could reduce portal pressure and portal territory blood flow and increase mean arterial pressure and splanchnic vascular resistance in common bile duct-ligated (CBDL) rats<sup>[18]</sup>. Because of GSFF's significant effect on decreasing portal pressure, clinical trial was approved by the FDA of China in 2015. During pre-clinical experiment, we found that GSFF could inhibit pseudo-lobule formation, restore the fenestrae of liver sinusoidal endothelial cells and reverse hepatic sinusoid capillarization in CBDL rats. Therefore, in this study, we aimed to observe the effects of GSFF on liver fibrosis *in vivo* and *in vitro* and explore whether GSFF-mediated alleviation of liver fibrosis is related to inflammation and the ERK signalling pathway.

## MATERIALS AND METHODS

### Animals

For the animal experiment, male Sprague-Dawley (SD) rats (SPF, Biotechnology Co., Ltd., Beijing, China) weighing 250-280 g were randomly divided into 3 groups: Sham group, CBDL group, and GSFF group. The rats underwent sham surgery or common bile duct ligation. The common bile ducts were exposed and ligated twice. The segment between the two ligations was resected, and the abdomen was sutured. The common bile ducts of sham rats were exposed but did not undergo ligation or resection. All experimental procedures were conducted in accordance with the guidelines for the use of experimental animals and were approved by the Institutional Review Committee on Animal Care and Use at the Experimental Animal Centre of Beijing University of Chinese Medicine [certificate of conformity: SCXK (2012-0001)].

SA-B (115939-25-8, purity  $\geq$  98%, Aladdin Biochemical Technology Co., Ltd.) and DG (s101148, purity  $\geq$  98%, Nature Standard Technical Service Co., Ltd.) were used at a ratio of 1:1. Before use, SA-B and DG were diluted with distilled water. After 1 wk of CBDL, rats in the GSFF group were treated with GSFF (25 mg/kg/d) by gavage. The dosage of GSFF depended on previous pharmacokinetic experimental results<sup>[18]</sup>. Rats in the sham and CBDL groups were administered the same amount of distilled water. The rats were sacrificed at 2 and 4 wk. Liver, spleen and body weights were recorded and used to calculate the liver and spleen coefficients as follows: Liver or spleen coefficient = liver or spleen weight/body weight. Liver tissues and blood were collected for subsequent analysis.

### HSC-T6 cell culture and treatment

The HSC-T6 rat HSC line (purchased from Kunming Cell Bank, Chinese Academy of Sciences) was cultured in high-glucose DMEM containing 10% FBS at 37°C with

5% CO<sub>2</sub>. First, to observe the effect of GSFF on HSC-T6 cell viability, based on some previous studies<sup>[19,20]</sup>, we chose and used a gradient of 6 concentrations, 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1 µmol/L, to select the appropriate concentration of GSFF to inhibit HSC-T6 cell viability. Second, to further verify the effect of GSFF on HSC-T6 cell viability, HSC-T6 cells were stimulated with TGF-β1 (2 ng/mL, Peprotech, United States) for 1 h, and the cells were then treated with GSFF. Third, to evaluate the effect of GSFF on ERK, HSC-T6 cells were pre-treated with PDGF-BB (10 ng/mL, Peprotech) for 1 h and then incubated with the ERK antagonist PD98059 (50 µmol/L, Abmole) or 0.125, 0.25, or 0.5 µmol/L GSFF for 24 h. Cells were starved of serum for 12 h before stimulation.

### **Histological and biochemical analysis and hydroxyproline assay**

Hematoxylin and eosin staining and Masson staining were performed according to our previous protocol<sup>[18]</sup>. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL) levels in the serum were measured using a commercial colorimetric kit (Randox Laboratories, Antrim, United Kingdom). The hydroxyproline (Hyp) content in the liver was measured with Hyp assay kits (Nanjing JianCheng, China). The Hyp content is expressed as micrograms of Hyp per milligram of wet liver weight.

### **Enzyme-linked immunoassorbent assay**

The concentration of collagen I (Col. I) in the culture supernatant of HSC-T6 cells was analysed using a commercial enzyme-linked immunoassorbent assay kit (CSB-E09243r, S-ABC kit). The concentrations of TNF-α and IL-1β in the liver tissue were analyzed according to the manufacturer's protocol.

### **CCK-8 assay**

The CCK-8 assay was performed to test cell viability. HSC-T6 cells were seeded in 96-well plates at  $5 \times 10^4$  cells/mL. After 24 h, the cells were treated with different concentrations of GSFF for 24 h. Subsequently, 10 µL of CCK-8 reagent was added to each well, followed by incubation at 37°C for 2 h. To further observe the inhibitory effect of GSFF on HSC-T6 cell activation, HSC-T6 cells were stimulated with TGF-β1 (2 ng/mL) for 1 h as described before and then treated with 0.125, 0.25, or 0.5 µmol/L GSFF for 24 h. The absorbance at 450 nm was measured with a microplate reader (Bio-Rad, United States).

### **Immunofluorescence to test α-SMA expression in HSC-T6 cells**

Cells were seeded at a density of  $2 \times 10^5$  cells/mL in laser confocal dishes. After 24 h, the HSC-T6 cells were stimulated with TGF-β1 for 1 h, and the cells were then treated with 0.125, 0.25, or 0.5 µmol/L GSFF for 24 h. The following day, the cells were fixed with 4% paraformaldehyde for 20 min. Then, the cells were treated with anti-α-SMA antibody (1:50, a gift from Christine Chaponnier, Geneva University) overnight at 4°C and incubated for 1 h with a secondary antibody (Alexa Fluor-594 donkey-anti-rabbit IgG secondary antibody). Finally, nuclei were stained with DAPI (1:1000) for 2 min in the dark. Images were taken using a confocal microscope (Olympus Fv1000). The percentage of α-SMA-positive cells was determined using Image J software.

### **Western blot analysis**

Col.I, NF-κB, ERK, and p-ERK protein expression levels were detected by western blotting as previously described<sup>[18]</sup>. In brief, liver samples or cell lysates were scraped in ice-cold lysis buffer. The protein content was quantified with a BCA protein assay reagent kit. Total liver and cell lysates were separated on 10% polyacrylamide gels by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. After blocking in a 5% nonfat powdered milk solution or bovine serum albumin for 1 h, the membranes were incubated with primary antibodies overnight at 4°C. The following primary antibodies were used for western blotting: mouse anti-collagen I (ab90395, Abcam, United States, 1:4000), rabbit anti-rat monoclonal NF-κB (p65) (8242S, Cell Signaling, United States, 1:4000), rabbit anti-rat monoclonal ERK (16443-1-AP, Proteintech Group, United States, 1:4000), and rabbit anti-rat monoclonal phospho-p44/42 mitogen-activated protein kinase (Erk1/2) (4370S, Cell Signaling Technology, MA, United States, 1:6000). Then, the membranes were incubated with the appropriate secondary antibody for 2 h at room temperature. GAPDH (60004-1-Ig, Proteintech Group, United States, 1:40000) was used as an internal reference/control.

### **Statistical analysis**

The experimental data were analysed using SPSS 22.0 statistical software, and the data are expressed as the mean ± SD. To compare differences in groups of data with a normal distribution and uniform variance, one-way ANOVA was used; if the data did



not conform to a normal distribution or the variance was not uniform, a nonparametric test was used.  $P < 0.05$  indicated a statistically significant difference.

## RESULTS

### **GSFF decreased ALT, AST, and TBIL levels and the liver and spleen coefficients in CBDL rats**

After 2 and 4 wk of CBDL, ALT, AST and TBIL were increased significantly in CBDL rats compared with sham rats, which indicated hepatocyte injury after cholestasis (Figure 1A-C). GSFF decreased ALT, AST and TBIL levels at 4 wk, but not at 2 wk (Figure 1A-C). In addition, GSFF reduced the liver and spleen coefficients at 2 and 4 wk (Figure 1D and E).

### **GSFF alleviated liver fibrosis and the inflammatory response in CBDL rats at 2 and 4 wk**

Hematoxylin and eosin staining showed a normal liver structure and hepatic cords arranged radially around the central vein in rats in the sham group (Figure 2A). After 2 wk, the proliferation of small bile ducts in CBDL rats was clearly observed. In addition, many fibroblasts and inflammatory cells had infiltrated to the area around the newly proliferated bile ducts. After 4 wk, with the progression of cholestasis, bile duct proliferation in CBDL rats was more severe, and a destroyed histological structure, the infiltration of a large number of inflammatory cells and fibrotic septa formation were observed. The increased inflammatory response was also confirmed by increased production of the cytokines TNF- $\alpha$  and IL-1 $\beta$  (Figure 2D and E). Masson staining indicated the extensive proliferation of fibrotic tissue in CBDL rats after 2 and 4 wk (Figure 2A and B). Particularly at 4 wk, the fibrotic tissue was linked, and the normal liver structure had been destroyed. Compared with CBDL rats, GSFF-treated rats showed alleviated bile duct proliferation, reduced inflammatory cell infiltration and TNF- $\alpha$  and IL-1 $\beta$  synthesis, and decreased fibrotic tissue accumulation after 2 and 4 wk of CBDL. Consistent with the results of Masson staining, the Hyp concentration was increased after 2 and 4 wk of CBDL, and GSFF reduced Hyp synthesis (Figure 2C).

### **GSFF inhibited HSC-T6 cell viability and collagen synthesis**

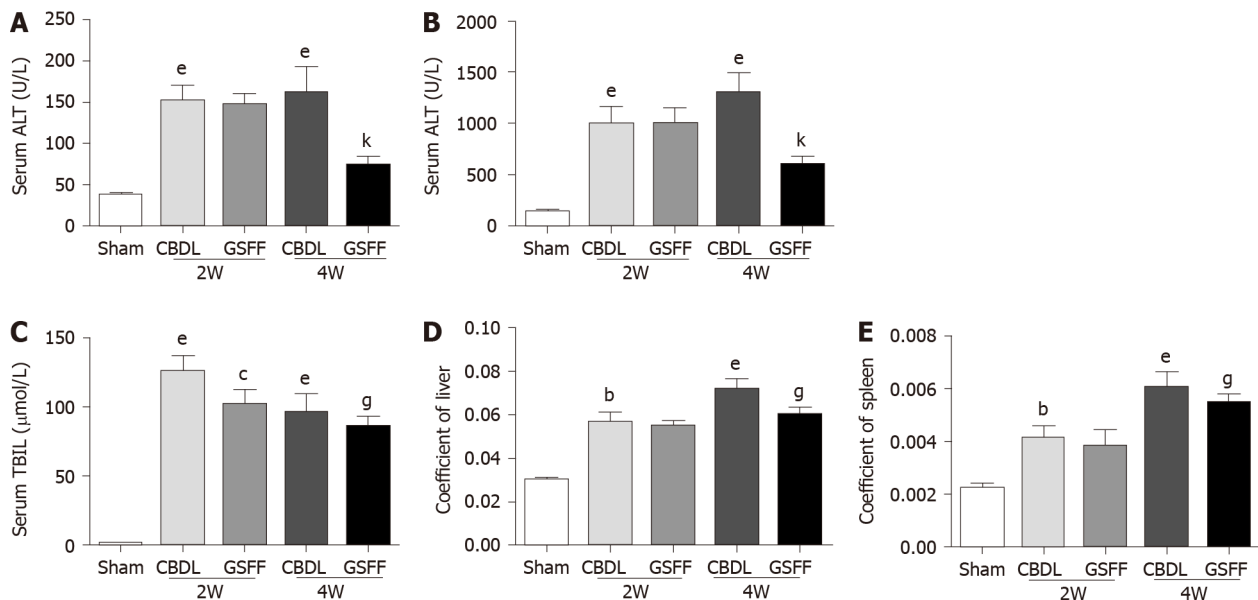
Treatment with 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1  $\mu\text{mol/L}$  GSFF for 24 h had an obvious inhibitory effect on the viability of HSC-T6 cells (Figure 3A). From this result, we identified the following two points. First, GSFF at a concentration from 0.03125 to 0.5  $\mu\text{mol/L}$  had a substantial dose-dependent inhibitory effect. In particular, the dose-dependent effect of GSFF at 0.125, 0.25, and 0.5  $\mu\text{mol/L}$  was evident ( $P < 0.001$ ). Second, although 1  $\mu\text{mol/L}$  GSFF also inhibited cell viability, the effect of GSFF at this dose was not as pronounced as that of GSFF at 0.5  $\mu\text{mol/L}$ . We also tested the effect of GSFF at other doses (5  $\mu\text{mol/L}$ , 10  $\mu\text{mol/L}$ ) and found that the inhibitory effect of GSFF was decreased with increasing dose (data not shown). Based on these results, GSFF at concentrations of 0.125, 0.25, and 0.5  $\mu\text{mol/L}$  was selected for the following experiments. The enzyme-linked immunoassay results showed that 0.125, 0.25, and 0.5  $\mu\text{mol/L}$  GSFF could inhibit their release of Col.I and that the effect of 0.5  $\mu\text{mol/L}$  GSFF was most obvious (Figure 3B).

To further confirm the effect of GSFF on HSC-T6 cell activation, the groups of cells except the control cells were stimulated with TGF- $\beta$ 1. Cell viability and proliferation were significantly promoted by TGF- $\beta$ 1. Treatment with GSFF at 0.125, 0.25 and 0.5  $\mu\text{mol/L}$  inhibited cell viability more than treatment with TGF- $\beta$ 1 alone (Figure 3C). Both Col. I synthesis and  $\alpha$ -SMA expression were significantly increased after stimulation with TGF- $\beta$ 1 (Figure 3D-F), and GSFF could downregulate collagen synthesis and  $\alpha$ -SMA expression, with the effect being most obvious at a concentration of 0.5  $\mu\text{mol/L}$ .

### **GSFF reduced ERK phosphorylation and NF- $\kappa$ B expression in CBDL rats and HSC-T6 cells**

NF- $\kappa$ B is an important mediator involved in the inflammatory responses of various organs. In this study, after 2 and 4 wk of CBDL, the level of NF- $\kappa$ B (p65) expression was increased in the CBDL rats, and GSFF inhibited NF- $\kappa$ B (p65) expression (Figure 4A and D). The levels of p-ERK were dramatically increased in the livers of CBDL rats. GSFF inhibited p-ERK expression at 2 and 4 wk (Figure 4A and B). Col. I synthesis was also examined by western blotting and found to be significantly increased after CBDL and inhibited by GSFF at 2 and 4 wk (Figure 4A and C).

*In vitro*, GSFF inhibited ERK phosphorylation in HSC-T6 cells (Figure 4E and F).



**Figure 1** Gan Shen Fu Fang decreased serum levels of alanine aminotransferase, aspartate aminotransferase and total bilirubin in common bile duct-ligated rats and the liver and spleen coefficients ( $n = 8$ ). A: Alanine aminotransferase level; B: Aspartate aminotransferase level; C: Total bilirubin level; D: Liver coefficient; E: Spleen coefficient. The data are presented as the mean  $\pm$  SD. <sup>a</sup> $P < 0.01$ , <sup>e</sup> $P < 0.001$ , compared with the sham group; <sup>c</sup> $P < 0.05$ , compared with the 2W-CBDL rats; <sup>g</sup> $P < 0.05$ , <sup>k</sup> $P < 0.001$ , compared with the 4W-CBDL rat. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; CBDL: Common bile duct-ligated; GSFF: Gan Shen Fu Fang.

After stimulating the cells with PDGF-BB, ERK phosphorylation was increased and inhibited by PD98059 and GSFF, respectively (Figure 4H). GSFF also inhibited the expression of NF- $\kappa$ B (p65) in HSC-T6 cells (Figure 4E and G).

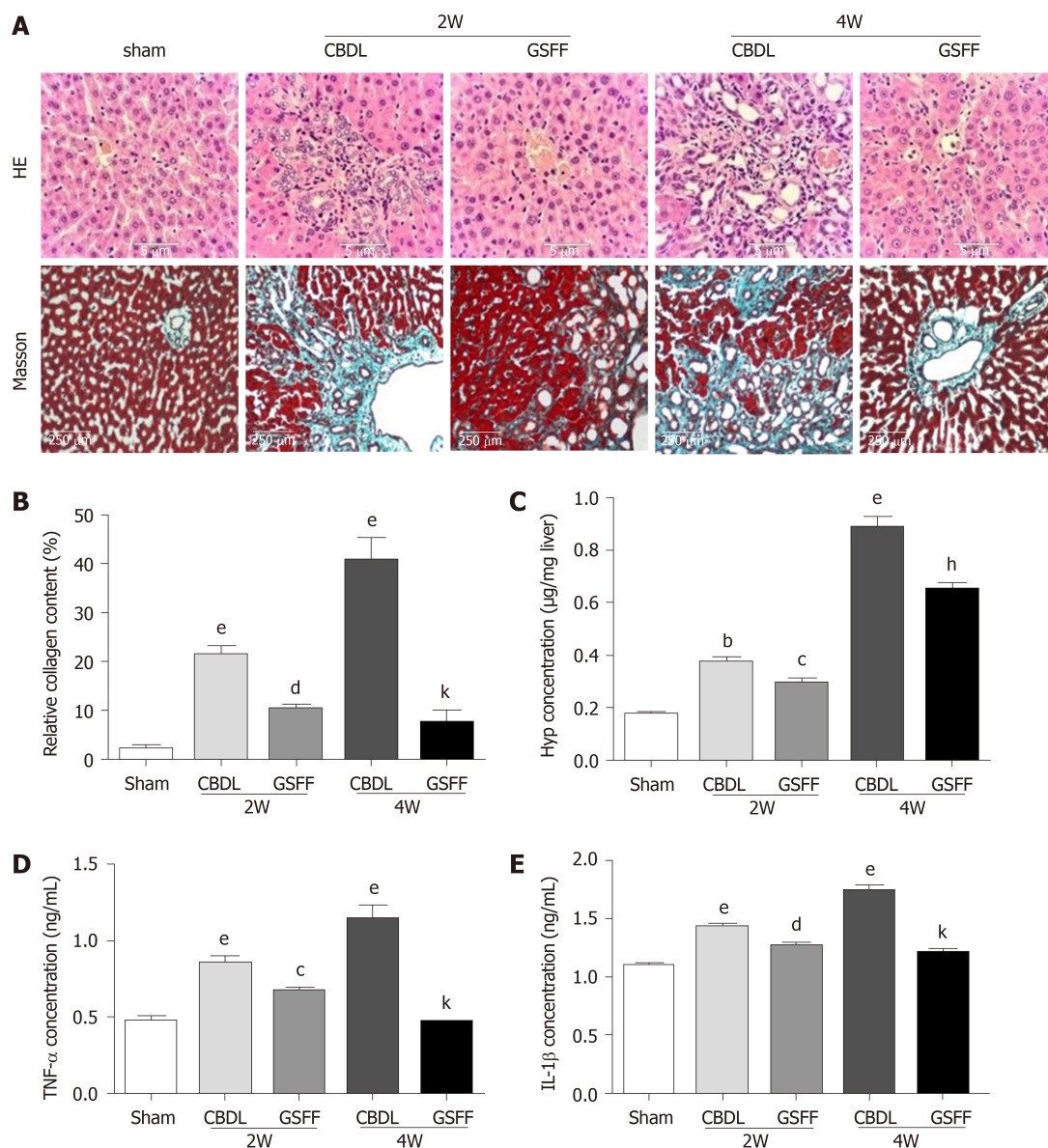
## DISCUSSION

Liver fibrosis, the result of most types of chronic liver diseases, is a common health problem worldwide. Though important progress has been made in basic research on liver fibrosis, there is still a lack of effective clinical medicines. In this study, we showed that the herbal medicine GSFF alleviated liver fibrosis and inhibited HSC-T6 cell activation, which were related to a decreased inflammatory response and reduced ERK phosphorylation.

First, GSFF was shown to inhibit liver fibrosis progression and attenuate cholestatic liver injury. Common bile duct ligation in rats or mice is a classic method to produce an animal model of liver fibrosis<sup>[21-23]</sup>. When bile was blocked within the livers of our model rats, hepatocytes underwent necrosis. To repair this injury, tissue damage and concomitant inflammation triggered fibrotic tissue proliferation and lead to excessive ECM accumulation, as evidenced by histological changes; increased serum ALT, AST, and TBIL levels; and increased Hyp content in the liver tissue after 2 and 4 wk of CBDL.

GSFF comprises two ingredients, SA-B and DG. SA-B was shown to inhibit liver fibrosis induced by CCl<sub>4</sub><sup>[24]</sup>. DG was found to have prominent anti-inflammatory effects and improve liver function. Diammonium glycyrrhizin (DG, known as Gan Li Xin in China) is commonly used to treat chronic viral hepatitis. Hyp assays and Masson staining verified the anti-fibrotic effect of GSFF after 2 and 4 wk of treatment. GSFF decreased ALT, AST and TBIL levels at 4 wk but not at 2 wk. This may be because the 1-wk GSFF treatment time was too short. A longer 3-wk GSFF treatment time was long enough for GSFF to protect hepatocytes. In this study, we also found that the livers and spleens of CBDL rats were larger than those of the sham rats and had shrunk after GSFF treatment. Enlargement of the liver and spleen may have resulted from inflammatory congestion and cholestasis.

Second, GSFF inhibited HSC activation, as indicated by their reduced viability and collagen synthesis. Activated HSCs experience structural and functional changes and are the main source of myofibroblasts in liver fibrosis<sup>[25,26]</sup>. Functionally, activated HSCs acquire enhanced viability (hyper-proliferation, hyper-migration) and exhibit the increased secretion of ECM, which is very important in liver fibrosis formation and progression<sup>[27,28]</sup>. Because the effect of GSFF on HSCs had not been tested *in vitro*,

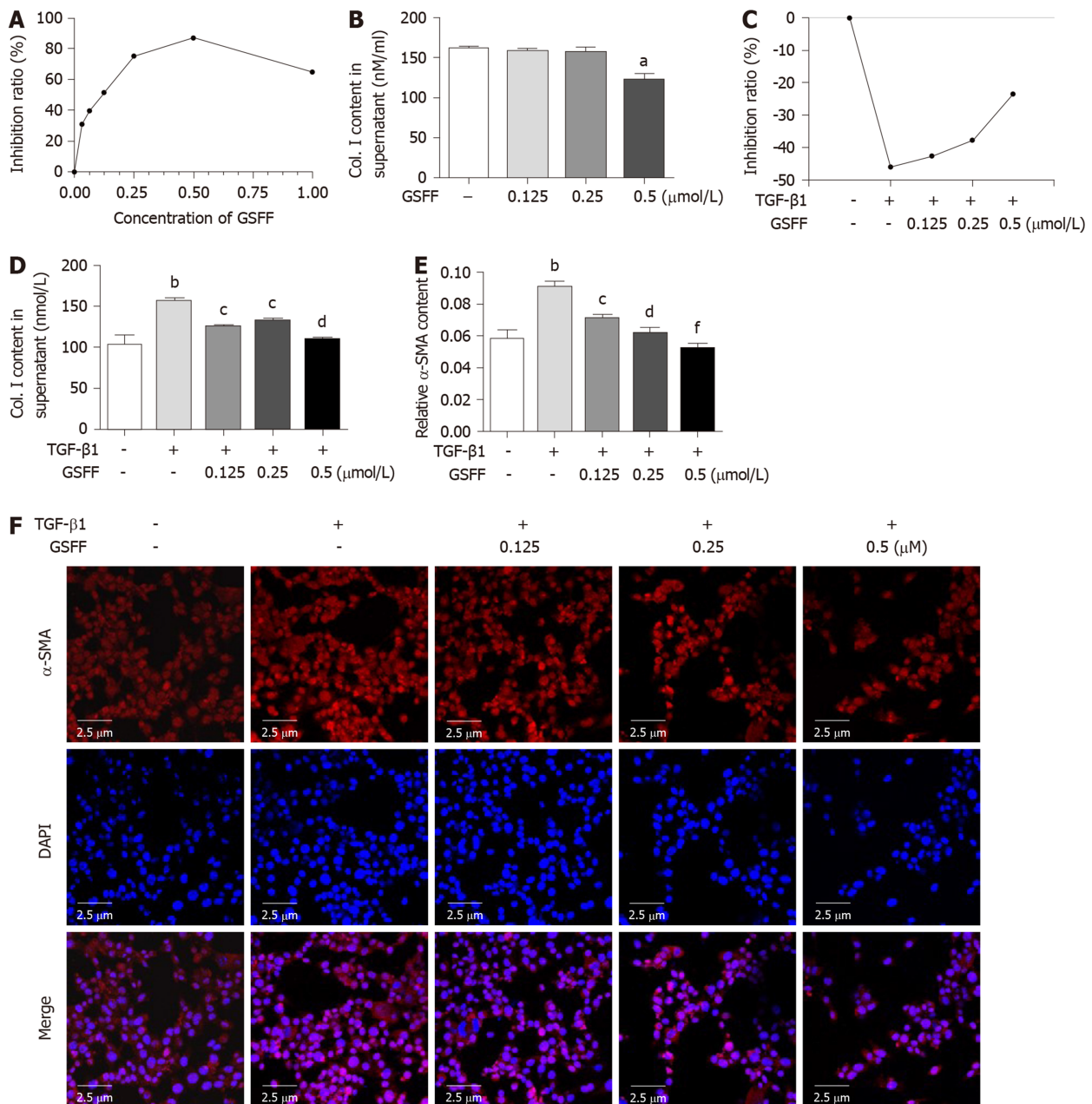


**Figure 2 Gan Shen Fu Fang inhibited liver fibrosis and alleviated the inflammatory response.** A: Hematoxylin and eosin staining and Masson staining of the liver tissue; B: The collagen content was measured by quantitative histomorphometry; C: Hydroxyproline concentration in the liver; D and E: Tumour necrosis factor- $\alpha$  and IL-1 $\beta$  levels in the liver. The data are presented as the mean  $\pm$  SD. <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ , compared with the sham group; <sup>e</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , compared with the 2W-CBDL rats; <sup>h</sup> $P < 0.01$ , <sup>k</sup> $P < 0.001$ , compared with the 4W-CBDL rats. HE: Hematoxylin and eosin; CBDL: Common bile duct-ligated; GSFF: Gan Shen Fu Fang; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; IL-1 $\beta$ : Interleukin-1 $\beta$ ; Hyp: Hydroxyproline.

we first designed a 6-concentration gradient of GSFF and used this concentration series to determine the dose of GSFF with an effect on HSC activation. GSFF at concentrations of 0.125, 0.25, 0.5  $\mu\text{mol/L}$  was selected for subsequent experiments. We found that 0.125, 0.25, and 0.5  $\mu\text{mol/L}$  GSFF inhibited HSC-T6 cell viability and Col.I synthesis with and without stimulation by TGF- $\beta$ 1. In addition, the effect of GSFF was most pronounced at a concentration of 0.5  $\mu\text{mol/L}$ . Zhang *et al.*<sup>[29]</sup> found that SA-B (1  $\mu\text{mol/L}$ ) inhibited the expression of  $\alpha$ -SMA and Col. I in human HSCs. However, in this study, though 1  $\mu\text{mol/L}$  GSFF (containing 0.5  $\mu\text{mol/L}$  SA-B) could also inhibit HSC-T6 cell viability, GSFF at this concentration was not as effective as that at 0.5  $\mu\text{mol/L}$ . We also tested higher doses of GSFF (5  $\mu\text{mol/L}$  and 10  $\mu\text{mol/L}$ ), and the results showed no effect on cell viability. Therefore, we concluded that GSFF can be used at an appropriate range of doses to inhibit HSC viability and a higher dose did not mean a more pronounced effect. In addition, SA-B and DG may enhance each other's effects when combined, so a lower dose of SA-B with DG had a satisfactory effect.

Third, the anti-fibrotic effect of GSFF may be related to an alleviated inflammatory response and reduced ERK phosphorylation. Liver fibrosis is considered an abnormal wound healing response fuelled by a vicious pathogenic circle of hepatocyte necrosis,



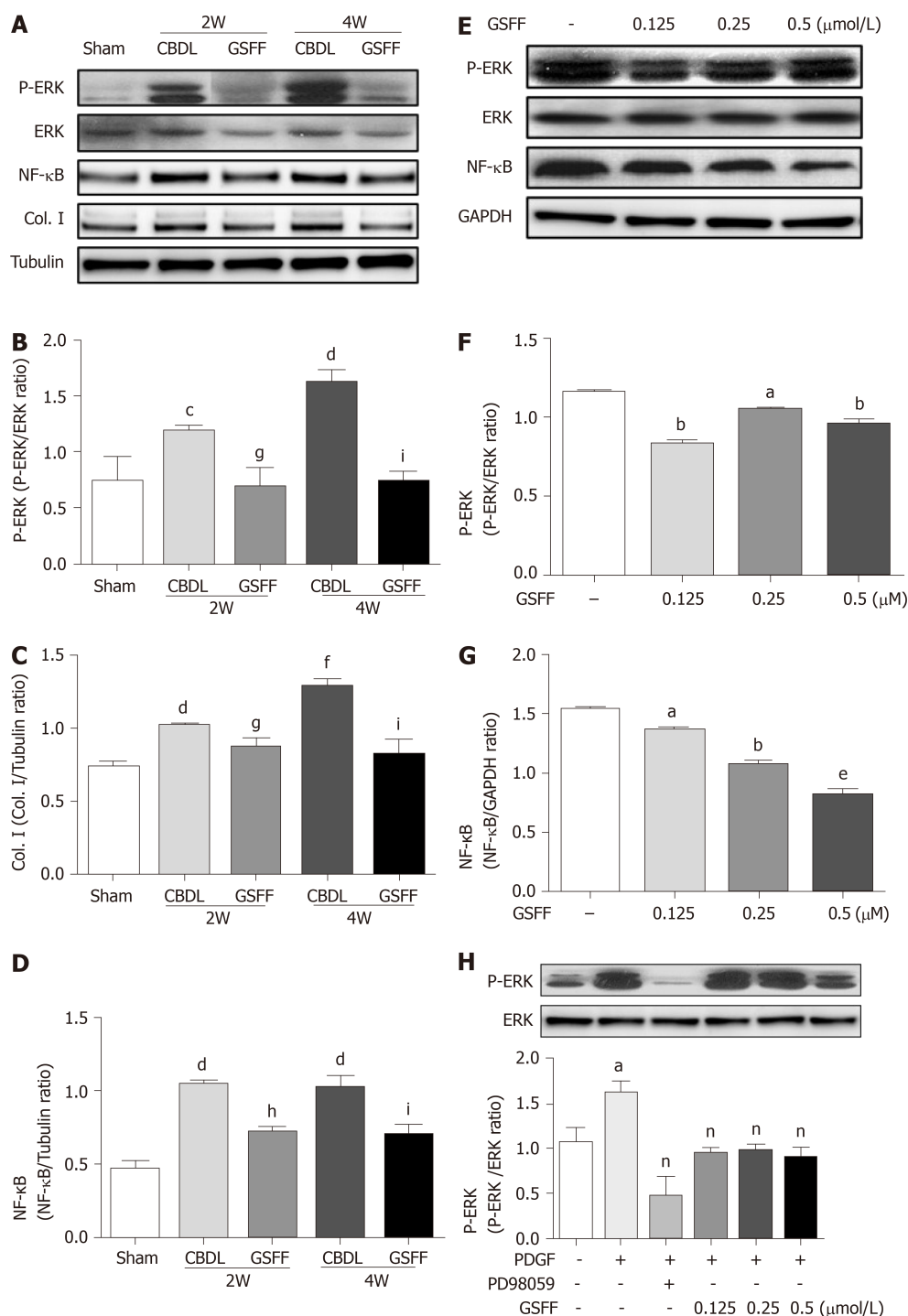


**Figure 3** Gan Shen Fu Fang inhibited HSC-T6 cell viability and collagen synthesis. A and B: GSFF inhibited HSC-T6 cell viability and Col. I release; C and D: GSFF inhibited the viability and Col. I release of HSC-T6 cells stimulated with transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1); E and F: TGF- $\beta$ 1 promoted  $\alpha$ -SMA expression in HSC-T6 cells, which was decreased by GSFF. The relative  $\alpha$ -SMA content was measured by quantitative histomorphometry, and the results are shown in panel E. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , compared with the control cells. <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , <sup>f</sup> $P < 0.001$ , compared with cells stimulated with TGF- $\beta$ 1 only. GSFF: Gan Shen Fu Fang; TGF- $\beta$ 1: Transforming growth factor  $\beta$ 1.

inflammation and excessive ECM deposition<sup>[30]</sup>. Though inflammation is not a prerequisite for liver fibrosis<sup>[31]</sup>, in most cases, a persistent inflammatory response is one of the main characteristics of liver fibrosis and participates in HSC activation and collagen synthesis<sup>[7,32,33]</sup>. GSFF could reduce inflammatory cell infiltration and decrease the release of pro-inflammatory cytokines (IL-1 $\beta$  and TNF- $\alpha$ ) as well as NF- $\kappa$ B synthesis *in vivo* and *in vitro*. This finding is consistent with that of a previous study showing that SA-B inhibited IL-1 $\beta$ , IL-6, and TNF- $\alpha$  synthesis in a cholestatic liver injury rat model<sup>[34]</sup>. The ERK cascade is crucial for HSC activation. GSFF inhibited ERK phosphorylation *in vivo* and *in vitro*, which is also consistent with the results of a previous study<sup>[35]</sup>. GSFF also inhibited ERK phosphorylation in HSC-T6 cells stimulated with PDGF-BB *in vitro*.

In conclusion, the Chinese herbal medicine GSFF alleviates liver fibrosis and HSC-T6 cell activation through inhibiting the inflammatory response and ERK phosphorylation. This study focuses on only one mechanism. Further studies are still needed to analyse the mechanism of GSFF and determine why SA-B in combination





**Figure 4** Gan Shen Fu Fang reduced extracellular signal-regulated kinase phosphorylation and NF-κB expression in common bile duct-ligated rats and HSC-T6 cells. A-D: Liver tissue homogenates were subjected to immunoblotting as indicated. Representative bands and quantitative data are shown.  $N = 4$ ; E-G: HSC-T6 cells were not stimulated, and whole-cell lysates were used. Representative images and quantitative data are shown; H: HSC-T6 cells were stimulated with platelet-derived growth factor-BB to further evaluate the effect of GSFF on extracellular signal-regulated kinase phosphorylation. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ , compared with control cells; <sup>d</sup> $P < 0.05$ , <sup>e</sup> $P < 0.01$ , <sup>f</sup> $P < 0.001$  compared with sham rats. <sup>g</sup> $P < 0.05$ , <sup>h</sup> $P < 0.01$  compared with the 2W-CBDL rats; <sup>i</sup> $P < 0.05$ , <sup>j</sup> $P < 0.001$ , compared with the 4W-CBDL rats; <sup>n</sup> $P < 0.01$ , compared with cells stimulated with only platelet-derived growth factor-BB. PDGF: Platelet-derived growth factor; GSFF: Gan Shen Fu Fang; ERK: Extracellular signal-regulated kinase; CBDL: Common bile duct-ligated.

with DG has a more pronounced effect than SA-B alone.

## ARTICLE HIGHLIGHTS

### Research background

Liver fibrosis is a common healthy problem worldwide and there is still a lack of specific medicine. The Chinese herbal medicine, Gan Shen Fu Fang (GSFF) is composed of salvianolic

acid B and diammonium glycyrrhizinate. In this study, we observe the effects of GSFF on liver fibrosis *in vivo* and *in vitro* in an attempt to provide some hope for the treatment.

### Research motivation

During pre-clinical experiment, we found that GSFF could inhibit pseudo-lobule formation in common bile duct-ligated (CBDL) rats. However, the mechanisms remain unclear. Therefore, in this study, we aimed to observe the effects of GSFF on liver fibrosis *in vivo* and *in vitro* and determine whether GSFF-mediated alleviation of liver fibrosis is related to inflammation and the ERK signalling pathway.

### Research objectives

The present study aimed to observe the effects of GSFF on liver fibrosis *in vivo* and *in vitro* and investigate the mechanism from the perspective of the inflammatory response and extracellular signal-regulated kinase (ERK) phosphorylation.

### Research methods

CBDL rats were used for *in vivo* experiments. Hepatic stellate cells-T6(HSC-T6) cells were used for *in vitro* experiments. Hematoxylin and eosin and Masson staining, biochemical assays, hydroxyproline assays, enzyme-linked immunosorbent assay and western blotting were performed to evaluate the degree of liver fibrosis, liver function, the inflammatory response and ERK phosphorylation. The CCK8 assay, immunofluorescence and western blotting were applied to test the effect of GSFF on HSC-T6 cell activation and determine whether GSFF had an effect on ERK phosphorylation in HSC-T6 cells.

### Research results

GSFF improved liver function and inhibited liver fibrosis in CBDL rats after 3wk of treatment, as demonstrated by histological changes, hydroxyproline assays and collagen I concentrations. GSFF alleviated inflammatory cell infiltration and reduced the synthesis of pro-inflammatory cytokines (tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$ ) and NF- $\kappa$ B. In addition, GSFF decreased ERK phosphorylation. *In vitro*, GSFF inhibited the viability of HSC-T6 cells with and without transforming growth factor  $\beta$ 1 stimulation and decreased the synthesis of collagen I. GSFF had the greatest effect at a concentration of 0.5  $\mu$ mol/L. GSFF inhibited the expression of  $\alpha$ -smooth muscle actin, a marker of HSC activation, in HSC-T6 cells. Consistent with the *in vivo* results, GSFF also inhibited the phosphorylation of ERK and downregulated the expression of NF- $\kappa$ B.

### Research conclusions

GSFF inhibited liver fibrosis progression *in vivo* and HSC-T6 cell activation *in vitro*. These effects may be related to an alleviated inflammatory response and downregulated ERK phosphorylation.

### Research perspectives

The definite anti-liver fibrosis effect and clear mechanism of GSFF provide hope for the treatment of liver fibrosis.

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## Retrospective Cohort Study

# Periportal thickening on magnetic resonance imaging for hepatic fibrosis in infantile cholestasis

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**Author contributions:** Lee MH and Lee MJ designed the research; Lee MH, Shin HJ, Yoon H and Lee MJ performed the research and wrote the manuscript; Lee MH and Lee MJ analyzed the data; Han SJ and Koh H contributed analytic tools; Lee MH, Shin HJ, Yoon H, Han SJ, Koh H, and Lee MJ revised and approved the final version.

**Institutional review board statement:** This study was reviewed and approved by the local ethics committee of the Severance Hospital, Yonsei University.

**Informed consent statement:** Because of the retrospective and anonymous character of this study, the need for informed consent was waived by the institutional review board.

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## Abstract

### BACKGROUND

Untreated neonatal cholestasis can progress to liver cirrhosis and end stage liver disease in infancy due to prolonged hepatocyte and biliary tree injury and may require liver transplantation. Therefore, non-invasive evaluation of hepatic fibrosis is important in infants with cholestasis.

### AIM

To investigate the usefulness of periportal thickening (PT) measured on liver magnetic resonance imaging (MRI) for the assessment of hepatic fibrosis in infants with cholestasis including biliary atresia (BA).

### METHODS

This retrospective study included infants less than 6 mo who underwent liver MRI and biopsy for the evaluation of infantile cholestasis. PT and spleen size were measured on MRI. Serologic assessment was based on aspartate transaminase to platelet ratio index (APRI). The grade of histopathologic fibrosis was assessed by the METAVIR grading system. Correlation and diagnostic performance of PT, normalized spleen size ratio (SR, using the upper normal size limit), and APRI for diagnosing hepatic fibrosis were obtained by receiver-operating characteristic (ROC) curve analysis.

### RESULTS

A total of 155 patients were included, 110 of which were diagnosed with BA. Mean age at the time of MRI was  $57.6 \pm 34.4$  d. There were positive correlations



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between fibrosis grade and PT and SR, even after adjusting age (all,  $P < 0.001$ ). For the diagnosis of significant fibrosis (METAVIR grade F2-F4), the area under the ROC curve was 0.899 (95%CI: 0.840–0.941) for PT (cutoff, 4.2 mm), which was higher than 0.741 (95%CI: 0.664–0.808) for SR and 0.712 (95%CI: 0.634–0.782) for APRI (both,  $P < 0.001$ ). For the diagnosis of cirrhosis (F4), the area under the ROC curve was the highest with SR as 0.790 (95%CI: 0.718–0.852).

## CONCLUSION

Liver MRI findings of PT and SR are useful to assess clinically significant hepatic fibrosis (F2 and higher) in infants with cholestasis including BA.

**Key words:** Infants; Cholestasis; Biliary atresia; Liver; Fibrosis; Magnetic resonance imaging

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**Core tip:** Non-invasive evaluation of hepatic fibrosis is important in infants with cholestasis including biliary atresia. Periportal thickening (PT) and normalized spleen size ratio (SR) measured on liver magnetic resonance imaging (MRI) showed positive correlations with hepatic fibrosis grade, even after adjusting age. For the diagnosis of significant fibrosis (F2-F4), PT using the cutoff of 4.2 mm showed higher diagnostic performance than SR or aspartate transaminase to platelet ratio index. For the diagnosis of cirrhosis (F4), SR was the best. Therefore, liver MRI findings of PT and SR can be useful to assess clinically significant hepatic fibrosis (F2 and higher) in infants with cholestasis.

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## INTRODUCTION

Neonatal cholestasis, an intrahepatic or extrahepatic disorder, is defined as persistent conjugated hyperbilirubinemia more than 2 wk and may be caused by various diseases<sup>[1]</sup>. Untreated neonatal cholestasis can progress to liver cirrhosis or end stage liver disease in infants due to prolonged hepatocyte and biliary tree injury and may require liver transplantation. Ultimately, the degree of hepatic fibrosis in infantile cholestasis is a major determinant of the patient's outcome<sup>[2]</sup>. For biliary atresia (BA), a previous study reported that severe hepatic fibrosis at the time of Kasai operation, irrespective of age, carried a poor outcome and the degree of hepatic fibrosis, as well as the time of operation, is an important factor for survival after Kasai operation<sup>[3,4]</sup>. Therefore, knowing the degree of hepatic fibrosis before the operation can help predict the treatment methods and outcomes of patients and prepare for subsequent liver transplants.

Hepatic fibrosis in neonates and infants can be measured by serology, ultrasound elastography, and liver biopsy<sup>[5]</sup>. Ultrasound elastography is non-invasive, but has the disadvantage that the accuracy of the test may be impaired if the practitioner lacks experience or if ascites exists in the perihepatic space. Liver biopsy is necessary for confirmation of biliary disease including BA as an accurate diagnostic tool and assessment of hepatic fibrosis, but it is an invasive method and has the disadvantage that it cannot be repeatedly performed due to complications of the test itself<sup>[6,7]</sup>.

Magnetic resonance imaging (MRI) has been reported to be useful for differential diagnosis of cholestatic jaundice in infants with high resolution and quantitative evaluation<sup>[8]</sup>. Several previous studies investigating diagnosis of BA by MRI evaluated visibility of the extrahepatic bile duct on MR cholangiopancreatography (MRCP)<sup>[9-11]</sup>, but it has been reported that the accuracy of BA diagnosis was improved when the triangular cord thickness or periportal thickening (PT), which is usually measured on ultrasonography, was applied to MRI<sup>[12]</sup>. We thought if these MRI findings were associated with hepatic fibrosis, degree of hepatic fibrosis could be predicted

noninvasively and reproducibly by making less susceptible to practitioner's skill than ultrasonography. However, there was no previous study showing that MRI findings including PT reflect the degree of hepatic fibrosis in patients with infantile cholestasis. Therefore, we investigated whether the PT and spleen size measured on MRI is associated with pathologically assessed hepatic fibrosis in patients with infantile cholestasis.

## MATERIALS AND METHODS

This retrospective study was approved by our Institutional Review Board, with a waiver for informed consent for reviewing medical records and images of the patients. However, written informed consent for sedation and MRI was received before each examination as routine clinical practice.

### *Patients and basic characteristics*

We included all consecutive patients between July 2009 and October 2017 who (1) underwent both liver MRI and liver biopsy to evaluate the cause of infantile cholestasis which was defined as prolonged hyperbilirubinemia longer than 2 wk; (2) were younger than 6 mo at the time of MRI as identified by chart review; and (3) had an interval between liver MRI and liver biopsy within 1 mo. We do liver MRI and liver biopsy in infants with persistent jaundice after conservative treatment. We also do liver biopsy during BA and choledochal cyst operation in neonates and infants as a routine protocol. Patient's sex and age at the time of MRI, laboratory results at the time of MRI and etiology of cholestasis confirmed by pathology and/or operative cholangiography (BA *vs* non-BA) were evaluated through medical chart review.

### *MRI acquisition and interpretation*

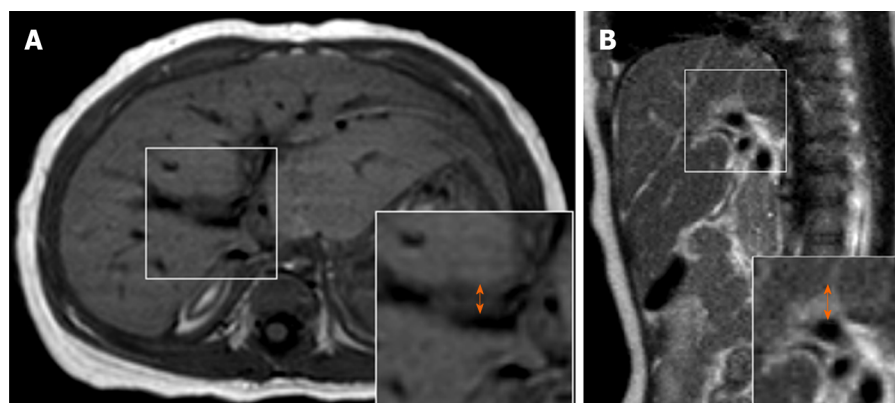
All MR images were obtained using a 1.5-T unit (Intera Achieva; Philips Healthcare) with a cardiac coil and patients were sedated by general anesthesia under the supervision of anesthesiologists. MR sequences used at our hospital included respiratory gated T2-weighted fast spine echo (FSE) axial, T1-weighted spin echo axial, T2-weighted single shot FSE sagittal, and respiratory gated FSE 3 dimensional MRCP coronal images. Repetition time/echo time was 2000/100 ms for FSE T2, 600/11 for T1, 700/80 for single shot FSE T2, and 1500/650 for MRCP. Matrix was 256 × 256. Section thickness was 2 mm with 1 mm gap for MRCP and 3 mm without gap for the others. Field of view was 18-24 cm. The total scan time for our protocol was approximately 15 -20 min for most of the cases.

Two radiologists (with 15 and 4 years of clinical experience in pediatric radiology, respectively) who were unaware of the final diagnosis analyzed the MR images in consensus on the picture archiving and communication system (Centricity; GE Healthcare). The PT measured on MRI was defined as the maximal thickness of periportal signal change on MRI which was measured (1) either along the right main or left main portal vein; and (2) on either side of the portal vein where its thickness was the greatest. The maximum thickness of the periportal signal change was separately measured on T1-weighted axial, T2-weighted axial and T2-weighted sagittal images, and then the largest value among the three measurements was selected (Figure 1)<sup>[12]</sup>. The size of the spleen was defined as the maximum length of spleen either measured on T2-weighted axial or sagittal images, and the larger value was used for analysis. Then we analyzed the normalized spleen size ratio (SR)<sup>[13]</sup> by calculating the maximum spleen length divided by the upper normal limit of size according to the patient's age and sex<sup>[14]</sup>.

### *Liver biopsy and histologic grade*

Basically, ultrasonography-guided liver biopsy was performed using an 18-gauge core biopsy needle (TSK Stericut Standard type with Co-axial; TSK) with a free-hand technique under sedation supervised by anesthesiologists in patient with infantile cholestasis. However, if BA or choledochal cyst was strongly suspected, liver biopsy was performed during operation.

All specimens were evaluated for histopathologic diagnosis of cholestasis and grading hepatic fibrosis by hepatobiliary pathologists. The fibrosis grades of pathologic specimens of all cases were evaluated by METAVIR fibrosis grade, ranging from grade F0 to F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis). Clinically significant fibrosis was defined as grade F2 to F4 and advanced fibrosis was defined as grade F3 to F4<sup>[15,16]</sup>.



**Figure 1** Example of measuring of periportal thickening on magnetic resonance imaging. Pancreaticobiliary magnetic resonance imaging of a 3-mo-old girl diagnosed as biliary atresia. A: On T1-weighted axial image, the maximal thickness of periportal signal change was measured on right main portal vein where its greatest thickness was 5.5 mm; B: On T2-weighted sagittal image, the maximal thickness of periportal signal change was 6.1 mm. Therefore, the larger of the two, which was measured on T2-weighted sagittal image, was defined as periportal thickening of this patient.

### Serologic evaluation

Laboratory results included aspartate aminotransferase (AST), alanine transaminase and platelet count. We calculated the AST-to-platelet ratio index (APRI), known as a noninvasive fibrosis score by serology, which is calculated as  $[(AST/\text{upper limit of normal AST}) \times 100]/\text{platelet count } (10^9/L)$ .

### Statistical analysis

Statistical analyses were performed using SPSS version 23 (IBM Corp.). The independent two-sample *t* test for continuous variables and  $\chi^2$  test for categorical variables were used. Analysis of variance was used for the comparison of parameters with different hepatic fibrosis grades. Correlation between laboratory or imaging parameters and hepatic fibrosis grades was analyzed using Kendall's tau correlation coefficient. The diagnostic performances of parameters for clinically significant or advanced fibrosis and cirrhosis were evaluated by a receiver operating characteristic (ROC) curve analysis. The optimal cut-off values from the ROC curves were determined by the Youden index. The area under the ROC curve (AUC) of the parameters was compared with a Z test. In all tests, a *P* value less than 0.05 was considered statistically significant.

## RESULTS

### Subjects

During the study period, 155 patients with infantile cholestasis were included. Baseline characteristics of our study are summarized in Table 1. The mean age at the time of MRI was  $57.6 \pm 34.4$  d and 94 patients (60.6%) were female. Among the patients, 110 infants (71%) were diagnosed with BA and the remaining 45 (29%) were diagnosed with diseases other than BA by pathologic confirmation. The final diagnoses of the non-BA patients were choledochal cyst ( $n = 29$ ), hepatitis ( $n = 8$ ), paucity of intrahepatic bile ducts ( $n = 3$ ), and other metabolic diseases ( $n = 5$ ).

The mean age at the time of MRI was not different between the BA and non-BA groups ( $P = 0.303$ ). The PT and SR were significantly higher in the BA group than in the non-BA group ( $P < 0.001$ ), but APRI was not different (Table 1). The number of patients with clinically significant fibrosis (F2–F4) was 113 (72.9%), with 105 from the BA group and 8 from the non-BA group. There were 47 patients (30.3%) with advanced fibrosis (F3–F4), with 46 from the BA group and 1 from the non-BA group. The distribution of each hepatic fibrosis grade is detailed in Table 1. There were 9 BA and 1 non-BA patients with liver cirrhosis (F4).

### Comparison and correlation between parameters and hepatic fibrosis grades

Table 2 summarizes the results of parameters in each fibrosis grading group. Age, APRI, PT, and SR were different in the fibrosis group comparison. Correlation analysis also demonstrated positive correlation between these parameters and fibrosis grades (all,  $P < 0.001$ ). However, after adjusting age, partial correlation results

**Table 1 Clinical summary of the patients with infantile cholestasis**

	Overall (n = 155)	BA (n = 110)	Non-BA (n = 45)	P value
Demographics				
Age (d)	57.6 ± 34.4	59.7 ± 30.3	52.4 ± 42.8	0.303
Gender (M:F)	61:94	41:69	20:25	0.470
Laboratory results				
APRI	1.32 ± 1.61	1.47 ± 1.66	0.95 ± 1.42	0.069
Imaging findings				
PT (mm)	4.9 ± 1.8	5.7 ± 1.4	2.9 ± 0.9	< 0.001
SR	1.02 ± 0.20	1.07 ± 0.19	0.91 ± 0.17	< 0.001
METAVIR grades (Patients' number)				
F0/F1/F2/F3/F4	32/10/66/37/10	1/4/59/37/9	31/6/7/0/1	< 0.001

P values from independent *t*-test except for gender and fibrosis grades from  $\chi^2$  test. BA: Biliary atresia; APRI: Aspartate transaminase to platelet ratio index; SR: Normalized spleen size ratio; PT: Periportal thickening.

demonstrated that only PT ( $\tau = 0.518$ ,  $P < 0.001$ ) and SR ( $\tau = 0.340$ ,  $P < 0.001$ ) showed positive correlations with the hepatic fibrosis grades. Representative images of each hepatic fibrosis grade are shown in Figure 2.

### Assessment of diagnostic performance of parameters for clinically significant or advanced fibrosis and cirrhosis

The diagnostic performances in diagnosing clinically significant fibrosis (F0–F1 *vs* F2–F4), advanced fibrosis (F0–F2 *vs* F3–F4), and cirrhosis (F0–F3 *vs* F4) for each parameter are shown in Table 3. The AUCs of PT were 0.899 (95% CI: 0.840–0.941) using a cutoff value of 4.2 mm for clinically significant fibrosis and 0.734 (95% CI: 0.657–0.801) with a cutoff of 5.3 mm for advanced fibrosis ( $P < 0.001$ ). The AUCs of SR and APRI were 0.741 with a cutoff value of 0.85 and 0.712 with a cutoff of 0.44 for the diagnosis of clinically significant fibrosis, respectively. For diagnosing advanced fibrosis, the AUC of SR was 0.742 with a cutoff value of 1.1 and that of APRI was 0.724 with a cutoff value of 0.78. For diagnosing cirrhosis, the AUC was the highest with SR as 0.790 (95% CI: 0.718–0.852) with a cutoff value of 1.04. However, the AUC of PT was not significant ( $P = 0.058$ ).

### Comparison of diagnostic performance of parameters for clinically significant or advanced fibrosis and cirrhosis

For diagnosing clinically significant fibrosis, there was a significant difference in the AUC between PT and SR (difference between area, 0.158;  $P < 0.001$ ) and PT and APRI (difference between area, 0.186;  $P < 0.001$ ), but no significant difference between SR and APRI (difference between area, 0.029;  $P = 0.613$ ). The AUC of PT (0.899) was significantly higher than those of SR (0.741) and APRI (0.712) (Figure 3A).

In contrast, there were no significant differences in the AUCs between PT, SR and APRI for diagnosing advanced fibrosis. The differences between areas were 0.008 ( $P = 0.873$ ) between PT and SR, 0.009 ( $P = 0.859$ ) between PT and APRI, and 0.018 ( $P = 0.724$ ) between SR and APRI (Figure 3B).

In cirrhosis, the AUC with SR was higher than that with PT (difference between area, 0.135;  $P = 0.041$ ), but not that with APRI (difference between area, 0.075;  $P = 0.427$ ). There was no significant difference in the AUCs between PT and APRI (difference of 0.060,  $P = 0.626$ ) (Figure 3C).

## DISCUSSION

Serologic marker of APRI and splenomegaly are well known markers for the evaluation of hepatic fibrosis in adults, but there is limited study about these parameters in infants. Moreover, this is the first study suggested the usefulness of PT for investigating hepatic fibrosis in infants with cholestasis. We demonstrated that PT measured on MRI showed positive correlation with pathologically assessed hepatic fibrosis grades and had good performance to diagnose both clinically significant fibrosis and advanced fibrosis with cutoff values of 4.2 mm and 5.3 mm, respectively. In addition, hepatic fibrosis was also significantly correlated with SR and APRI, but the diagnostic performance of PT measured on MRI for clinically significant hepatic



**Table 2 Comparison and correlation analyses for fibrosis grading**

	Age (d)	APRI	PT (mm)	SR
Hepatic fibrosis group comparison				
F0 ( <i>n</i> = 32)	40.8 ± 34.8	1.12 ± 1.63	3.1 ± 1.1	0.89 ± 0.15
F1 ( <i>n</i> = 10)	60.6 ± 44.0	0.46 ± 0.38	3.3 ± 1.3	0.94 ± 0.20
F2 ( <i>n</i> = 66)	52.3 ± 31.0	1.14 ± 1.23	5.2 ± 1.4	1.01 ± 0.17
F3 ( <i>n</i> = 37)	71.9 ± 28.6	2.00 ± 2.27	5.9 ± 1.6	1.12 ± 0.20
F4 ( <i>n</i> = 10)	89.7 ± 26.6	1.52 ± 0.55	5.7 ± 1.6	1.21 ± 0.17
<i>P</i> value	< 0.001	0.026	< 0.001	< 0.001
Correlation analysis				
Overall correlation				
τ	0.313	0.326	0.448	0.361
<i>P</i> value	< 0.001	< 0.001	< 0.001	< 0.001
Partial correlation with adjusting age				
τ		0.071	0.518	0.340
<i>P</i> value		0.405	< 0.001	< 0.001

*P* values from analysis of variance for group comparisons and Kendall's tau for correlation analyses. APRI: Aspartate transaminase to platelet ratio index; PT: Periportal thickening, SR: Normalized spleen size ratio.

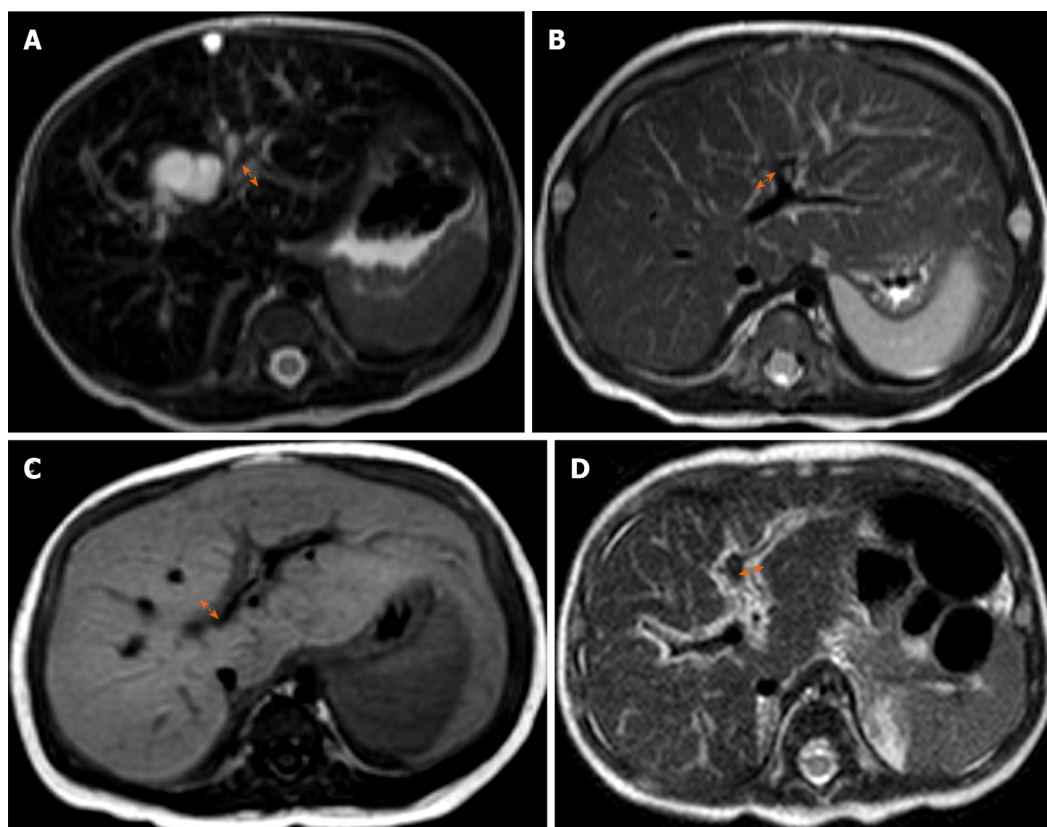
fibrosis was superior to that of SR and APRI. Moreover, after adjusting age, only PT and SR showed positive correlations with the hepatic fibrosis grades. Therefore, our study shows that MRI findings of PT and SR are useful in predicting the degree of hepatic fibrosis in infants with cholestasis who cannot easily undergo liver biopsy.

To determine grade of hepatic fibrosis in patients with neonatal cholestasis, liver biopsy is the gold standard. However, it is invasive and can result in severe, life-threatening complications in infants and neonates<sup>[17-19]</sup>. Moreover, this procedure is associated with significant sampling error<sup>[20]</sup>. Therefore, several methods for non-invasive assessment of hepatic fibrosis in infants and neonates have been proposed. APRI by laboratory test could be a reliable method to assess advanced fibrosis (F3-F4) and liver cirrhosis (F4) at the time of Kasai operation and postoperative follow-up care<sup>[21]</sup>. APRI has shown significant correlation with hepatic fibrosis grade in children with chronic liver disease<sup>[5]</sup>. These studies are consistent with our study, in which APRI of our patients showed significant correlation with hepatic fibrosis grade and good performance to diagnose clinically significant fibrosis and advanced fibrosis. However, APRI was not correlated with hepatic fibrosis grades after adjusting age.

Another tool is transient elastography, which could suggest optimal time of Kasai operation in patients with BA<sup>[22]</sup>. There have been several reports on the utility of this technique to evaluate hepatic fibrosis and cirrhosis in patients with BA<sup>[23-25]</sup>. However, there are limitations in the aforementioned studies in that they could not evaluate the diagnostic performance of clinically significant fibrosis (F2-F4) and operator dependency of this method<sup>[7,23,25,26]</sup>.

Triangular cord sign or PT was a concept originally designed for ultrasound, which is represented by triangular cone-shaped fibrous tissue in the bifurcation of the portal vein at the porta hepatis. However, due to operator dependence on ultrasound, the concept of PT measured on MRI has been proposed and defined as a thickening of periportal signal change on T1 or T2-weighted images<sup>[27,28]</sup>. A recent study also proposed that an MRI-based decision tree for diagnosis of BA using PT on MRI with a cutoff value of 5.1 mm showed high sensitivity (97.3%) and specificity (94.8%)<sup>[12]</sup>. PT measured on ultrasonography was associated with hepatic fibrosis in BA, but there was no significant association between PT and hepatic fibrosis grade<sup>[23]</sup>. And there was a few previous study on whether PT measured on MRI is associated with hepatic fibrosis in patients with neonatal cholestasis with relatively small population<sup>[28]</sup>. Because this was a retrospective study and there were limited data about PT on ultrasonography in non-biliary atresia patients, we could not evaluate the diagnostic performance of PT on ultrasonography compared with that on MRI. However, as ultrasonography is a more available and easily accessible technique, further study is needed for this topic.

Degree of hepatic fibrosis at time of Kasai operation is known to be one of the prognostic factors after Kasai operation in patients with BA<sup>[3,29]</sup>. Many previous studies have focused on age at time of surgery, but a few studies have suggested that severe fibrosis at the time of Kasai operation, irrespective of age, carries a poor



**Figure 2** Representative images of periportal thickening for each hepatic fibrosis grade. The maximal thickness (double arrow in each figure) of periportal signal intensity change was measured. A: 2.7 mm on axial T2-weighted image in a 7-d-old girl with non-biliary atresia (non-biliary atresia, choledochal cyst) and hepatic fibrosis grade 1 (F1); B: 4.2 mm on T2-weighted axial image in a 2-mo-old girl with BA and F2; C: 5.5 mm on T1-weighted axial image in a 3-mo-old girl with biliary atresia and F3; D: 5.4 mm on T2-weighted axial image in a 2-mo-old girl with non-biliary atresia (metabolic disease) and F4.

outcome<sup>[3,30]</sup>. Severe fibrosis was associated with unsuccessful outcome of Kasai operation without biliary drainage and all patients with cirrhosis had no biliary drainage after Kasai operation in a previous study<sup>[4]</sup>. Therefore, in patients with advanced fibrosis and liver cirrhosis at the time of diagnosis, early liver transplantation may improve prognosis over Kasai portoenterostomy<sup>[23,31]</sup>. A few retrospective studies suggested that anti-inflammatory and immunomodulatory drugs such as steroids might alter progression of hepatic fibrosis and improve biliary drainage and survival rate of native liver of patients<sup>[32]</sup>. Considering that hepatic fibrosis progresses even after surgery, degree of hepatic fibrosis both before and after surgery could be associated with postoperative complications and prognosis in BA. A prospective study is needed on whether the imaging findings of PT are also relevant to the patient's prognosis.

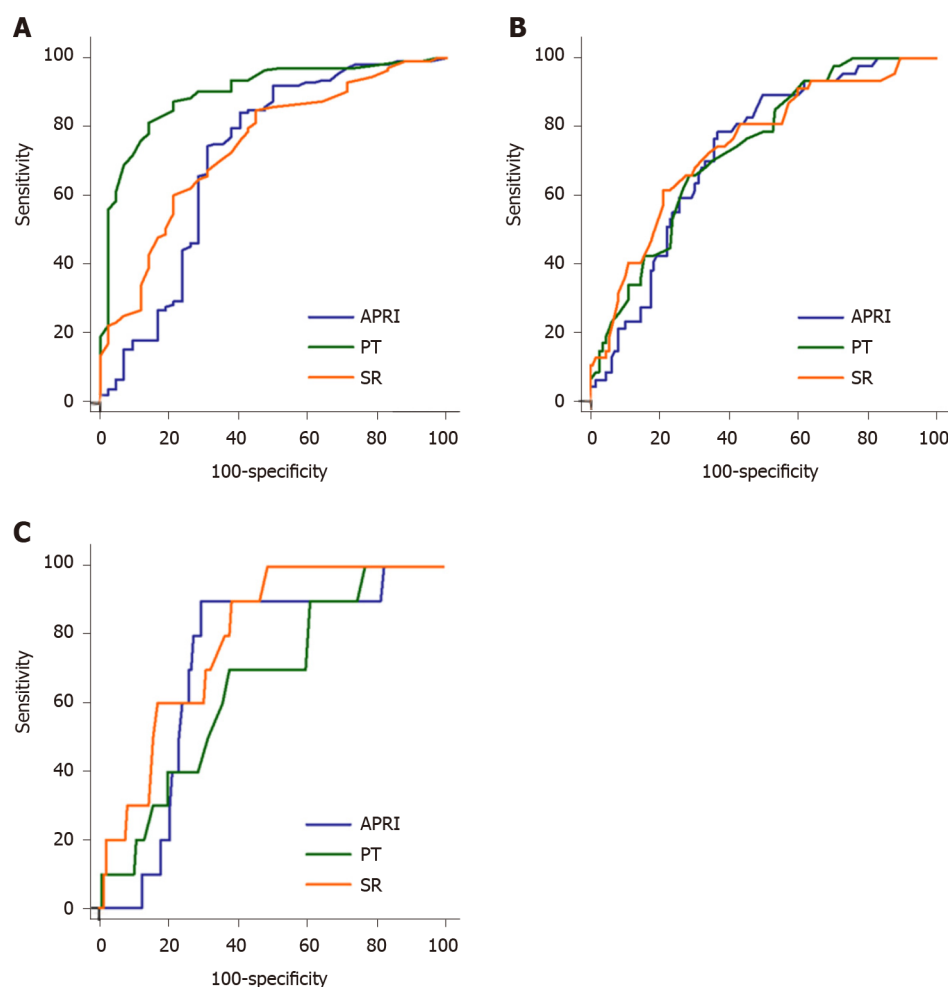
There were several limitations in this study. First, this study was performed retrospectively, so inherent possibility of bias exists. Second, reproducibility of PT measurements could be problematic, even though we used consensus reading. Third, we could not evaluate the pathologic finding of PT. The meaning of PT as a direct or indirect finding of hepatic or periportal fibrosis should be evaluated. Finally, the number of patients with F1 and F4 was relatively small, leading to unreliable data from these groups.

In conclusion, liver MRI findings of PT and SR can be useful to assess hepatic fibrosis in infants with cholestasis including BA. The finding of PT with a cutoff value of 4.2 mm has better diagnostic performance to predict clinically significant fibrosis than either SR or APRI. And SR had the highest diagnostic performance value for differentiating cirrhosis. The degree of hepatic fibrosis is an important prognostic factor after Kasai operation in patients with BA. Therefore, PT and SR measured on MRI may help predict prognosis of BA and suggest more effective non-invasive treatment options.

**Table 3** Diagnostic performance of the parameters

Variable	Cutoff value	Sensitivity (%)	Specificity (%)	AUC	95%CI	P value
Clinically significant fibrosis (F2-F4)						
APRI	0.44	84.1	59.5	0.712	0.634-0.782	0.001
PT	4.2 mm	81.4	85.7	0.899	0.840-0.941	< 0.001
SR	0.85	85.0	54.8	0.741	0.664-0.808	< 0.001
Advanced fibrosis (F3-F4)						
APRI	0.78	78.7	63.0	0.724	0.647-0.793	< 0.001
PT	5.3 mm	66.0	71.3	0.734	0.657-0.801	< 0.001
SR	1.1	61.7	78.7	0.742	0.666-0.809	< 0.001
Cirrhosis (F4)						
APRI	1.24	90.0	70.3	0.715	0.637-0.785	0.002
PT	5.3 mm	70.0	62.1	0.656	0.675-0.730	0.058
SR	1.04	90.0	61.4	0.790	0.718-0.852	< 0.001

AUC: Area under the curve; APRI: Aspartate transaminase to platelet ratio index; PT: Periportal thickening; SR: Normalized spleen size ratio.



**Figure 3** Comparisons of receiver operating characteristic curves. The comparisons of area under the curve (AUC) for diagnosing A: Clinically significant fibrosis (F2-F4); B: Advance fibrosis (F3-F4); C: Cirrhosis (F4) with periportal thickening (PT, green line), normalized spleen size ratio (SR, orange line), and aspartate aminotransferase to platelet ratio index (APRI, blue line). The AUC of PT for diagnosing clinically significant fibrosis was higher than that of SR or APRI. However, the AUCs were not different for differentiating advanced fibrosis. For diagnosing cirrhosis, SR had higher AUC value than PT. APRI: Aspartate aminotransferase to platelet ratio index; SR: Normalized spleen size ratio; PT: Periportal thickening.

## ARTICLE HIGHLIGHTS

**Research background**

Untreated neonatal cholestasis can progress to liver cirrhosis or end stage liver disease in infants due to prolonged hepatocyte and biliary tree injury and may require liver transplantation. Therefore, non-invasive evaluation of hepatic fibrosis is important in infants with cholestasis including biliary atresia.

**Research motivation**

Some serologic tests and splenomegaly are known markers for the evaluation of hepatic fibrosis in adults, but there is limited study about these parameters in infants. Periportal thickening (PT) was only considered as the finding of biliary atresia and has not been evaluated for assessing hepatic fibrosis in infants with cholestasis.

**Research objectives**

We investigated whether the PT and spleen size measured on magnetic resonance imaging (MRI) is associated with pathologically assessed hepatic fibrosis in patients with infantile cholestasis including biliary atresia.

**Research methods**

This retrospective study included infants less than 6 mo with liver MRI and biopsy for the evaluation of infantile cholestasis. Not only PT and spleen size measured on MRI, but also serologic assessment of aspartate transaminase to platelet ratio index (APRI) were evaluated and compared with histopathologic METAVIR grading of hepatic fibrosis. We calculated normalized spleen size ratio (SR) using the upper normal size limit.

**Research results**

A total of 155 patients were evaluated including 110 with biliary atresia. Mean age at the time of MRI was  $57.6 \pm 34.4$  d. There were positive correlations between fibrosis grade and PT and SR, even after adjusting age. The finding of PT with a cutoff value of 4.2 mm has better diagnostic performance to predict clinically significant fibrosis than either SR or APRI. And SR had the highest diagnostic performance value for differentiating cirrhosis.

**Research conclusions**

Liver MRI findings of PT and SR are useful to assess clinically significant hepatic fibrosis (F2 and higher) in infants with cholestasis including biliary atresia.

**Research perspectives**

The degree of hepatic fibrosis is an important prognostic factor after Kasai operation in patients with biliary atresia. Therefore, PT and SR measured on MRI may help predict prognosis of biliary atresia and suggest more effective non-invasive treatment options.

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## Retrospective Study

# Successful robotic radical resection of hepatic echinococcosis located in posterosuperior liver segments

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## Abstract

### BACKGROUND

Radical resection is an important treatment method for hepatic echinococcosis. The posterosuperior segments of the liver remain the most challenging region for laparoscopic or robotic hepatectomy.

### AIM

To demonstrate the safety and preliminary experience of robotic radical resection of cystic and alveolar echinococcosis in posterosuperior liver segments.

### METHODS

A retrospective analysis was conducted on the clinical data of 5 patients with a median age of 37 years (21-56 years) with cystic and alveolar echinococcosis in difficult liver lesions admitted to two centers from September to December 2019. The surgical methods included total pericystectomy, segmental hepatectomy, or hemihepatectomy.

### RESULTS

Among the 5 patients, 4 presented with cystic echinococcosis and 1 presented with alveolar echinococcosis, all of whom underwent robotic radical operation successfully without conversion to laparotomy. Total caudate lobectomy was performed in 2 cases, hepatectomy of segment VII in 1 case, total pericystectomy of segment VIII in 1 case, and right hemihepatectomy in 1 case. Operation time was 225 min (175-300 min); blood loss was 100 mL (50-600 mL); and postoperative hospital stay duration was 10 d (5-19 d). The Clavien-Dindo complication grade was I in 4 cases and II in 1 case. No recurrence of echinococcosis was found in any patient at the 3 mo of follow-up.

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## CONCLUSION

Robotic radical surgery for cystic and selected alveolar echinococcosis in posterosuperior liver segments is safe and feasible.

**Key words:** Cystic echinococcosis; Alveolar echinococcosis; Robotic surgery; Posterosuperior segment; Caudate lobe; Liver

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**Core tip:** This study aimed to elucidate the safety and preliminary experience of robotic radical resection of cystic and alveolar echinococcosis in posterosuperior liver segments. Our results demonstrated that robotic transabdominal approach can be an option of treatment and feasible for resection of cystic and selected alveolar echinococcosis located in the posterosuperior hepatic segments.

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## INTRODUCTION

Echinococcosis is a zoonotic disease caused by *Echinococcus* tapeworms, which can lead to damage to tissues and organs such as the liver, lungs, and brain. Human liver echinococcosis mainly includes cystic echinococcosis (CE) caused by *Echinococcus granulosus* infection and alveolar echinococcosis (AE) caused by *Echinococcus multilocularis* infection<sup>[1]</sup>. The worldwide spread of echinococcosis has made the disease a serious threat to public health, especially in Western China. The World Health Organization (WHO) has listed echinococcosis as one of the 17 diseases to be controlled or eliminated by 2050<sup>[2]</sup>.

According to the classification by the WHO Informal Working Group on Echinococcosis<sup>[3,4]</sup>, the treatment methods for hepatic echinococcosis include surgery, medication, and puncture and drainage. Radical resection remains the primary treatment for CE2, CE3, and AE<sup>[5]</sup>. Since the first laparoscopic pericystectomy for hepatic echinococcosis carried out in France in 1991 by Katkhouda *et al*<sup>[6]</sup>, there have been increasing reports on the laparoscopic treatment of CE<sup>[7-10]</sup>. The posterosuperior region of the liver – such as the caudate lobe (segment I), segment VII, and segment VIII – is considered to be the site where difficult lesions reside and remains the most challenging region for complex laparoscopic hepatectomy. Thus, extensive experience in open laparoscopic hepatectomy is imperative<sup>[11,12]</sup>.

The da Vinci Surgical System (Intuitive Surgical Inc., United States) is an advanced and minimally invasive surgery tool, which has been shown to have advantages in complex hepatectomy<sup>[13,14]</sup>. Since Giulianotti *et al*<sup>[15]</sup> reported two cases of robotic hepatic hydatid surgery in 2011, the literature on robotic hepatic hydatid surgery in difficult lesions has mostly been presented in case reports<sup>[16]</sup>. The aim of this study was to retrospectively analyze the data on robotic surgery for hepatic echinococcosis in difficult lesions from two centers in China and to explore the experience of performing robotic surgery for CE and AE.

## MATERIALS AND METHODS

### Study population and data collection

A retrospective analysis was conducted of the clinical data of patients with hepatic echinococcosis who underwent robotic surgery at the Second Department of Hepatopancreatobiliary Surgery, The First Medical Center, Chinese People's Liberation Army General Hospital as well as the Department of Hepatobiliary Surgery, The People's Hospital of Xinjiang Uygur Autonomous Region, from September to December 2019. The inclusion criteria were: (1) Patients who had pathologically confirmed CE or AE; (2) Patients who had lesions located in the

caudate lobe of the liver (segment I), segment VII, and segment VIII; and (3) Patients who underwent total pericystectomy, segmental hepatectomy, or hemihepatectomy. The exclusion criteria were: (1) Patients who had multiple organ echinococcoses; and (2) Patients who could not tolerate anesthesia or surgery.

Overall, 5 patients (all men, aged 21-56 years, with a median age of 37 years) were enrolled. The body mass index was 21.22-30.1 kg/m<sup>2</sup>, with a median body mass index of 25.54 kg/m<sup>2</sup>. The classification by the American Society of Anesthesiologists was I-III. Among them, 4 cases presented with CE alongside 1 case of AE, with a median tumor diameter of 75 mm (51-80 mm) (Table 1).

All patients completed abdominal ultrasound, computed tomography and magnetic resonance imaging examinations (Figure 1) before operation, and multidisciplinary discussion and WHO Informal Working Group on Echinococcosis classification were performed. All patients provided written informed consent. The research was approved by the hospital ethics committee, which complies with medical ethics regulations. All operations were performed by surgeons with vast experience in laparotomy and robotic hepatectomy.

### **Surgical procedure**

**Position and trocar hole layout:** Tracheal intubation integrated with intravenous general anesthesia was performed in a 25° reverse Trendelenburg position and a lithotomy position. When resection of segment of VII or VIII was required, the patient was turned 45° to his left side. The CO<sub>2</sub> pneumoperitoneum pressure was controlled at 14 mmHg, and the central venous pressure was maintained at 0-5 cm H<sub>2</sub>O<sup>[17]</sup>. Robotic procedures were performed using the da Vinci Si or Xi Surgical Systems (Intuitive Surgical Inc., United States).

The trocar layout consists of the optic port: 12 mm trocar, located at 3 cm on the right side of the umbilicus; assistant port: 12 mm trocar, located on the umbilicus (3 cm above the umbilicus when segment VII is excised); robotic arm 1: 8 mm trocar, located 3-5 cm below the xiphoid processes (below the xiphoid process when segment VII is excised); robotic arm 2: 8 mm trocar, located below the costal margin of the right anterior axillary line; and robotic arm 3: 8 mm trocar, located below the costal margin of the left anterior axillary line.

**Operation methods:** Intraoperative ultrasound and the Pringle maneuver were routinely used. According to previous reports<sup>[18,19]</sup>, the operation methods can be summarized as follows. (1) Caudate lobectomy: The left lateral and Spiegel's lobes were dissociated, and the short hepatic vessels of the caudate lobe were ligated using Hemolock clip (TFX Medical, RTP Durham, NC, USA). After the caudate lobe was separated from the inferior vena, the Arantius ligament and the hepatic pedicle branch of the caudate lobe were ligated using Hemolock clip. The hepatic parenchyma of the caudate lobe was detached using an ultrasonic scalpel, and the hydatid cyst was detached from the middle and the right hepatic veins. During intraoperative hepatic vein hemorrhage, the CO<sub>2</sub> pneumoperitoneum pressure was increased to 15 mmHg at first; the hemorrhage of branches < 2 mm could be bipolarly occluded; and the ethmoidal foramina > 2 mm in hepatic veins were treated with 6-0 prolene suture. After the detachment of the hepatic parenchyma, wound surface was fully exposed in the inferior vena cava as well as the left, middle, and right hepatic veins (Figure 2A and B). (2) Hepatectomy of segment VII: The right liver needed complete mobilization. The hepatic parenchyma was detached along the cephalic approach of the right hepatic vein; the right hepatic vein branch of segment VII and the hepatic pedicle branch of segment VII were ligated using Hemolock clip; and the hydatid cyst was removed from the right hepatic vein (Figure 2B and C). (3) Pericystectomy of segment VIII: After appropriate mobilization of the right liver, the hepatic parenchyma was detached at 2 cm from the margin of the hydatid cyst; the branches of the right and middle hepatic veins as well as the hepatic pedicle branch of segment VIII were ligated using Hemolock clip; and the focus was resected completely. and (4) Right hemihepatectomy: The right gallbladder was resected, and the right hepatic artery and the right branch of the portal vein were ligated. After complete right liver mobilization, the right hepatic parenchyma was detached with an ultrasound scalpel; the branches of segment V and segment VIII of the middle hepatic vein were ligated using Hemolock clip; and the right hepatic duct and right hepatic vein were detached, respectively.

The drainage tube was routinely placed on the cross section of the liver or through the Winslow hole, which was led out from robotic arm 2. The specimens were taken using the extended assistant port in the midline of the upper abdomen.

### **Perioperative results and follow-up**

The operation time, intraoperative blood loss and blood transfusion rate,



**Table 1** Demographic and clinical characteristics of the patients

Patient No.	Age (yrs)	Gender (M/F)	BMI (kg/m <sup>2</sup> )	ASA (I/II/III)	Tumor size (mm)	Pathology (WHO-IWGE)
1	37	M	23.84	II	75	CE3a
2	24	M	25.54	I	80	CE3a
3	31	M	21.22	I	80	CE3a
4	56	M	30.1	III	68	CE3a
5	45	M	27.7	II	51	AE(P1N0M0)
Median	37 (24, 56)	-	25.54 (21.22, 30.1)	-	75 (51, 80)	-

BMI: Body mass index; ASA: American society of anesthesiologists; CE: Cystic echinococcosis; AE: Alveolar echinococcosis; WHO-IWGE: World Health Organization Informal Working Group on Echinococcosis.

postoperative complications, drainage tube removal time, and postoperative hospital stay were assessed. The data were expressed as median and analyzed *via* IBM SPSS Statistics 20 statistical software. No patient took albendazole orally after the operation, and all patients underwent abdominal ultrasound or computed tomography 1 and 3 months after operation.

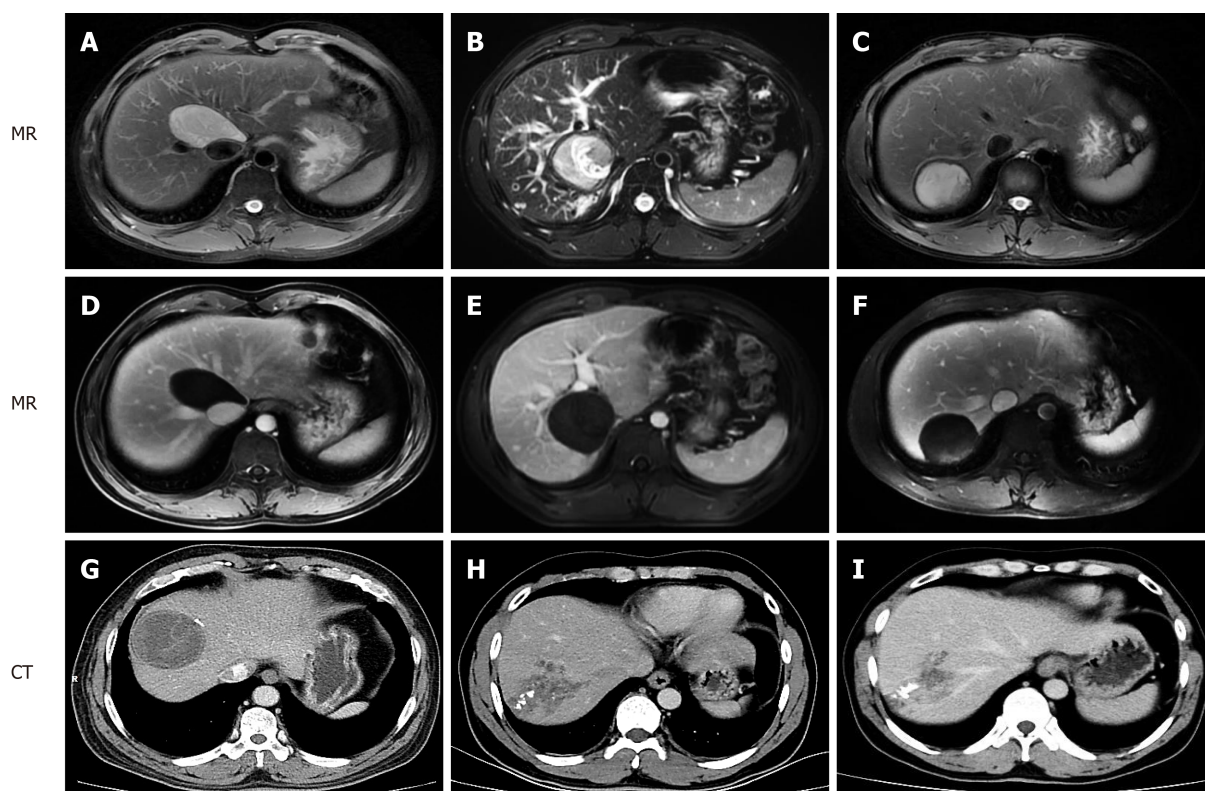
## RESULTS

All 5 patients successfully underwent robotic radical hepatectomy for hepatic echinococcosis, including total caudate lobectomy ( $n = 2$ ), hepatectomy of segment VII ( $n = 1$ ), total pericystectomy of segment VIII ( $n = 1$ ), and right hemihepatectomy ( $n = 1$ ), without conversion to laparotomy or perioperative deaths. The median operation time was 225 min (175-300 min); the median blood loss was 100 mL (50-600 mL), with 4 U of red blood cell suspension transfused in 1 patient because of an intraoperative blood loss of 600 mL; the median postoperative hospital stay was 10 d (5-19 d); and the median drainage tube removal time was 10 d (4-16 d). No bile leakage was found in any of the patients. According to the Clavien-Dindo complication grading, there were 4 cases of grade I and 1 case of grade II complications. The case of grade II complications with CE of the caudate lobe had postoperative right hepatic subcapsular hematoma; however, the patient recovered after bed rest and symptomatic treatment (Table 2). No recurrence of echinococcosis was found in any of the patients at 3 mo of follow-up.

## DISCUSSION

Surgery is an important treatment method for hepatic echinococcosis, and the postoperative recurrence rate of hepatic echinococcosis has been reported to be 2%-25%<sup>[20]</sup>. The surgery for CE includes radical surgery and palliative surgery. In accordance with the report of Georgiou *et al*<sup>[21]</sup>, the complication rate and 3-year postoperative recurrence rate of radical surgery are 10.95% and 6.9%, respectively, whereas the complication rate of palliative surgery is 24.13%. At present, the preferred radical resection approach for CE includes pericystectomy and segmental or partial hepatectomy<sup>[2]</sup>. The imaging findings of AE are similar to those of liver malignancies, and AE is associated with a 90% mortality rate in untreated patients. Hepatectomy is the main surgical method for AE, and liver transplantation can be considered as the last resort for end-stage AE<sup>[22]</sup>.

In 2016, Di Benedetto *et al*<sup>[23]</sup> reported a case of caudate lobectomy (Spiegel's lobe) for CE for the first time. The focus diameter was 5.6 cm; the operation time was 280 min; the blood loss was 200 mL; and the postoperative hospital stay was 3 d. To date, robotic total caudate lobectomy alone has not been reported in the literature. During traditional laparoscopic anatomical caudate lobectomy, due to the defects in two-dimensional visual field and endoscopic instruments, the dorsal part of the middle hepatic vein is often poorly exposed, and tearing of the small branches of the paracaval portion and the middle hepatic vein often results in catastrophic hemorrhage, which is difficult to treat under endoscopy<sup>[24]</sup>. In the present study, lesions in the caudate lobe in 2 cases of CE involved the Spiegel's lobes, caudate processes, and paracaval portion; the focus diameter was 8 cm; and the hydatid cyst was adjacent to the hepatic vein, inferior vena cava, and hepatic pedicle. We adopted the left approach, completely removed the hydatid cyst from the surface of the hepatic



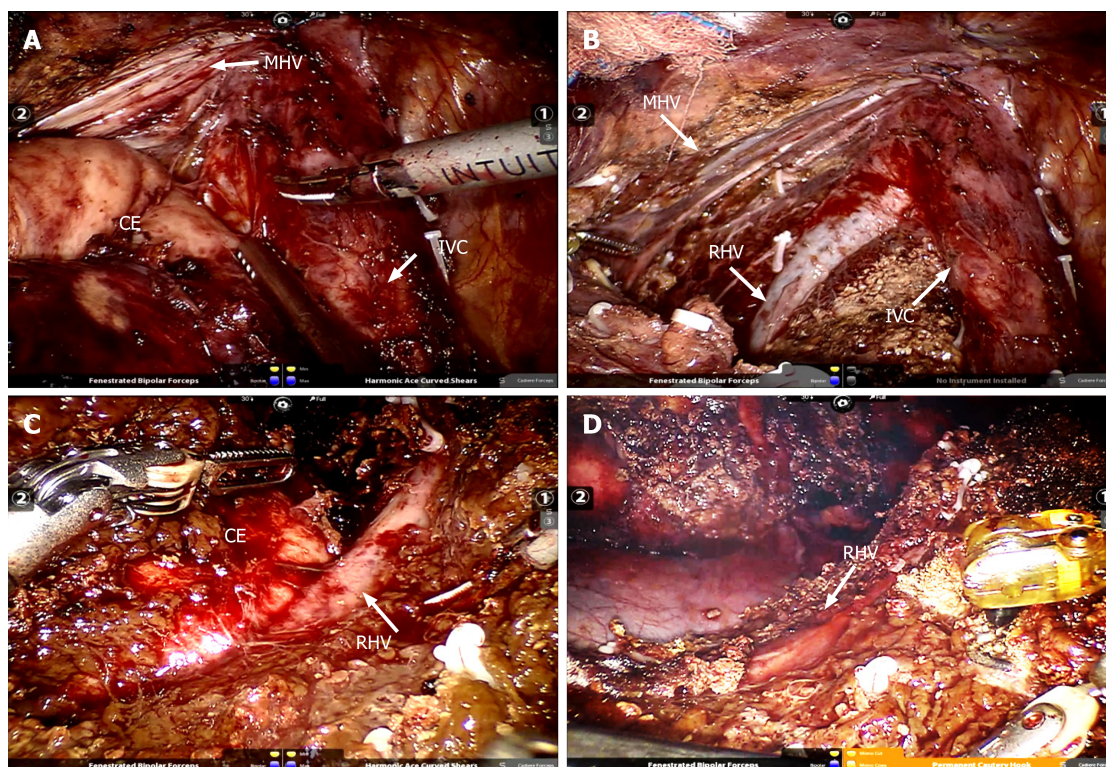
**Figure 1** Contrast-enhanced magnetic resonance imaging and computed tomography manifestations of hepatic cystic and alveolar echinococcosis. A, D: Patient 1, cystic echinococcosis in caudate lobe; B, E: Patient 2, cystic echinococcosis in caudate lobe; C, F: Patient 3, cystic echinococcosis in segment VII; G: Patient 4, cystic echinococcosis in segment VIII; H, I: Patient 5, alveolar echinococcosis in segment VII/VIII. MR: Magnetic resonance; CT: Computed tomography.

vein, and successfully performed robotic total caudate lobectomy.

In 2011, Casciola *et al*<sup>[25]</sup> reported robotic partial hepatectomy of segments I–VIII, including pericystectomy of segment IVa in 1 case and segment VII in 1 case. The study showed that robots had certain advantages in partial hepatectomy in the posterosuperior region of the liver or in cases with the focus adjacent to the hepatic vein. In 2013, Troisi *et al*<sup>[26]</sup> also found that compared with laparoscopic hepatectomy, robotic surgery is safe and feasible in hepatectomy for difficult lesions of the posterosuperior region of the liver, and thus, has certain advantages in hepatectomy with the preservation of the liver parenchyma. In the current study, the focus of CE at segment VII was adjacent to the right hepatic vein in 1 case. After the focus was located by ultrasound during the operation, the hydatid cyst and the right hepatic vein were finely detached via the cephalic approach of the right hepatic vein, and segment VII was resected completely. In addition, pericystectomy was successfully performed in 1 case of CE at segment VIII. The operation time in 2 patients were 175 min and 300 min, respectively; the intraoperative blood loss was 100 mL; the postoperative hospital stay was 11 d and 5 d, respectively; and the Clavien-Dindo complication grades were grade I in both.

In 2019, Magistri *et al*<sup>[16]</sup> reported the results of robotic surgery in 15 cases of CE from 3 centers in Italy, including resection of the Spiegel's lobe (as reported by Di Benedetto *et al*<sup>[23]</sup>), and other difficult segments. Consequently, we believe that the robot's video imaging system, which provides a magnified high-definition three-dimensional visual field, can ensure full exposure of the deep space of the caudate lobe, segment VII, and segment VIII. With the advantages of simulating the flexibility of the human wrist, eliminating hand tremor, and providing continuous and stable traction, the robotic arm can assist in the fine separation of hydatid cyst from the hepatic vein and suturing of the hepatic vein hiatus, and reduce uncontrollable hemorrhage.

Radical hepatectomy for AE requires that the normal liver tissues should be more than 1 cm above the edge of the focus, so as to eliminate the active hyperplasia region of the focus. Laparoscopic and robotic hepatectomy for AE, however, has not been reported in the literature. In the present study, robotic right hepatectomy was successfully performed in 1 case of AE involving segment VII and segment VIII. The operation time was 225 min; the blood loss was 200 mL; the postoperative hospital stay was 9 d; and the Clavien-Dindo complication grade was grade I. As such, we



**Figure 2** Intraoperative visual field and status after removal of the specimen. A: Cystic echinococcosis was located in caudate lobe adjacent to the inferior vena and hepatic vein; B: Complete caudate resection; C: cystic echinococcosis was located in segment VII adjacent to the right hepatic vein; D: Segment VII resection. CE: Cystic echinococcosis; IVC: Inferior vena cava; RHV: Right hepatic vein; MHV: Middle hepatic vein.

believe that robotic hepatectomy is safe and feasible for AE partially confined to the hemiliver or hepatic segment.

In summary, robotic radical surgery for cystic and selected alveolar echinococcosis in difficult liver lesions is safe and feasible.



Table 2 Perioperative data and outcomes

Patient No.	Resection	Operation time (min)	EBL (mL)	Transfusion (U)	POS (d)	Drainage (d)	Conversion	Complication grade (Clavien-Dindo)
1	I	210	50	0	19	16	-	II
2	I	300	600	4	10	10	-	I
3	VII	175	100	0	11	10	-	I
4	VIII	300	100	0	5	4	-	I
5	VII, VIII	225	200	0	9	6	-	I
Median	-	225 (175, 300)	100 (50, 600)	-	10 (5, 19)	10 (4, 16)	-	-

EBL: Estimated blood loss; POS: Postoperative hospital stay.

## ARTICLE HIGHLIGHTS

### Research background

Radical resection is an important treatment method for hepatic echinococcosis. The posterosuperior segments of the liver remain the most challenging region for laparoscopic or robotic hepatectomy.

### Research motivation

This study intended to retrospectively analyze the clinical data on robotic surgery for hepatic echinococcosis in difficult lesions from two centers in China and to explore the experience of performing robotic surgery for cystic and alveolar echinococcosis.

### Research objectives

The aim of this study was to demonstrate the safety and preliminary experience of robotic radical resection of cystic and alveolar echinococcosis in posterosuperior liver segments.

### Research methods

A retrospective analysis was conducted on the clinical data of patients with hepatic echinococcosis who underwent robotic surgery from September to December 2019.

### Research results

All 5 patients successfully underwent robotic radical hepatectomy for hepatic echinococcosis, including total caudate lobectomy, hepatectomy of segment VII, total pericystectomy of segment VIII, and right hemihepatectomy, without conversion to laparotomy or perioperative deaths. The operation time was 225 min; the blood loss was 100 mL; and the postoperative hospital stay duration was 10 d. The Clavien-Dindo complication grade was grade I in 4 cases and grade II in 1 case. No recurrence of echinococcosis was found in any of the patients after 3 mo of follow-up.

### Research conclusions

This study suggested that robotic radical surgery for cystic and selected alveolar echinococcosis in difficult liver lesions is safe and feasible.

### Research perspectives

Robotic transabdominal approach can be an option for resection of cystic and selected alveolar echinococcosis located in the posterosuperior hepatic segments.

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## Retrospective Study

# Non-invasive prediction model for high-risk esophageal varices in the Chinese population

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**Institutional review board statement:** This study has been approved by the Ethics Committee of The Second Affiliated Hospital of Xi'an Jiaotong University.

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## Abstract

### BACKGROUND

There are two types of esophageal varices (EVs): high-risk EVs (HEVs) and low-risk EVs, and HEVs pose a greater threat to patient life than low-risk EVs. The diagnosis of EVs is mainly conducted by gastroscopy, which can cause discomfort to patients, or by non-invasive prediction models. A number of non-invasive models for predicting EVs have been reported; however, those that are based on the formula for calculation of liver and spleen volume in HEVs have not been reported.

### AIM

To establish a non-invasive prediction model based on the formula for liver and spleen volume for predicting HEVs in patients with viral cirrhosis.

### METHODS

Data from 86 EV patients with viral cirrhosis were collected. Actual liver and spleen volumes of the patients were determined by computed tomography, and their calculated liver and spleen volumes were calculated by standard formulas. Other imaging and biochemical data were determined. The impact of each parameter on HEVs was analyzed by univariate and multivariate analyses, the data from which were employed to establish a non-invasive prediction model. Then the established prediction model was compared with other previous prediction models. Finally, the discriminating ability, calibration ability, and clinical efficacy of the new model was verified in both the modeling group and the external validation group.

### RESULTS

Data from univariate and multivariate analyses indicated that the liver-spleen volume ratio, spleen volume change rate, and aspartate aminotransferase were correlated with HEVs. These indexes were successfully used to establish the non-invasive prediction model. The comparison of the models showed that the

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established model could better predict HEVs compared with previous models. The discriminating ability, calibration ability, and clinical efficacy of the new model were affirmed.

## CONCLUSION

The non-invasive prediction model for predicting HEVs in patients with viral cirrhosis was successfully established. The new model is reliable for predicting HEVs and has clinical applicability.

**Key words:** Cirrhosis; High-risk esophageal varices; Non-invasive prediction model; Liver volume; Spleen volume

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**Core tip:** The non-invasive prediction model for predicting high-risk esophageal varices in patients with viral cirrhosis was successfully established based on the standard formula for calculation of liver and spleen volumes. It is a novel model that has not been reported. The model was shown to be better than previous prediction models. The new model had clinical efficacy and the ability to predict high-risk esophageal varices.

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## INTRODUCTION

Esophageal varices (EVs) are highly common in patients with cirrhosis. The risk of rupture depends mainly on severity of liver disease, level of portal pressure, diameter of varices, red sign, and the presence or absence of coagulation abnormalities<sup>[1-4]</sup>. Diagnosis of EVs relies mainly on endoscopy, which has been described in various documentations. Among all reported recording methods, the location, diameter and risk of bleeding classification is the most commonly used method for recording EVs. This classification records location, diameter, red sign, and local condition of EVs<sup>[5]</sup>.

Rupture of EVs, which can potentially occur in high-risk EVs (HEVs), is dangerous and life threatening. Current guidelines recommend that patients with HEVs should be treated with non-selective beta-blockers or endoscopic variceal ligation to reduce the risk of bleeding. Although these treatments can significantly reduce risk of esophageal variceal bleeding, because of difficulty in the early identification of HEVs in patients with cirrhosis, many of these patients have not benefited from these treatments<sup>[6,7]</sup>. The identification of HEVs is particularly important, and the currently used method is gastroscopy<sup>[8,9]</sup>, a method that can conveniently be used for visual determination of diameter and red sign of EVs. Gastroscopy is considered the gold standard for the diagnosis of EVs. However, patients with cirrhosis may not receive gastroscopic examination until there is a sign of esophageal variceal bleeding, or when low-risk EVs (LEVs) progress to become HEVs. Thus, identification of HEVs is of great significance, and establishment of a non-invasive predictive model that can predict patients with HEVs should help to solve this problem.

Liver volume and spleen volume measurements are essential for patients with cirrhosis and liver transplantation<sup>[10,11]</sup>. To date, the measurement of liver and spleen volume remains a very complex process. Although computed tomography (CT) or magnetic resonance imaging (MRI) can be used to calculate the liver and spleen volume, these methods are time-consuming and laborious. To address these problems, in our previous study, we conducted Pearson correlation analysis and stepwise multiple linear regression analysis of sex, height, weight, body surface area (BSA), body mass index (BMI), and actual liver volume and actual spleen volume. The same approach has also been introduced into many other studies<sup>[12,13]</sup>. We successfully applied the BSA of the patient to establish the formulas for calculation of standard liver volume (SLV) and standard spleen volume (SSV) as follows:  $SLV = 858.186 \times BSA - 393.349$  ( $R^2 = 0.350$ ); and  $SSV = 188.813 \times BSA - 140.981$  ( $R^2 = 0.126$ ). These

formulas were achieved using the data from 207 Chinese healthy adults and were verified using the data from another 98 healthy adults. The formulas were demonstrated to have higher accuracy and less error than other commonly used formulas<sup>[12-15]</sup>.

## MATERIALS AND METHODS

### Patients

The data were collected from all patients with viral cirrhosis who were admitted to the Second Affiliated Hospital of Xi'an Jiaotong University (Xi'an, Shaanxi Province of China) from October 2017 to December 2018 and underwent upper abdominal CT examination. The inclusion criteria were as follows: (1) Age > 18 years; (2) Have hepatitis B viral and hepatitis C viral cirrhosis; and (3) Underwent biochemical examination, upper abdominal CT examination, and gastroscopy, and interval between examinations of no more than 3 mo. Patients with the following criteria were excluded: (1) Other types of cirrhosis such as alcoholic cirrhosis, autoimmune cirrhosis, occult cirrhosis, *etc*; (2) Cirrhosis patients with medium to large ascites; (3) Suspected liver tumors; (4) History of liver or spleen resection; (5) Benign diseases that may affect the size of the liver or spleen such as cysts (diameter > 1 cm; number ≥ 2); (6) Other conditions that can possibly affect the hemodynamic of portal vein or splenic vein such as thrombosis, embolism, or spongiform degeneration; (7) Other conditions that may affect liver stiffness measurement (LSM), such as BMI > 35 kg/m<sup>2</sup>; (8) Unreliable liver hardness measurement: Interquartile range/median > 0.3, success rate < 60%, or effective measurement times < 10 times; (9) Patients with viral cirrhosis but without EVs; (10) Patients with severe weight loss or malnutrition; (11) Patients with hematological disease that may affect the spleen volume; and (12) Patients with a history of bleeding from the esophagus and receiving endoscopic or surgical treatment.

A total of 86 patients, including 56 patients with HEVs and 30 patients with LEVs, who met the inclusion criteria were enrolled in this study as the modeling group. Fifty other patients who met the inclusion criteria were enrolled in the study as the external validation group. Data collection for patients in the external validation group was performed after the new model was established. The application of the new model and the collection of gastroscopy results of the external validation group are independent processes. According to the Baveno V standard, the patients were divided into HEV and non-LEV groups. Basic information of each patient such as gender, age, height, weight, and BSA was recorded. The BSA was calculated by the Mosteller formula, which is more suitable for the Chinese population, as follows:  $BSA = \sqrt{[BW \text{ (kg)} \times BH \text{ (cm)}] / 3600}$ . HEVs are defined by the Baveno V standard as large EVs (diameter ≥ 5 mm), small EVs (diameter < 5 mm) with red signs, or EVs for Child C patients; and LEVs are EVs that do not meet these criteria<sup>[8,16]</sup>. The grading and scoring of patients with cirrhosis were performed following the Child-Pugh scoring system. This study was approved by the Ethics Committee of The Second Affiliated Hospital of Xi'an Jiaotong University. This was a retrospective study; thus, the Ethics Committee waived the requirement to obtain informed consent from the patients.

### Measurement of actual liver and spleen volume

All patients underwent CT examination. The upper abdominal CT examination was performed using a multi-slice spiral CT scanner (GE 128-slice spiral CT scanner; Linux Medical System, United States) with a reconstructed layer thickness of 5 mm and at a time interval of 5 s.

The CT data from the patients were retrospectively collected. The patients' actual liver and spleen volumes, portal vein diameter (PVD), portal vein surface area (PVSA), and spleen vein diameter (SVD), spleen long diameter (SLD) were measured using an image analysis program (Linux Imaging Workstation; Linux Medical Systems), which was performed by experienced radiologists who were unaware of the patients' basic condition. The surface area of the liver and spleen was manually tracked at each level. The actual volume of the liver and spleen was calculated by summing the surface area of each layer and multiplying it by the layer thickness. Large blood vessels, gallbladders, and fissures were avoided throughout the entire measurement. The PVD and PVSA were measured at the midpoint of the portal vein bifurcation and portal vein confluence site. The SVD was measured at 1 cm from the portal vein and splenic vein junction site. SLD is defined as the longest radial line of the layer with the largest surface area of the spleen.



### Calculation of parameters for liver and spleen

The SLV and SSV were calculated by the formulas established in our previous study as follows: (1)  $SLV = 858.186 \times BSA - 393.349$  ( $R^2 = 0.350$ ); and (2)  $SSV = 188.813 \times BSA - 140.981$  ( $R^2 = 0.126$ ). Other formulas included: Live *r* volume change rate =  $(CTLV - SLV)/SLV$ ; spleen volume change rate =  $(CTSV - SSV)/SSV$ ; change of liver volume =  $CTLV - SLV$ ; and change of spleen volume =  $CTSV - SSV$ , where CTLV and CTSV are the actual liver volume and spleen volume calculated by CT, respectively.

### Biochemical tests

All patients underwent biochemical tests, in which the data including blood routine analysis, liver function, renal function, and hepatitis detection were retrospectively collected from the patients. The blood test was performed using the XN-9000 analyzer (Xisen Meikang Medical Electronics Co. Ltd., Shanghai, China), the coagulation function test was performed using the Sysmex Co-CS-1500 system, and the liver function test was performed using the cobas 8000 analyzer (Roche Diagnostics, Mannheim, Germany).

### LSM by transient elastography

All patients underwent LSM using FibroScan (Echosens, Paris, France) and FibroTouch (Haishkell Medical Technology Center, Beijing, China). The results from the transient elastography (TE) were retrospectively collected and expressed in kilopascals (kPa). For patients with more than one LSM result during the study, only the result with a lower interquartile or lower median variability was selected. Several studies have shown that FibroTouch and FibroScan can detect liver fibrosis with high accuracy and consistency, and there are no statistically significant differences between them<sup>[17,18]</sup>.

### Gastroscopy

EVs were examined using an Olympus electronic gastroscope (Olympus, Tokyo, Japan) by experienced doctors and were divided into three categories: (1) No EVs; (2) Small EVs (diameter < 5 mm); and (3) Large EVs (diameter ≥ 5 mm). Observation of red sign was also recorded.

The published and currently used non-invasive prediction models are as follows: Liver stiffness-spleen diameter to platelet (PLT) ratio score (LSPS) =  $[LSM \text{ (KPa)} \times SLD \text{ (cm)}]/PLT \times 10^9/L$ <sup>[19]</sup>; variceal risk index (VRI) =  $-4.364 + 0.538 \times SLD - 0.049 \times PLT - 0.044 \times LSM + 0.001 \times (LSM \times PLT)$ <sup>[20]</sup>; aspartate transaminase (AST) to PLT ratio index (APRI) =  $[AST(U/L)/AST \text{ (normal upper limit)}] \times 100/PLT \times 10^9/L$ <sup>[21]</sup>; AST/alanine aminotransferase ratio (AAR) =  $AST/ALT$ <sup>[21]</sup>.

To evaluate the performance of the present and previous predictive models in identification of HEVs, the results from gastroscopy were used as the gold standard, and the receiver operating characteristic (ROC) curve of each model was plotted, and the area under curve (AUC) of ROC curve, sensitivity, specificity and Youden's index were calculated. The cutoff value of the point at which the sum of sensitivity and specificity was largest was selected as the optimal cutoff value in the diagnosis of HEVs or LEVs. The validity of the prediction model was evaluated by consistency (c) statistic (corresponding to AUC), and  $c > 0.7$  was considered effective.

### Evaluation of new prediction models

The discriminating ability of the model was determined by the ROC of the model in both the modeling group and external validation group. The difference in the ROC was evaluated by the Z test. If the ROC in both groups was not different and was higher than 0.7, the discriminating ability of the model was considered good. The calibration ability of predictive models was evaluated by the Hosmer-Lemeshow test and calibration scatter plot of the two groups. Decision curve analysis (DCA) of the two groups was carried out to evaluate the clinical efficacy of the new model.

### Statistical analysis

Statistical analysis was performed by SPSS 19.0 and R software (IBM SPSS, Chicago, IL, United States). Data are expressed as mean ± SD. The  $\chi^2$  test was employed to compare between the measured data of the HEVs and LEVs groups, and the Mann-Whitney *U* test was used to conduct the univariate analysis. The multivariate analysis was performed by backward WALD regression analysis. The ROC curve was obtained using SPSS 19.0. The Hosmer-Lemeshow test results, calibration plot figures, and DCA were obtained using R software. All statistical analyses were two-tailed, and  $P < 0.05$  was considered statistically significant.

## RESULTS

### **Characteristics of patients**

Based on the endoscope result and the Baveno V standard, we divided the patients into two groups: HEV group and LEV group. Age and gender of patients in the HEV group and LEV group were not significantly different ( $P > 0.05$ ), and the two groups were comparable. General characteristics of the modeling group and external validation group are shown in Tables 1 and 2.

### **Univariate analysis of HEVs**

The *t*-test and Mann-Whitney U test were used in the univariate analysis. The results summarized in Table 3 show that PVSA, PVD, SVD, CTSV, liver-spleen volume ratio, spleen volume change rate, spleen volume change, spleen diameter, ALT, AST, and thromboplastin time of the HEV group and LEV group were significantly different ( $P < 0.05$ ). By contrast, CTLV, SSV, SLV, the change rate of liver volume, liver volume change, total bilirubin, prothrombin time, PLT, and LSM of the two groups were not significantly different ( $P > 0.05$ ).

### **Multivariate analysis of HEVs**

The parameters shown in Table 3, which show that the HEV and LEV groups were significantly different, were subjected to multivariate analysis, which was carried out using the backward WALD regression analysis. As illustrated in Table 4, the three factors related to HEVs including ratio of liver volume to spleen volume, rate of spleen volume change, and AST in patients with HEVs were significantly different ( $P < 0.05$ ) from those with LEVs.

### **Establishment of non-invasive prediction model**

Based on the multivariate analysis results, all parameters that were not significantly different between the two groups were eliminated, whereas those that were significantly different, which included the ratio of liver volume to spleen volume, rate of spleen volume change, and AST, were employed to establish the non-invasive predictive model. As shown in Table 5, the non-invasive prediction model was obtained as follows:  $\ln [P/(1 - P)] = 8.342 - 2.162 \times (\text{CTLV}/\text{CTSV}) - 0.314 \times [(\text{CTSV} - \text{SSV})/\text{SSV}] - 0.07 \times \text{AST}$ . The ratio of liver volume to spleen volume, the rate of spleen volume change, and the AST were negatively associated with HEVs.

### **Comparison of prediction models**

The non-invasive predictive model for predicting HEVs in patients with viral cirrhosis established in the present study was compared with other previously reported models, namely LSPS, VRI, APRI, and AAR, which have been widely used for assessing EVs in patients with cirrhosis. Sensitivity, specificity, and AUC of the four indicators and of the established non-invasive prediction model for the modeling group were calculated. The cutoff value of the non-invasive prediction model was defined based on the maximum value of the sum of sensitivity and specificity. When the *P* value calculated by the established formula was larger than the cutoff value, the patients were considered to have HEVs. The results depicted in Figure 1A and Table 6 show that the AUC of the present model was 0.865, whereas that of the ROC curves of LSPS, VRI, APRI, and AAR were 0.591, 0.717, 0.431, and 0.445, respectively. A model with an AUC of higher than 0.7 was considered to have good discriminating ability. The higher the AUC, the better the discriminating ability of the model.

### **Comparison of accuracy of the models**

Accuracy, positive predictive value, and negative predictive value of all models (non-invasive predictive model, and LSPS, VRI, APRI, and AAR models) were calculated in all 86 patients enrolled in the modeling group. As shown in Table 7, the present non-invasive prediction model had high accuracy of 84.9% and high positive predictive value of 96.4%. The accuracy and the positive predictive value indicate the possibility of correctly diagnosing HEVs: the higher their values, the more likely the diagnosis is correct.

### **Evaluation of discriminating ability of the new model**

To evaluate the discriminating ability of the new model, we generated ROC curves of the external validation group using the new models and compared between the AUC curve of the modeling group and the external validation group using the Z test. The results showed that the AUC of the external validation group was 0.879. The Z test result also showed that the *P* value was 0.17, which indicates that the modeling group and the external validation group were not significantly different. This also indicates that the discriminating ability of the new prediction model was similar for both the

**Table 1 Comparison of general characteristics in the modeling group, *n***

Parameter	Patients with HEVs, <i>n</i> = 56	Patients with LEVs, <i>n</i> = 30	All patients, <i>n</i> = 86	<i>P</i> value
Age in yr	52.93 ± 11.61	54.70 ± 12.24	53.55 ± 11.79	0.35
Male (%)	33 (58.9)	14 (46.7)	47 (54.7)	0.43
Etiology, HBV/HCV	51/5	22/8	73/13	0.47
Course of disease in mo	48.3 ± 12.1	46.7 ± 11.3	48.1 ± 11.6	0.45
Child-Pugh class, A/B/C	31/19/6	9/16/0	40/35/6	< 0.05
Diameter of EVs in mm	0.8 ± 0.2	0.3 ± 0.1	0.6 ± 0.2	< 0.01
Red sign	39	0	39	< 0.01

*P* < 0.05 is considered statistically significant. HEVs: High-risk esophageal varices; LEVs: Low-risk esophageal varices; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

external verification group and the modeling group. The ROC curve of external validation group is shown in [Figure 1B](#).

### **Evaluation of calibration ability of the new model**

To evaluate the calibration ability of the new model, we used the Hosmer-Lemeshow test to calculate the  $\chi^2$  for the modeling group and the external validation group. The results showed that the  $\chi^2$  of the modeling group was 4.86, and that of the external validation group was 4.69; their *P* values were 0.746 and 0.790, respectively. The *P* values of both groups were higher than 0.05, indicating that the new model accurately predicted HEVs in both groups. The calibration scatter plots of both groups are shown in [Figure 2](#). According to the plots, all scattered points fluctuated around the reference line without significant deviation, which was due to the fact that the *P* values of both groups were higher than 0.05 and there was no statistical difference between the two groups. This result suggests that using the new model, the predicted HEV patients were in good agreement with the actual HEV patients.

### **Evaluation of clinical efficacy of the new model**

We used the DCA to evaluate the clinical efficacy of the new model. The DCA was drawn using the predicted probability of the model group and the external validation group and the actual occurrence of HEVs. The predicted probability of the model group was represented by  $P_{in}$  and that of the external validation group was represented by  $P_{out}$ . The DCA of the two groups are shown in [Figure 3](#). In the DCA curve, the black line indicated that in extreme cases, the new model predicted that there were no HEVs in all patients with viral cirrhosis and the clinical net benefit was 0. The gray line, which had a negative slope and was the clinical net benefit, indicated that in extreme cases, the new model predicted that there were HEVs in all patients with viral cirrhosis. The red line was the DCA of the new model. According to the DCA curves, the red line was higher than the black and gray lines, suggesting that both groups of patients could benefit from the new model when it is applied to two cohorts. It also suggests that the new model has clinical efficacy.

## **DISCUSSION**

In China, there are a large number of patients with liver cirrhosis due to the high infection rates of hepatitis B and C<sup>[22,23]</sup>. Although gastroscopy is the gold standard diagnosis technique for EVs, its procedure is invasive, and thus can cause discomfort to patients. Painless gastroscopy can significantly reduce the discomfort, and most gastroscopy in China is performed without anesthesia. The risk of bleeding in patients with EVs is different, and according to the Baveno V standard, EVs are divided into high bleeding risk EVs and low bleeding risk EVs. For patients with HEVs, taking preventive measures early significantly reduces the risk of esophageal varices bleeding. In China, many patients with liver cirrhosis do not receive the first gastroscopy until the esophageal varices rupture and bleed. Thus, it is highly important to accurately identify patients at high risk of bleeding caused by esophageal varices.

Other than gastroscopy, CT or MRI can also be used to predict the HEVs, and thus are often used to make preliminary judgments about the presence of EVs<sup>[24]</sup>. However, these two techniques cannot visually observe the red sign, making it difficult to correctly diagnose HEVs. There are many non-invasive models that can be used to

**Table 2 Comparison of general characteristics in the external validation group, *n***

Parameter	Patients with HEVs, <i>n</i> = 31	Patients with LEVs, <i>n</i> = 19	All patients, <i>n</i> = 50	<i>P</i> value
Age in yr	51.86 ± 10.93	55.33 ± 11.98	54.15 ± 10.38	0.37
Male (%)	18 (58.1)	10 (62.6)	28 (56.0)	0.46
Etiology, HBV/HCV	21/10	16/3	37/13	0.51
Course of disease in mo	47.6 ± 11.3	45.8 ± 12.1	46.9 ± 10.9	0.43
Child-Pugh class, A/B/C	11/18/2	7/12/0	18/30/2	< 0.05
Diameter of EVs in mm	0.8 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	< 0.01
Red sign	11	0	11	< 0.01

*P* < 0.05 is considered statistically significant. HEVs: High-risk esophageal varices; LEVs: Low-risk esophageal varices; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

predict HEVs, and the most commonly used models include LSPS, VRI, APRI, and AAR. The indexes used in these models include AST, ALT, PLT, PLD, and LSM. According to various studies, these models have proven to be effective in predicting HEVs<sup>[19-21]</sup>. The liver and spleen volume ratio is also an effective index that can be used to establish a non-invasive model to predict the hepatic vein pressure gradient<sup>[25]</sup>. Although many studies have reported the formula for calculating liver volume, few have reported its clinical application. Unlike these models, in this study, we used the volume calculation formulas established in previous studies to calculate the standard liver and spleen volumes, and used CT data to calculate the actual liver and spleen volumes. The differences between the calculated volumes and the actual volumes were considered the pathological change. Change rates of volume and other indexes related to liver and spleen volume were adopted to establish the non-invasive model for predicting HEVs. This approach has not been reported.

We successfully constructed a non-invasive prediction model that can predict HEVs, as follows:  $\ln [P/(1 - P)] = 8.342 - 2.162 \times (\text{CTLV}/\text{CTSV}) - 0.314 \times [(\text{CTSV} - \text{SSV})/\text{SSV}] - 0.07 \times \text{AST}$ . We selected the cutoff value, the point at which the sum of sensitivity and specificity was largest, as the optimal cutoff value, which was 0.571. When the value of *P* in the model was higher than the optimal value, HEVs were considered present. In validation of the new model, we compared AUC, sensitivity, specificity, Youden's index, accuracy, positive predictive value, and negative predictive value of the new model with those of LSPS, VRI, APRI, and AAR in 86 cirrhosis patients with EVs. The new model had an AUC of 0.865, a Youden's index of 0.71, an accuracy of 84.9%, and a positive predictive value of 96.4%. The results obtained from the new model were better than those obtained from LSPS, VRI, APRI, and AAR. High AUC (> 0.7) and Youden's index indicated that the model can accurately predict HEVs. The positive predictive value was an important index that reflected the model's ability to make a positive diagnosis of HEVs, which was the primary aim of this research: The higher the positive predictive value, the greater the probability of a positive diagnosis. In summary, we conclude that the new model can better predict HEVs compared to other previously reported models.

We further evaluated the discriminating ability, calibration ability, and clinical efficacy of the new model in predicting HEVs in both the modeling group and the external validation group. The discriminating ability of the model was determined based on the AUC of ROC curve. According to the results, the AUC of the model was higher than 0.8 in the two groups, indicating that the model had good discrimination ability (the AUC between 0.7 to 0.9 indicated that the model had good discrimination ability). The calibration ability of the model was analyzed by the Hosmer-Lemeshow test and the calibration scatter plot. In prediction of patients in both groups, the *P* values of the model were higher than 0.05, and the scattered points fluctuated around the reference line without significant deviation, indicating that the model had good calibration ability. The DCA can be used to evaluate the clinical efficacy of the model<sup>[26,27]</sup>. The model was considered to have clinical efficacy only when its DCA was higher than the extreme line. According to the DCA figures, the DCA of the new model was higher than the extreme line, indicating that the new model had good clinical efficacy. Therefore, the new model can accurately predict HEVs and has clinical application value.

Taken together, we successfully developed a non-invasive predictive model that can predict HEVs in patients with viral cirrhosis using the liver and spleen volume calculation formulas, which has not been reported. The model was compared with other previous models including LSPS, VRI, APRI and AAR. Comparisons of AUC of



**Table 3** Univariate analysis of parameters of patients with high-risk esophageal varices and low-risk esophageal varices

Parameter	Patients with HEVs, <i>n</i> = 56	Patients with LEVs, <i>n</i> = 30	<i>P</i> value
PVSA, mm <sup>3</sup>	227.04 ± 76.66	183.81 ± 69.10	< 0.01
PVD, mm	14.35 ± 2.64	12.67 ± 2.58	< 0.01
SVD, mm	10.08 ± 3.36	8.52 ± 2.67	0.02
CTLV, cm <sup>3</sup>	901.95 ± 219.00	935.18 ± 299.83	0.66
CTSV, cm <sup>3</sup>	917.30 ± 394.37	546.00 ± 375.35	< 0.01
Ratio of liver and spleen volume	1.18 ± 0.57	3.16 ± 5.25	< 0.01
SSV, cm <sup>3</sup>	177.03 ± 33.41	175.34 ± 29.76	0.81
SLV, cm <sup>3</sup>	1052.08 ± 151.88	1044.40 ± 135.25	0.80
Rate of change of liver volume, %	-0.14 ± 0.20	-0.10 ± 0.29	0.68
Rate of change of spleen volume, %	4.21 ± 2.11	2.06 ± 2.07	< 0.01
Change of liver volume, cm <sup>3</sup>	-150.13 ± 224.10	-109.22 ± 300.13	0.56
Change of spleen volume, cm <sup>3</sup>	740.27 ± 382.77	370.66 ± 365.27	< 0.01
Spleen diameter, cm	15.81 ± 2.67	12.67 ± 2.58	< 0.01
ALT, IU/L	57.68 (15-176)	40.97 (13-88)	< 0.01
AST, IU/L	36.38 (10-81)	61.26 (18-164)	< 0.01
TBIL, μmol/L	27.89 (3.5-73.69)	34.12 (6-119.7)	0.59
PT in s	13.69 (9.9-24)	13.50 (9.1-18.2)	0.85
TT in s	20.44 (17.2-27.9)	20.28 (16.9-23.1)	0.01
PLT, × 10 <sup>9</sup> /L	57.68 (15-176)	73.77 (29-159)	0.05
LSM, kPa	20.45 (9.4-36.1)	26.8 (7.6-75)	0.26

*P* < 0.05 is considered statistically significant. HEVs: High-risk esophageal varices; LEVs: Low-risk esophageal varices; PVSA: Portal vein surface area; PVD: Portal vein diameter; SVD: Splenic vein diameter; CTLV: Actual liver volume measured by CT; CTSV: Actual spleen volume measured by CT; SSV: Standard spleen volume calculated by equation; SLV: Standard liver volume calculated by equation; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; PT: Prothrombin time; TT: Thrombin time; LSM: Liver stiff measurement.

ROC curve, sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of each model showed that the established non-invasive prediction model better identified patients with HEVs than other models. Evaluation of the new model showed that it had high discriminative ability, calibration ability, and clinical efficacy. Moreover, the subjects included in this study were patients with viral cirrhosis; this can minimize the bias of the results while providing good consistency. This research had some limitations, such as the small sample size. In addition, the application of the model relied only on CT, a technique that can cause harm to patients. In China, CT is used for routine examination of patients with cirrhosis, with the aim of excluding the liver tumor. Other countries may use different techniques. All patients enrolled in this study were Chinese; thus, it remains unclear whether the model is applicable to patients in other ethnic groups. Moreover, the change in liver and spleen volume in viral cirrhosis patients was different from that in alcohol cirrhosis patients, and the new model can only be used in viral cirrhosis patients. These limitations may affect the promotion and application of the new model.

**Table 4 Multivariate analysis of parameters of patients with high-risk esophageal varices and low-risk esophageal varices**

Parameter	Patients with HEVs, <i>n</i> = 56	Patients with LEVs, <i>n</i> = 30	<i>P</i> value
PVSA, mm <sup>3</sup>	227.04 ± 76.66	183.81 ± 69.10	0.52
PVD, mm	14.35 ± 2.64	12.67 ± 2.58	0.60
SVD, mm	10.08 ± 3.36	8.52 ± 2.67	0.20
CTSV, cm <sup>3</sup>	917.30 ± 394.37	546.00 ± 375.35	0.26
Ratio of liver and spleen volume, %	1.18 ± 0.57	3.16 ± 5.25	< 0.01
Rate of change of spleen volume, %	4.21 ± 2.11	2.06 ± 2.07	0.047
Change of spleen volume, cm <sup>3</sup>	740.27 ± 382.77	370.66 ± 365.27	0.30
Spleen diameter, cm	15.81 ± 2.67	12.67 ± 2.58	0.58
ALT, IU/L	57.68 (15-176)	40.97 (13-88)	0.71
AST, IU/L	36.38 (10-81)	61.26 (18-164)	< 0.01
TT in s	20.44 (17.2-27.9)	20.28 (16.9-23.1)	0.93

*P* < 0.05 is considered statistically significant. HEVs: High-risk esophageal varices; LEVs: Low-risk esophageal varices; PVSA: Portal vein surface area; PVD: Portal vein diameter; SVD: Splenic vein diameter; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PT: Prothrombin time; TT: Thrombin time.

**Table 5 Parameters used to establish the non-invasive prediction model**

Parameter	B	SE	Wals	df	Sig	Exp (B)	95%CI of exp (B)
Ratio of liver and spleen volume	-2.162	0.683	10.028	1	0.002	0.115	0.030-0.439
Rate of spleen volume change	-0.314	0.246	1.619	1	0.203	0.731	0.451-1.185
AST	-0.070	0.020	12.672	1	0.000	0.933	0.898-0.969
Constant	8.342	2.413	11.946	1	0.001	4194.879	

AST: Aspartate aminotransferase; df: Degree of freedom; CI: Confidence interval.

**Table 6 Comparison of various parameters of each model**

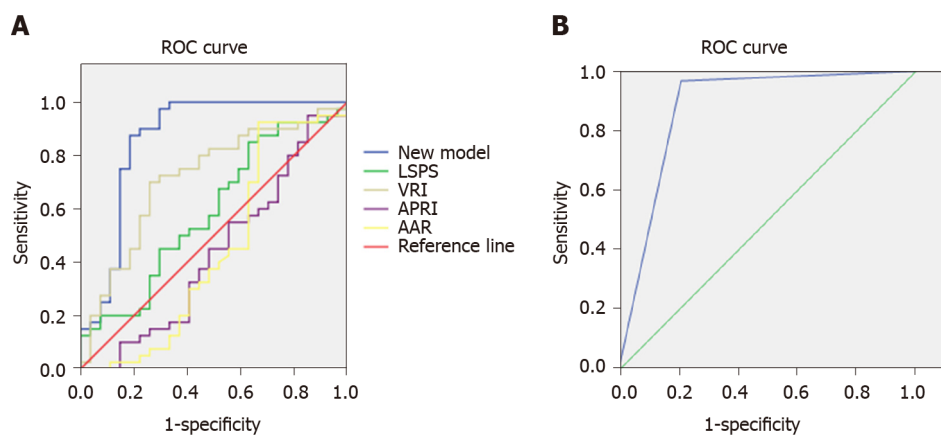
	Area	SE	Sig	95%CI of exp (B)	Sensitivity	Specificity	Youden's index
The new model	0.865	0.054	0.000	0.759-0.970	0.91	0.80	0.71
LSPS	0.591	0.072	0.210	0.450-0.732	0.85	0.37	0.22
VRI	0.717	0.065	0.003	0.589-0.844	0.70	0.74	0.44
APRI	0.431	0.074	0.344	0.285-0.577	0.95	0.15	0.10
AAR	0.445	0.080	0.447	0.288-0.601	0.93	0.33	0.26

CI: Confidence interval.

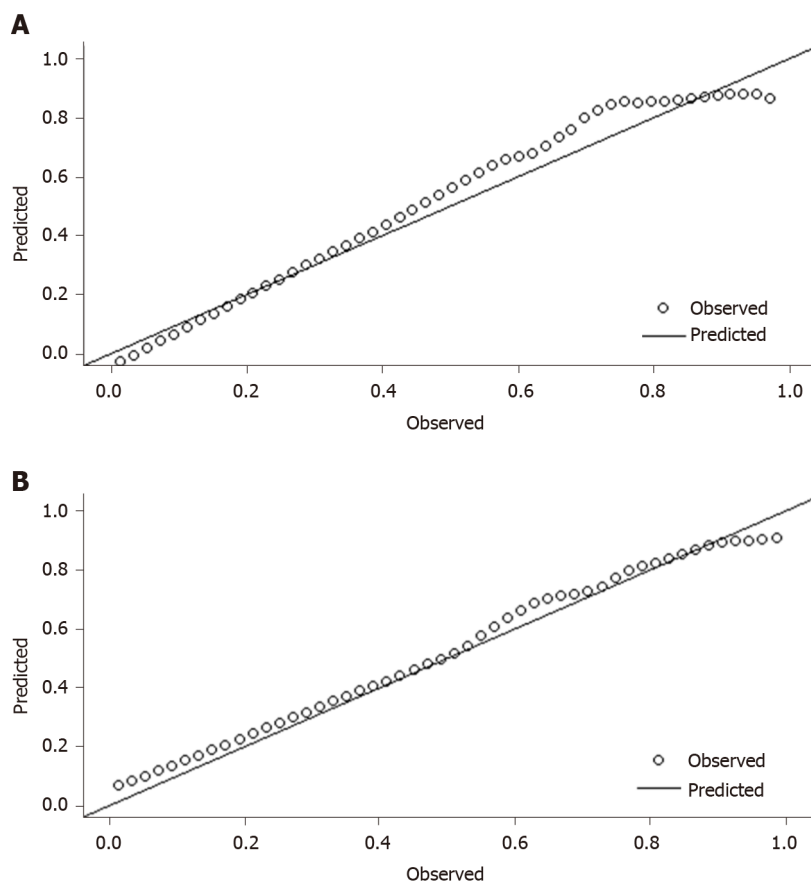
**Table 7 Comparison of accuracy of each model in predicting high-risk esophageal varices of patients in the modeling group**

	Accuracy, %	Positive predictive value, %	Negative predictive value, %	Cutoff value
New model	84.9	96.4	63.3	0.5713638
LSPS	82.1	85.0	37.0	3.0852585
VRI	70.1	70.0	74.1	0.52695
APRI	67.4	96.4	13.3	0.5671263
AAR	68.6	89.3	30	0.9861111

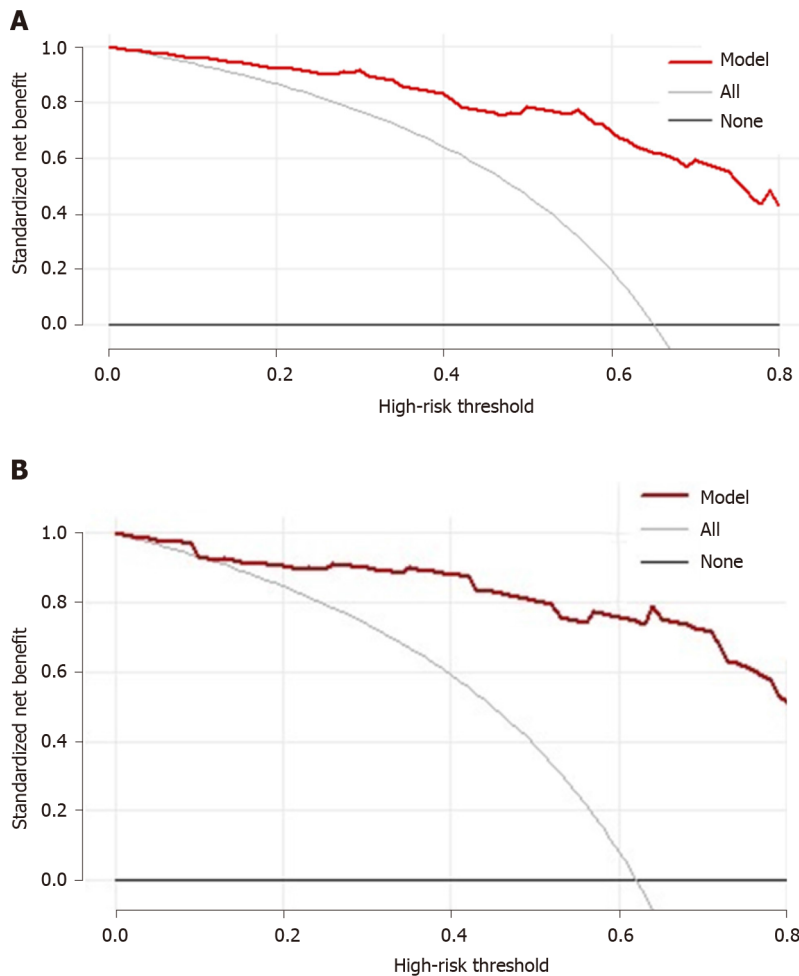
LSPS: Liver stiffness-spleen diameter to platelet ratio score; VRI: Variceal risk index; APRI: Aspartate transaminase to platelet ratio index; AAR: Aspartate transaminase/alanine aminotransferase ratio.



**Figure 1** Area under the curve of various models in predicting high-risk esophageal varices of patients. A: Modeling group; B: External validation group. The area under the curve of the new model in predicting high-risk esophageal varices of patients was 0.865 in the modeling group, which was higher than that of liver stiffness-spleen diameter to platelet ratio score, variceal risk index, aspartate transaminase to platelet ratio index, and aspartate transaminase /alanine aminotransferase ratio; and it was 0.879 in the external validation group. ROC: Receiver operating characteristic.



**Figure 2** Calibration scatter plot of data of patients. A: Modeling group; B: External validation group. In predicting patients in the modeling group and external validation group, the scattered points fluctuated around the reference line without significant deviations.



**Figure 3 Adjusted decision curve analysis of data of patients.** A: Modeling group; B: External validation group. The black line indicates that in extreme cases, the new model predicted that there were no high-risk esophageal varices in all patients with viral cirrhosis, and the clinical net benefit was 0. The gray curve indicates that in extreme cases, the new model predicts there are high-risk esophageal varices in all patients with viral cirrhosis, the clinical net benefit is the negative slope. The red line indicates that the new model has a clinical net benefit. The red line is higher than the black and gray lines, indicating that patients in the modeling group can benefit from the new model.

## ARTICLE HIGHLIGHTS

### Research background

Several models for predicting high-risk esophageal varices (HEVs) have been reported; however, models that are based on liver and spleen volume calculation formula in HEVs have not been reported.

### Research motivation

HEVs are EVs that have a high risk of bleeding, and the establishment of a non-invasive predictive model will be useful for the early identification of HEVs. These patients will benefit if necessary measures are taken in a timely manner.

### Research objectives

This present study established a non-invasive prediction model based on the liver and spleen volume calculation formula for predicting HEVs in patients with viral cirrhosis.

### Research methods

Eighty-six EVs patients with viral cirrhosis, from October 2017 to December 2018, were included at the Second Affiliated Hospital of Xi'an Jiaotong University. By reviewing the medical records, required data were collected for. The impact of each parameter on HEVs was analyzed by univariate and multivariate analyses, the data from which were employed to establish a non-invasive prediction model. Then the established prediction model was compared with LSPS, VRI, APRI, and AAR. The discriminating ability, calibration ability, and clinical efficacy of the established model were verified in both the modeling group and the external validation group.

### Research results

After univariate and multivariate analysis, liver-spleen volume ratio, spleen volume change rate,



and aspartate aminotransferase were successfully used to establish the non-invasive prediction model for HEVs. The new model could better predict HEVs compared with LSPS, VRI, APRI, and AAR. The discriminating ability, calibration ability, and clinical efficacy of the new model were verified.

### Research conclusions

The non-invasive prediction model for predicting HEVs is a reliable model for predicting HEVs and has clinical applicability.

### Research perspectives

The predictive value of the new model needs to be confirmed in a large number of virus cirrhosis patients with EVs. Predictive models with high accuracy need to be established taking into account the limitations of the new model.

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## Observational Study

# Golimumab in real-world practice in patients with ulcerative colitis: Twelve-month results

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## Abstract

### BACKGROUND

The introduction of biologics has revolutionized the management of the chronic inflammatory bowel disease, ulcerative colitis (UC), with many patients experiencing significant improvements not only in their symptoms but in other outcomes relevant to individuals and society as a whole. In Germany, there are no prospective data > 3 mo that assess the work productivity, daily activities and quality of life (QoL) of patients with moderate-to-severe UC treated with golimumab.

### AIM

To assess change in work productivity, capacity for daily activities and QoL in UC patients treated with golimumab in Germany.

### METHODS

The validated Work Productivity Activity Impairment (WPAI) Questionnaire was used to analyze the change in work productivity, the capacity for daily activities after three months (primary endpoint) and disease specific and health related QoL (HRQoL) up to 1 year (secondary endpoints). The changes in work productivity and activity impairment were evaluated every three months until month twelve compared to baseline. Disease-specific and health-related QoL

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were assessed with the inflammatory bowel disease questionnaire and with the short-form 12 health survey questionnaire (SF-12).

## RESULTS

This prospective non-interventional study included 287 patients. The analysis population was comprised of 282 patients who had completed at least two visits. At baseline, 61% of patients had moderate UC and 18% had severe UC. Furthermore, 75% of patients worked full-time or part-time at baseline. A total of 212 patients who were employed at the start of the study (employed population) were evaluated for the primary endpoint. Golimumab significantly reduced all WPAI sub-scores compared to baseline after three, six, nine and twelve months after the start of treatment ( $P < 0.0001$ ). In addition, disease-specific QoL and HRQoL, as measured by the SF-12 questionnaire, improved significantly with golimumab at all evaluation times ( $P < 0.0001$  in each case *vs* baseline).

## CONCLUSION

Treatment of moderate-to-severe UC with golimumab leads to significant improvements in patient's work productivity, daily activity and QoL over twelve months.

**Key words:** Ulcerative colitis; Quality of life; Work productivity; Non-interventional study; Golimumab; Work Productivity Activity Impairment (WPAI) Questionnaire

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**Core tip:** Systematic reviews indicate that ulcerative colitis (UC) causes a significant socioeconomic burden, but prospective studies are scarce. The aim of this study is to evaluate the changes in work productivity and quality of life (QoL) of UC patients in Germany treated with the human monoclonal tumor necrosis factor alpha antibody golimumab in order to assess the specific benefit of this treatment option. This prospective non-interventional study included 287 patients. Based on these data we were able to show that treatment of moderate-to-severe UC with golimumab leads to significant improvements in patient's work productivity, daily activity and QoL over twelve months.

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## INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disease of the gastrointestinal tract [inflammatory bowel disease (IBD)] with a peak onset between ages 15 and 30 years<sup>[1,2]</sup>. The main features are mucosal inflammation, which spreads proximally from the rectum, and the development of extensive superficial ulcerations<sup>[3]</sup>. The symptoms include recurrent episodes of bloody diarrhea with excretion of liquid bloody stools several times daily, a strong urge to defecate, abdominal pain, incontinence, weight loss and general malaise. In about 50%-80% of patients, UC follows a relapsing course with variable disease activity. In a further 15%-30% of patients it is difficult to achieve permanent remission<sup>[4]</sup>. Health-related quality of life (HRQoL) is severely impaired in moderate-to-severe UC. In addition, a negative association between HRQoL on the one hand and unemployment, sick days and claiming of disability pensions on the other, is described in patients with IBD<sup>[5]</sup>. Most perturbing is that the risk of work disability is highest among the youngest patients, when pioneering career steps generally need to be taken<sup>[6]</sup>.

The early disease onset of UC with frequent hospitalizations as well as extraintestinal manifestations is associated with high utilization of health services<sup>[7]</sup>. UC patients often suffer from a significant impairment in their quality of life (QoL) and an overall poor general condition due to chronic recurrent disease. In addition to



gastrointestinal symptoms, this also includes disturbances in social interaction, sleep and emotional behavior. The majority of patients also suffer from concentration problems; the lower working speed and lower productivity can lead to problems in the workplace. Incapacity for work is in turn associated with lower QoL and a higher rate of depressive and anxious symptoms, which further worsen functional status<sup>[8]</sup>. Many UC patients require continuous or intermittent treatment. Achieving and sustaining at least partial remission might help to improve the patient's functional status and ability to work<sup>[9]</sup>.

Systematic reviews indicate that UC causes significant socioeconomic burden<sup>[10,11]</sup>. Long-term analyses by the European Collaborative Study on IBD over a 10-year period show large differences in the annual direct costs per patient for Europe depending, among other things, on geographical location, therapy practice, hospitalization duration and disease duration<sup>[12]</sup>. It is therefore necessary, not only for quality assurance of medical interventions, to evaluate the use of medical services in a health care system specific, naturalistic setting, including health economic endpoints<sup>[13]</sup>. Several studies show that biologic treatment of patients with IBD affects both the direct and indirect costs of healthcare<sup>[14,15]</sup>. For example, a profound assessment of the healthcare costs and productivity losses in a large cohort of IBD patients revealed that productivity losses accounted for 39% of the total costs in patients with UC<sup>[15]</sup>.

The primary aim of UC therapy is to rapidly achieve clinical remission and maintain long-term steroid-free clinical and endoscopic remission<sup>[16]</sup>. Anti-tumor necrosis factor alpha (anti-TNF $\alpha$ ) therapy offers a way of escalating treatment to induce and gradually maintain remission in UC. The current S3 UC guideline of the German Society for Gastroenterology, Digestive and Metabolic Diseases recommends the use of thiopurines or anti-TNF $\alpha$  antibodies (in the case of infliximab in combination with thiopurine where appropriate), vedolizumab or tofacitinib in patients with steroid dependent UC<sup>[16]</sup>. The human monoclonal TNF $\alpha$  antibody golimumab is indicated for the treatment of moderately to severely active UC in adult patients who have responded insufficiently to conventional therapy or who have an intolerance of or contraindication to such therapies<sup>[17]</sup>.

Until now, there have been few data on how anti-TNF $\alpha$  therapy affects work productivity in patients with moderate-to-severe UC. In particular, there are no systematic data on the use of golimumab in patients with moderate-to-severe UC in Germany, containing insight on outcome parameters, QoL and health economics.

The aim of the GO-CUTE study is to evaluate the changes in work productivity and QoL of UC patients treated with golimumab in Germany in order to assess the specific benefit of this treatment option. The analysis is presented after a treatment period of three months (primary endpoint), six, nine and twelve months.

## MATERIALS AND METHODS

### *Study design and ethical considerations*

The non-interventional, multicenter, prospective study GO-CUTE (project ID: MK8259-031) is conducted in fifty gastroenterological practices in Germany. Patients will be observed for 2 years. The presented analysis is based on full one-year data from the third interim analysis [March 21, 2014 (first patient first visit) to August 16, 2019 (last patient last visit)].

GO-CUTE complies with all legal and regulatory requirements for non-interventional studies, including EU Directive 2001/20/EC, and in accordance with Section 67(6) of the German Medicinal Products Act (AMG), it was notified to the Paul Ehrlich Institute, the National Association of Statutory Health Insurance Physicians, the Central Federal Association of Health Insurance Funds and the Association of Private Health Insurance Companies. In accordance with AMG Section 4 No.23, the treatment of the patients presented here followed exclusively the individual medical decision in daily practice. Informed consent was obtained from all individual participants prior to study enrollment.

The observation plan and patient information of the GO-CUTE study were approved by the Institutional Review Board Ethics Committee of the Bavarian State Medical Association (Bayrische Landesärztekammer).

### *Study endpoints*

The primary endpoint was evaluation of the changes in work productivity or activity impairment (WPAI) in month three (from baseline) compared to baseline in UC patients treated in Germany with golimumab in clinical practice using the WPAI questionnaire.

Secondary endpoints of the study were as follows: (1) Evaluation of the change in the short-form 12 health survey questionnaire (SF-12) in months three, six, nine, twelve, eighteen and twenty-four after baseline compared to baseline in UC patients treated in Germany with golimumab in clinical practice; and (2) Evaluation of the change in the IBD questionnaire (IBDQ) in months three, six, nine, twelve, eighteen and twenty-four after baseline compared to baseline in UC patients treated in Germany with golimumab in clinical practice.

### Patients

The study included 287 patients aged  $\geq 18$  years with UC, diagnosed by a gastroenterologist, who were suitable for golimumab therapy in accordance with the approved product information and clinical standards.

The following patients were excluded: (1) Patients with a contraindication according to the current Simponi® Summary of Product Characteristics (SmPC)<sup>[17]</sup>; (2) Patients previously treated with golimumab; (3) Patients with previous biologic treatment whose treatment was changed due to a serious adverse event (SAE), an opportunistic infection or hypersensitivity reaction; and (4) Patients currently participating in another clinical trial (with the exception of register studies).

Treatment was carried out in accordance with the current Simponi® (golimumab) SmPC. Simponi® is approved in the European and is indicated for the treatment of moderately to severely active UC in adult patients who have had an inadequate response to conventional therapy, including corticosteroids and 6-mercaptopurine or azathioprine, or who are intolerant of or have medical contraindications to such therapies. It is administered subcutaneously: (1) In the induction phase: golimumab 200 mg in week 0 and golimumab 100 mg in week 2; and (2) In the maintenance phase: in patients with body weight  $< 80$  kg: 50 mg every 4 weeks; in patients with body weight  $\geq 80$  kg: 100 mg every four weeks. The dosages and times of subcutaneous administration were documented throughout the study.

### Assessments

**Work productivity:** The validated WPAI questionnaire was used to analyze the primary endpoint. The WPAI is considered to be the psychometrically best validated instrument for determining health-related work productivity and is widely used<sup>[18]</sup>. The WPAI has previously been studied and validated in various chronic inflammatory diseases, including in patients with IBD<sup>[19,20]</sup>. In UC, it has proved its worth in randomized, controlled trials as well as in non-interventional observational studies<sup>[21]</sup>. Changes in work productivity and the capacity for daily activities were evaluated in months three (Visit 1, V1), six (V2), nine (V3), and twelve (V4) compared to baseline. All four WPAI subscores<sup>[22]</sup> were assessed: (1) Absence from work due to illness (absenteeism); (2) Reduced performance due to health problems (presenteeism); (3) Total work productivity impairment (TWPI); and (4) Daily activity based on general health problems or specific health problems. Patients were asked about a recall period of seven days.

**SF-12:** The HRQoL in UC patients was assessed using the SF-12 in months three, six, nine, and twelve after baseline. The SF-12 was developed as a shorter alternative to the more extensive SF-36, and can be used regardless of the patient's disease and age<sup>[23]</sup>. The SF-12 includes twelve questions on physical and mental status [physical component score (PCS-12), and mental component score (MCS-12)] to assess the overall state of health and evaluate the ability to engage in moderate activities. Patients were asked about a recall period of four weeks.

**IBDQ:** Disease-specific QoL was assessed by the IBDQ<sup>[24]</sup>. Systemic symptoms such as sleep disorders and fatigue (systemic symptoms), specific bowel functions [frequency of pain and cramps (bowel systems)], impairment in social activities (social function) and emotional consequences of the disease (emotional health) are recorded in thirty-two items. Patients were asked about a recall period of two weeks.

### Statistical analysis

Data were analyzed using SAS version 9.4 (NC, United States). All data were expressed as mean  $\pm$  SD or as  $n$  and %. The primary analyses were carried out in the full analysis population (mITT) comprised of patients having data for at least two visits ( $n = 282$ ). All patients who started treatment with golimumab were considered for analyses, regardless of whether they remained on golimumab at the time of evaluation.

For the WPAI, a one-sample  $t$  test for the change from baseline was used to compare the values obtained at months three, six, nine, and twelve (V1-V4), or the Wilcoxon signed rank test was used if the assumption of a normal distribution was

doubtful. All differences with a *P* value less than 0.05 were considered as statistically significant and should be interpreted in an explorative manner. The statistical methods of this study were reviewed by Ulrich Elsasser, MedPharmTec GmbH, Munich, Germany.

## RESULTS

### *Patient disposition*

A total of 287 patients were included in the study. The mITT was comprised of 282 patients who had data from at least two visits. A total of 212 patients who were employed at the start of the study (mITTe) were evaluated for the primary endpoint. The mITT and mITTe populations were well balanced in terms of their demographic and disease-specific characteristics (Table 1). Table 2 shows the occupational status in the analysis and employed populations. Table 3 summarizes immune-modulating medications concomitant to golimumab in the analysis and employed populations.

### *Primary endpoint*

From three months after the start of treatment up to twelve months after the start of treatment there was a significant reduction in all WPAI sub-scores compared to baseline ( $P < 0.0001$ ; Wilcoxon signed rank test) with golimumab (Figure 1).

The mean value in the absenteeism sub-score as observed decreased after three months (V1) from  $27.6\% \pm 37.7\%$  to  $10.3\% \pm 25.3\%$  (mean change from baseline:  $-13.8\% \pm 38.8\%$ ;  $P < 0.0001$ ; Wilcoxon signed rank test, Figure 1A). A quarter of patients achieved a reduction of at least 25%; 15% of patients did not record any improvement. After twelve months (V4), the mean difference compared to baseline was  $19.9\% \pm 42.0\%$  ( $P < 0.0001$ ; Wilcoxon signed rank test). About 35% of patients achieved a reduction of at least 40%, and there was no improvement in 20% of patients.

The mean value in the presenteeism sub-score as observed decreased after three months (V1) from  $45.3\% \pm 26.0\%$  to  $29.4\% \pm 25.1\%$  (mean change from baseline:  $-14.9\% \pm 28.8\%$ ;  $P < 0.0001$ ; Wilcoxon signed rank test, Figure 1B). A quarter of the patients achieved a reduction of at least 40%; 20% of patients did not record any improvement. After twelve months (V4), the mean difference compared to baseline was  $22.5\% \pm 29.2\%$  ( $P < 0.0001$ ; Wilcoxon signed rank test). A quarter of the patients achieved a reduction of at least 50%; there was no improvement in 15% of patients.

The mean value in the TWPI sub-score as observed decreased after three months (V1) from  $49.7\% \pm 27.7\%$  to  $31.9\% \pm 26.9\%$  (mean change from baseline:  $-17.3 \pm 32.3$ ;  $P < 0.0001$ ; Wilcoxon signed rank test, Figure 1C). About one fifth of the subjects reached a reduction of at least 45%, 30% of the subjects had no improvement. After twelve months (V4), the mean difference compared to baseline was  $23.7\% \pm 30.8\%$  ( $P < 0.0001$ ; Wilcoxon signed rank test). About 35% of patients achieved a reduction of at least 40%, and there was no improvement in 20% of patients.

The mean value in the activity impairment sub-score as observed decreased after three months (V1) from  $52.8\% \pm 26.9\%$  to  $36.3\% \pm 27.8\%$  (mean change from baseline:  $14.4 \pm 28.5$ ;  $P < 0.0001$ ; Wilcoxon signed rank test, Figure 1D). A quarter of the patients achieved a reduction of at least 40%; 25% of patients did not record any improvement. After twelve months (V4), the mean difference compared to baseline was  $27.5\% \pm 29.3\%$  ( $P < 0.0001$ ; Wilcoxon signed rank test). A quarter of the patients achieved a reduction of at least 55%; there was no improvement in 10% of patients.

### *Secondary endpoints*

Significant improvements were also obtained in the secondary endpoints for disease-specific QoL, as measured by the IBDQ, as well as the HRQoL as measured by the SF-12, during treatment with golimumab ( $P < 0.0001$  vs baseline; Wilcoxon signed rank test).

**IBDQ:** The average IBDQ score was  $124.8 \pm 35.8$  points at baseline and  $168.0 \pm 32.3$  points after 1 year (V4). This was equivalent to an increase of  $42.3 \pm 38.3$  points. About 85% of participants achieved an improvement in their average IBDQ total score compared to baseline. The IBDQ total score improved by more than 50 points in 60% of participants. The significant improvement in the average IBDQ total score was already seen after three months (V1) with an increase of  $26.5 \pm 36.4$  points, and in subsequent visits after six months (V2:  $29.9 \pm 36.7$  points), nine months (V3:  $34.8 \pm 39.2$  points) and after twelve months (V4:  $43.3 \pm 38.3$  points). The difference from baseline in the total IBDQ score was statistically significant for all visits ( $P < 0.0001$ ; Wilcoxon signed rank test, Figure 2).

**SF-12:** The mean SF-12 physical component score (PCS-12) increased continuously

**Table 1** Demographic and disease-specific properties

	mITT (n = 282)	mITTe (n = 212)
Gender (male/female), %	47.2/52.8	47.6/52.4
Age (mean $\pm$ SD)	40.8 ( $\pm$ 13.9)	39.7 ( $\pm$ 12.1)
Marital status		
Single	116 (41.1)	88 (41.5)
Married/living together	156 (55.3)	117 (55.2)
Divorced	8 (2.8)	7 (3.3)
Widowed	2 (0.7)	-
Rectal bleeding		
No blood seen	95 (33.7)	68 (32.1)
Streaks of blood with stool less than half the time	81 (28.7)	55 (25.9)
Obvious blood with stool most of the time	81 (28.7)	69 (32.5)
Blood alone passes	25 (8.9)	20 (9.4)
Physician global assessment		
Normal	8 (2.8)	7 (3.3)
Mild disease	54 (19.1)	39 (18.4)
Moderate disease	173 (61.3)	131 (61.8)
Severe disease	47 (16.7)	35 (16.5)
Alcohol consumption (actual)		
Yes/no	142 (50.4)/140 (49.6)	114 (53.8)/98 (46.2)
Concomitant disease		
Yes/no	121 (42.9)/161 (57.1)	82 (38.7)/130 (61.3)
Frequency of alcohol consumption		
Occasional	117 (82.4)	95 (83.3)
Once a week	11 (7.7)	10 (8.8)
Several times a week	9 (6.3)	5 (4.4)
Daily	5 (3.5)	4 (3.5)
Nicotine consumption (actual)		
Yes/no	28 (9.9)/254 (90.1)	22 (10.4)/190 (89.6)
Abuse of other substances		
Yes/no	1 (0.4)/281 (99.6)	1 (0.5)/281 (99.5)
Cannabis	1 (100)	1 (100.0)

Data are shown as *n* (%), except where otherwise mentioned. mITT: Total analysis population; mITTe: Employed analysis population.

from  $43.1 \pm 8.7$  points at baseline (V0) to  $48.7 \pm 7.2$  points after 1 year (V4). This corresponded to an average difference of  $6.1 \pm 8.9$  points (median 5.9, range -13.6 to 36.5). The significant improvement in the mean PCS-12 was firstly seen after three months (V1) with an increase of  $3.3 \pm 8.3$  points, and in subsequent visits after six months (V2:  $3.9 \pm 8.3$  points) and nine months (V3:  $4.8 \pm 9.7$  points). The difference from baseline in the PCS12 score was statistically significant for all visits ( $P < 0.0001$ ; Wilcoxon signed rank test, [Figure 3](#)).

The mean SF-12 mental component score (MCS-12) increased continuously from  $39.7 \pm 10.1$  points at baseline (V0) to  $46.4 \pm 9.0$  points after 1 year (V4). This corresponded to an average difference of  $6.5 \pm 10.8$  points (median 6.3, range -24.1 to 31.9). The significant improvement in the mean MCS-12 was firstly seen after three months (V1) with an increase of  $4.2 \pm 10.1$  points, and in subsequent visits after six months (V2:  $4.7 \pm 10.5$  points) and nine months (V3:  $5.1 \pm 11.7$  points). The difference from baseline in the MCS-12 score was statistically significant for all visits ( $P < 0.0001$ ; Wilcoxon signed rank test, [Figure 3](#)).

## DISCUSSION

In this prospective study, we showed that golimumab leads to a significant improvement in work productivity, daily activity and QoL in UC patients after three



**Table 2 Occupational status**

	mITT (n = 282)	mITTe (n = 212)
Occupational status		
Employed (full time)	172 (61.0)	172 (81.1)
Employed (part time)	39 (13.8)	39 (18.4)
Retired	13 (4.6)	1 (0.5) <sup>1</sup>
Full reduced	12 (4.3)	
Not working	46 (16.3)	
Part time employment due to		
Ulcerative colitis	7 (17.9)	7 (17.9)
Other reason	32 (82.1)	32 (82.1)
Full reduced due to		
Ulcerative colitis	8 (66.7)	N/A
Other reason	4 (33.3)	N/A

<sup>1</sup>One subject who stated “retired” on the electronic case report form was included, because she had entries in the work productivity and activity impairment. It was assumed that this subject actually worked part-time. Data are shown as *n* (%). mITT: Total analysis population; mITTe: Employed analysis population; N/A: Not applicable.

months of golimumab induction. These benefits persisted for twelve months. Our data contribute to fulfilling the recent recommendation from a systematic literature review that WPAI should be used for measuring work outcomes in UC patients<sup>[21]</sup>.

Our results may illustrate that golimumab is able to restore patients' QoL; a well-accepted therapeutic goal beyond achieving induction and maintenance of remission<sup>[25,26]</sup>. Furthermore, our study provides correlative data on QoL in a real world setting which were limited to date<sup>[27,28]</sup>. Comparable to our data, a prospective multicenter study of golimumab effectiveness and QoL in a real-life population showed a marked improvement of QoL measured by IBDQ<sup>[29]</sup>. From baseline (start of induction) to week eight and week thirty-two a significant IBDQ mean increase (32.9; mean value: 172; and 25.2, mean value 170; respectively) was observed ( $P < 0.05$ ), respectively<sup>[29]</sup>. In an interim analysis of a prospective cohort study from Sweden, QoL improved in golimumab treated patients, with a significant reduction in the overall short health scale score ( $P = 0.04$ )<sup>[30]</sup>. To date, there have been no systematic data on subjectively-assessed outcome parameters, QoL and health economics regarding the use of golimumab in patients with moderate-to-severe UC in Germany.

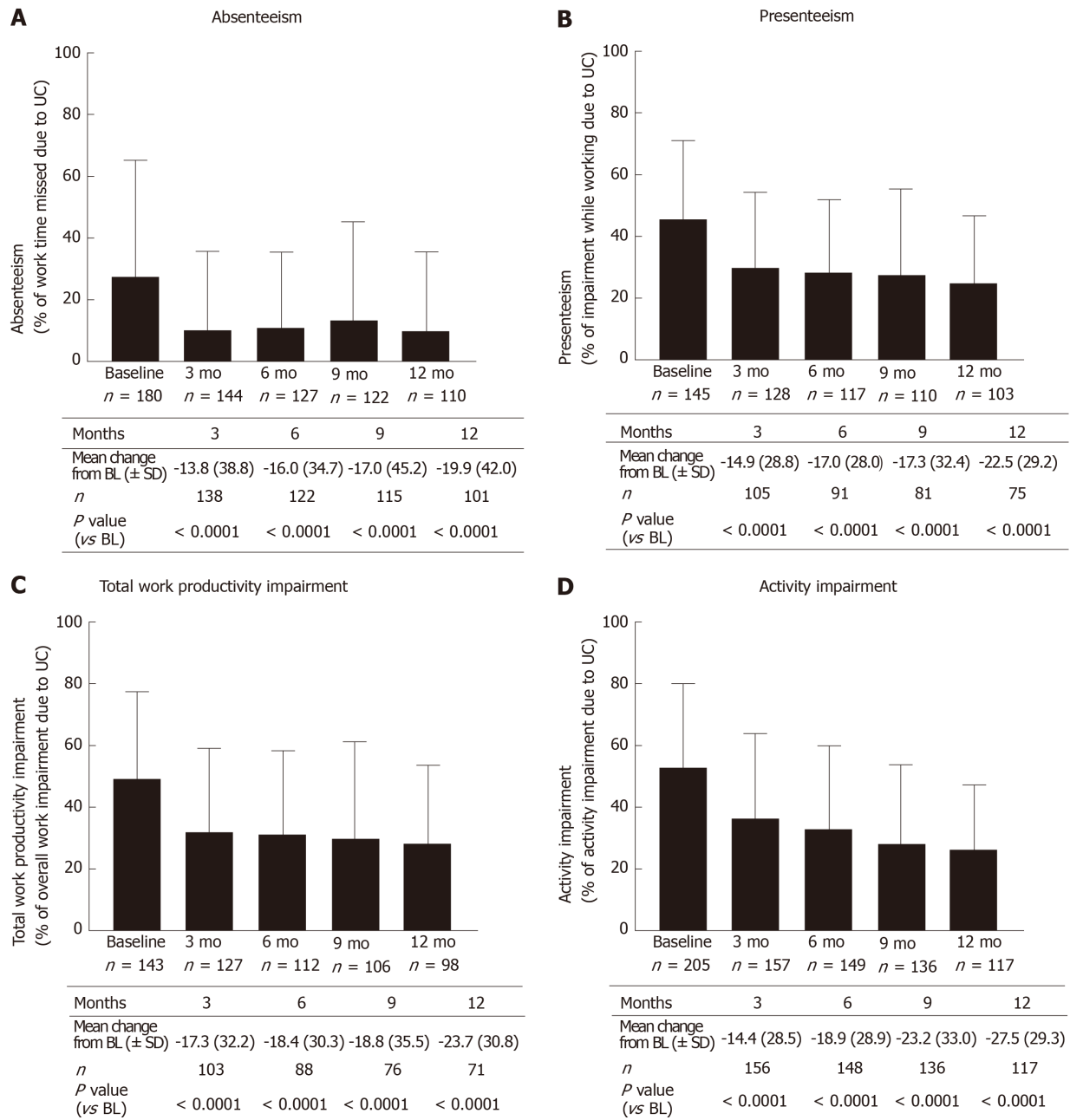
Our data compare well to the non-interventional QUO-VADIS study that evaluated the health-economic aspects of anti-TNF $\alpha$  therapy for ankylosing spondylitis<sup>[31]</sup>. This study, reporting on 963 ankylosing spondylitis patients, showed a gain in work productivity and activity and fewer disease-related absences in patients newly treated with golimumab or infliximab within six months of treatment<sup>[31]</sup>. Evidence from randomized controlled trials is needed to directly evaluate WPAI's responsiveness to treatment.

To conclude, our study shows a strong WPAI's responsiveness of the treatment of moderate-to-severe UC with golimumab. Golimumab induction resulted in significant improvements in the work productivity, daily activity and QoL of patients over full treatment duration of 12 mo.

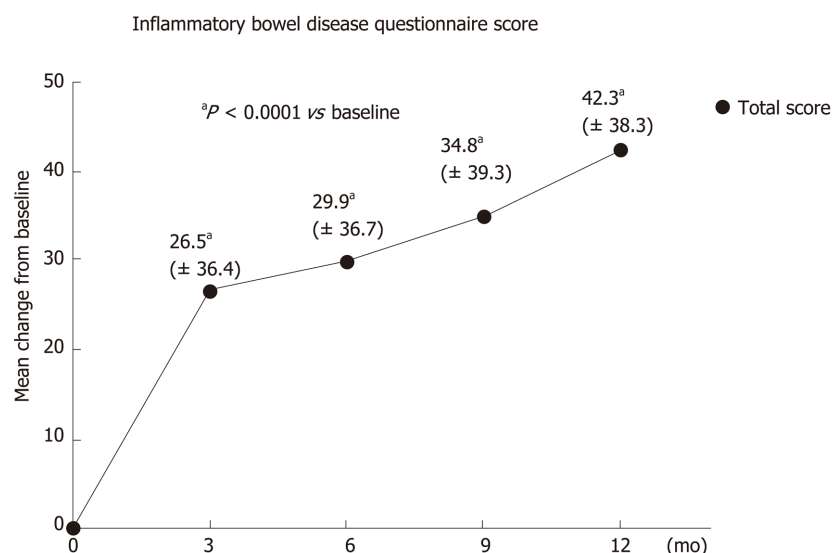
**Table 3** Selected concomitant medications

	mITT (n = 282)	mITTe (n = 212)
Any medication	125 (44.3)	90 (42.5)
Corticosteroids	123 (43.6)	88 (41.5)
Thiopurines		
Azathioprin	4 (1.4)	4 (1.9)

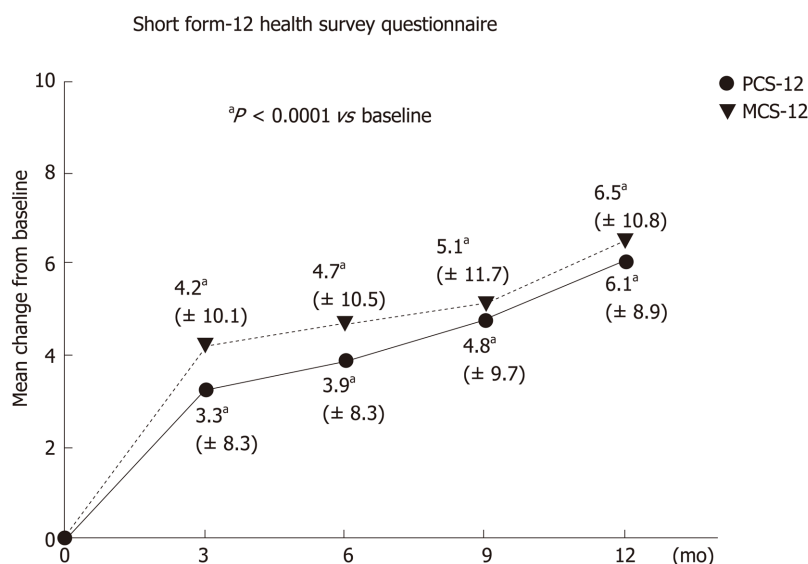
Data are shown as n (%). mITT: Total analysis population; mITTe: Employed analysis population.



**Figure 1** Overview of mean work productivity and activity impairment (WPAI) domain scores comparison vs baseline in patients employed at baseline (employed analysis population). A: Patients with absenteeism; B: Patients with presenteeism; C: Patients' work impairment; D: Patients' activity impairment. Bars represent the standard deviation. A significant reduction in all work productivity and activity impairment sub-scores after onset of golimumab therapy was detectable for each time point in comparison to baseline (for each visit  $P < 0.0001$ , Wilcoxon signed rank test). UC: Ulcerative colitis; BL: Baseline; n: Number of patients; SD: Standard deviation.



**Figure 2 Inflammatory bowel disease questionnaire (IBDQ) score – change from baseline over time (total analysis population).** The difference from baseline in the total IBDQ score was statistically significant for all visits ( $^aP < 0.0001$ , Wilcoxon signed rank test). Standard deviation is given in brackets.



**Figure 3 Short form-12 health survey questionnaire - physical component score (PCS-12) and short form-12 health survey questionnaire - mental component score (MCS-12) – change from baseline over time (total analysis population).** The difference from baseline in the PCS-12 as well as in the MCS-12 was statistically significant for all visits ( $^aP < 0.0001$ , Wilcoxon signed rank test). Standard deviation is given in brackets. PCS-12: Short form-12 health survey questionnaire - physical component score; MCS-12: Short form-12 health survey questionnaire - mental component score.

## ARTICLE HIGHLIGHTS

### Research background

Ulcerative colitis (UC) represents a chronic inflammatory bowel disease with recurrent episodes of debilitating symptoms leading to an impaired health-related quality of life (HRQoL), especially in those patients with moderate-to-severe UC. Besides HRQoL, in most of the patients, work productivity is negatively affected and an increased incapacity to work is reported due to UC. Therefore, UC causes additionally a substantial socioeconomic burden. Consequently, it is a considerable necessity to evaluate the impact of treatment options on work productivity and work life impairment. Golimumab, a human monoclonal tumor necrosis factor alpha (TNF $\alpha$ ) antibody is indicated to treat moderate-to-severe UC in adult patients without effective response to conventional therapies and its use has led to significant decrease of symptom burden in treated subjects.

### Research motivation

Until now, it is rarely evaluated how anti-TNF $\alpha$  therapy affected work life in patients with

moderate-to-severe UC. In particular, there are no systematic data on the use of golimumab in patients with moderate-to-severe UC in Germany with regard to work life impairment, quality of life (QoL) and health economics.

### Research objectives

The GO-CUTE study aimed to evaluate the changes in work productivity and HRQoL in UC patients treated with golimumab in Germany in order to assess the specific benefit of this treatment option. Changes in work productivity and the capacity for daily activities after three months represented the primary endpoint. The changes in HRQoL and disease specific QoL up to 1 year during golimumab treatment were defined as secondary endpoints.

### Research methods

This non-interventional, observational, prospective study was conducted in fifty gastroenterological practices in Germany. Work productivity and activity impairment were analyzed using the validated Work Productivity Activity Impairment (WPAI) Questionnaire. Short-form 12 health survey questionnaire (SF-12) and inflammatory bowel disease questionnaire (IBDQ) were used to complete HRQoL and disease-specific QoL assessment.

### Research results

Our results showed a significant reduction in all WPAI sub-scores after the start of treatment with golimumab for each time point (month three, six, nine and twelve) when compared to baseline data (for each visit  $P < 0.0001$ , Wilcoxon signed rank test). A quarter of patients achieved a reduction of at least 25% in the absenteeism sub-score and a reduction of at least 40% in the presenteeism sub-score after three months of golimumab treatment. After twelve months, in 80% of the subjects the absenteeism sub-score and in 85% of the patients the presenteeism sub-score was enhanced. Significant improvements were also detected for disease-specific QoL as well as for HRQoL during treatment with golimumab ( $P < 0.0001$  vs baseline, Wilcoxon signed rank test) assessed by IBDQ and SF-12, respectively.

### Research conclusions

The results of the GO CUTE study demonstrated that golimumab treatment in patients suffering from moderate-to-severe UC significantly improves both patient's work productivity and daily activity as well as HRQoL and disease-specific QoL. Furthermore, our data revealed that these benefits persisted over twelve months of treatment.

### Research perspectives

We were able to show a strong responsiveness of the WPAI to the treatment of moderate-to-severe UC with golimumab, but evidence from randomized controlled trials is additionally needed for final conclusions.

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## Observational Study

# M2BPGi for assessing liver fibrosis in patients with hepatitis C treated with direct-acting antivirals

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**Author contributions:** Saleh SA designed the research; Alhusseini MM participated in the acquisition of data; Saleh SA, Salama MM, Alhusseini MM, Mohamed GA participated in the analysis and interpretation of the data; Saleh SA, Salama MM, Mohamed GA revised the article critically for important intellectual content; Mohamed GA wrote the paper.

### Institutional review board

**statement:** The study was reviewed and approved by the institutional review board of Faculty of Medicine, Ain Shams University, Cairo, Egypt.

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

**Conflict-of-interest statement:** All authors have nothing to disclose.

**Data sharing statement:** The statistical code and dataset are available from the corresponding author at [ghadaabdelrahman@med.asu.edu.eg](mailto:ghadaabdelrahman@med.asu.edu.eg). The participants gave informed consent for the data sharing.

**STROBE statement:** The authors have read the STROBE Statement-checklist of items, and the manuscript was prepared and revised according to the STROBE Statement-checklist of items.

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## Abstract

### BACKGROUND

Assessing liver fibrosis is important for predicting the efficacy of direct-acting antivirals (DAAs) and patient prognosis. Non-invasive techniques to assess liver fibrosis are becoming important. Recently, serum Mac-2 binding protein glycosylation isomer (M2BPGi) was identified as a non-invasive marker of liver fibrosis.

### AIM

To investigate the diagnostic accuracy of M2BPGi in assessing liver fibrosis in patients with chronic hepatitis C (CHC) treated with DAAs.

### METHODS

From December 2017 to August 2018, 80 treatment-naïve adult patients with CHC who were eligible for DAAs therapy were consecutively enrolled in this observational cohort study. For 12 weeks, 65 patients were treated with sofosbuvir/daclatasvir, and 15 patients were treated with sofosbuvir/daclatasvir and a weight-based dose of ribavirin at knowledge and technology association for hepatitis C management clinic, Cairo, Egypt. We measured serum M2BPGi levels, PAPAS index, fibrosis-4 (FIB-4) score and liver stiffness measurements (LSM) at baseline and 12 weeks after the end of treatment. Serum M2BPGi levels were measured using enzyme-linked immunosorbent assay.

### RESULTS

All patients achieved sustained virologic response (SVR12) (100%). Serum M2BPGi levels, LSM, FIB-4 score and PAPAS index decreased significantly at SVR12 ( $P < 0.05$ ). Serum M2BPGi levels correlated positively with LSM at baseline and SVR12 ( $P < 0.001$ ). At baseline, compared with the FIB-4 score and PAPAS index, M2BPGi was the best marker to distinguish patients with grade F4 fibrosis (AUC = 0.801,  $P < 0.001$ ), patients with grade F2 from grade F0-1 fibrosis (AUC = 0.713,  $P = 0.012$ ), patients with grade F3-4 from grade F0-2 fibrosis (AUC = 0.730,  $P < 0.001$ ), and patients with grade F2-4 from grade F0-1 fibrosis (AUC =

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0.763,  $P < 0.001$ ). At SVR12, M2BPGi had the greatest AUCs for differentiating patients with grade F4 fibrosis (AUC = 0.844,  $P < 0.001$ ), patients with grade F3 from grade F0-2 fibrosis (AUC = 0.893,  $P = 0.002$ ), patients with grade F3-4 from grade F0-2 fibrosis (AUC = 0.891,  $P < 0.001$ ), and patients with grade F2-4 from grade F0-1 fibrosis (AUC = 0.750,  $P < 0.001$ ).

## CONCLUSION

M2BPGi is a reliable marker for the non-invasive assessment and prediction of liver fibrosis regression in patients with CHC who achieved an SVR with DAAs therapy.

**Key words:** Hepatitis C virus; Liver fibrosis; Human Mac-2 binding protein; Antiviral agents; Sustained virologic response; Elastography

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**Core tip:** For 12 wk, 80 chronic hepatitis C patients received a sofosbuvir/daclatasvir/± ribavirin treatment. All patients achieved sustained virologic response (SVR12). Serum Mac-2 binding protein glycosylation isomer (M2BPGi) levels, liver stiffness measurements, fibrosis-4 (FIB-4) and PAPAS index decreased significantly at SVR12. At baseline, M2BPGi was the best marker to distinguish patients with grade F4 fibrosis, patients with grade F2 from grade F0-1 fibrosis, patients with grade F3-4 from grade F0-2 fibrosis, and patients with grade F2-4 from grade F0-1 fibrosis. At SVR12, M2BPGi had the greatest AUCs for differentiating patients with grade F4 fibrosis, patients with grade F3 from grade F0-2 fibrosis, patients with grade F3-4 from grade F0-2 fibrosis, and patients with grade F2-4 from grade F0-1 fibrosis.

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## INTRODUCTION

Hepatitis C virus (HCV) is considered a public health problem, as approximately 3% of the global population is infected with HCV<sup>[1]</sup>. It is imperative to assess the degree of liver fibrosis in patients with chronic hepatitis C (CHC) because fibrogenesis causes all the clinical events, including decompensated liver disease and hepatocellular carcinoma (HCC), affecting the prognosis of and treatment strategies used in patients with CHC<sup>[2]</sup>.

Although a liver biopsy is considered the gold standard for stratifying hepatic fibrosis, its clinical utility is substantially limited because of the invasiveness and the sampling variability<sup>[3]</sup>. Additionally, a liver biopsy is impractical particularly during follow-up due to its invasive nature<sup>[4,5]</sup>.

Consequently, non-invasive methods have been previously proposed and validated for the assessment of hepatic fibrosis, such as ultrasound or magnetic resonance imaging<sup>[6]</sup>, elastographic techniques<sup>[7]</sup>, serum biomarkers including hyaluronic acid, type IV collagen, and type III procollagen-N-peptide<sup>[8]</sup>, and surrogate markers, *e.g.*, the aspartate aminotransferase (AST)-to-platelet ratio index<sup>[9]</sup>, the fibrosis-4 (FIB-4) score<sup>[10]</sup>, AST to alanine aminotransferase (ALT) ratio<sup>[11]</sup> and PAPAS [platelets/age /phosphatase/alpha fetoprotein (AFP)/AST] index<sup>[12]</sup>.

Mac-2 binding protein glycosylation isomer (M2BPGi) is a glycoprotein that is produced by hepatic stellate cells (HSCs). It functions as a messenger between HSCs and Kupffer cells to promote fibrogenesis<sup>[13]</sup>. The feasibility of monitoring serum M2BPGi levels to assess hepatic fibrosis was evaluated, and some studies recommended it as an accurate method for staging hepatic fibrosis<sup>[14,15]</sup>.

Subsequently, several investigators validated the usefulness of M2BPGi in various aetiologies of liver diseases, such as viral hepatitis<sup>[16-19]</sup>, mortality in liver cirrhosis<sup>[20]</sup>, biliary atresia<sup>[21]</sup>, non-alcoholic fatty liver disease<sup>[22,23]</sup>, non-alcoholic steatohepatitis<sup>[24]</sup>, primary biliary cirrhosis<sup>[25]</sup>, autoimmune hepatitis<sup>[26]</sup> and primary sclerosing

cholangitis<sup>[27]</sup>. Furthermore, it was investigated as a marker to assess the risk of HCC development<sup>[28,29]</sup>. According to recent studies<sup>[17,18,28,30-32]</sup>, M2BPGi is a useful marker for monitoring the improvement of patients with liver fibrosis who have achieved a sustained virologic response (SVR) after antiviral therapy.

Recently, interferon (IFN)-based treatment has been replaced by direct-acting antivirals (DAAs). The approval of DAAs was a revolution in HCV eradication, with SVR rates exceeding 90%, good tolerability, and increased efficacy with shorter treatment durations<sup>[33]</sup>. However, a few reports have documented the improvement in liver fibrosis in patients treated with IFN-free DAAs<sup>[31,34-37]</sup>.

We aimed to investigate the diagnostic accuracy of serum M2BPGi levels in assessing the grade of liver fibrosis in patients with CHC before and after DAAs-based treatment, as well as to compare its diagnostic value with the FIB-4 score and PAPAS index.

## MATERIALS AND METHODS

From December 2017 to August 2018, 80 treatment-naïve adult patients with CHC who were eligible for DAAs therapy were consecutively enrolled in this observational cohort study. For 12 weeks, 65 patients were treated with sofosbuvir/daclatasvir, and 15 patients were treated with sofosbuvir/daclatasvir and a weight-based dose of ribavirin at Knowledge and Technology Association for Hepatitis C Management Clinic, Cairo, Egypt. The exclusion criteria were (1) positivity for antibodies against human immunodeficiency virus or positivity for hepatitis B surface antigen; (2) other causes of liver disease (autoimmune hepatitis, primary biliary cirrhosis, haemochromatosis, sclerosing cholangitis, Wilson's disease, or an  $\alpha$ 1-antitrypsin deficiency); (3) clinical or biochemical evidence of hepatic decompensation (ascites, bleeding varices or encephalopathy); (4) suspected HCC or other cancers; (5) excessive alcohol consumption (> 40 g/d) or intravenous drug abuse; or (6) a previous liver transplantation.

This study was approved by the Research Ethics Committee of our institution. Written informed consent was obtained from every patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

We evaluated the liver stiffness measurement (LSM), serum M2BPGi levels, FIB-4 score, PAPAS index, biochemical data, haematological data, virologic data and abdominal ultrasound at baseline and 12 weeks after the end of treatment (EOT), namely, the time SVR12 was achieved, of every patient.

### Measurement of HCV RNA levels

Plasma HCV RNA levels were measured using the Roche TaqMan real-time reverse transcriptase-PCR assay version 2.0, with lower limits of quantification and detection of 15 IU/mL. SVR12 was defined as a lack of detectable HCV RNA at week 12 after EOT.

### Measurement of the serum M2BPGi level

Serum M2BPGi levels were measured using human M2BPGi enzyme-linked immunosorbent assay kits, with a detection range of 0.625 - 200 ng/mL, sensitivity 0.1 ng/mL, and intra-assay and inter-assay coefficients of variation less than 15%.

### LSM

The LSM was performed using Fibroscan® (Echosens, 502 Touch, Paris, France). It was conducted by an experienced examiner after the patient had fasted for at least six hours, and 10 valid measurements were recorded. The median LS in kilopascals (kPa) was reported. Only examinations with a success rate > 60% and IQR < 25% were included and considered reliable. According to Tsochatzis *et al*<sup>[38]</sup>, the following fibrosis staging cut-off values were used: F0-F1 < 7 kPa; F2 7 - 9.4 kPa; F3 9.5 - 11.9 kPa; and F4 > 12 kPa.

### Non-invasive liver fibrosis assessment

The PAPAS index and FIB-4 score were calculated using the following formulas:

PAPAS index<sup>[12]</sup> =  $\text{Log}(\text{index} + 1) = 0.0255 + 0.0031 \times \text{age (year)} + 0.1483 \times \log[\text{ALP (U/L)}] + 0.004 \times \log[\text{AST (U/L)}] + 0.0908 \times \log[\text{AFP (ng/L)} + 1] - 0.028 \times \log[\text{platelets count (10}^9\text{/L)}]$ .

FIB-4 score<sup>[39]</sup> =  $\text{Age (yr)} \times \text{AST (IU/L)} / [\text{platelets count (10}^9\text{/L)} \times [\text{ALT (IU/L)}]^{1/2}]$ .

A FIB-4 score < 1.45 indicates no or minimal fibrosis.

A FIB-4 score > 3.25 indicates significant fibrosis.



### Statistical analysis

Statistical analyses were performed using Stata® version 13.1 software (StataCorp. 2013, College Station, TX: StataCorp LP). Patients' characteristics are presented as mean  $\pm$  SD, median (IQR) or number (percentage), as appropriate. Accordingly, paired *t* test, Wilcoxon matched-pairs signed rank test or chi squared test was used, as appropriate. Values were compared between different grades of liver fibrosis using one-way ANOVA test. Pearson's correlation analysis was used to study the correlation between serum M2BPGi levels and the characteristics of the study population. A receiver operating characteristic (ROC) curve analysis was used to identify the best cut-off value for the serum M2BPGi level with maximum sensitivity and specificity for the differentiation of different grades of fibrosis. A *P* value < 0.05 was considered significant.

The statistical methods of this study were performed by Hazem M. El-Hariri from Department of Community Medicine, National Research Centre, Cairo, Egypt.

## RESULTS

### Patients' characteristics

The studied patients included 40 males (50%) and 40 females (50%), with a mean age of  $52.3 \pm 10.7$  years and BMI ( $\text{kg}/\text{m}^2$ ) =  $28 \pm 5.4$ . Patients' characteristics at baseline and SVR12 are shown in [Table 1](#).

### Safety and adherence to therapy

All patients completed the scheduled course of treatment with follow up until 12 wk after EOT. SVR12 was achieved in all patients (100%). Overall, the treatment was well tolerated. The most commonly reported adverse events were fatigue (5%), followed by pruritus (4.2%), rash (2.3%), headache (2%), and a loss of appetite (1%), all of which were mild in severity.

### Impact of SVR12 on the serological data

Haemoglobin levels, WBC, total bilirubin levels and international normalized ratio (INR) did not change significantly after patients achieved SVR12. Platelets count and albumin levels were significantly higher at SVR12. ALT, AST, ALP and creatinine levels decreased significantly after patients achieved SVR12. AFP levels decreased after patients achieved SVR12, but the difference was not statistically significant ([Table 1](#)).

### Effect of SVR12 on liver fibrosis

Serum M2BPGi levels, LSM, FIB-4 score and PAPAS index decreased significantly after patients achieved SVR12 ([Table 1](#)). The improvement in LSM was more noticeable in patients with grade F4 fibrosis ([Table 2](#)). Only serum M2BPGi levels were significantly different between patients with different grades of fibrosis at baseline and SVR12 ([Table 3](#)).

### Correlations with serum M2BPGi levels

At baseline, serum M2BPGi levels correlated positively with total bilirubin levels and negatively with AST levels. At SVR12, serum M2BPGi levels correlated positively with INR and negatively with platelets count ([Table 4](#)).

### Correlations between serum M2BPGi levels, LSM, FIB-4 score and PAPAS index

At baseline, LSM correlated with serum M2BPGi levels and FIB-4 score. In addition, a significant correlation was observed between FIB-4 score and PAPAS index. At SVR12, LSM correlated with serum M2BPGi levels, FIB-4 score and PAPAS index ([Table 5](#) and [Figure 1](#)).

### ROC curve analysis for the assessment and differentiation of the grades of liver fibrosis

At baseline, compared with the FIB-4 score and PAPAS index, M2BPGi was the best marker to distinguish patients with grade F4 fibrosis (AUC = 0.801, *P* < 0.001), patients with grade F2 from grade F0-1 fibrosis (AUC = 0.713, *P* = 0.012), patients with grade F3-4 from grade F0-2 fibrosis (AUC = 0.730, *P* < 0.001), and patients with grade F2-4 from grade F0-1 fibrosis (AUC = 0.763, *P* < 0.001) ([Supplementary Table 1](#), [Figures 1](#) and [3](#)).

At SVR12, M2BPGi had the greatest AUCs for differentiating patients with grade F4 fibrosis (AUC = 0.844, *P* < 0.001), patients with grade F3 from grade F0-2 fibrosis (AUC = 0.893, *P* = 0.002), patients with grade F3-4 from grade F0-2 fibrosis (AUC =

**Table 1 Patients' characteristics at baseline and sustained virologic response 12**

Measures	Baseline	SVR12	P value
Age (yr)	52.3 ± 10.7		
Gender	40 (50%) males and 40 (50%) females		
BMI	28 ± 5.4		
HCV RNA (log copies/mL)	6.0 ± 0.7	Non-detectable	0.000 <sup>b</sup>
Platelets count (× 10 <sup>3</sup> /mL)	220.3 ± 65.3	245.3 ± 77.8	0.005 <sup>b</sup>
ALT (IU/L)	40.5 (29-54)	32 (26-38)	< 0.001 <sup>b</sup>
AST (IU/L)	39 (29-51)	30 (24-37)	< 0.001 <sup>b</sup>
ALP (IU/L)	138.5 (95.5-196)	107 (86-141)	< 0.001 <sup>b</sup>
Albumin (g/dL)	3.8 ± 0.3	4.2 ± 0.4	< 0.001 <sup>b</sup>
Total bilirubin (mg/dL)	0.7 (0.5-0.8)	0.7 (0.5-0.95)	0.8
Creatinine (mg/dL)	0.86 ± 0.19	0.75 ± 0.23	0.002 <sup>b</sup>
INR	1.06 ± 0.09	1.08 ± 0.15	0.222
AFP (ng/mL)	4.5 ± 2.1	3.7 ± 1.9	0.128
LSM (kPa)	11.4 ± 4.5	9.5 ± 3.3	0.002 <sup>b</sup>
FIB-4 score	1.8 ± 0.5	1.3 ± 0.7	< 0.001 <sup>b</sup>
PAPAS index	2.2 ± 0.5	2.1 ± 0.3	0.010 <sup>a</sup>
Serum M2BPGi (ng/mL)	9 ± 3.8	6.7 ± 2.3	< 0.001 <sup>b</sup>

The values are expressed as mean ± SD or median (IQR).

<sup>a</sup>P < 0.05.

<sup>b</sup>P < 0.01. SVR: Sustained virologic response; WBC: White blood cell count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; INR: International normalized ratio; AFP: Alpha fetoprotein; LSM; Liver stiffness measurement; M2BPGi: Mac-2 binding protein glycosylation isomer.

0.891,  $P < 0.001$ ), and patients with grade F2-4 from grade F0-1 fibrosis (AUC = 0.750,  $P < 0.001$ ) (Supplementary Table 1, Figures 2 and 3).

## DISCUSSION

Currently, non-invasive methods for detecting liver fibrosis are used much more frequently than liver biopsies<sup>[40]</sup>. M2BPGi has been shown to be a useful predictor of liver fibrosis<sup>[14,17,41]</sup>. Previous reports have documented a substantial improvement in liver fibrosis after patients achieve SVR12 through treatment with DAAs for HCV<sup>[42-44]</sup>. Here, we aimed to investigate the diagnostic accuracy of serum M2BPGi levels for assessing the grade of liver fibrosis in patients with CHC before and after DAAs-based treatment, as well as to compare its diagnostic value with the FIB-4 score and PAPAS index.

The present study confirms the efficacy and safety of sofosbuvir + daclatasvir ± ribavirin in real-world situations. The SVR12 is 100%.

Similar to previous studies<sup>[33,45-48]</sup>, we observed an improvement in liver functions after patients achieved SVR12, as indicated by a significant decrease in the levels of ALT, AST and ALP, and a significant increase in the serum albumin levels and platelets count. Additionally, in consistency with previous literature<sup>[37,45,47,49-52]</sup>, serum M2BPGi levels, LSM, FIB-4 score and PAPAS index decreased significantly after patients achieved SVR12. These findings suggest the possibility of liver fibrosis regression after viral eradication was achieved.

In agreement with previous reports<sup>[3,16,31,30,53]</sup>, we observed a significantly increasing trend of serum M2BPGi levels with the progression of liver fibrosis both at baseline and SVR12 ( $P < 0.001$ ). Moreover, in accordance with Akahane *et al.*<sup>[46]</sup> and Ishikawa *et al.*<sup>[36]</sup>, serum M2BPGi levels decreased significantly at SVR12 ( $P < 0.001$ ). In addition, in a study by Miyaki *et al.*<sup>[31]</sup>, serum M2BPGi levels did not change in the non-SVR group ( $P = 0.715$ ), but decreased significantly in the SVR group ( $P < 0.0001$ ). These results suggest that serum M2BPGi would be a good surrogate marker for predicting and differentiating liver fibrosis stages.

In the present study, pre-treatment serum M2BPGi levels correlated with bilirubin and AST levels, while serum M2BPGi levels correlated with platelets count and INR at SVR12. These findings suggest that M2BPGi reflects not only the severity of liver fibrosis but also the severity of liver inflammation in CHC patients<sup>[18]</sup>. This may be

**Table 2** Liver stiffness measurements of all patients at baseline and sustained virologic response 12

Total baseline	Changes in the fibrosis grade at SVR12			
	F0-1 (%)	F2 (%)	F3 (%)	F4 (%)
F0-1 29 (36.3%)	23 (79.3)	5 (17.2)	1 (3.4)	0 (0.0)
F2 20 (25%)	10 (50)	10 (50)	0 (0.0)	0 (10)
F3 5 (6.3%)	1 (20)	2 (40)	1 (20)	1 (20)
F4 26 (32.5%)	1 (3.8)	6 (23.1)	4 (15.4)	15 (57.7)
Total SVR12	35 (43.7)	23 (28.7)	6 (7.5)	16 (20)

Total patients number = 80. The values are expressed as numbers (%). SVR: Sustained virologic response.

attributed to the role of M2BPGi as a messenger between HSCs and Kupffer cells and its accompanying inflammation<sup>[13]</sup>.

Similar to our results, Ura *et al*<sup>[30]</sup> reported a significant negative correlation between serum M2BPGi levels and platelets count ( $r = -0.47$ ,  $P < 0.0001$ ). Additionally, Yamasaki *et al*<sup>[54]</sup> observed a significant positive correlation between serum M2BPGi and bilirubin levels ( $r = 0.091$ ,  $P = 0.001$ ) and a significant negative correlation with platelets count ( $r = -0.147$ ,  $P < 0.001$ ). However, in contrast to our results, Yasui *et al*<sup>[55]</sup> observed a positive correlation between serum M2BPGi and AFP levels ( $r = 0.428$ ,  $P < 0.001$ ), and a negative correlation with albumin levels ( $r = -0.471$ ,  $P < 0.001$ ).

In agreement with the present study, Tawara *et al*<sup>[53]</sup> reported a correlation coefficient between serum M2BPGi levels and FIB-4 score of less than 0.4, suggesting that the correlation between serum M2BPGi levels and FIB-4 score was weak. In contrast, Ura *et al*<sup>[30]</sup> and Yasui *et al*<sup>[55]</sup> detected a significant positive correlation between serum M2BPGi levels and FIB-4 score ( $r = 0.66$ ,  $P < 0.0001$  and  $r = 0.546$ ,  $P < 0.001$ , respectively). This discrepancy can be attributed to the different sample size.

In terms of differentiation of liver fibrosis grades, consistent with the present study, Xu *et al*<sup>[16]</sup> reported that the AUC values of M2BPGi for predicting fibrosis grade  $\geq$  F2 and F4 were significantly superior to the values of FIB-4 score (0.774 *vs* 0.702,  $P < 0.001$  and 0.892 *vs* 0.818,  $P < 0.05$ ), respectively. In contrast, Tawara *et al*<sup>[53]</sup> reported that the FIB-4 score had a greater AUC value for differentiating of fibrosis grades than M2BPGi (AUC values were 0.768, 0.827 and 0.876 for fibrosis grade  $F \geq 2$ ,  $F \geq 3$  and F4, respectively), while the AUC values of M2BPGi were 0.747, 0.733 and 0.796 for fibrosis grade  $F \geq 2$ ,  $F \geq 3$  and F4, respectively.

The limitations of the present study are the absence of a paired histological evaluation due to the invasiveness of liver biopsy, and the short duration of follow up after completion of treatment. Further large-scale studies with a longer follow-up period should be performed.

In conclusion, M2BPGi is a reliable marker for the non-invasive assessment and prediction of liver fibrosis regression in patients with CHC who achieved an SVR with DAAs therapy.

**Table 3 Non-invasive assessment data obtained at baseline and sustained virologic response 12 from patients stratified according to the fibrosis grade**

Parameter					P value
Baseline (Total <i>n</i> = 80)	F0-1 ( <i>n</i> = 29)	F2 ( <i>n</i> = 20)	F3 ( <i>n</i> = 5)	F4 ( <i>n</i> = 26)	
LSM (kPa)	5.9 ± 0.6	7.8 ± 0.5	10.8 ± 1.2	20.6 ± 7.7	< 0.001 <sup>b</sup>
FIB-4 score	1.7 ± 1.4	1.4 ± 0.7	1.8 ± 0.4	2.3 ± 1.5	0.108
PAPAS index	2.1 ± 0.4	2.2 ± 0.6	2.3 ± 0.3	2.3 ± 0.5	0.303
Serum M2BPGi (ng/mL)	4.5 ± 2.2	5.3 ± 2.8	9.4 ± 4	14.5 ± 6.7	0.001 <sup>b</sup>
SVR 12 (Total <i>n</i> = 80)	F0-1 ( <i>n</i> = 35)	F2 ( <i>n</i> = 23)	F3 ( <i>n</i> = 6)	F4 ( <i>n</i> = 16)	
LSM (kPa)	5.8 ± 1.2	7.6 ± 0.6	9.9 ± 0.8	15 ± 2.3	< 0.001 <sup>b</sup>
FIB-4 score	1.1 ± 0.5	1.5 ± 1	1.3 ± 0.6	1.6 ± 0.5	0.075
PAPAS index	2 ± 0.3	2.1 ± 0.3	2.1 ± 0.3	2.3 ± 0.3	0.069
Serum M2BPGi (ng/mL)	3.4 ± 1.6	4.9 ± 2.1	12.7 ± 5.1	13.3 ± 5.2	0.001 <sup>b</sup>

<sup>b</sup>*P* < 0.01. The values are expressed as mean ± SD. SVR: Sustained virologic response; LSM: Liver stiffness measurement; M2BPGi: Mac-2 binding protein glycosylation isomer.

**Table 4 Correlations of serum Mac-2 binding protein glycosylation isomer levels with laboratory data**

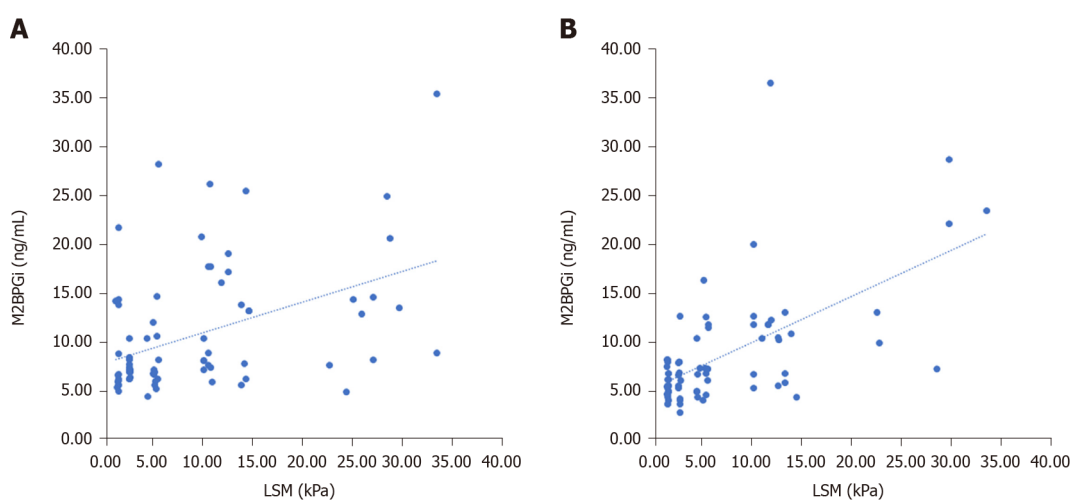
Variables	Baseline		SVR12	
	r	P value	r	P value
Age	0.050	0.662	0.107	0.346
BMI	-0.007	0.951	0.057	0.617
HCV RNA	0.043	0.702	0.025	0.827
Hb	0.143	0.205	0.131	0.246
WBC	0.047	0.677	-0.152	0.178
Platelets count	-0.118	0.297	-0.299	0.007 <sup>b</sup>
ALT	-0.090	0.425	-0.129	0.256
AST	-0.227	0.043 <sup>a</sup>	0.006	0.961
ALP	-0.024	0.831	0.106	0.350
Albumin	0.016	0.888	0.070	0.535
Total bilirubin	0.268	0.016 <sup>a</sup>	0.111	0.326
Creatinine	0.158	0.162	0.088	0.439
INR	0.063	0.582	0.220	0.049 <sup>a</sup>
AFP	-0.098	0.388	0.026	0.822

<sup>a</sup>*P* < 0.05.

<sup>b</sup>*P* < 0.01. SVR: Sustained virologic response; BMI: Body mass index; Hb: Haemoglobin; WBC: White blood cell count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; INR: International normalized ratio; AFP: Alpha fetoprotein.

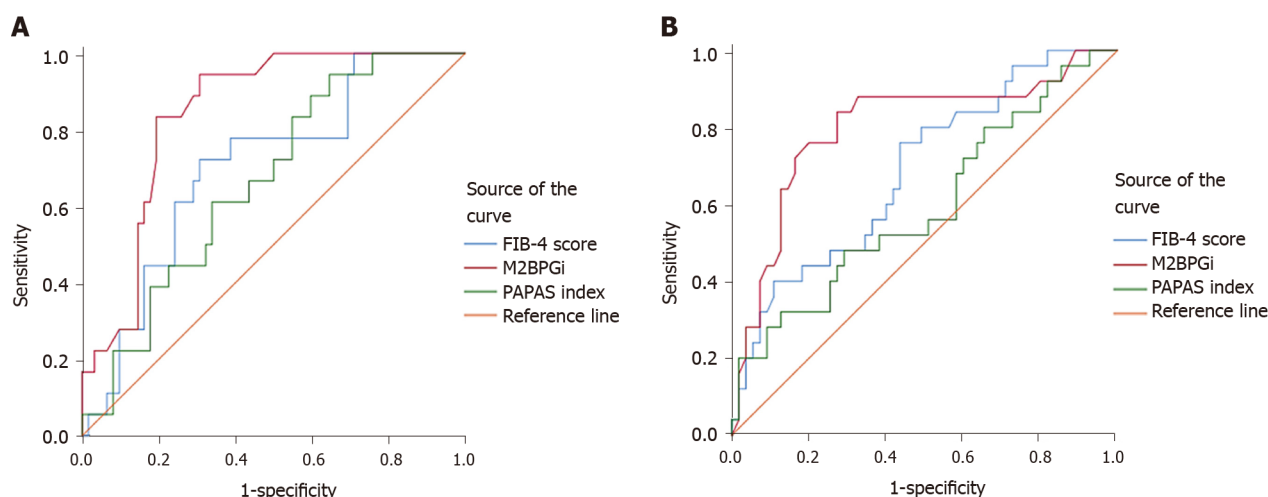
**Table 5** Correlations between non-invasive assessment methods at baseline and sustained virologic response 12

Variables		LSM	Serum M2BPGi	FIB-4 score
At baseline				
Serum M2BPGi level	r	0.453		
	P value	< 0.001 <sup>b</sup>		
FIB-4 score	r	0.328	-0.099	
	P value	0.003 <sup>b</sup>	0.384	
PAPAS index	r	0.110	-0.049	0.444
	P value	0.330	0.664	< 0.001 <sup>b</sup>
At SVR12				
Serum M2BPGi level	r	0.517		
	P value	< 0.001 <sup>b</sup>		
FIB-4 score	r	0.231	0.188	
	P value	0.039 <sup>a</sup>	0.095	
PAPAS index	r	0.328	0.185	0.188
	P value	0.003 <sup>b</sup>	0.100	0.095

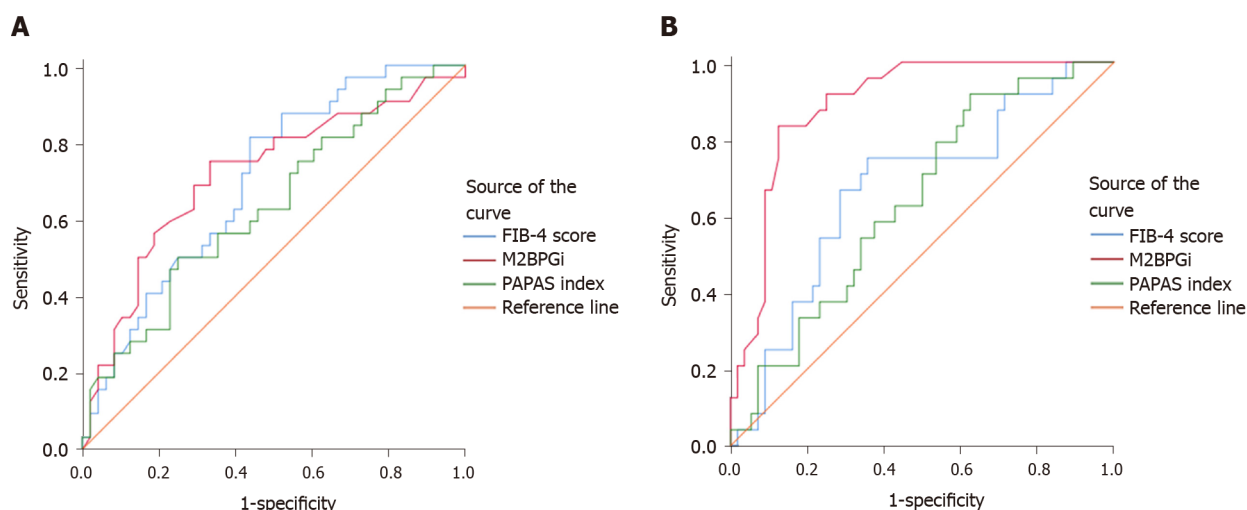
<sup>a</sup>P < 0.05.<sup>b</sup>P < 0.01. SVR: Sustained virologic response; LSM: Liver stiffness measurement; M2BPGi: Mac-2 binding protein glycosylation isomer.

**Figure 1** Correlation between serum Mac-2 binding protein glycosylation isomer levels and liver stiffness measurement at baseline and after patients achieved sustained virologic response 12. A: Correlation between serum Mac-2 binding protein glycosylation isomer (M2BPGi) levels and liver stiffness measurement (LSM) at baseline; B: Correlation between serum M2BPGi levels and LSM After patients achieved sustained virologic response 12. M2BPGi: Mac-2 binding protein glycosylation isomer; LSM: Liver stiffness measurement; SVR: Sustained virologic response.





**Figure 2** Diagnostic performance of non-invasive parameters in differentiating grades F4 from F0-3 at baseline and after patients achieved sustained virologic response 12. A: Diagnostic performance of non-invasive parameters in differentiating grades F4 from F0-3 at baseline; B: Diagnostic performance of non-invasive parameters in differentiating grades F4 from F0-3 after patients achieved sustained virologic response 12. FIB-4: Fibrosis-4; M2BPGi: Mac-2 binding protein glycosylation isomer.



**Figure 3** Diagnostic performance of non-invasive parameters in differentiating grades F3-4 from F0-2 at baseline and after patients achieved sustained virologic response 12. A: Diagnostic performance of non-invasive parameters in differentiating grades F3-4 from F0-2 at baseline; B: Diagnostic performance of non-invasive parameters in differentiating grades F3-4 from F0-2 After patients achieved sustained virologic response 12. FIB-4: Fibrosis-4; M2BPGi: Mac-2 binding protein glycosylation isomer.

## ARTICLE HIGHLIGHTS

### Research background

Assessing liver fibrosis is important for predicting the efficacy of direct-acting antivirals (DAAs) and patient prognosis. Non-invasive techniques to assess liver fibrosis are becoming important. Recently, serum Mac-2 binding protein glycosylation isomer (M2BPGi) was identified as a non-invasive marker of liver fibrosis.

### Research motivation

The approval of DAAs was a revolution in hepatitis C virus eradication, with sustained virologic response (SVR) rates exceeding 90%. However, a few reports have documented the improvement in liver fibrosis in patients treated with DAAs. Although liver biopsy is considered the gold standard for stratifying hepatic fibrosis, its clinical utility is substantially limited because of the invasiveness and the sampling variability. Accordingly, serum M2BPGi was evaluated as a non-invasive marker for assessing the grade of hepatic fibrosis in patients who have achieved SVR after antiviral therapy.

### Research objectives

We aimed to investigate the diagnostic accuracy of serum M2BPGi levels in assessing the grade

of liver fibrosis in patients with chronic hepatitis C (CHC) before and after DAAs-based treatment, as well as to compare its diagnostic value with the FIB-4 score and PAPAS index.

### Research methods

Eighty treatment-naïve adult patients with CHC who were eligible for DAAs therapy were consecutively enrolled in this observational cohort study. For 12 weeks, 65 patients were treated with sofosbuvir/daclatasvir, and 15 patients were treated with sofosbuvir/daclatasvir and a weight-based dose of ribavirin. We measured serum M2BPGi levels, PAPAS index, FIB-4 score and liver stiffness measurements (LSM) at baseline and 12 weeks after the end of treatment. Serum M2BPGi levels were measured using enzyme-linked immunosorbent assay.

### Research results

All patients achieved SVR12 (100%). Serum M2BPGi levels, LSM, FIB-4 score and PAPAS index decreased significantly at SVR12 ( $P < 0.05$ ). Serum M2BPGi levels correlated positively with LSM at baseline and SVR12 ( $P < 0.001$ ). At baseline, compared with the FIB-4 score and PAPAS index, M2BPGi was the best marker to distinguish patients with grade F4 fibrosis (AUC = 0.801,  $P < 0.001$ ), patients with grade F2 from grade F0-1 fibrosis (AUC = 0.713,  $P = 0.012$ ), patients with grade F3-4 from grade F0-2 fibrosis (AUC = 0.730,  $P < 0.001$ ), and patients with grade F2-4 from grade F0-1 fibrosis (AUC = 0.763,  $P < 0.001$ ). At SVR12, M2BPGi had the greatest AUCs for differentiating patients with grade F4 fibrosis (AUC = 0.844,  $P < 0.001$ ), patients with grade F3 from grade F0-2 fibrosis (AUC = 0.893,  $P = 0.002$ ), patients with grade F3-4 from grade F0-2 fibrosis (AUC = 0.891,  $P < 0.001$ ), and patients with grade F2-4 from grade F0-1 fibrosis (AUC = 0.750,  $P < 0.001$ ).

### Research conclusions

M2BPGi is a reliable marker for the non-invasive assessment and prediction of liver fibrosis regression in patients with CHC who achieved an SVR with DAAs therapy.

### Research perspectives

Non-invasive methods have been previously proposed and validated for the assessment of hepatic fibrosis. In this study, we confirm that serum M2BPGi is a reliable marker for liver fibrosis. Further studies are needed to investigate its therapeutic potential and ultimate clinical utility.

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## Observational Study

# Selective lateral lymph node dissection after neoadjuvant chemoradiotherapy in rectal cancer

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## Abstract

### BACKGROUND

Lateral lymph node metastasis is one of the leading causes of local recurrence in patients with advanced mid or low rectal cancer. Neoadjuvant chemoradiotherapy (NCRT) can effectively reduce the postoperative recurrence rate; thus, NCRT with total mesorectal excision (TME) is the most widely accepted standard of care for rectal cancer. The addition of lateral lymph node dissection (LLND) after NCRT remains a controversial topic.

### AIM

To investigate the surgical outcomes of TME plus LLND, and the possible risk factors for lateral lymph node metastasis after NCRT.

### METHODS

This retrospective study reviewed 89 consecutive patients with clinical stage II-III mid or low rectal cancer who underwent TME and LLND from June 2016 to October 2018. In the NCRT group, TME plus LLND was performed in patients with short axis (SA) of the lateral lymph node greater than 5 mm. In the non-NCRT group, TME plus LLND was performed in patients with SA of the lateral lymph node greater than 10 mm. Data regarding patient demographics, clinical workup, surgical procedure, complications, and outcomes were collected. Multivariate logistic regression analysis was performed to evaluate the possible risk factors for lateral lymph node metastasis in NCRT patients.

### RESULTS

LLN metastasis was pathologically confirmed in 35 patients (39.3%): 26 (41.3%) in

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the NCRT group and 9 (34.6%) in the non-NCRT group. The most common site of metastasis was around the obturator nerve (21/35) followed by the internal iliac artery region (12/35). In the NCRT patients, 46% of patients with SA of LLN greater than 7 mm were positive. The postoperative 30-d mortality rate was 0%. Two (2.2%) patients suffered from lateral local recurrence in the 2-year follow up. Multivariate analysis showed that cT4 stage (odds ratio [OR] = 5.124, 95% confidence interval [CI]: 1.419-18.508;  $P = 0.013$ ), poor differentiation type (OR = 4.014, 95%CI: 1.038-15.520;  $P = 0.044$ ), and SA  $\geq 7$  mm (OR = 7.539, 95%CI: 1.487-38.214;  $P = 0.015$ ) were statistically significant risk factors associated with LLN metastasis.

## CONCLUSION

NCRT is not sufficient as a stand-alone therapy to eradicate LLN metastasis in lower rectal cancer patients and surgeons should consider performing selective LLND in patients with greater LLN SA diameter, poorer histological differentiation, or advanced T stage. Selective LLND for NCRT patients can have a favorable oncological outcome.

**Key words:** Rectal neoplasms; Neoadjuvant therapies; Lateral lymph node dissection; Locoregional recurrence; Lymphatic metastasis; Total mesorectal excision

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**Core tip:** Lateral lymph node metastasis is one of the leading causes of local recurrence in patients with advanced mid or low rectal cancer. Lateral local recurrence remains a significant clinical problem associated with severe morbidity and low salvage likelihood. There is an East (mainly Japan)-West divide regarding the management of lateral lymph nodes associated with lower rectal cancer. Preoperative chemoradiotherapy followed by total mesorectal excision is a standard procedure in the west. Our study shows that lateral lymph node metastasis cannot be eradicated by neoadjuvant chemoradiotherapy. Selective total mesorectal excision plus lateral lymph node dissection should be performed in advanced mid or low rectal cancer patients.

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## INTRODUCTION

Lateral lymph node metastasis in mid and low rectal cancer was first described in 1895 by Gerota<sup>[1]</sup> using dye injection. Since then, many anatomical and pathological studies have divided the rectal lymphatic drainage into three main directions: Upward, lateral, and downward. Among them, lateral lymphatic drainage nodes comprise an important rectal approach below the peritoneal reflection<sup>[2-4]</sup>. According to the Japanese Classification of Colorectal, Appendiceal, and Anal Carcinoma: The 3<sup>rd</sup> English Edition, lateral lymph nodes are two groups of lymph nodes: One group along the internal iliac arteries and the obturator vessels and nerves, and the other along the common iliac, external iliac, and median sacral arteries<sup>[5]</sup>. The incidence of lateral lymph node metastasis from lower rectal cancer is about 15%<sup>[6]</sup>, whereas the incidence of lateral lymph node metastasis in T3 and T4 patients is reported in more than 20% of cases<sup>[7,8]</sup>. Local recurrence of rectal cancer, specifically lateral local recurrence, remains a significant clinical problem associated with severe morbidity, low salvage likelihood, and eventual death in the majority of patients<sup>[9]</sup>. There is a lack of consensus leading to an East (mainly Japan)-West division concerning the management of lateral lymph nodes associated with lower rectal cancer. In western practice, patients with locally advanced (stage II-III) rectal cancer are treated with neoadjuvant chemoradiotherapy (NCRT) and total mesorectal excision (TME) as the standard. This is based on the interpretation that lymph node involvement is considered a systemic disease<sup>[10]</sup>. Furthermore, lateral lymph node dissection (LLND)

inevitably results in a longer operative time and increased blood loss compared to TME alone. The adoption of NCRT followed by TME has demonstrated increased local control resulting in local recurrence in less than 10% of cases<sup>[11]</sup>. On the other hand, the Japanese Society for Cancer of the Colon and Rectum cites different guidelines for the treatment of rectal cancer and recommends LLND for advanced rectal cancer that extends below the peritoneal reflection to address the possibility of LLN metastasis<sup>[12]</sup>. Several studies from Japan argue that LLN metastasis should be considered a local disease rather than systemic disease, and that LLND can significantly reduce local recurrence rates<sup>[6,13]</sup>. In recent years, a growing body of evidence has supported conflicting standard strategies in both Japan and Western countries, culminating in similar local recurrence rates<sup>[14]</sup>. Recent studies have suggested that perhaps a middle-ground selective LLND should be considered after preoperative chemoradiotherapy based on magnetic resonance imaging/computed tomography (MRI/CT) findings<sup>[15,16]</sup>.

In China, preoperative chemoradiotherapy followed by TME is still the standard of care, as most surgeons do not perform an LLND most commonly citing extended operative time and potential nerve damage as the reason. We collected data from 89 consecutive patients with mid or low rectal cancer who underwent TME plus LLND in this study to investigate the therapeutic effect of preoperative CRT on LLN metastasis and identify the associated risk factors.

## MATERIALS AND METHODS

### Patients

The study was approved by the ethics committee of the National Cancer Center and conformed to the ethical standards of the World Medical Association Declaration of Helsinki. All patients signed an informed consent form. A total of 89 mid or low rectal cancer patients who underwent TME plus LLND at the National Cancer Center/National Sciences Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College from June 2016 to October 2018 were consecutively collected in this study. The inclusion criteria of this study were as follows: (1) Histologically confirmed adenocarcinoma of the middle or low rectum (the distal verge of the tumor located below the peritoneal reflection); (2) All patients were confirmed to have clinical tumor-node-metastasis stage II-III by MRI/CT at the time of diagnosis; and (3) All patients underwent TME plus LLND. Patients with distant metastasis were excluded.

### Treatment strategy

Treatment strategies for each patient were determined by a multidisciplinary meeting and the patient's wishes. In the NCRT group, patients received short-course radiotherapy for a total dose of 25 Gy or received 5-fluorouracil-based NCRT, with a total dose of 45 Gy or 50.4 Gy before surgery. Both the obturator and internal iliac compartments were included in the standard radiation field. MRI after 4 wk of NCRT were done to evaluate swollen lymph nodes. Patients with swollen lymph nodes with an SA  $\geq$  5 mm underwent LLND plus TME. The operation was carried out within 1 wk after short-course radiotherapy or 8 wk after the end of a long-course NCRT. For patients without NCRT, if the lateral swollen lymph nodes with an SA  $\geq$  10 mm, TME plus LLND were performed.

All patients were placed in the modified lithotomy position after anesthesia. They all underwent TME with LLND. The pelvic peritoneum was opened, and the hypogastric nerves were identified and preserved. LLND included six regions: Common iliac, internal iliac, external iliac node, obturator, aortic bifurcation, and median sacral regions<sup>[17]</sup>. Typically, the external iliac node and median sacral region are not dissected because of a low metastatic incidence. The probability of bilateral lymph node metastasis is extremely low and results in a significantly higher postoperative complication rate, longer operation time, and more bleeding. Thus, bilateral lymph node dissection is not routinely performed unless the MRI/CT strongly suggests bilateral lymph node involvement<sup>[18-20]</sup>.

### Pathological diagnosis

After resection of the surgical specimens, LLNs are separated according to the anatomical position, fixed in formalin, and sent for pathological examination. The tumor stage was decided by professional pathologists according to the 7<sup>th</sup> and 8<sup>th</sup> edition of the American Joint Committee on Cancer.

### Statistical analysis

The Statistical Package for the Social Sciences version 21.0 for Windows (IBM Corp,

Armonk, NY, United States) was used for data analyses. Quantitative data are shown as the mean  $\pm$  standard deviation and were analyzed by a *t*-test. Categorical data are shown as frequencies and percentages and were analyzed by the  $\chi^2$  or Fisher's exact test as appropriate. Univariate logistic regression analysis was used to evaluate the association between lateral lymph node metastasis and various parameters. Multivariate logistic regression analysis was performed to examine the predictors of lateral lymph node metastasis to calculate the 95% confidence intervals (CIs) for each risk factor, and differences were considered statistically significant when  $P < 0.05$ . The data were statistically reviewed by a biomedical statistician from the National Cancer Center.

## RESULTS

The demographics of 89 rectal cancer patients treated with TME plus LLND at the National Cancer Center/Chinese Academy of Medical Sciences are summarized in [Table 1](#). Clinical T3 and T4 rectal cancer accounted for 60.7% and 39.3% of the cases, respectively, and clinical N1 and N2 stage accounted for 56.2% and 43.8%, respectively. Among the 89 patients, 63 received neoadjuvant therapy. Of those, three received short-course radiotherapy (25 Gy administered doses of 5 Gy over 5 d) and underwent TME plus LLND within 1 wk. Sixty patients received 5-fluorouracil-based long-course NCRT (45-50.4 Gy administered in 25-28 fractions), and then surgery after an 8-wk interval. Twenty-six patients were treated with TME plus LLND directly without receiving any NCRT. [Table 2](#) shows the surgery-related data. Two patients initially received laparoscopic surgery that was subsequently converted to open surgery, while the others underwent a laparoscopic TME plus LLND. Low anterior resection was done in 44 (49.4%) patients. Unilateral and bilateral LLNDs were performed in 76 (85.4%) and 13 (14.6%) patients, respectively. Nine (10.1%) patients received a temporary ileum stoma during the surgery.

Fifteen patients (16.8%) had postoperative complications reported after LLND ([Table 3](#)). According to the Clavien-Dindo classification<sup>[21]</sup>, most of the patients developed to Grade II or Grade III complications, there were no grade IV or grade V postoperative complications. Four (4.5%) patients suffered an anastomotic leakage, three of which received an ileostomy while the fourth recovered after conservative treatment. Two (15.4%) of thirteen bilateral LLND patients were discharged from the hospital with an indwelling catheter due to urinary retention. In both cases, after 4 wk of bladder training, the catheter was successfully removed. Tissue liquefaction occurred in three (3.4%) patients, after a careful dressing change, and the wound finally healed well. Four (4.5%) patients had small bowel obstruction, and they all recovered with conservative medical treatment.

The median follow-up duration was 24.5 mo (range 6-38 mo). During follow-up, mortality occurred in 8 (9.0%) patients due to distant metastasis and the 2-year disease-free survival was 80.9%. Two (2.2%) patients suffered lateral local recurrence during follow-up and both underwent unilateral LLND.

[Table 4](#) describes the pathological outcomes. Thirty-five (39.3%) patients were pathologically confirmed with lateral lymph node metastasis. Of these, 26 (41.3%, 26/63) patients received NCRT before surgery, while 9 (34.6%, 9/26) did not receive NCRT. Moreover, the pathological results revealed that the obturator region was the location with the highest lymph node metastasis involvement, accounting for 60.0% in the 35 patients. Twelve (34.3%) cases of LLNs metastasis were in the internal iliac region and two (5.7%) were located at the bifurcation of the abdominal aorta. An R0 margin status was observed in 87 (97.8%) cases, while the other 2 patients had a positive circumferential resection margin.

[Table 5](#) depicts an LLN metastatic rate for different cutoff values along the short axis (SA) in patients who received preoperative (chemo)radiotherapy. Patients with a SA of LLN  $\geq 10$  mm after NCRT had the highest positive LLN metastasis rate (51.9%). The pathological positive rates of the SA of 5-7 mm and 7-10 mm were 23.1% and 39.1%, respectively.

[Table 6](#) summarizes the univariate analysis, which revealed that the clinical T stage ( $P = 0.003$ ), histological type ( $P = 0.183$ ), and the SA diameter of LLN after NCRT ( $P = 0.135$ ) were candidate variables that may be associated with LLN metastasis. After multivariate analysis, clinical T4 stage (95% CI: 1.419-18.508;  $P = 0.013$ ), poor histological type (95% CI: 1.038-15.520;  $P = 0.044$ ), and SA diameter of LLN after NCRT (95% CI: 1.487-38.214;  $P = 0.015$ ) were independent risk factors associated with LLN metastasis.



**Table 1 Patient demographics, *n* = 89**

Variables	Value
Gender, <i>n</i> (%)	
Male	51 (57.3)
Female	38 (42.7)
Age in yr, mean $\pm$ SD	54.4 $\pm$ 10.1
BMI in kg/m <sup>2</sup> , mean $\pm$ SD	24.9 $\pm$ 4.6
ASA score, <i>n</i> (%)	
ASA I	13 (14.6)
ASA II	56 (62.9)
ASA III	20 (22.5)
cT stage	
cT3	54 (60.7)
cT4	35 (39.3)
cN stage	
N1	50 (56.2)
N2	39 (43.8)
Neoadjuvant chemoradiotherapy	
No	26 (29.2)
Short-course radiotherapy	3 (3.4)
Long-course radiotherapy + chemotherapy	60 (67.4)

ASA: American Society of Anesthesiologists; BMI: Body mass index; SD: Standard deviation.

## DISCUSSION

This study supports the importance of selective LLND after preoperative chemoradiotherapy. Clinical T4 stage, poor histological type, and an SA diameter of LLNs  $\geq 7$  mm after NCRT were independent and significant risk factors associated with LLNs metastasis in patients with advanced mid or low rectal cancer treated with NCRT. Since the LLN metastatic rate in NCRT patients can be as high as 41.3%, we suggest that selective LLND be performed.

It has been reported that LLN metastasis occurs in 10%-25% of all mid or low rectal cancer patients who did not receive preoperative chemoradiotherapy treatment<sup>[8,22,23]</sup>. Fujita *et al*<sup>[6]</sup> suggested that the incidences of local recurrence in patients without NCRT were 7% and 13% after TME plus LLND or TME alone, respectively. The European MERCURY Study Group similarly reported that 11.7% of rectal cancer patients suffer from LLN metastasis<sup>[24]</sup>. Still, in western surgical practice, it remains uncommon to perform an LLND in advanced rectal cancer patients as preoperative CRT and TME is the standard protocol<sup>[25]</sup>. The addition of NCRT has decreased 5-year local recurrence rates from  $> 25\%$  to approximately 5% to 10%<sup>[26]</sup>. Yet, a study in South Korea enrolled 366 patients with advanced rectal tumor and showed that TME following preoperative CRT is not enough to control LLNs metastasis. The reported incidence of LLNs metastasis was 12.5% in patients with lymph node SA of 5-9.9 mm and 68.8% in patients with an SA  $\geq 10$  mm. The LLNs accounted for 82.7% of all local recurrences<sup>[27]</sup>. Oh *et al*<sup>[28]</sup> published a multicenter retrospective cohort study that included 36 patients with lateral lymph nodes greater than 5 mm after NCRT. All patients received LLND and the pathological results showed 22 (61.1%) patients had LLNs metastasis. These findings indicate that NCRT plus TME or TME plus LLND alone is not sufficient to eradicate LLN metastasis, and LLND should be considered if LLN metastasis is suspected even after chemoradiotherapy. Our data show that the incidence of LLNs metastasis in NCRT patients with SA  $\geq 5$  mm is 41.3% (26 of 63) and 51.9% (14 of 27) in patients with lymph nodes SA  $\geq 10$  mm. If these pathological metastases had not been removed by LLND, they may subsequently lead to local recurrence<sup>[29]</sup>. In the 2-year follow-up period, 2 (2.2%) patients developed local recurrence. Thus, our results suggest that there is an oncological benefit when performing LLND for patients with clinically suspected LLN metastasis after preoperative CRT<sup>[29]</sup>. In addition, in the present study, after LLND 80.9% of patients did not have a systemic recurrence. Therefore, we believe that LLN metastasis can be regarded as a locoregional disease rather than a systemic one<sup>[30]</sup>.

Laparoscopic LLND for rectal cancer patients after NCRT is a challenging



Table 2 Surgery-related data

Variables	Value
Type of operation, <i>n</i> (%)	
Low anterior resection	44 (49.4)
Intersphincteric resection	2 (2.2)
Hartmann's procedure	6 (6.7)
Abdominoperineal resection	37 (41.6)
Conversion to open, <i>n</i> (%)	2 (2.2)
Operation time in min, mean $\pm$ SD	290.7 $\pm$ 89.5
Estimated blood loss in mL, mean $\pm$ SD	79.2 $\pm$ 146.7
Temporary stoma, <i>n</i> (%)	9 (10.1)
Type of LLND, <i>n</i> (%)	
Unilateral	76 (85.4)
Bilateral	13 (14.6)
Hospital stay after operation (d, mean $\pm$ SD)	8.5 $\pm$ 4.2
30 d post-operative mortality, <i>n</i> (%)	0
2-yr lateral local recurrence, <i>n</i> (%)	2 (2.2)
2-yr disease-free survival	80.90%
2-yr overall survival	91.00%

LLND: Lateral lymph node dissection; SD: Standard deviation.

procedure because of the complicated anatomy of the pelvic sidewall. The JCOG0212 study showed that the operation time was significantly longer in the TME + LLND group compared with the TME alone group (360 min *vs* 254 min,  $P < 0.0001$ ), and also blood loss was significantly higher in the TME + LLND group (576 mL *vs* 337 mL,  $P < 0.0001$ ). In addition, the overall postoperative complication in the LLND + TME group was higher than that in the TME alone group, but without statistical difference (22% *vs* 16%,  $P = 0.007$ )<sup>[31]</sup>. In our study, the most common postoperative complications were anastomotic leakage (4.5%) and bowel obstruction (4.5%), and the overall postoperative complication rate was 16.8%, similar to a previously reported study (18%-38%)<sup>[32]</sup>.

Some studies have pointed out that longer operative time and increased blood loss may increase the postoperative complication rate and thus the criteria for selecting patients for LLND is crucial<sup>[6]</sup>. Several studies have suggested that LLN size after NCRT is a powerful indicator of pathological LLNs metastasis. The European Society of Gastrointestinal and Abdominal Radiology recommended that size (SA diameter) is a reliable criterion for lymph node staging after neoadjuvant treatment, and should remain the prime criterion for malignancy in that location<sup>[33]</sup>. Akiyoshi *et al*<sup>[34]</sup> analyzed the data of 77 patients with advanced low rectal cancer and suspected LLNs involvement were undergone NCRT and LLND. LLNs metastasis was confirmed in 40.3% of patients. They showed that LLN metastasis was significantly higher in patients with LLN SA  $> 5$  mm. Oh *et al*<sup>[28]</sup> as previously described, demonstrated that an LLN greater than 5 mm on post-NCRT MRI was significantly associated with residual tumor metastasis as 61.1% (22 of 36) of patients were found to be pathologically positive. This was comparable to the 41.2% positive rate found in our center where the criteria for LLND if the SA of LLNs greater than 5 mm after NCRT or greater than 10 mm without NCRT. Furthermore, we performed receiver operating characteristic analysis for the sizes of dissected LLNs, and the area under the curve value was 0.686 for the prediction of pathological metastasis (data not shown), which was regarded as low accuracy. In order to identify risk factors correlated with LLN metastasis after CRT, we performed multivariate analysis that revealed that clinical T4 stage (95%CI: 1.419-18.508;  $P = 0.013$ ), poor histological type (95%CI: 1.038-15.520;  $P = 0.044$ ), and SA diameter of LLN after NCRT  $\geq 7$  mm (95%CI: 1.487-38.214;  $P = 0.015$ ) were independently associated with LLN metastasis.

The performance of TME plus LLND dates back to the 1970s when it was associated with favorable oncological results, but had a high urinary and sexual dysfunction rate<sup>[35,36]</sup>. To preserve the function of the autonomic nerves, nerve-preserving LLND was developed in the mid-1980s in order to obtain local control with an acceptable quality of life<sup>[37]</sup>. Georgiou *et al*<sup>[38]</sup> conducted a meta-analysis investigating the outcomes of an extended lymphadenectomy *versus* conventional surgery for rectal

**Table 3** Postoperative complications, *n* = 89

Variables	Value, <i>n</i> (%)
Anastomotic leakage	4 (4.5)
Urinary retention	2 (2.2)
Wound infection	3 (3.4)
Bowel obstruction	4 (4.5)
Lymphatic leakage	1 (1.1)
Pelvic hemorrhage	1 (1.1)

cancer. Their results suggested that LLND was associated with increased urinary and sexual dysfunction incidence, as one of its included studies suggested that the urinary retention happened in the LLND + TME and TME along group were 16% and 4%, respectively. However, many of the retrospective studies included did not perform nerve-preservation surgeries. The Japanese Research Committee for Colorectal Cancer has emphasized that an autonomic nerve-preserving technique results in better urinary and sexual function<sup>[17]</sup>. JCOG0212 was the largest randomized clinical trial that has compared postoperative urinary and sexual dysfunction between TME and TME plus LLND in lower rectal cancer patients. They suggested that blood loss was the only independent predictor of early urinary dysfunction and that LLND did not increase sexual dysfunction incidence after rectal cancer surgery. Sexual dysfunction was independently associated with increased age<sup>[39,40]</sup>. In our study, 2 (2.2%) patients experienced urinary retention, both received bilateral lymph node metastasis and after 4 wk of bladder practice, their catheters were successfully removed. These acceptable functional results might be explained by the relatively mature nerve-preserving techniques in the laparoscopic rectal cancer surgeries. Longer operative time and increased blood loss may associate with higher postoperative complication rates according to some study results<sup>[41,42]</sup>. Thus, we do not recommend routine bilateral lymph nodes dissection unless there is strong suspicion of bilateral LLNs metastasis<sup>[6]</sup>.

This study had several limitations. First, it was a single-center retrospective analysis and the sample size was relatively small; thus, a multi-center study should be conducted to confirm our conclusions. Additionally, we did not evaluate lateral lymph nodes metastasis in non-NCRT patients due to the small sample size. Next, the rectal cancer patients received either short-course or long-course radiotherapy, which might have caused heterogeneity in the pathological outcomes of the lateral lymph nodes. Third, the follow-up duration was short, because of the low local recurrence rate after NCRT, so longer follow-up may be necessary to evaluate more recurrences. Fourth, we did not study the effect of LLND on sexual functions because of poor medical records.

In conclusion, the present study showed that LLN metastasis cannot eradicate by NCRT and that selective TME plus LLND should be considered in mid or low rectal cancer patients. Our results showed satisfying perioperative and oncological outcomes.

**Table 4 Pathological outcomes**

Variables	Value
Tumor size in cm, mean $\pm$ SD	4.9 $\pm$ 2.3
Differentiation degree, <i>n</i> (%)	
Poor	28 (31.5)
Moderate/well	61 (68.5)
Pathological LLN metastasis, <i>n</i> (%)	
With NCRT	26 (41.3)
Without NCRT	9 (34.6)
Position of metastasis, <i>n</i> (%)	
Internal iliac	12 (34.3)
Obturator	21 (60.0)
Bifurcation of abdominal aorta	2 (5.7)
R status, <i>n</i> (%)	
R0	87 (97.8)
R1	2 (2.2)

LLN: Lateral lymph node; NCRT: Neoadjuvant chemoradiotherapy; SD: Standard deviation.

**Table 5 Lateral lymph node metastatic rate for different cutoff values in short-axis in patients who received (chemo)radiotherapy, *n* = 63**

Variables	Positive, <i>n</i> = 26	Negative, <i>n</i> = 37	<i>P</i> value
SA 5-7 mm, <i>n</i> (%)	3 (23.1)	10 (76.9)	0.216
SA 7-10 mm, <i>n</i> (%)	9 (39.1)	14 (60.9)	
SA $\geq$ 10 mm, <i>n</i> (%)	14 (51.9)	13 (48.1)	

SA: Short-axis.

**Table 6 Risk factors for pathological lateral lymph node metastasis after neoadjuvant chemoradiotherapy, *n* = 63**

Variables	Univariate analysis	Multivariate analysis	
	<i>P</i> value	95%CI	<i>P</i> value
Sex	0.259	0.184-2.697	0.609
Male			
Female			
Age	0.987	0.242-3.269	0.889
≥ 60			
< 60			
cT stage	0.003	1.419-18.508	0.013
cT3			
cT4			
Histological type	0.183	1.038-15.520	0.044
Poor			
Moderate/well			
Short-axis	0.135	1.487-38.214	0.015
5-7 mm			
≥ 7 mm			
Mixed signal intensity of LLN	0.739	0.342-4.894	0.705
Yes			
No			
Border irregularity of LLN	0.315	0.119-1.675	0.232
Yes			
No			

LLN: Lateral lymph node.

## ARTICLE HIGHLIGHTS

### Research background

Lateral lymph node metastasis is one of the leading causes for local recurrence in patients with advanced mid or low rectal cancer. The addition of lateral lymph node dissection (LLND) after neoadjuvant chemoradiotherapy (NCRT) remains a controversial topic.

### Research motivation

There is a lack of consensus leading to an East (mainly Japan)-West division concerning the management of lateral lymph nodes after NCRT associated with lower rectal cancer. There are few data regarding surgical outcomes of total mesorectal excision (TME) plus LLND after NCRT.

### Research objectives

The main aim of this study was to investigate the surgical outcomes of TME plus LLND, and the possible risk factors for lateral lymph node metastasis after NCRT.

### Research methods

We performed an observational study and enrolled patients who underwent TME plus LLND. Information regarding the clinicopathologic features and clinical outcomes was collected and analyzed. Multivariate logistic regression analysis was performed to evaluate the possible risk factors for lateral lymph node metastasis in the NCRT patients.

### Research results

Lateral lymph node metastasis can be found in lower rectal cancer patients with enlarged lymph node size. Advanced T stage, poor differentiation type, and short axis ≥ 7 mm were statistically significant risk factors associated with LLN metastasis.

### Research conclusions

Preoperative chemoradiotherapy is not sufficient as a stand-alone therapy to eradicate LLN metastasis in lower rectal cancer patients and surgeons should consider performing selective LLND in patients with greater lateral lymph node short axis diameter, poorer histological differentiation or advanced T stage. Selective LLND for NCRT patients can have a favorable oncological outcome.

## Research perspectives

Larger prospective multicenter clinical studies need to be performed so that standard managements regarding lateral lymph nodes in rectal cancer can be established.

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