

# World Journal of *Gastroenterology*

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## Gallbladder carcinoma: Prognostic factors and therapeutic options

Thorsten Oliver Goetze

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### Abstract

The outcome of gallbladder carcinoma is poor, and the overall 5-year survival rate is less than 5%. In early-stage disease, a 5-year survival rate up to 75% can be achieved if stage-adjusted therapy is performed. There is wide geographic variability in the frequency of gallbladder carcinoma, which can only be explained by an interaction between genetic

factors and their alteration. Gallstones and chronic cholecystitis are important risk factors in the formation of gallbladder malignancies. Factors such as chronic bacterial infection, primary sclerosing cholangitis, an anomalous junction of the pancreaticobiliary duct, and several types of gallbladder polyps are associated with a higher risk of gallbladder cancer. There is also an interesting correlation between risk factors and the histological type of cancer. However, despite theoretical risk factors, only a third of gallbladder carcinomas are recognized preoperatively. In most patients, the tumor is diagnosed by the pathologist after a routine cholecystectomy for a benign disease and is termed "incidental or occult gallbladder carcinoma" (IGBC). A cholecystectomy is performed frequently due to the minimal invasiveness of the laparoscopic technique. Therefore, the postoperative diagnosis of potentially curable early-stage disease is more frequent. A second radical re-resection to complete a radical cholecystectomy is required for several IGBCs. However, the literature and guidelines used in different countries differ regarding the radicality or T-stage criteria for performing a radical cholecystectomy. The NCCN guidelines and data from the German registry (GR), which records the largest number of incidental gallbladder carcinomas in Europe, indicate that carcinomas infiltrating the muscularis propria or beyond require radical surgery. According to GR data and current literature, a wedge resection with a combined dissection of the lymph nodes of the hepatoduodenal ligament is adequate for T1b and T2 carcinomas. The reason for a radical cholecystectomy after simple CE in a formally R0 situation is either occult invasion or hepatic spread with unknown lymphogenic dissemination. Unfortunately, there are diverse interpretations and practices regarding stage-adjusted therapy for gallbladder carcinoma. The current data suggest that more radical therapy is warranted.

**Key words:** Gallbladder carcinoma; Stage-adjusted therapy; Radical cholecystectomy; Gallbladder polyps;

Cholecystitis; Gallstones; Laparoscopic cholecystectomy

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**Core tip:** The outcome of gallbladder carcinoma is poor. In patients with early-stage disease, a 5-year survival rate of 75% is possible. Stage-adjusted therapy is key for improving survival. Despite the theoretical risk factors of gallbladder malignancies, only a third of gallbladder carcinomas are recognized preoperatively, and radical re-resection in cases of incidental discoveries of incidental or occult gallbladder carcinomas is often crucial to complete a so called radical cholecystectomy. Unfortunately, there are diverse interpretations and practices regarding stage-adjusted therapy for gallbladder carcinoma patients. The current data suggest that more radical therapy is warranted.

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## GENERAL DATA

Gallbladder carcinoma is the fifth most common neoplasm of the digestive tract and has an overall incidence of 3 per 100000 people. Gallbladder carcinoma is the most common cancer of the biliary tract<sup>[1]</sup>. A gallbladder carcinoma is found in 0.2%-3% of all cholecystectomies and 0.09%-2% of all laparoscopic cholecystectomies<sup>[2,3]</sup>. A gallbladder carcinoma is suspected preoperatively in only 30% of all patients<sup>[1]</sup>. The other 70% of cases are diagnosed using postoperative incidental findings by a pathologist. These cancers are termed incidental or occult gallbladder carcinomas. Only 15%-47% of the preoperatively known gallbladder carcinomas are suitable for resection<sup>[4]</sup>. The majority of symptomatic patients with malignant gallbladder disease have an incurable tumor. The outcome of gallbladder carcinoma is poor, and the overall 5-year survival rate is less than 5%. In early-stage disease, a 5-year survival rate of 75% can be achieved if stage-adjusted therapy is performed<sup>[5]</sup>.

Gallbladder carcinoma is described in up to 3.4% of autopsies conducted on cholelithiasis patients over 60 years of age<sup>[6]</sup>. The risk of gallbladder carcinoma increases with age. There are 2 peaks observed in gallbladder tumor incidence. The first peak occurs at 50-60 years of age. The second peak occurs at 70-80 years of age and has a higher prevalence among women<sup>[7-9]</sup>.

There is a wide geographic variance in the fre-

quency of gallbladder carcinoma. The incidence rates are extraordinarily high in Mapuche Indians in Chile, South America. This population exhibits the highest rate of gallbladder cancer: 12.3/100000 for males and 27.3/100000 for females<sup>[10]</sup>. The women of north India have an incidence of 22/100000. In North American Indians (New Mexico) and Pakistan, the incidence is 11/100000. Europe has a low overall incidence of 0-4/100000. There are also relatively high rates observed in several Eastern European countries such as Poland, which has an incidence of 14/100000. The literature reports Japan as having a high incidence rate at 7/100000, though this value is low compared with that of Poland<sup>[11]</sup>. Epidemiological studies suggest that the mortality rates are related to the incidence. Countries with the highest incidence have the highest mortality rates. There is an inverse relationship regarding the cholecystectomy rate and incidence of gallbladder carcinoma. Thus, countries with a higher rate of cholecystectomy have a lower rate of gallbladder carcinomas because the patients with risk factors have their gallbladders removed before carcinoma develops. Therefore, a survey of disease risk factors is important.

## RISK FACTORS

Gallstones represent an important risk factor in the formation of gallbladder malignancies. Concrements are present in up to 85% of patients with gallbladder carcinomas<sup>[12]</sup>. Furthermore, gallbladder cancer rates are correlated with the prevalence of gallstone disease<sup>[12]</sup>. Increasing stone size elevates the risk of developing gallbladder cancer. Gallstones larger than 3 cm are associated with a greater than tenfold increased risk of cancer compared with that of small gallstones<sup>[13,14]</sup>. The type of concrement also matters. Cholesterol gallstones resulting from a distinct local mucosal irritation and chronic inflammation are associated with a higher risk of cancer.

Chronic inflammation is strongly associated with the malignant transformation of cells. Chronic inflammation causes DNA damage, which provokes repeated tissue proliferation and restoration attempts. This response involves the release of cytokines and growth factors and, thus, predisposes cells to oncogenic transformation<sup>[15]</sup>.

Chronic cholecystitis is typically caused by chronic irritation due to a cholelithiasis, which may provoke cancer development after many years. Chronic inflammation can also result in calcium enclosure in the gallbladder wall. Only those with punctual calcium enclosures are considered premalignant, cases with transmural enclosures are associated with a decreased risk of carcinoma<sup>[16]</sup>. Porcelain gallbladder is a rare type of chronic inflammation that occurs in approximately 0.8% of all cholecystectomies and is associated with an increased carcinogenic risk. Porcelain gallbladder



is a form of diffuse transmural calcification. Several authors have reported a 62% carcinogenic risk, which appears to be an overestimation<sup>[16]</sup>. Additionally, xanthogranulomatous cholecystitis is associated with the development of gallbladder cancer but is not considered a precancerous lesion<sup>[17]</sup>. A prophylactic cholecystectomy is recommended for patients showing these particular disease patterns. The diagnosis is often based on postoperative findings. Chronic bacterial infection of the biliary tract is also a risk factor for biliary malignancy (e.g., *Salmonella typhi*, *Paratyphi* and *Helicobacter bilis*). The bacterial colonization causes the degradation of bile, chronic irritation, and inflammation of the biliary wall. These changes may affect malignant transformation by altering tumor suppressor genes or proto-oncogenes<sup>[18,19]</sup>.

Primary sclerosing cholangitis (PSC) is a chronic inflammatory syndrome with a neoplastic "field effect" that further supports the role of chronic inflammation of the gallbladder and consecutive carcinogenesis as there is an increased rate of gallbladder tumors that occur *via* a metaplasia-dysplasia-carcinoma sequence<sup>[20]</sup>. The AASLD recommends an annual ultrasound to detect mass lesions in the gallbladder. A cholecystectomy is advised in patients found to have gallbladder mass lesions regardless of the lesion size<sup>[21]</sup>. According to the EASL, gallbladder mass lesions in PSC frequently (> 50%) represent adenocarcinomas regardless of their size. Therefore, a cholecystectomy is recommended in PSC patients with a gallbladder mass of even < 1 cm in diameter<sup>[22]</sup>.

The association between environmental exposures and gallbladder cancer are unclear. The risk factors for gallstones and gallbladder carcinoma include obesity, metabolic syndrome, and diabetes. There is a risk of malignancy in diabetes mellitus patients in the absence of concrements in the organ<sup>[23-27]</sup>.

An anomalous junction of the pancreaticobiliary duct is a congenital malformation that is rare in Western countries; however, the malformation occurs frequently in Asian populations and especially Japan<sup>[28]</sup>. The histological subtype is usually a papillary carcinoma. A prophylactic cholecystectomy is recommended for these patients.

When considering the risk factors for gallbladder cancer, it is important to assess the management of gallbladder polyps that are present in up to 5% of adults and are more frequently diagnosed due to better imaging modalities<sup>[24,29]</sup>. Approximately 60% of gallbladder polyps are cholesterol polyps and 25% have an adenomyosis with hyperplastic mucosa. An additional 10% of polyps are inflammatory polyps, and 4% of all gallbladder polyps harbor benign adenomas and have neoplastic potential<sup>[30]</sup>. It is not clear if benign adenomas progress to gallbladder carcinoma because the absence of adenomatous polyp residuum in gallbladder adenocarcinoma histology challenges an adenoma-carcinoma sequence. The following factors are signs of potential malignant growth: polyps greater

than 10 mm, rapidly increasing polyps, solitary or sessile polyps, association with gallstones, patients over 50 years of age, and K-ras positivity. The S3 Guidelines<sup>[31]</sup> in Germany recommend a conventional cholecystectomy by laparotomy for polyps larger than 18 mm. Polyps > 5 mm warrant an endoscopic ultrasound. Observation *via* transabdominal ultrasound is recommended for polyps < 1 cm without additional risk factors. A laparoscopic cholecystectomy is recommended for polyps < 1 cm with risk factors or polyps > 1 cm independent of the presence of risk factors.

The worldwide variation in the prevalence of gallbladder cancer can only be explained by genetic factors and their alteration. One method of assessing possible environmental influences on the risk of developing gallbladder cancer is to examine changes in the cancer incidence after immigration events. First-generation immigrants in Sweden were studied by Hemminki *et al*<sup>[32]</sup> using the nationwide Swedish Family Cancer Database. Only women from India and Chile had an increased risk and Northern European immigrants showed decreased risks of developing gallbladder malignancies. The increased rate of gallbladder carcinomas in Chilean and Indian immigrants suggests that carcinogenesis susceptibility was present before emigration and was responsible for the cancer<sup>[9]</sup>. A study by Kim *et al*<sup>[33]</sup> identified potential markers of GBC. The close genetic similarity between early and advanced gallbladder carcinoma cases highlights the aggressive biology of early-stage gallbladder carcinomas<sup>[33]</sup>.

Gallstones are one of the most important risk factors for developing cancer. The genetic alterations that occur in the gallbladder wall are important for understanding cancer development. The gallbladder wall is altered by gallstones. The molecular pathogenesis results in an accumulation of mutations that may lead to malignancy. The common genetic mutations responsible for carcinogenesis include the activation of oncogenes, deactivation of tumor suppressor genes, microsatellite instability, and methylation of gene promoter regions<sup>[34]</sup>.

Several of these genetic changes are associated with particular risk factors. For example, cases with papillary carcinomas are 100% K-ras positive and K-ras is increased in cases of an anomalous pancreatobiliary ductal junction. Squamous-cell carcinomas and adenocarcinomas are K-ras positive in 33% and 66% of cases, respectively. There was no detectable K-ras mutation in undifferentiated adenocarcinomas<sup>[35]</sup>.

The genetic mutation profile is interesting in the context of chemotherapy for different histological types of gallbladder carcinomas. Papillary tumors are more responsive to EGFR tyrosine kinase inhibitors. Thus, EGFR targeted therapy could be an option. Adenocarcinoma histology carcinomas should be treated with gemcitabine and cisplatin. However, squamous-cell and adenosquamous-cell carcinoma are

not sensitive to chemotherapeutics<sup>[35-39]</sup>.

There is also an interesting correlation between risk factors and the histological cancer type. Approximately 80%-97% of gallbladder carcinomas are adenocarcinomas. The remaining 3%-20% of tumor types include squamous-cell, adenosquamous-cell carcinomas, or papillary carcinomas. Additionally, gallstones and sludge are coexistent in 96% of cases. There are gallstones present in nearly 100% of squamous-cell and adenosquamous-cell carcinomas. In particular, large (> 1.5 cm) cholesterol, composite, or combination gallstones were found more frequently in gallbladders with squamous-cell and adenosquamous-cell carcinomas. In nearly 88% of gallbladder adenocarcinoma cases, there are also gallstones present. In particular, large, cholesterol, composite, or combination gallstones (> 1.5 cm) have been found in 68.2% of adenocarcinomas. Furthermore, small cholesterol, mixed, or pigmented gallstones and biliary sludge are found in 31.8% of adenocarcinomas<sup>[35]</sup>.

The association between gallstones and carcinoma in cases of SCC requires longer periods of time. Thus, the patients with SCC are often older. SCC is more locally aggressive and is less sensitive to chemotherapeutics. In locally advanced stages, the prognosis of SCC is worse than adenocarcinomas. However, it has also been shown that R0 resection of an intramucosal pure squamous-cell carcinoma has a comparable prognosis to adenocarcinomas<sup>[35]</sup>.

Papillary adenocarcinoma normally has K-ras mutations that are associated with pancreatobiliary reflux, but not with gallstones. Papillary adenocarcinoma patients are younger and have an abnormal pancreatobiliary channel, cystic duct dilatation, long common pancreatobiliary channel and adenomatous polyps. The patients are common in Eastern countries and Japan.

Approximately one-third of gallbladder carcinomas are known preoperatively despite understanding the theoretic risk factors. In the majority of cases, the tumor is diagnosed by the pathologist after a routine cholecystectomy for a benign disease<sup>[40,41]</sup>, and these tumors are termed "incidental or occult gallbladder carcinomas".

## SURGICAL APPROACH AND STAGE ADJUSTED THERAPY

The gallbladder is currently removed laparoscopically in more than 75% of cases<sup>[5,40]</sup>. In western countries such as Germany more than 90% of gallbladders are removed by the laparoscopic technique. However, the laparoscopic approach for treating gallbladder carcinoma remains controversial. In cases of preoperative suspicion, the laparoscopic approach is contraindicated for gallbladder carcinoma because of an increased risk of organ perforation due to grasping instruments, bile spillage, and port-site recurrences<sup>[7,42-44]</sup>. Consequently,

when GBC is suspected preoperatively, an open technique is recommended for performing a radical cholecystectomy. However, due to the minimally invasive nature of the laparoscopic technique, a cholecystectomy is performed more frequently. As a result, the postoperative diagnosis of early gallbladder carcinoma is more frequent<sup>[45]</sup>. A second surgery for radical re-resection is required for IGBCs, depending on tumor stage<sup>[31]</sup>. Several studies have suggested that laparoscopy for IGBC is associated with a greater risk of tumor dissemination than is the open approach<sup>[46-48]</sup>. However, these conclusions are based on small sample sizes, inhomogeneous patient groups, and older data. Multiple studies including the GR, which has the largest number of IGBCs in Europe with more than 900 IGBC cases<sup>[40,41,45,49]</sup>, indicated the primary access technique (laparoscopy vs the primary open technique) did not affect prognosis. A study by Cho *et al*<sup>[50]</sup> showed that the laparoscopic approach is feasible for suspected early-stage gallbladder carcinoma. However, stage-adjusted therapy should be performed regardless of the primary access technique<sup>[49]</sup>.

Although there are guidelines in different countries, the literature, guidelines, and the compliance with those guidelines can vary for stage-adjusted therapy<sup>[49]</sup>. Stage-adjusted therapy includes radical surgery of the liver and a lymphadenectomy. According to the S3 Guidelines in Germany<sup>[31]</sup>, the recommended treatment for gallbladder carcinoma is liver resection in the form of wedge resection of the gallbladder bed or a resection of liver segments 4b and 5. This surgery is always combined with a lymphadenectomy of the hepatoduodenal ligament in cases of T2 and more advanced T-stages. A similar procedure is recommended even for T1b and more advanced carcinomas by the Guidelines of the National Comprehensive Cancer Network, which is an alliance of 25 of the world's leading cancer centers<sup>[51]</sup>.

The reason for a so called radical cholecystectomy after simple CE in a formally R0 situation is an occult invasion or hepatic spread, respectively a not known lymphogenic dissemination. A radical resection following R1 or R2 surgery should always be an individual decision based on the opinion of several surgical and oncologic specialists.

The types of liver invasion were described by Nagai *et al*<sup>[52]</sup> and include liver-bed and hepatic-hilar type. Ogura *et al*<sup>[53]</sup> further defined a gallbladder confined type, a liver bed type, and a hepatic-hilar type with an expansive and infiltrating pattern with continuous or discontinuous spread.

Endo *et al*<sup>[54]</sup> described a venous and lymphogenic pathway of microscopic tumor cell seeding in the liver in T2 GBCs, which is a T-stage without direct infiltration in the liver. The use of venous drainage in the liver is well described by Boerma *et al*<sup>[55]</sup>, in form of a drainage in portal system of both lobes of the liver and direct drainage in segments IV and V through so called vesicohepatic vessels.

Different modes of lymphatic spread were described by Fahim *et al*<sup>[56]</sup> based on the anatomical work in fetuses of Clermont in 1909 and the 3 pathways described by Ito *et al*<sup>[57]</sup> in adult cadavers. Shirai *et al*<sup>[58]</sup> identified the regional lymphatic system of the gallbladder by intraoperative vital staining. Uesaka *et al*<sup>[59]</sup> visualized the routes of lymphatic drainage in the gallbladder with a carbon particle suspension and found 3 different pathways. The final destination of all of the lymphatic routes is the confluence in the abdominal aortic nodes near the left vena renal location and the paraaortic nodes. Thus, preoperative knowledge regarding the paraaortic nodes is crucial if planning an ultraradical resection termed HPD (hepato-pancreatic-duodenectomy) as shown by Sasaki *et al*<sup>[60]</sup> or Kondo *et al*<sup>[61]</sup>. Kondo *et al*<sup>[62]</sup> shows a combined (lymphatic and liver) mode of spread in GBC that could be subdivided into the following categories: a hepatic bed type, hepatic hilum type, bed and hilum type, lymph node type, cystic duct type and a localized type.

The T-stage that requires radical liver resection is still a matter of debate. The NCCN clinical practice guidelines in oncology recommend a liver resection and lymph node dissection in T1b and more advanced T-stages<sup>[51]</sup>. The literature<sup>[63-73]</sup> supports radical GBC surgical therapy for the T1b stage and above. The German registry contains more than 900 IGBC cases and supports radical resection for the T1b stage. The GR data highlight the importance of IRR in cases of T2 and T3 carcinomas<sup>[72,73]</sup>. The radical liver resection techniques are supported by evidence from the GR and the literature<sup>[74]</sup>. The data support a wedge-resection technique with respect to prognosis and preoperative morbidity in T1b and even T2 cases. The prognostic impact of positive lymph nodes in stage T1b to T3 incidental gallbladder<sup>[75]</sup> and node dissection is important. The rate of positive nodes is 15.7% in T1b carcinomas, 46% in T2, and 75% in T3 GBCs.

Despite the many known risk factors, only 1/3 of all carcinomas are detected preoperatively. Less than half of these carcinomas are suitable for resection. Thus, it is important that the early-stage gallbladder carcinomas found in the 2/3 of patients who are diagnosed postoperatively as an incidental finding are treated with stage-adjusted therapy that includes liver resection and lymph node dissection.

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## 2015 Advances in Colorectal Cancer

# Stage migration vs immunology: The lymph node count story in colon cancer

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## Abstract

Lymph node staging is of crucial importance for the therapy stratification and prognosis estimation in colon cancer. Beside the detection of metastases, the number of harvested lymph nodes itself has prognostic relevance in stage II/III cancers. A stage migration effect caused by missed lymph node metastases has

been postulated as most likely explanation for that. In order to avoid false negative node staging reporting of at least 12 lymph nodes is recommended. However, this threshold is met only in a minority of cases in daily practice. Due to quality initiatives the situation has improved in the past. This, however, had no influence on staging in several studies. While the numbers of evaluated lymph nodes increased continuously during the last decades the rate of node positive cases remained relatively constant. This fact together with other indications raised doubts that understaging is indeed the correct explanation for the prognostic impact of lymph node harvest. Several authors assume that immune response could play a major role in this context influencing both the lymph node detectability and the tumor's behavior. Further studies addressing this issue are need. Based on the findings the recommendations concerning minimal lymph node numbers and adjuvant chemotherapy should be reconsidered.

**Key words:** Colon cancer; Lymph node harvest; Stage migration; Understaging; Will Rogers; Immune response

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**Core tip:** The number of evaluated lymph nodes is prognostic in stage II and III colon cancers. Understaging due to inadequate lymph node harvest causing a stage migration effect is a widely accepted explanation for this. However, there is growing evidence that understaging plays only a minor role in this context. It seems much more likely that immune response has influence on the lymph nodes' detectability and is associated with outcome in colon cancer.

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## INTRODUCTION

Lymph node staging is still of crucial importance for the prognosis and the therapy stratification in colon cancer. The occurrence of lymph node metastases is associated with an adverse clinical course with an indication for adjuvant chemotherapy. In contrast, patients with stage I / II colon cancers show a considerable better outcome with a high rate of long-term survivors. Because only a small number of these patients benefit from adjuvant chemotherapy it is restricted to high risk situations like T4-stage or emergency resections<sup>[1,2]</sup>. In order to ensure high quality in staging colon cancer several national guidelines recommend the histopathological evaluation of at least 12 lymph nodes<sup>[3,4]</sup>. On the other hand it is well known that this recommendation is achieved only partially sometimes only in a minority of cases<sup>[5,6]</sup>. Low lymph node yields, however, are associated with an adverse outcome<sup>[7]</sup>. Cases with low lymph node harvests might be prone to the missing of positive lymph nodes and understaging. In contrast, high numbers of evaluated lymph nodes could prevent from understaging. Actually, high numbers of investigated lymph nodes are associated with favorable outcome in colon cancer. A stage migration effect also called Will Rogers phenomenon introduced by Feinstein *et al*<sup>[8]</sup> would take place resulting in improved survival curves both for stage II and III cancers. The elimination of false node negative cases within the collective of stage I / II cases and the shift of relatively early nodal positive cases into the correct stage III category is believed to cause such a phenomenon.

This prognostic impact of high lymph node yields prompted the demand of more intensive lymph node evaluations with up to 30 lymph nodes or even more<sup>[9-11]</sup>. Because insufficient lymph node harvest has been identified as an adverse prognostic factor adjuvant chemotherapy is recommended for patients with less than 12 identified lymph nodes regardless of the nodal status<sup>[1,2]</sup>.

However, the achievement of the 12 lymph node threshold is not only of prognostic and therapeutic relevance. It has also implications in terms of the quality measurement in surgical oncology. It is widely accepted that the identification of at least 12 lymph nodes is a good surrogate marker for an adequately performed surgical resection<sup>[12]</sup>. This is still the case today although it could be shown by several investigation that the number of elevated lymph nodes is not only influenced by the surgeon but by many other very different factors including the pathologist, the age of the patient and the molecular alterations

of the tumor<sup>[13,14]</sup>. The attempt to improve the quality of colon cancer therapy is very likely the reason for the increased rate of sufficiently staged cases in the past<sup>[9,15]</sup>. This development to an improved lymph node staging should influence the outcome statistics not only mathematically but also effectively because of a higher rate of correct stage adapted therapy. This would be a strong argument that understaging of cases with a poor lymph node yield is the reason for its prognostic impact. However, several authors express doubts that the Will Rogers phenomenon is really the correct explanation for this effect<sup>[16,17]</sup>. An alternative thesis is that immune response plays a major role in this context<sup>[16,18,19]</sup>. A strong immunologic reaction against the tumor could result in local lymph node hyperplasia with enlargement and enhanced lymph node detectability.

This review discusses the current literature in order to elucidate the biological nature of the prognostic impact of lymph node count in colon cancer.

For that a broad literature research within the MEDLINE Database was performed. The search terms included "lymph node" in combination with "colon" or "colorectal". Additionally previously published reviews<sup>[7,20,21]</sup> were screened for relevant references that might have been missed by the initial MEDLINE search. Because this review emphasizes on colon cancer articles that solely deal with rectal cancers were excluded. Articles in English and German language were considered for integration. In order to answer the question of this review articles providing information about the following topics were of particular interest: Factors influencing the lymph node harvest; Prognostic impact of lymph node harvest; Lymph node positivity rates; Upstaging from N0 to N+ after secondary lymph node dissection; Effect of advanced dissection techniques; Effect of improved lymph node recovery over time; Comparison of differently performing hospitals; Indications for the role of immune response.

## FACTORS INFLUENCING THE LYMPH NODE HARVEST

Analyzing the literature an increasing interest in identifying factors that influence the lymph node harvest is recognizable. It seems that a search is ongoing for the one who is to blame when the 12 lymph nodes rule could not be achieved and for answer of the question whether it is justified to demand this rule in all situations. Forty-four studies investigating such potential factors, published between 2003 and 2014, are included in this review. Before discussing these factors it might be worth to consider how many lymph nodes can be expected within a colonic specimen. Two studies reporting the results of entire submission of mesenteric tissue (ESMT). Brown *et al*<sup>[22]</sup> found about 90 lymph nodes per colonic specimen on average while Kim *et al*<sup>[23]</sup> detected about 43 lymph

**Table 1 Factors with influence on lymph node harvest in colorectal cancers**

Surgery	Pathology	Patient	Tumor	Other
Experience Volume	Experience Technique	Age Gender BMI	Location T-stage N-stage Lymph node size MSI	Specimen length Hospital status Year of operation

BMI: Body mass index; MSI: Microsatellite instability.

nodes in colorectal cancers. Anecdotally, the authors group found 360 lymph nodes in one specimen of a total colectomy using methylene blue assisted lymph node dissection (unpublished case). It is clear that the vast majority of these nodes are very tiny and barely visible. Nevertheless, these reports indicate that the theoretically achievable numbers are by far above the 12 recommended lymph nodes. The main different factor categories are given in Table 1 and discussed below.

### Surgery

The resection of the complete lymphatic basin is an essential part of the oncological adequate surgical therapy of colon cancer. As mentioned before the total number of evaluated lymph nodes is a well-established but also controversial debated marker for the surgery's quality. Many of the published studies show significant differences between individual surgeons and/or positive associations between, surgeon's experience/qualification and/or surgical volume and the number of harvested lymph nodes<sup>[13,24-30]</sup>. A few studies, however, found no influence of surgery related variables<sup>[31,32]</sup>. Open and laparoscopic technique were shown to be equal in terms of lymph node retrieval in two meta-analyses<sup>[33,34]</sup>.

### Pathology

The independent influence of the pathologist on lymph node retrieval is also reported in several studies<sup>[13,25,27,29,30,35,36]</sup>. Interestingly, an inverse association between qualification or level of training and number of identified lymph nodes is reported. Kuijpers *et al*<sup>[37]</sup> reported better results of pathology assistants compared to pathologists and Bamboat *et al*<sup>[35]</sup> showed that residents in their first year of training are more successful in dissecting lymph nodes than there more experienced colleagues. To pathologists these results are probably less surprising as the might be to others. Dissecting lymph nodes of a surgical specimen is certainly one of the most unpopular task in pathology. Diligence and lack of time play a major role in this context. Pathology assistance and young residents may have more time and patience to do a better job.

The usage of special techniques like fat clearance,

methylene blue technique or ESMT improves the lymph node yield effectively in comparison to the conventional manual technique<sup>[38]</sup>. The same two studies that did not find lymph node retrieval influenced by the surgeon also reported a lacking influence of pathology related factors on<sup>[31,32]</sup>. This, however, is probably more the result of homogenous performance levels of these specialties within these single centers.

### Patient

Patient related factors are unmodifiable and therefore different from the former discussed points. Patient's age is mentioned by many authors as an independent predictor for the lymph node count<sup>[19,28,39-47]</sup>. All these studies reported consistently an inverse association between higher age and lymph node harvest. To our knowledge there are no studies available that investigated the underlying reasons for that. One can speculate that surgical aggressiveness differ between different age groups. On the other hand increasing age could be accompanied by a diminishing immunologic response resulting in smaller lymph nodes.

The role of patient's gender is somewhat more controversial. Gender was identified only by a minority of studies as a lymph node yield influencing factor. Nevertheless, three of the four of these studies reported an association of female sex with higher lymph node count<sup>[19,41,48]</sup>. Only Horzic *et al*<sup>[49]</sup> found a higher lymph node count in males.

Whether the body mass index (BMI) plays a role or not remains unclear. There are studies that found a positive association between particular low BMI and lymph node harvest<sup>[31,50]</sup> others report an association between high BMI and poor harvest<sup>[13,32]</sup>. Explanations again are speculative and point in the same direction as in age. In several other studies, however, no effect was seen<sup>[25,51,52]</sup>.

### Tumor

Tumor associated factors are also unmodifiable. There is strong evidence from 15 studies<sup>[14,19,26,28,31,36,39,42-44,53-57]</sup> that right location of the tumor is associated with significant higher lymph node counts compared to left sided tumors. This might be related to anatomic differences between the different parts of the colon. A higher rate of microsatellite instability (MSI) positive cancers - which are mainly located in the right colon - could be another explanation at least in certain a part of cases.

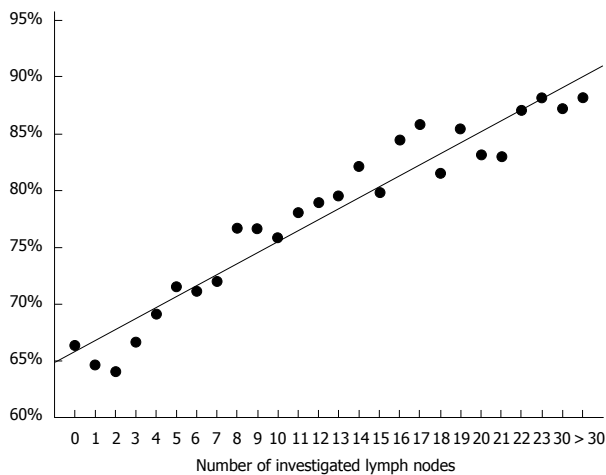
Like location, T-stage and/or tumor size were found to be predictive for the lymph node yield in colon cancer very often<sup>[13,31,36,41-43,46,49,55,56,58-60]</sup>. The immunogenicity of advanced tumors seems to be higher compared to low stages inducing a stronger reaction in lymph nodes. A more aggressive surgical treatment in advanced diseases is also thinkable.

Several authors report a positive correlation between lymph node number and the detection of lymph node metastases<sup>[41,44,56,61]</sup>. Nevertheless, it

**Table 2** Prognostic relevance of lymph node harvest in stage II colon cancers

First author	Year	n	Insuff.-rate	pT3/4	Prognostic	Endpoints	Cut off	Survival
Swanson	2003	35787	60%	100%	Yes	5yOS	No cut off	linear increase of 5yOS-rate
Law	2003	115	NA	100%	Yes	5yOS, 5yDFS	$\geq 7$	5yOS: < 7LN 69% vs > 6LN 89%
Bui	2006	453 <sup>1</sup>	NA	NA	Yes	OS	1-3 vs 10-36	HR = 0.6 (CI: 0.4-1.0), $P = 0.03$
Bilimoria	2008	142009	NA	59%	Yes	5yOS	$\geq 12$	HR = 0.75 (CI: 0.71-0.8), $P < 0.0001$
Maggard	2009	11263	NA	69%	Yes	5yOS	4 (T1) and 10 (T2)	T1: HR = 0.76 (CI: 0.641-0.902), $P = 0.002$ T2: 0.853 (CI: 0.776-0.937), $P = 0.001$
Stocchi	2011	901	NA	100%	Yes	OS, DFS, CsS	$\geq 12$	< 12 LN: HR = 1.93 (1.27-2.94), $P = 0.002$
Sato	2011	1476	56%	100%	Yes	5yOS	> 12	ACT: improved 5yOS for LNs $\leq 12$

<sup>1</sup>Sub-group of the study collection. NA: No available data; 5yOS: 5 years overall survival; 5yDFS: 5 years disease-free survival; OS: Overall survival; CsS: Cancer specific survival; ACT: Adjuvant chemotherapy; LN: Lymph node.



**Figure 1** Linear correlation between between 5-year overall survival rates and lymph node count in T3N0 colon cancers calculated on the data published by Swanson *et al*<sup>[77]</sup>.

seems questionable whether this means that a greater lymph node yield necessarily results in higher detection of metastases. The opposite linkage - the metastatic involvement induce a stronger lymph node reaction - is at least as plausible similar to advanced T-stages.

Lymph node size was recently reported as being associated with total lymph node count by the authors group, Märkl *et al*<sup>[62]</sup> and Sloothak *et al*<sup>[63]</sup>. MSI is also believed to interfere with the lymph node count. However, we found only four articles addressing this issue. Three authors report higher lymph node numbers in MSI positive cancers<sup>[14,53,64]</sup>. MacQuarrie *et al*<sup>[65]</sup> did not find such an association focusing on stage III cancers. Both factors lymph nodes size and MSI might indicate an immunologic association.

### Other factors

Other factors that influence lymph node yields are the specimen length, the status of the hospital and the year of diagnosis/operation. The latter will be discussed in detail in one of the following paragraphs. The specimen length is probably multifactorial influenced by the surgeon, the tumor and patient's individual anatomy. Several studies report this factor as lymph node count

influencing<sup>[19,26,29,47,55,56,60]</sup>.

A view studies investigated the impact of hospital status on lymph node count retrieval<sup>[60,66-68]</sup>. The results indicate that teaching and high volume centers are more successful in identifying a sufficient number of lymph nodes.

## PROGNOSTIC RELEVANCE OF LYMPH NODE COUNT IN COLON CANCER

We identified 49 studies published between 1998 and 2014 including total 625279 (range: 94-194459) that investigated the prognostic impact of lymph node count in colon and colorectal cancers, respectively. These studies show a very high heterogeneity in many respects. The study endpoint differ as cut offs, included locations and stages, case numbers and study designs do. Two nested cohort studies<sup>[69,70]</sup> and 10 register studies<sup>[45,71-79]</sup> were found. The other studies are mainly performed in single centers.

### Stage I / II colon cancers

All seven studies that were restricted to stage I / II colon cancers showed survival advantages with considerable risk reductions for the groups with higher lymph node counts (Table 2)<sup>[28,76,77,79-82]</sup> or increased risk for patients with low lymph node yields. Most authors used defined cut offs for their analyses. Swanson *et al*<sup>[77]</sup> however showed a linear increase of the 5 year overall survival rates with increasing numbers of evaluated lymph nodes (Figure 1). The work of Sato and coworkers<sup>[82]</sup> seems unique because of addressing the issue of adjuvant chemotherapy in patients with poor lymph node yield. The authors reported an improved outcome in the chemotherapy group.

### Stage I - III colon cancers

Twelve studies performed between 2002 and 2014 investigated colon cancers with and without lymph node metastases (stage I - III or stage III colon cancers exclusively) (Table 3)<sup>[44,45,54,69-71,73,74,78,83-85]</sup>. Both nested cohort studies<sup>[69,70]</sup> which are retrospective analyzes from two large multicenter studies belong to this group. All ten studies that included stage II cases



**Table 3** Prognostic relevance of lymph node harvest in stage II and III colon cancers

First author	Year	n	N+	Insuff rate	pT3/4	Endpoints	Cut off	Prognostic stage II	Prognostic stage III
Prandi <sup>1</sup>	2002	3491	48%	50 <sup>1</sup> %	n.m.	OS, PFS	8-12 (RR = 0.46) <i>vs</i> 13-17 (RR = 0.76) <i>vs</i> > 17 (RR = 0.79)	Yes	No
Le Voyer <sup>2</sup>	2003	3411	81%	NA	89%	CsS	N1: ≥ 12 <i>vs</i> > 10 <i>vs</i> > 40; N2: > 35; N0: ≥ 12 <i>vs</i> ≥ 12 <i>vs</i> > 20 and < 35	Yes	Yes
Jestin	2004	3735	31%	NA	NA	OS	≥ 12	Yes	/ <sup>3</sup>
Johnson	2006	20702	100%	NA	92%	5yCsS	< 4 neg LN <i>vs</i> > 12 neg LN	/	Yes
Kelder	2009	2281	32.4%	79%	79%	5yOS	< 6; 6-11; > 11	Yes	N
Tsikitis	2009	329	100%	49%	NA	CsS/DFS	> 12	/	N
Vather	2009	4309	NA	NA	NA	5yOS	4 LN wide steps	Yes	Yes
Dillman	2009	574	NA	NA	NA	OS	≥ 12	Yes	No
Shanmugam	2011	490	46.9%	24%	NA	5yCsS/CsS	≥ 20	Yes	Yes
Chang	2012	9644	41%	27.7%	80.2%	5yOS	≥ 12	Yes	Yes
Gleisner	2013	154208	34% <sup>4</sup>	NA	69.4%	OS	Linear risk reduction up to 25 LN in N- and up 10 LN in N+	Yes	Yes
Khan	2014	194459	NA	41%	NA	CsS	≥ 12 LN	Yes	Yes

<sup>1</sup>Intergroup Trial INT-0089; <sup>2</sup>INTAC-Trial; <sup>3</sup>Lymph node ratio is prognostic; <sup>4</sup>Mean out of two collectives. NA: No available data; 5yOS: 5 year overall survival; 5yDFS: 5 year disease-free survival; OS: Overall survival; CsS: Cancer specific survival; HR: Hazard rate; ACT: Adjuvant chemotherapy; LN: Lymph node; PFS: Progression-free survival; DFS: Disease-free survival; N-: Node negative; N+: Node positive.

**Table 4** Prognostic relevance of lymph node harvest in stage II colorectal cancers

First author	Year	n	Insuff rate	pT3/4	Prognostic	Endpoint	Cut off	Survival
Cserni	2002	8574	NA	100%	Yes	OS	No cut off	Continuously improved survival
Cianchi	2002	140	min 40% <sup>1</sup>	n.m.	Yes	5yOS	≥ 9	54.9% <i>vs</i> 79.9%, <i>P</i> < 0.001
Wong	2002	345	NA	NA	≥ 68	DFS	22.6 <i>vs</i> 11.3 <sup>2</sup>	40% <i>vs</i> 90% <sup>1</sup> , <i>P</i> < 0.001
Berberoglu	2004	301	53.5% <sup>1</sup>	69%	Yes	5yOS	≤ 10	RR = 2.8 (CI: 1.6-5.2), <i>P</i> = 0.0008
Yoshimatsu	2005	94	35%	100%	Yes	5yOS	≥ 9	66.7% <i>vs</i> 86.7%
Tsai	2007	180	NA	70%	Yes	OS	≥ 18	5yOS: 70 <i>vs</i> 98% <sup>1</sup> , <i>P</i> = 0.015
Norwood	2009	2449	NA	NA	Yes	OS	< 12	about 15% difference <sup>1</sup> , <i>P</i> = 0.001
Ishizuka	2010	205	min 36% <sup>1</sup>	100%	Yes	CsS	≤ 9 <i>vs</i> > 9	44.5 mo <i>vs</i> 66 mo, <i>P</i> = 0.0042
Nir	2010	117	28%	100%	No	5yOS, 5yDFS	≥ 12	No difference
La Torre	2012	204	16%	100%	Yes	5yDFS, 5yCsS, and 5yOS	> 12	5yOS 78.5% <i>vs</i> 53.1%, <i>P</i> = 0.001
Iachetta	2013	657	22%	100%	Yes	CsS/PFS	< 12 <i>vs</i> ≥ 20	HR = 0.49 (CI: 0.30-0.79), <i>P</i> = 0.003
Xingmao	2013	729	NA	100%	Yes	OS	> 12	88.7% <i>vs</i> 64.9%, <i>P</i> = 0.000

<sup>1</sup>Estimated based on Kaplan-Meier-curve; <sup>2</sup>Comparison of different cohorts. NA: No available data; 5yOS: 5 year overall survival; 5yDFS: 5 year disease-free survival; OS: Overall survival; CsS: Cancer specific survival; HR: Hazard rate; LN: Lymph node; PFS: Progression-free survival.

found favorable outcomes of the groups with higher lymph node counts. Again the chosen cut offs differed considerable. In 8 of 12 studies superior survival rates were found in stage III cancers also. One study showed an advantage for cases with low lymph node ratio (number metastatic lymph node divided by total lymph node number). Three groups including Kelder *et al*<sup>[44]</sup>, Prandi *et al*<sup>[70]</sup> and Tsikitis *et al*<sup>[85]</sup> however, found no significant association between lymph node harvest and outcome in stage III cancers.

### Stage I / II colorectal cancers

Twelve publication between 2002 and 2013 were restricted to stage I / II cases but included both colon and rectal cancers (Table 4)<sup>[47,72,86-95]</sup>. Despite the very different cut off points and endpoints, all except one paper reported favorable outcomes for the groups

with better lymph node harvests. Nir *et al*<sup>[91]</sup> were not able to show such an effect. This, however, might be the result of a relatively small sample number with only 117 cases. The authors reported at least a non-significant trend (*P* = 0.15) towards better disease free survival in the group of ≥ 12 lymph nodes.

### Stage I - III colorectal cancers

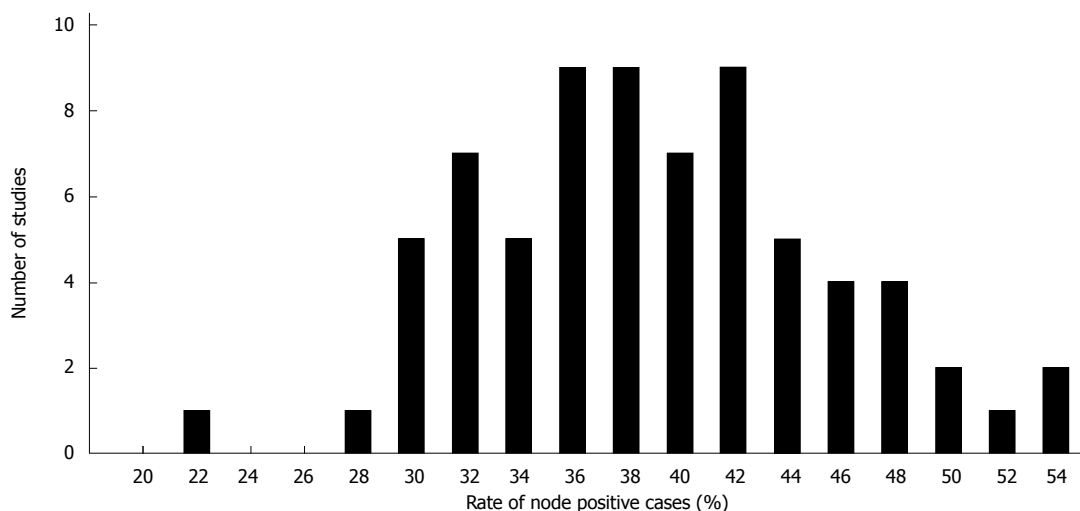
Node negative and positive colorectal cancers were investigated in 17 studies between 1998 and 2014 (Table 5)<sup>[19,26,75,96-109]</sup>. Again all but one study reported better clinical courses for cases with higher lymph node counts in stage II cancers. The half of the studies, however, found no significant difference in stage III cancers. An explanation for this discrepancy to the findings in other constellations could be that fact locally advanced rectal cancers are usually treated



**Table 5 Prognostic relevance of lymph node harvest in stage II and III colorectal cancers**

First author	Year	n	N+	Insuff-rate	pT3/4	Endpoints	Cut off	Stage II	Stage III
Caplin	1998	377	NA	NA	NA	OS	> 6	Yes	No
Sarli	2005	1040	NA	NA	100%	5yOS	< 10	Yes	No
Wong	2005	2149 <sup>1</sup>	37%	NA	67%	OS	> 13	Yes	<sup>1</sup>
George	2006	3592	NA	79%	NA	5yOS	0-4; 5-10; > 10	Yes	Yes
Edler	2007	125	51%	87%	NA	OS	0-11 vs > 11	Yes	Yes <sup>1</sup>
Evans	2008	381	45.3%	47% <sup>1</sup>	82%	5yOS	≥ 9	Yes	<sup>2</sup>
Choi	2010	664	NA	NA	100%	DFS	> 20	Yes	No
Desolneux	2010	362	NA	NA	72.4%	OS	< 8 vs ≥ 8 and < 12 vs ≥ 12	Yes	No
Ogino	2010	716	38%	63% <sup>1</sup>	68.3%	CsS/OS	0-3 negative LN, 7-12 and > or = 13 negative LN	Yes	Yes
Fretwell	2010	351	48%	min 20%	95%	5yOS	≥ 9 (Dukes B); > 9 (Dukes C)	Yes	Yes
Wong	2011	8521	About 30%	32%	66%	CsS	medians: 4 vs 8 vs 10	Yes	No
Kotake	2011	16865	46%	24% <sup>1</sup>	100%	5yOS	< 10 vs > 27	Yes	Yes
Kritsanasakul	2012	533	43%	59.1%	82%	5yOS	≥ 12	Yes	<sup>1</sup>
Moro-Valdezate	2013	1166 <sup>2</sup>	39.7%	65% <sup>1</sup>	79.7%	5yOS/5yCsS	≥ 12	No	No
Zhang	2013	265	42.3%	75.1%	79.2%	OS	< 12	Yes	Yes
Onitilo	2013	1397	37%	26%	67%	OS	≥ 12	Yes	Yes
Duraker	2014	461	NA	51%	74%	CsS	≥ 12	Yes	No <sup>1</sup>

<sup>1</sup>Survival analysis only in the nodal negative sub group ( $n = 1348$ ); <sup>2</sup>Based on cases with available survival data ( $n = 359$ ). NA: No available data; 5yOS: 5 year overall survival; 5yDFS: 5 year disease-free survival; OS: Overall survival; CsS: Cancer specific survival; HR: Hazard rate; LN: Lymph node; PFS: Progression-free survival.

**Figure 2** Mean or median lymph node positivity reported in 71 studies.

by neoadjuvant radiochemotherapy which itself is associated with decreased lymph node yields<sup>[13]</sup>. Govindarajan *et al*<sup>[110]</sup> found that lymph node harvests beyond the 12 lymph node rule in neoadjuvantly treated rectal cancers were not associated with understaging or inferior survival.

## LYMPH NODE POSITIVITY RATES

For an estimation of the lymph node positivity rate that can be expected by conventional pathological examination technique 57 studies including about 750000 cases of colon and colorectal cancers were analyzed published between 1987 and 2015 [5,6,10,14,15,17,24,26,40-44,46,48,53,55,57,59,61,63,64,66,71,73,75,83,84,95,100,101,103-109,111-127].

The mean and median rates of lymph node positivity rate on the basis of the selected studies were 39%

and 38% (range: 28-53) (Figure 2). Based on patients the mean percentage of node positive cases was 37%. There was a significant correlation between the portion of pT3/4 cancers and the occurrence of lymph node metastases (Figure 3A). The rate of inadequately staged cancers, however, had no influence on the rate node positive cases (Figure 3B). The results of the five largest studies are given in Table 6.

## UPSTAGING AFTER RE-EVALUATION AND INFLUENCE OF ADVANCED TECHNIQUES

### Influence of re-evaluation on staging

Fourteen studies were identified that evaluated the effect of secondary or even tertiary lymph node

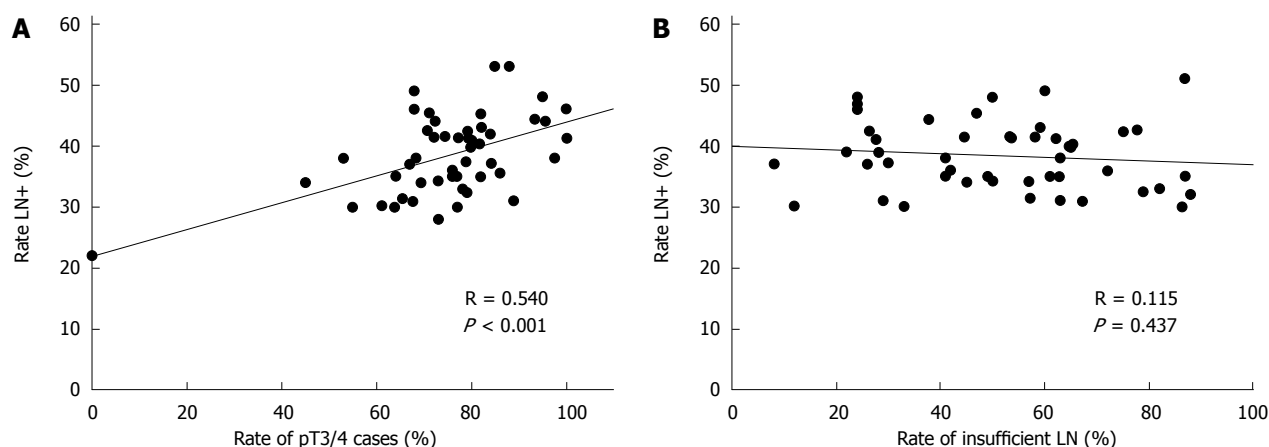


Figure 3 Association between the rate of pT3/4 cancers and the lymph node positivity rate in 51 studies (A) and rate of cases with inadequate lymph node harvest ( $< 12$  LN) and lymph node positivity (B).

Table 6 Lymph node positivity rates of the five largest studies

First author	Year	n	Register	N+ rate	Insuff rate	Rate T3/4
Gleisner	2013	154208	SEER	34%	NA	69.4%
Baxter	2010	110444	SEER	41%	53.6%	100%
Ricciardi	2006	106900	SEER	34%	57%	73%
Gonsalves	2011	19240	VACCR	30%	NA	61.1%
Chang	2012	9644	Taiwan Cancer Database	41%	27.7%	80.2%

SEER: Surveillance, Epidemiology and End Results cancer registry; VACCR: Veteran's Affairs Central Cancer Registry; NA: No available data.

dissection in colorectal cancer most often with aid of clearance techniques (Table 7)<sup>[22,23,48,128-137]</sup>. Only the authors study was restricted to colon cancers. All these studies are limited by relatively small case numbers ranging from 15 to 188. Nevertheless, nine studies report upstaging from N0 to N+ after re-evaluation of the specimens in up to 31%. All studies reporting relatively high upstaging rates between 5%-31%, however, show poor harvest results after the initial dissection step with mean numbers between 3 and 10 lymph nodes. In studies with adequate or high primary lymph node counts upstaging occurs almost exclusively in single cases.

#### Influence of advanced dissection techniques on staging

Eleven studies published between 1999 and 2015 which compared the result of advanced techniques like fat clearing or methylene blue injection with conventional manual dissection were selected to investigate the influence of these techniques on staging (Table 8)<sup>[16,138-147]</sup>. Only three of these studies<sup>[141,145,146]</sup> report significant higher node positivity rates in the study groups compared to the control groups. Despite acceptable or even excellent lymph node yields the rates of node involvement was considerable low in the control groups of these studies. The study groups showed results comparable to the values achieved by standard technique as shown in the section above. This indicates that the reported

differences might be caused more by the especially low metastatic rates in the control arms than by the effect of the advanced technique. A study performed by the authors group including more than 1300 cases revealed no differences regarding the local metastatic rate<sup>[16]</sup>.

## CHANGES OF LYMPH NODE HARVEST OVER TIME

Several studies report an improvement concerning the lymph node yields over time with increasing mean/median lymph node numbers per case and increasing rates of adequately staged cases. Twelve studies were identified investigating the development from the 50ies to present<sup>[9,15,17,95,119,120,126,148-151]</sup>. An increase in evaluated lymph nodes per case is shown in all these investigations. However, only three report an associated increase of the lymph node positivity rate. Analyzing data from 750 patients with pT3 colorectal cancers from the SEER database Goldstein *et al*<sup>[149]</sup> found an almost continuous increase concerning the mean lymph node count from 3.3 in the 1950ies to 19.4 in 1990ies. Reaching a mean count of 8.4 lymph nodes the metastases rate increased relatively abruptly from rates  $\leq 35\%$  to 38%-53%. A rate of 70% found in the latest investigation period is very likely a statistical outlier. In 2002 Goldstein published an analysis of an enlarged group from the SEER database

**Table 7 Upstaging rates after re-evaluation**

First author	Year	n	Mean LN before	Mean LN after	Upstaging NO/N+	Up-rate	Location	Technique	Comment
Scott	1989	103	6.2	12.4	Yes	8.6%	CR	Fat clearing	5yFU available
Haboubi	1992	41	6.7	58.2	Yes	28% <sup>1</sup>	CR	Fat clearing	Based on HE;
Cohen	1994	41	13	17	? <sup>1</sup>	<sup>1</sup>	CR	Xylene	higher up-staging with ICH <sup>1</sup>
Koren	1997	30	2.6	8.6	Yes	31%	CR	Fat clearing	Upstaging in 1 single case;
Brown	2005	15	20.8	89.6	Yes	<sup>1</sup>	CR	ESMT	primary N-stage (N0/1) not given;
Kim	2007	48	19.4	43	No	/	CR	ESMT	%tage N+ not given <sup>1</sup>
Richter	2007	188	n.m.	n.m.	Yes	min 4%	CR	Fat clearing	1 of 7; however unclear whether it was a
Vogel	2008	80	6.9	11.3	Yes	2%	CR	Fat clearing	LN metastasis or a deposit <sup>1</sup>
Märkl	2008	30	17	25	Yes	3%	C	Fat clearing	Initial insuff rate 59; after 9
Märkl	2008	30	35	40	No	/	C	Fat clearing	Primarily conventional technique
Fan	2010	115	9.1	14.2	Yes	5%-10% <sup>1</sup>	CR	Re-evaluation	Primarily methylen technique
Hernanz	2010	50	13.9	23.9	Yes	4% <sup>1</sup>	CR	Fat clearing	Insuff Rate 79%; Up Staging rate not
Chapman	2012	94	22.5	29	Yes	<sup>1</sup>	CR	Schwartz-clearing	exactly calculatable
Chen	2014	83	7.2	14.1	No	/	CR	Re-evaluation: partly Fat clearing	based on own calculation
Ma	2014	55	9.8	18.4	Yes <sup>1</sup>	<sup>1</sup>	CR	GEWF	1 single case upstaged <sup>1</sup>
									Upstaging in cases with primary
									insufficient LNY; 3 cases N0 to N+ <sup>1</sup>

<sup>1</sup>See comment in the same row. CR: Colorectal; C: Colon; ESMT: Entire submission of mesenteric tissue; GEWF: Glacial acetic acid, ethanol, distilled water, formaldehyde; LN: Lymph node.

**Table 8 Results of advanced pathological lymph node dissection techniques in colorectal cancers**

First author	Year	n	Mean/median LN-Conv	Mean/median LN-Spec	N+ Konv	N+ Spec	T3/4 Konv	T3/4 Spec	Technique	P value
Ratto	1999	801	11.4%	29.4%	30.2%	37.5%	76.9%	84.5%	Fixing Technique	< 0.05
Newell	2001	67	6.8%	10.2%	31%	46%	81%	85%	GEWF	NS
Kukreja	2009	701	12.8%	17.3%	36.9%	32.4%	65.8%	62.8%	Fat clearance	NS
Törnroos	2009	32	22%	61%	56.3%	37.5%	100%	100%	MB	NS
van Steenberg	2010	170	11%	14%	42%	41%	80%	79%	mesent. Patent Blue Injection	ND
Frasson	2012	473	20.6%	37.1%/47.6%	38.9%	48%	80.9%	72%	MB	NS
Jepsen	2012	428	24%	37%	9.4% <sup>1</sup>	26.7% <sup>1</sup>	82%	81%	MB	0.040
Märkl	2013	1332	13%	34%	37%	37%	65%	63%	MB	ND
Kir	2014	180	21.5%	24.5%	28%	47.9%	91.6%	84.9%	MB	0.006
Borowski	2014	100	15%	23%	34% <sup>1,2</sup>	40% <sup>1,2</sup>	NA	NA	MB	NS
Iversen	2015	120	9.5%	16.5%	44%	36%	81%	71%	GEWF	NS

<sup>1</sup>Subgroup of T1/2 cases; <sup>2</sup>Based on the number of % Dukes C cases. LN-Conv.: Lymph node harvest of conventional dissected cases; LN-Spec: Lymph node harvest of cases using advanced techniques; NA: No data available; GEWF: Glacial acetic acid, ethanol, distilled water, formaldehyde; MB: Methylene blue assisted lymph node dissection; NS: Not significant; ND: No difference.

including 2427 pT3 cases<sup>[9]</sup>. Again, the author found a similar association. Noteworthy, despite a temporary decrease of the lymph node yield in the 1980ies the trend of an increasing rate of lymph node metastases was not affected. Wong *et al*<sup>[95]</sup> investigated a cohort of total 345 patient between 1995 and 1999. They found an inverse association between increasing numbers of investigated lymph nodes and the percentage of node negative cases. However, similar mean lymph node numbers in 1995 and 1997 corresponded to considerable differing node negative rates of 65% and 55.4%, respectively. This indicates that random

changes could play a major role. All other studies including total about 250000 patients found no change in the rate of lymph node metastases over time although the lymph node yield could be improved significantly.

## COMPARISON OF DIFFERENTLY PERFORMING HOSPITALS

Hospitals belong to the factors that inhere with the lymph node harvest in colon cancers. Several investigations addressed this issue particular with

respect on its impact on the detection of lymph node metastases. Nine such studies published between 2004 and 2014 were identified<sup>[68,80,105,109,122,125,129,152,153]</sup>. Miller *et al*<sup>[152]</sup> evaluated the performance of low-, medium and high volume hospitals and found significant differences concerning the rate of poor lymph node harvest (< 7 lymph nodes) and lymph node positivity rates of 15.2% vs 35.6% and 42.6%, respectively between the low volume hospitals and the other hospital categories. Chen *et al*<sup>[129]</sup> analyzed two branches of the same institution and found significant differences with rates of inadequate staging in 20% vs 75% with corresponding lymph node positivity rates of 40.5% vs 30.6%. This was also associated with an increased long term survival. In contrast, all other seven studies, did not identify an association between the number of identified lymph nodes and the rate of stage III cancers on the hospital level. Despite the lacking impact on staging, an influence of lymph node count on survival could be shown. Wong *et al*<sup>[68]</sup> showed a favorable outcome for cases with  $\geq 12$  evaluated lymph nodes on the patients' level. Survival difference for N0 patients between differently performing hospital despite similar rates of lymph node positivity were reported by Wong *et al*<sup>[105]</sup>.

## INDICATIONS FOR THE ROLE OF IMMUNE RESPONSE

Facing limitations of the current explanation of the prognostic impact of lymph node count a possible link between immune response and the number of detected lymph nodes was proposed by a number of authors discussing their results or commenting other's papers<sup>[19,42,68,102,150,154]</sup>. These authors suggest changes of the lymph nodes - either enlargement or a diminishing - that alter its detectability. The associated differences in the number of identified number of lymph node would display a surrogate marker of the immune response against the tumor. The important role of the immune system for the patient's prognosis is unquestionably. For instance tumor infiltrating lymphocytes are associated with a favorable prognosis<sup>[155]</sup>. The same is true for crohn-like reactions in colon cancer<sup>[156]</sup>.

To the authors knowledge, however, there are only a few studies published that investigated a direct connection between parameters representing the extent of an immune response and the numbers of investigated lymph nodes. Recently, Kim *et al*<sup>[18]</sup> as well as George *et al*<sup>[102]</sup> found an association between tumor infiltration lymphocytes and the number of retrieved lymph nodes. In 1980 Pihl *et al*<sup>[157]</sup> described favorable outcomes in colorectal cancer cases with germinal center- or paracortical hyperplasia in Dukes B and C stages. Dworak<sup>[158]</sup> reported 1991 the incidence of germinal center- and paracortical hyperplasia in non-involved lymph nodes in rectal cancers. The

author, however, did not perform a survival analysis. An association between the occurrence of  $\geq 7$  lymph nodes larger than 5 mm with the total number of dissected lymph node and with favorable outcome was shown by the authors group<sup>[62]</sup>. As mentioned above microsatellite instability is found to be associated with lymph node harvest by a several authors<sup>[14,53,64]</sup>. Moreover, it is a well-known predictor for a favorable prognosis and immunologic factors are believed to be the reason for that<sup>[159]</sup>.

## CONCLUSION

Lymph node harvest has a substantial impact on the prognosis in colon cancer and has been proven in many investigations as could be shown in this review and also in a systematic review by Chang *et al*<sup>[7]</sup>.

The number of harvested lymph nodes in colon cancers is influenced by a number of modifiable and unmodifiable factors. The pathologist, surgeon and the hospital volume belong to the modifiable factors. Age, tumor stage, location and genetic alterations of the tumor are unchangeable. Stage migration also known as Will-Rogers-phenomenon is believed to be the result of understaging of stage I / II cases caused by poor lymph node retrieval. If this is true surgeons and/or pathologist would be to blame for it.

Depending on the used technique and the extent of the operation surgeons doubtless can influence the number of identifiable lymph nodes in colonic specimens. Therefore, it is to assume that more restricted excisions are prone to miss involved lymph nodes. To the author's knowledge, however, there is no evidence for that. On the other hand there are some arguments at least against a relevant frequency of its occurrence. Complete mesocolic excision has shown to be associated with reduced local recurrence and superior overall and disease-free survival in stage II and III cancers<sup>[160,161]</sup>. Nevertheless, despite improved lymph node yields the rates of nodal positive cases did not increase by this technique and seem therefore unrelated to the improved outcome results. Moreover, the reported rates of local recurrence after curatively intended resections are low which seem not compatible with a relevant rate of missed positive lymph during surgical excision<sup>[162]</sup>. Law *et al*<sup>[81]</sup> reported a higher incidence of recurrence in stage II colon cancers with inadequate lymph node harvest. Interestingly, this was caused by a higher rate of distant metastases. The rates of local recurrence did not differ between well and poorly lymph node harvested cases.

On the other hand it seems obvious that pathologist in deed have a big influence on the number of reported lymph nodes. This can be stated based on the author's experience in daily practice and many reports in the literature. The fact that pathology assistants and young residents do a better job than pathologist by spending more time and diligence<sup>[35,37]</sup> as well as the fact that the same surgeons achieve



different results when he is collaborating with different pathology department<sup>[67]</sup> are only two examples. If pathologist missed positive lymph nodes by inadequate dissection of the specimens in a significant number differences regarding the lymph node positivity rates should be determinable. However, the analysis of the data provided in the literature shows no association between the rate of inadequate lymph node yields and the rate of lymph node positivity (Figure 2). Upstaging occurs after reevaluation, however, this is mainly restricted to cases with poor lymph node harvests or single cases as shown above. Techniques to improve lymph node harvest are highly effective but not associated with higher rates of stage III cancers<sup>[38]</sup>. This is remarkable. However, pathologists seem to be highly effective in picking the relevant lymph nodes from the correct area. In an experimental model the authors group could show that there is 63% chance to detect the first lymph node metastases within the first five dissected lymph node. This increases to 86% when analyzing the first nine lymph nodes<sup>[163]</sup>. The work of Mainprize *et al*<sup>[164]</sup> point in the same direction.

These arguments raise doubts that understaging is actually a relevant problem in the management of colon cancer. The comparison of differently performing hospitals as well as the analysis of the continuously improved lymph node harvest results over time show in the vast majority of studies no association between the numbers of investigated lymph nodes and the rate of lymph node positivity.

Another point is that lymph node count was shown to be prognostic in stage III cancers at least in a part of studies. Stage migration in these cases is ruled out, naturally. Based on these arguments stage migration as reason for the well investigated prognostic impact can be excluded in the author's point of view. If at all stage migration effects seem to be restricted to the very poorly staged node negative cases. Such cases show a prognosis similar to stage III cancers. If false negative diagnoses would be the reason for that logically 100% of these cases actually had to be node positive, which seems very unlikely.

In concordance with other authors<sup>[19,42,68,102,150,154]</sup> one can state that a confounder which is related to the both lymph node harvest and to outcome has to be searched. It seems very likely that immune response is this confounder. A strong reaction can cause lymphatic hyperplasia with enlargement of lymph nodes and enhanced detectability. On the other hand an intensive immune reaction can prevent the patient from tumor progression. This hypothesis, however, is not proven yet. Nevertheless, there are indications pointing in this direction. Emerging evidence is provided by studies addressing the impact of lymph node reactions on survival as well as the prognostic relevance of lymph node size and tumor infiltrating lymphocytes<sup>[16,18,102,157,158]</sup>. Microsatellite instable tumors seem to be especially immunogenic associated with both high lymph node counts and reduce risk of

progression. They could, therefore, serve as model helping to understand what happens.

This is of high clinical relevance. The role of lymph node number as reliable quality marker becomes more and more questionable. More important a low lymph node count is currently accepted as a risk factor and often prompts the administration of adjuvant chemotherapy<sup>[165]</sup>. In many cases such poor lymph node harvests are probably not the result of poorly performing physicians but the expression of an impaired immune response. With growing success of quality initiatives these cases will escape from a possibly necessary adjuvant therapy by forcing the 12 lymph nodes. It is therefore of crucial importance to close the existing knowledge gaps and reconsider the concerned recommendations.

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## 2015 Advances in Colorectal Cancer

# Irinotecan, a key chemotherapeutic drug for metastatic colorectal cancer

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## Abstract

Irinotecan hydrochloride is a camptothecin derivative that exerts antitumor activity against a variety of

tumors. SN-38 produced in the body by carboxylesterase is the active metabolite of irinotecan. After irinotecan was introduced for the treatment of metastatic colorectal cancer (CRC) at the end of the last century, survival has improved dramatically. Irinotecan is now combined with 5-fluorouracil, oxaliplatin and several molecularly-targeted anticancer drugs, resulting in the extension of overall survival to longer than 30 mo. Severe, occasionally life-threatening toxicity occurs sporadically, even in patients in relatively good condition who have a low risk of chemotherapy-induced toxicity, often causing the failure of irinotecan-based chemotherapy. Clinical pharmacological studies have revealed that such severe toxicity is related to exposure to SN-38 and genetic polymorphisms in UDP-glucuronosyltransferase *1A1* gene. The large inter- and intra-patient variability in systemic exposure to SN-38 is determined not only by genetic factors but also by physiological and environmental factors. This review first summarizes the roles of irinotecan in chemotherapy for metastatic CRC and then discusses the optimal dosing of irinotecan based on the aforementioned factors affecting systemic exposure to SN-38, with the ultimate goal of achieving personalized irinotecan-based chemotherapy.

**Key words:** Irinotecan; Metastatic colorectal cancer; Survival advantage; Personalized chemotherapy; Dosing; Severe renal failure

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**Core tip:** Irinotecan is a key anticancer drug for the treatment of metastatic colorectal cancer. By combining irinotecan with 5-fluorouracil, oxaliplatin, and a molecularly-targeted drug, overall survival of longer than 30 mo has been achieved. Exposure to SN-38, the active metabolite of irinotecan, shows large inter- and intra-patient variability and can cause



severe irinotecan-related toxicities. Many studies have recommended the dose reduction of irinotecan for patients with UDP-glucuronosyltransferase 1A1 polymorphisms and liver dysfunction. Surprisingly, dose reduction may be required in patients with severe renal failure, even though irinotecan is predominantly eliminated *via* the liver.

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## INTRODUCTION

Irinotecan hydrochloride is an analogue of camptothecin, an extract from the Chinese tree *Camptotheca acuminata*, with higher aqueous solubility than camptothecin<sup>[1]</sup>. Irinotecan was a pro-drug that is metabolically activated in the body to 7-ethyl-10-hydroxycamptothecin (SN-38). Irinotecan has a broad spectrum of antitumor activity both *in vitro* and *in vivo*<sup>[2]</sup> and is associated with more predictable and clinically manageable toxicity than the originally isolated structure. After clinical trials, irinotecan became commercially available in Japan for treatment of lung, cervical and ovarian cancers in 1994. Irinotecan was first approved for the treatment of metastatic colorectal cancer (CRC) refractory to 5-fluorouracil (5-FU) in the United States in 1996, followed by approval in combination with 5-FU/leucovorin (LV) for the first-line treatment of metastatic CRC. A wide variety of clinical trials performed to date have revealed a survival advantage of irinotecan-based regimens in patients with metastatic CRC, making irinotecan hydrochloride one of the key drugs for the treatment of metastatic CRC. Recently, overall survival (OS) longer than 30 mo was achieved in patients with metastatic CRC who received irinotecan-based combination chemotherapy<sup>[3]</sup>.

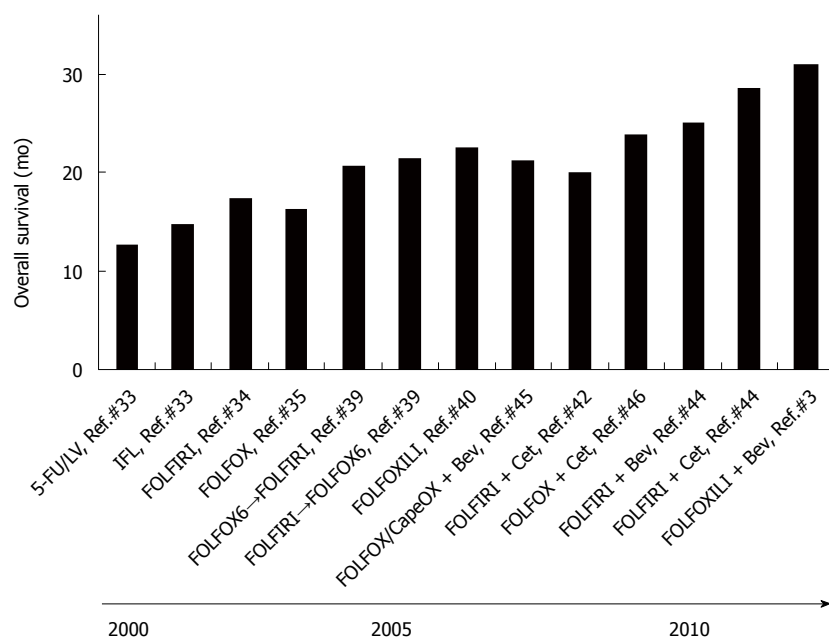
In early clinical development, the dose-limiting toxicity (DLT) of irinotecan hydrochloride was found to be severe neutropenia and delayed diarrhea. Severe, occasionally life-threatening toxicity occurs sporadically, even in patients with relatively good physical condition who have a low risk of chemotherapy-induced toxicity, who are eligible for enrollment in clinical trials of anticancer drugs<sup>[4,5]</sup>. Interindividual variability in the pharmacokinetics of SN-38 resulting from glucuronide formation is at least one of the major causes of irinotecan-induced severe toxicity<sup>[6-8]</sup>. Investigators have thus focused primarily on the polymorphic glucuronidation of SN-38 by UDP-glucuronosyltransferase (UGT) 1A1<sup>[9]</sup>, since UGT1A1 is the enzyme primarily involved in endogenous bilirubin glucuronidation as well as in irinotecan glucuronidation.

Such studies have shown that genetic polymorphisms in the *UGT1A1* gene, such as *UGT1A1*\*28 and *UGT1A1*\*6 are associated with irinotecan-induced severe toxicities<sup>[10-13]</sup>, resulting in revision of the package inserts in the United States, Japan, and other countries, including a recommendation to use a lower initial dose of irinotecan in patients with *UGT1A1*\*28/\*28, *UGT1A1*\*6/\*6 or *UGT1A1*\*6/\*28 genotype. However, pharmacogenetic factors other than *UGT1A1*, physiological factors, and environmental factors can also cause the large interindividual variability in SN-38 pharmacokinetics and contribute to irinotecan-induced toxicities. Therefore, clinical pharmacological studies are needed to establish personalized dosing strategy for irinotecan-based chemotherapy.

This review article first introduces the general pharmacokinetics and pharmacodynamics of irinotecan and then describes the development of clinical applications of irinotecan in patients with metastatic CRC. We next discuss the optimal dosing of irinotecan on the basis of factors affecting systemic exposure to irinotecan, such as pharmacogenetic factors, physiological factors, and environmental factors, with the ultimate goal of achieving personalized chemotherapy.

## GENERAL PHARMACOKINETICS

Irinotecan is unique among camptothecin analogs in that it must first be converted by a carboxylesterase (CES) to the active metabolite SN-38<sup>[14,15]</sup>. SN-38 is the major metabolite believed to be responsible for irinotecan's biologic effects, including efficacy and toxicity. It is subsequently detoxified, predominantly by UGT1A1 in the liver, to form inactive SN-38 glucuronide (SN-38G). Irinotecan is also metabolized in the liver by CYP3A4/5 to form inactive metabolites. In addition to these drug-metabolizing enzymes, transporters expressed in the liver are implicated in various aspects of SN-38 pharmacokinetics. A primary active transport system is involved in permeation of SN-38 across canalicular membranes in both humans and rats, and ATP-binding cassette (ABC) transporter, subfamily C, member 2 (ABCC2), ABC transporter, subfamily G, number 2 (ABCG2), and ABC transporter, subfamily B, number 1 (ABCB1) play roles in mediating its biliary excretion<sup>[16-18]</sup> (<http://www.pharmgkb.org/do/serve?objId=PA2001&objCls=Pathway>). A portion of SN-38 produced in the liver is thought to be transported to the systemic circulation across sinusoidal membranes by unidentified transporter(s), because SN-38 is detectable in plasma immediately after injection of irinotecan in patients with cancer. SN-38 is a substrate of the organic anion transporting polypeptides (OATPs) 1B1 and OATP1B3, which are localized on sinusoidal membranes in humans<sup>[19,20]</sup> and participate in the uptake of SN-38 into hepatocytes<sup>[21]</sup>. The contribution of OATP1B1 to the hepatic uptake of SN-38 was higher than that of OATP1B3<sup>[21]</sup>.



**Figure 1** Extension of overall survival and development of chemotherapy regimens in metastatic colorectal cancer. 5-FU: 5-fluorouracil; LV: Leucovorin; FOLFIRI: Irinotecan, bolus 5-FU/LV and infusional 5-FU; FOLFOX: Oxaliplatin, bolus 5-FU/LV and infusional 5-FU; FOLFIRI: Irinotecan, oxaliplatin, bolus 5-FU/LV and infusional 5-FU.

## GENERAL PHARMACODYNAMICS

The finding that camptothecin induced single-strand DNA breaks in the presence of topoisomerase I led to identifying this enzyme as a major target for the antitumor effects of camptothecin<sup>[22]</sup>. Subsequent genetic studies with yeast and mammalian cells revealed that the cellular effects of camptothecin can be attributed entirely to its action on topoisomerase I<sup>[1]</sup>. The lactone form of camptothecin and all its analogues, including irinotecan, appears to reversibly stabilize the topoisomerase I cleavable complex, resulting in single-strand DNA breaks and inhibition of DNA religation. DNA synthesis is thus blocked in the presence of topoisomerase I inhibitors, leading to irreversible inhibition of DNA synthesis with double-strand DNA breaks. These events induce arrest of the cell cycle in the S-G2 phase and ultimately cause cell death<sup>[23]</sup>. Because the cytotoxicity of topoisomerase I inhibitors is S-G2 phase specific, prolonged infusion times might theoretically enhance the efficacy of irinotecan<sup>[14]</sup>.

Many other factors can potentially affect the pharmacodynamics of irinotecan. Tyrosyl-DNA phosphodiesterase 1 (TDP1) participates in the repair of strand breaks by removing abortive topoisomerase I and DNA complexes. Thus, a role of TDP1 in counteracting DNA damage induced by camptothecins has been proposed<sup>[24]</sup>. X-ray repair cross complementing group 1 (XRCC1), a scaffolding protein, plays a critical role in base excision repair pathway by bringing together a complex of DNA repair proteins, including poly (ADP-ribose) polymerase I<sup>[25]</sup>. Overexpression of XRCC1 leads to camptothecin resistance in cells<sup>[26]</sup>. Cell

cycle division 45-like protein (CDC45L) is responsible for DNA replication. CDC45L was shown to be an important determinant of camptothecin sensitivity<sup>[27]</sup>. Small ubiquitin-like modifier-1 (SUMO1) has been demonstrated to compete with ubiquitin in conjugation of a protein at the same site. As a result, sumoylation, catalyzed by the sole E2-conjugating enzyme, UBC9, can stabilize the protein by preventing ubiquitin-proteasome-mediated degradation. Topoisomerase I sumoylation may inhibit ubiquitination and degradation of this enzyme, because topoisomerase I is modified by SUMO1 after camptothecin treatment<sup>[28]</sup>.

## ROLES OF IRINOTECAN TO EXTEND SURVIVAL IN PATIENTS WITH METASTATIC CRC

Since the introduction of 5-FU for the treatment of metastatic CRC, the survival had been gradually extended until the end of the last century. However, after the introduction of irinotecan and oxaliplatin, followed by molecularly-targeted anticancer agents such as bevacizumab, cetuximab, and panitumumab, a dramatic extension of survival has been achieved (Figure 1). We introduce the roles of irinotecan in the extension of survival in patients with metastatic CRC below (Figure 1 and Table 1).

### Monotherapy

The most popular schedules of irinotecan treatment are 30- or 90-min intravenous infusions of 125 mg/m<sup>2</sup> given weekly for 4 of every 6 wk or 350 mg/m<sup>2</sup>

**Table 1** Representative phase III clinical trials evaluating first-line chemotherapies in patients with metastatic colorectal cancer

Trials (primary endpoint)	Regimens	Overall survival (OS) (mo)	P value	Progression-free survival (PFS) (mo)	P value	Response rate (RR)	P value	Ref.
0038 (PFS)	5-FU/LV	12.6	0.040	4.3	0.004	21%	< 0.001	[33]
	IFL	14.8		7.0		39%		
V303 (RR)	5-FU/LV	14.1	0.031	4.4	< 0.001	22%	< 0.005	[34]
	FOLFIRI	17.4		6.7		35%		
(PFS)	5-FU/LV	14.7	0.120	6.2	< 0.001	22.3%	< 0.001	[35]
	FOLFOX	16.2		9.0		50.7%		
V308 (2 <sup>nd</sup> PFS) <sup>1</sup>	FOLFIRI→FOLFOX6	21.5	0.990	8.5	0.260	56%	> 0.05	[39]
	FOLFOX6→FOLFIRI	20.6		8.0		54%		
GONO (RR)	FOLFIRI	16.7	0.032	6.9	0.001	41%	< 0.001	[40]
	FOLFOXIRI	22.6		9.8		66%		
NO16966 (PFS)	FOLFOX/CapeOX	19.9	0.077	8.0	0.002	49%	0.310	[45]
	FOLFOX/CapeOX + Bev	21.3		9.4		47%		
CRYSTAL (PFS)	FOLFIRI	18.6	0.310	8.0	0.048	38.7%	0.004	[42]
	FOLFIRI + Cet	19.9		8.9		46.9%		
PRIME (PFS)	FOLFOX	19.7	0.072	8.0	0.020	48%	0.068	[46]
	FOLFOX + Pan	23.9		9.6		55%		
FIRE-3 (PFS)	FOLFIRI + Bev	25.0	0.017	10.3	0.550	58%	0.180	[44]
	FOLFIRI + Cet	28.7		10.0		62%		
TRIBE (PFS)	FOLFIRI + Bev	25.8	0.054	9.7	0.003	53.1%	0.006	[3]
	FOLFOXIRI + Bev	31.0		12.1		65.1%		

<sup>1</sup>Second PFS for FOLFIRI followed by FOLFOX6 and FOLFOX6 followed by FOLFIRI was 14.2 and 10.9 mo, respectively. Anticancer drugs; Bev: Bevacizumab; Cet: Cetuximab; 5-FU: 5-fluorouracil; LV: Leucovorin; Pan: Panitumumab; Regimens; CapeOX: Capecitabine and oxaliplatin; IFL: Irinotecan and bolus 5-FU/LV; FOLFIRI: Irinotecan, bolus 5-FU/LV and infusional 5-FU; FOLFOX: Oxaliplatin, bolus 5-FU/LV and infusional 5-FU; FOLFOXIRI: Irinotecan, oxaliplatin, bolus 5-FU/LV and infusional 5-FU.

given every 3 wk. The weekly-times-4 schedule is more popular in North America, and the every-3-wk schedule was developed predominantly in Europe. On the other hand, regimens of 100 mg/m<sup>2</sup> every week or 150 mg/m<sup>2</sup> every other week have been frequently used in Japan. In comparative clinical studies, none of these regimens was shown to be superior in terms of antitumor efficacy<sup>[14,29]</sup>. Remarkably, the dose intensity of all applied dosage regimens of irinotecan is approximately 100 mg/m<sup>2</sup> per week, which suggests that response does not depend on the specific treatment schedule. Phase II studies consistently obtained response rates (RR) of 10%-35% with single-agent irinotecan in metastatic CRC, independently of the administration schedule. There was also no apparent difference in the median remission duration or median survival time between different treatment schedules<sup>[30]</sup>.

Treatment with irinotecan given by intravenous infusion at a dose of 300 to 350 mg/m<sup>2</sup> every 3 wk was compared with best supportive care in a randomized phase III study in patients with metastatic CRC refractory to previous treatment with 5-FU-based chemotherapy. The 1-year survival rate was significantly higher in the irinotecan-treated group (36%) than in the control group (14%)<sup>[31]</sup>. Another randomized phase III study comparing irinotecan with three different continuous intravenous infusion schedules of 5-FU in patients with previously treated metastatic CRC demonstrated a survival advantage of irinotecan over 5-FU, regardless of the treatment schedule used<sup>[32]</sup>.

### Combination therapy

**5-FU combinations:** Based on the results of phase I/II studies showing that irinotecan combined with 5-FU/LV was active in patients with CRC, two randomized phase III studies were performed to compare irinotecan plus 5-FU/LV with 5-FU/LV alone as first-line treatment for metastatic CRC. Saltz *et al.*<sup>[33]</sup> showed that a weekly-times-4 regimen of 125 mg/m<sup>2</sup> irinotecan and 20 mg/m<sup>2</sup> LV, followed by an intravenous bolus of 500 mg/m<sup>2</sup> 5-FU (IFL) yielded a significantly longer OS than conventional low-dose 5-FU/LV (Table 1). Douillard *et al.*<sup>[34]</sup> investigated the efficacy of an intravenous bolus of 5-FU (400 mg/m<sup>2</sup>)/LV (200 mg/m<sup>2</sup> on days 1 and 2) plus continuous 5-FU infusion (600 mg/m<sup>2</sup> for 22 h on days 1 and 2), the so-called LV5FU2 regimen, with or without intravenous irinotecan infusion (180 mg/m<sup>2</sup> for 90 min) as first-line treatment for metastatic CRC. Irinotecan combined with infusional 5-FU (FOLFIRI) was well tolerated and increased the RR and prolonged the time to progression and OS (Table 1). The treatment schedules used in both studies were approved by the FDA as first-line chemotherapy for patients with metastatic CRC. However, because of the higher toxicity associated with the IFL regimen<sup>[33]</sup>, it has not been further used in the clinical practice.

In addition to irinotecan, oxaliplatin combined with infusional 5-FU has also extended OS in patients with metastatic CRC. A phase III study has shown that LV5FU2 combined with oxaliplatin (FOLFOX4) is beneficial as first-line therapy in metastatic CRC, prolonging progression-free survival (PFS), but not OS (Table 1), with acceptable tolerability and maintenance

of quality of life<sup>[35]</sup>. To simplify the treatment procedure and improve the quality of life of patients, a simplified LV5FU2 regimen has been combined with irinotecan (FOLFIRI) and with oxaliplatin (FOLFOX6) and evaluated as second-line therapy<sup>[36-38]</sup>. One phase III study compared two sequences of FOLFIRI and FOLFOX6, *i.e.*, first-line FOLFIRI followed by second-line FOLFOX6, and the reverse order<sup>[39]</sup>. Previously untreated patients were randomly assigned to receive a 2-h infusion of 200 mg/m<sup>2</sup> LV or 400 mg/m<sup>2</sup> dL LV followed by a 400 mg/m<sup>2</sup> bolus of 5-FU and 2400 to 3000 mg/m<sup>2</sup> of 5-FU as a 46-h infusion every 2 wk, either with 180 mg/m<sup>2</sup> irinotecan or with 100 mg/m<sup>2</sup> oxaliplatin as a 2-h infusion on day 1. On disease progression, irinotecan was replaced by oxaliplatin, or oxaliplatin was replaced by irinotecan. Median OS was almost equivalent in both arms (Table 1). The primary end point of the second PFS in each arm was 14.2 mo and 10.9 mo, respectively.

The Gruppo Oncologico Nord Ovest conducted a phase III study comparing 5-FU, LV, oxaliplatin, and irinotecan [FOLFOXIRI (165 mg/m<sup>2</sup> irinotecan, 85 mg/m<sup>2</sup> oxaliplatin, 200 mg/m<sup>2</sup> LV on day 1, and 3200 mg/m<sup>2</sup>, 5-FU as a 48-h continuous infusion starting on day 1, every 2 wk)] with infusional 5-FU, LV, and irinotecan (FOLFIRI) as first-line treatment for metastatic CRC<sup>[40]</sup>. The FOLFOXIRI regimen improved RR, PFS, and OS compared with FOLFIRI (Table 1), with an increased but manageable toxicity in patients with metastatic CRC with favorable prognostic characteristics.

### Molecularly targeted-drug combinations:

Currently, two promising classes of molecularly-targeted compounds have been introduced for the clinical management of metastatic CRC: epidermal growth factor receptor (EGFR) antagonists and angiogenesis inhibitors<sup>[41]</sup>. For example, cetuximab, a monoclonal antibody against the extracellular binding domain of EGFR, has single-agent activity against CRC, and augments the effects of irinotecan-based chemotherapy. In the CRYSTAL trial, the efficacy of cetuximab plus FOLFIRI as first-line treatment for metastatic CRC and associations between the mutation status of the *KRAS* gene in tumors and the clinical response to cetuximab were investigated<sup>[42]</sup>. The hazard ratio for PFS in the cetuximab-FOLFIRI group as compared with the FOLFIRI group was 0.85. There was no significant difference in OS between the two treatment groups (HR = 0.93). There was a significant interaction between treatment group and *KRAS* mutation status for tumor response, but not for PFS or OS. The hazard ratio for PFS among patients with wild-type-*KRAS* tumors was 0.68, in favor of the cetuximab-FOLFIRI group. These results indicate that first-line treatment with cetuximab plus FOLFIRI reduced the risk of progression of metastatic CRC as compared with FOLFIRI alone, although the benefit of

cetuximab was limited to patients with *KRAS* wild-type tumors.

The addition of bevacizumab to irinotecan also led to a statistically significant increase in the RR and a 4.7 mo prolongation of median OS (20.3 mo vs 15.6 mo for IFL and placebo, respectively)<sup>[43]</sup>. The addition of cetuximab or bevacizumab to FOLFIRI was compared in a phase III study (FIRE-3 trial) in patients with *KRAS* (exon 2) codon 12/13 wild-type metastatic CRC<sup>[44]</sup>. Although median PFS was almost equal in both cetuximab and bevacizumab groups (HR = 1.06), median OS in the cetuximab group was significantly longer than that in the bevacizumab group (HR = 0.77) (Table 1). The association with longer OS suggests that FOLFIRI plus cetuximab might be the preferred first-line regimen for patients with *KRAS* exon 2 wild-type metastatic CRC.

In the case of oxaliplatin, the addition of bevacizumab to first-line FOLFOX4 significantly improved PFS, but not OS and RR in patients with metastatic CRC (Table 1)<sup>[45]</sup>. Panitumumab, a fully humanized anti-EGFR monoclonal antibody, has been approved as monotherapy for patients with chemotherapy-refractory metastatic CRC because it improved PFS<sup>[46]</sup>. The efficacy and safety of panitumumab plus FOLFOX4 (panitumumab-FOLFOX4) were compared with those of FOLFOX4 alone as initial treatment for metastatic CRC in the PRIME study<sup>[47]</sup>. In the patients with wild-type *KRAS*, panitumumab-FOLFOX4 significantly improved PFS compared with FOLFOX4 (Table 1). A non-significant increase in OS was observed for panitumumab-FOLFOX4 vs FOLFOX4 (Table 1). In the patients with mutant *KRAS*, PFS and OS were reduced in the panitumumab-FOLFOX4 group vs the FOLFOX4 group. This study demonstrated that panitumumab-FOLFOX4 was well tolerated and significantly improved PFS in patients with wild-type *KRAS* tumors and emphasized the importance of *KRAS* testing for patients with metastatic CRC.

In a randomized phase III study (TRIBE)<sup>[3]</sup>, the median PFS was 12.1 mo in patients with metastatic CRC who received first-line FOLFOXIRI plus bevacizumab, as compared with 9.7 mo in those who received FOLFIRI plus bevacizumab group (HR = 0.75). OS in the FOLFIRI plus bevacizumab group was slightly but not significantly longer (31.0 mo vs 25.8 mo; HR for death = 0.79). FOLFOXIRI plus bevacizumab improved outcome in patients with metastatic CRC to achieve the OS of longer than 30 mo.

## IRINOTECAN OPTIMAL DOSE FOR PERSONALIZED CHEMOTHERAPY

### Factors influencing systemic exposure

Even after a specific dose is determined for a specific patient population on the bases of the results of clinical trials, this does not necessarily mean that the determined dose will be the optimal dose for each



**Table 2** Factors affecting SN-38 exposure and dosage recommendation of irinotecan summarized in this review

Factors	Exposure to SN-38	Irinotecan-induced toxicity	Dosage recommendation	Ref.
Pharmacogenetic factors				
<i>UGT1A1</i> *6 and *28	AUC ↑	Severe neutropenia (diarrhea)↑	Need to be reduced (prescription information in the US and Japan <i>etc.</i> )	[10-13,53,54,59,60]
<i>SLCO1B1</i> *15	AUC ↑	Severe neutropenia↑	No recommendation exists (need to be reduced?)	[62-64,67]
Physiological factors				
Age (elderly patients)	Comparable to younger	Comparable to younger	No need to be modified	[82,87-89]
Body size (obesity)	Similar in BSA-based and flat-fixed dosing	Similar in BSA-based and flat-fixed dosing	No need to be modified (flat-fixed dosing)	[48,54,68]
Organ dysfunctions				
Liver	AUC ↑	Severe neutropenia (diarrhea)↑	Need to be reduced	[95-97]
Kidney	AUC ↑	Mild to moderate, but prolonged neutropenia	Probably need to be reduced	[101,103]
Gender	Lower in female?	Severe hematologic toxicity in female?	No recommendation exists	[8,65,88,112,113]
Environmental factors				
Medication (drug-drug interactions)	No data available	Polypharmacy-related toxicity	No recommendation exists	[114]
Life style				
Smoking	AUC ↓	Lower toxicity	No recommendation exists	[115]

AUC: Area under the plasma concentration-time curve.

individual patient. The dose determined for a specific population will often be suboptimal for most patients. The large inter-patient and intra-patient variability in systemic exposure to a given drug is a limiting factor in determining the optimal dose, because of non-tumor-related differences in pharmacokinetics among individuals. These differences include pharmacogenetic factors, physiological factors and environmental factors (Table 2). In the case of irinotecan, the relation between systemic exposure to SN-38 and irinotecan-induced severe toxicity has received special attention, because toxicity often necessitates a decrease in planned dose intensity, resulting in the incomplete success of irinotecan treatment.

### Irinotecan pharmacogenetics

**UGT1A1:** Interindividual variability in the clearance of irinotecan is reported to be approximately 30%, whereas that of SN-38 is much higher (about 80%)<sup>[48]</sup>. Variability in SN-38 pharmacokinetics resulting from glucuronide formation is at least one of the major causes of irinotecan-induced severe toxicity<sup>[7,8]</sup>.

UGT1A1 is the enzyme primarily responsible for endogenous bilirubin glucuronidation as well as SN-38 glucuronidation<sup>[9]</sup>. Decreased bilirubin glucuronidation capacity of UGT1A1 is evident in patients with Gilbert's syndrome, for which the genetic basis has been elucidated. Gilbert's syndrome is most commonly related to homozygotes of the seven repeat of TA allele (*UGT1A1*\*28) in the proximal promoter region of *UGT1A1*<sup>[49]</sup>, causing decreased gene expression of *UGT1A1*<sup>[50]</sup>. In addition to this promoter-region polymorphism, missense polymorphisms in exon 1 and in the shared exons 2 to 5 have been found. Of particular relevance to East Asian populations, including Japanese, is a mutation in exon 1 (211G>A, G71R), referred to as *UGT1A1*\*6<sup>[51]</sup>. Homozygotes

for this mutation might have decreased catalytic activity by 60%<sup>[52]</sup>. Many clinical studies have linked *UGT1A1*\*28 and *UGT1A1*\*6 genotypes to irinotecan-induced toxicity, especially severe neutropenia<sup>[10-13,53]</sup> (Table 2). Frequencies of high-risk patients were nearly 10% in whites (*UGT1A1*\*28/\*28)<sup>[53,54]</sup> as well as Japanese (*UGT1A1*\*28/\*28, *UGT1A1*\*6/\*6, and *UGT1A1*\*6/\*28)<sup>[55]</sup>.

Stewart *et al.*<sup>[56]</sup> have demonstrated that severe toxicities such as grade 3 and 4 neutropenia and diarrhea did not increase in pediatric patients with the *UGT1A1*\*28/\*28 genotype when irinotecan was given according to a low-dose protracted schedule, although such patients tended to have higher area under the plasma concentration-time curve (AUC) of SN-38 and lower SN-38G to SN-38 AUC ratios. A meta-analysis has revealed that the risk of toxicity increased in an irinotecan dose-dependent fashion in patients with the *UGT1A1*\*28/\*28 genotype, but not in patients with the *UGT1A1*\*1/\*1 or *UGT1A1*\*1/\*28 genotype<sup>[57]</sup>, probably because the glucuronidation of SN-38 by UGT1A1 might be saturated in patients harboring two genetic variations, *i.e.*, *UGT1A1*\*6/\*6, *UGT1A1*\*28, or *UGT1A1*\*6/\*28, when higher doses of irinotecan were given<sup>[58]</sup>. Subsequent dose escalation studies have demonstrated a higher recommended dose of irinotecan in white cancer patients with *UGT1A1*\*1/\*1 and *UGT1A1*\*1/\*28 than in those with *UGT1A1*\*28/\*28<sup>[54,59,60]</sup>. These results indicate that the *UGT1A1*\*28 genotype can be used to individualize dosing of irinotecan (Table 2).

**Transporters:** In addition to drug metabolism, kinetic processes relevant to irinotecan disposition are highly depending on the interplay with drug transport in organs such as the liver. In this context, an area of investigation that remains relatively poorly explored

and understood in connection with irinotecan includes hepatocellular uptake transporters such as OATP1B1 and OATP1B3<sup>[19-21]</sup>, and active transport systems involved in permeation of SN-38 across canalicular membranes, including ABCC2 and ABCG2<sup>[16-18]</sup>. The International Transporter Consortium presented two polymorphisms for which there is compelling evidence supporting their clinical relevance: *SLCO1B1* (521T>C, V174A, rs4149056) and *ABCG2* (421C>A, Q141K, rs2231142)<sup>[61]</sup>. Given this important finding, we first summarize the relevance of these two polymorphisms in *SLCO1B1* and *ABCG2* to pharmacokinetics and clinical outcomes of irinotecan.

Life-threatening toxicities and higher exposure to SN-38 were observed in a Japanese patient with cancer harboring both *UGT1A1*\*6/\*28 and *SLOC1B1*\*15/\*15<sup>[62]</sup>. *SLCO1B1*\*15 haplotype consists of G allele at 388A>G and C allele at 521T>C. Significantly higher exposure to SN-38 was observed in Asian cancer patients with *SLCO1B1*\*15<sup>[63]</sup>. In a phase II study of chemotherapy with irinotecan and cisplatin in Korean patients with non-small cell lung cancer<sup>[64]</sup>, the 521TC or CC and -11187AA genotypes were associated with increased AUC of SN-38. Patients with *SLCO1B1*\*15 showed significantly higher AUC of SN-38 than those harboring haplotypes without 521T>C. Grade 4 neutropenia was associated with the 521TC or CC genotypes, whereas grade 3 diarrhea was associated with 388GG genotype. However, in a comprehensive pharmacogenetics analysis of irinotecan-induced neutropenia and pharmacokinetics<sup>[65]</sup>, there was no apparent relation between *SLCO1B1* 521T>C and the pharmacokinetics of SN-38 or irinotecan-induced toxicities. Negative results were also observed in a study performed by De Mattia *et al*<sup>[66]</sup>. Possible reasons for the discordant results include heterogeneous subjects enrolled in these studies and design of studies, *i.e.*, patients were prospectively enrolled, and the pharmacogenetic analyses were performed retrospectively. In a recent prospective study in patients with advanced cancer who received irinotecan-based regimens, *SLCO1B1* 521T>C allele was found to be significantly associated with increased SN-38 exposure<sup>[67]</sup>. However, because exposure to SN-38 was evaluated on the basis of the plasma concentration obtained immediately after the 90-min infusion of irinotecan, the relation between the *SLCO1B1* 521T>C allele and the AUC or clearance of SN-38, which have been proposed to be related to clinical outcome of irinotecan treatment<sup>[1]</sup>, was unclear. Available evidence thus suggest that the *SLCO1B1* 521T>C and the related haplotype are involved in SN-38 disposition and predictive marker for severe toxicity of irinotecan (Table 2), although further prospective studies are needed to draw definitive conclusion.

As for the polymorphism *ABCG2* 421C>A, no positive association with increased exposure to

SN-38 or with severe irinotecan-related toxicities was observed<sup>[65,66,68-70]</sup>.

Since SN-38 is a substrate of ABCC2, de Jong *et al*<sup>[71]</sup> explored associations of *ABCC2* polymorphisms and haplotypes with irinotecan disposition and diarrhea. The haplotype *ABCC2*\*2 was found to be associated with lower irinotecan clearance and with a reduced incidence of severe diarrhea, probably because of reduced hepatobiliary secretion of irinotecan. Han *et al*<sup>[72]</sup> and Fujita *et al*<sup>[73]</sup> also found that specific polymorphisms in *ABCC2* can influence disposition or tumor responses to irinotecan by regulating transporter activity.

**Other drug-metabolizing enzymes:** Previous *in vitro* experiments revealed that CES2 is associated with a high-affinity and high-velocity to catalyze irinotecan hydrolysis to form SN-38<sup>[74]</sup>. However, CES2 expression is high in the intestine and kidney, but low in the liver, whereas CES1 is abundantly expressed in the liver<sup>[75]</sup>. Although minor genetic variations in *CES2* found in Japanese were functionally deficient<sup>[76]</sup>, and some of them were associated with lower irinotecan metabolism *in vitro* and *in vivo*<sup>[76,77]</sup>, major *CES2* haplotypes (\*1b and \*1c) did not affect irinotecan pharmacokinetics<sup>[77]</sup>. Interestingly, a gene-dose effect of functional *CES1A* genes on SN-38 formation was observed in irinotecan-treated Japanese patients with cancer<sup>[78]</sup>, probably because CES1 is expressed at higher levels in the liver, a major organ for activating of irinotecan, although metabolic intrinsic clearance of CES1 is much lower than that of CES2<sup>[74]</sup>.

Because genetic diversity is observed in the genes encoding CYP3A4 and CYP3A5<sup>[79]</sup>, it has been suggested that genotyping for variants in these genes may be useful for predicting the pharmacokinetic profiles of irinotecan. However, multiple studies have demonstrated that this approach does not lead to significant correlations of *CYP3A* polymorphisms with irinotecan pharmacokinetics or clinical outcomes<sup>[80]</sup>. This failure to demonstrate clinically meaningful correlations may be due to the low allele frequency of most *CYP3A* variant genotypes or may reflect the relatively lower impact of these variants on enzyme activity *in vivo*<sup>[79]</sup>. CYP3A4 activity is determined not only by genetic variants, but also by complex regulations at the transcriptional and posttranscriptional levels, physiological factors, and environmental interactions. The role of *CYP3A4* and *CYP3A5* genotyping in improving treatment with irinotecan remains doubtful.

### Physiological factors affecting irinotecan exposure

**Age (elderly patients):** The elderly population has been increasing in recent years because of the prolongation of average life expectancy. The longer the average life expectancy becomes, the higher is the incidence of cancer. Consequently, the number of elderly patients with cancer is increasing. Although many cancers arise in elderly individuals, elderly

patients have been underrepresented in clinical trials designed to establish new anticancer treatment<sup>[81]</sup>, leading to inadequate data to support evidence-based decisions with respect to chemotherapy<sup>[82]</sup>. Older cancer patients show considerable heterogeneity in their handling of drugs as a result of age-related changes in body composition, including decreased muscle mass, increased adipose tissue, and decreased liver and renal functions. Aging is accompanied by an about 30% decrease in liver volume and an about 40% decrease in hepatic blood flow<sup>[83]</sup>. Thus, the clearance of drugs with a high hepatic elimination rate, which is limited by blood flow, might decrease in the elderly<sup>[84,85]</sup>. Age-related decreases in the functions of some drug-metabolizing enzymes have also been identified, but their clinical significance remains uncertain<sup>[83,86]</sup>.

Pharmacokinetic variables such as the maximum concentration and AUC of irinotecan, SN-38, and SN-38G in patients 65 years or older were comparable to the respective values in younger patients (within 3% of difference)<sup>[82,87]</sup>. A phase II trial was performed to evaluate the antitumor activity and toxicity of irinotecan in patients with metastatic CRC that had recurred or progressed after 5-FU-based chemotherapy<sup>[88]</sup>. This trial included patients 65 years or older. Elderly patients were twice as likely to develop grade 3 or 4 diarrhea as compared with younger patients when all courses of therapy were evaluated, suggesting that older patients are more sensitive to irinotecan-induced diarrhea than younger patients. However, older age did not significantly predict a higher incidence of first-course diarrhea. In addition, RRs do not depend on age<sup>[88]</sup>. On the basis of these findings Lichtman *et al.*<sup>[82,87]</sup> concluded that currently available evidence does not support a specific dose modification of irinotecan in elderly patients. A systematic review also concluded that pharmacokinetic and clinical data suggest that fit elderly patients may tolerate irinotecan as well as younger population. RR and survival achieved in elderly patients who receive irinotecan-based combination chemotherapy appear to be equivalent to those obtained in younger patients. For the subgroup of fit elderly patients, irinotecan may be used similarly to younger patients<sup>[89]</sup> (Table 2). However, a reduced starting dose of irinotecan has been recommended for patients older than 70 years who have received prior pelvic irradiation or have a poor performance status<sup>[29,32]</sup>.

**Body size (obesity):** According to the World Health Organization, worldwide obesity has nearly doubled since 1980 and now represents the fifth leading risk factor for global mortality, involved in the deaths of at least 2.8 million adults per each year (<http://www.who.int/mediacentre/factsheets/fs311/en/>). The proportion of the overweight population is projected to increase over the coming years in many industrialized countries, making obesity a major public health issue<sup>[90]</sup>.

Considerable lines of evidence suggest that the dose intensity of chemotherapy in overweight and obese patients with cancer in actual clinical practice is often lower than the recommended dose intensity<sup>[91]</sup>; nevertheless, retrospective and prospective clinical data have indicated an association of dose intensity with both clinical efficacy and toxicity. Because of the variability and uncertainty about the appropriate dose regimens of chemotherapy in obese patients, the American Society of Clinical Oncology has issued clinical practice guidelines for appropriate chemotherapy dosing in obese adults with cancer<sup>[92]</sup>. The guidelines recommend that after considering any comorbidities chemotherapy dosing should be calculated on the basis of body surface area (BSA) using actual weight, rather than an estimate or idealization of weight<sup>[92]</sup>.

However, Mathijssen *et al.*<sup>[48]</sup> have advocated for many years that BSA is not significantly related to the marked differences among patients in exposure to SN-38. Dosing based on BSA did not reduce clearance variability<sup>[93]</sup>, as compared with an unadjusted dose. Patients who received a flat-fixed irinotecan dose of 600 mg did not show greater interindividual pharmacokinetic variability than a control group who received the registered dose of 350 mg/m<sup>2</sup><sup>[68]</sup>. Because toxicity also did not significantly differ, it was concluded that flat-fixed dosing could safely be used to supplant the BSA-based dosing strategy of irinotecan. A recent study by Innocenti *et al.*<sup>[54]</sup> also demonstrated that with flat dosing of irinotecan BSA was not a significant predictor of the absolute neutrophil count nadir, a measure of irinotecan-induced myelosuppression. Collectively, flat dosing of irinotecan might be recommended (Table 2). Despite these observations, many prescribers and regulators maintain the erroneous belief that a patient with a larger BSA will always require a higher dose to induce the same drug effects<sup>[94]</sup>. Therefore, it is unfortunately unlikely that this strategy will be globally abandoned any time soon.

**Organ dysfunction:** Liver failure - since irinotecan and its metabolites are extensively eliminated *via* the liver, impaired liver function should be critical to the disposition of irinotecan as well as to the clinical outcomes of irinotecan-based chemotherapy.

In a phase I study, irinotecan was administered by an every-3-wk schedule to patients with varying degrees of liver dysfunction<sup>[95]</sup>. High bilirubin and alkaline phosphatase levels were associated with an exponentially decreased clearance of irinotecan. Drug toxicity was correlated with the serum bilirubin concentration. Patients who had total bilirubin levels less than 1.5 times the upper limit of normal (ULN) tolerated full-dose therapy (350 mg/m<sup>2</sup> every 3 wk). The maximum tolerated dose for patients who had total bilirubin levels 1.5 to 3.0 times the ULN was 200 mg/m<sup>2</sup> every 3 wk. Three patients with bilirubin levels higher than 3.0 times the ULN received one cycle of

irinotecan at a dose of 100 mg/m<sup>2</sup>. Although none of three patients had DLT, 2 patients had rapid hepatic tumor progression associated with exacerbation of liver dysfunction and worsening of performance status. Therefore, no dosing recommendations could be made for such patients. The most common DLTs in patients with hyperbilirubinemia were grade 4 febrile neutropenia and diarrhea. A separate phase I study confirmed that irinotecan dose reductions are required in patients with liver impairment<sup>[96]</sup>. Twelve patients with hyperbilirubinemia (median serum bilirubin, 2.1 mg/dL) were given irinotecan according to an every-3-wk schedule. Three of five patients had DLT at a dose of 145 mg/m<sup>2</sup>, while none of seven patients had DLT at a dose of 115 mg/m<sup>2</sup>. Two of the DLTs were neutropenia, and one was exacerbation of liver function. The recommended starting doses and the pharmacokinetics of irinotecan in a weekly schedule were also examined in patients with solid tumors who had impaired liver function, evaluated on the basis of the baseline serum total bilirubin level, and aspartate aminotransferase and alanine aminotransferase<sup>[97]</sup>. Irinotecan was given as a 90-min intravenous infusion weekly for the first 4 wk of each 6-wk cycle at starting doses ranging from 40 to 75 mg/m<sup>2</sup>. Hepatic dysfunction reduced irinotecan clearance while increasing relative exposure to SN-38. SN-38 exposures in patients who received doses of 40 to 75 mg/m<sup>2</sup> irinotecan were comparable to the level of exposure in patients with normal liver function who received a starting dose of 125 mg/m<sup>2</sup>. The administered starting doses of irinotecan seemed to be safe for patients with hepatic impairment who received irinotecan according to a weekly schedule. At these starting doses, exposure to SN-38 and the adverse event profile are similar to those in patients with normal liver function, and antitumor activity can be expected. These results indicate that patients with impaired liver function should be received a reduced dose of irinotecan because of increased exposure to SN-38 and an increased risk of irinotecan-induced toxicity (Table 2).

**Impaired renal function** - Even in cancer patients with severe renal failure, chemotherapeutic agents are given when patient's life expectancy is most likely to be determined by malignancy, not by renal dysfunction. Such patients are typically given anticancer drugs that are predominantly metabolized in the liver or eliminated into bile (or both), instead of drugs that are mainly excreted renally. Irinotecan is therefore administered to cancer patients with severe renal dysfunction, because it is extensively subjected to hepatic metabolism and excreted into bile. Urinary excretion of SN-38 accounts for less than 1% of the total administered dose of irinotecan<sup>[98-100]</sup>.

In a prospective clinical pharmacological study of irinotecan performed by us, the plasma concentration of SN-38, but not irinotecan or SN-38G, was significantly higher in patients with severe renal failure

who had a creatinine clearance (CL<sub>cr</sub>) of less than 20 mL/min and received hemodialysis than in patients without renal failure (terminal elimination rate constant, 0.0084 h<sup>-1</sup> vs 0.081 h<sup>-1</sup>)<sup>[101]</sup>, even though irinotecan is predominantly eliminated by the liver *via* glucuronidation and biliary excretion. The mean AUC of SN-38 calculated from 0 to 24 h in the patients with severe renal failure was 1.7-fold greater than that in the patients without renal failure (1.31 μmol/L·h vs 0.77 μmol/L·h)<sup>[101]</sup>. It should be pointed out that all patients with severe renal failure had mild or moderate but prolonged neutropenia even though they were receiving dialysis. The second course of irinotecan was delayed according to the prolonged neutropenia<sup>[101]</sup>. Because SN-38 concentrations have been reported to be still detectable even 500 h after administration of irinotecan in patients with normal renal function<sup>[102]</sup>, a long period of exposure to relatively high concentrations of SN-38 was postulated to be one of the causes for the prolonged neutropenia in such patients. A previous study has demonstrated that patients with slower CL<sub>cr</sub> (35-66 mL/min) had a four-fold higher risk of grade 3 or 4 neutropenia, although the pharmacokinetics of irinotecan and its metabolites did not differ from those in patients with normal kidney function<sup>[103]</sup>. Increased plasma SN-38 concentrations were found only in patients with severe renal failure associated with a CL<sub>cr</sub> of less than 20 mL/min in our studies<sup>[101]</sup>. These findings suggest that irinotecan is not necessarily safe in cancer patients with renal failure, even though this anticancer drug is predominantly eliminated *via* the liver (Table 2).

We have previously investigated potential mechanism(s) for delayed SN-38 elimination and found that SN-38 uptake by human hepatocytes was significantly inhibited by a mixture of organic anion uremic toxins [3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF), indoxyl sulfate, hippuric acid, and indole acetate], when the concentrations of these toxins were clinically relevant<sup>[21]</sup>. CMPF directly inhibited the uptake of SN-38 by human hepatocytes and most potently decreased SN-38 uptake mediated by cDNA-expressed OATP1B1 among the uremic toxins tested. Furthermore, of *SLCO1B1* and *SLCO1B3* gene expression in hepatocytes was significantly down-regulated by treatment with human uremic plasma. The inhibition of OATP1B1-mediated SN-38 uptake by uremic toxins and the down-regulation of *SLCO1B1* gene expression may thus at least partly contribute to the mechanisms responsible for the delayed SN-38 elimination in patients with severe renal dysfunction. Because no differences in the pharmacokinetics of irinotecan or SN-38G were found between patients with and those without severe renal failure, changes in CES or UGT1A1 activity appear unlikely, although further studies are needed.

To determine whether these findings in patients with severe renal failure are generally applicable, we have to examine whether similar findings are



obtained for other drugs that are predominantly taken up into liver by OATP1B1. Repaglinide is a nonrenally eliminated drug, which is a substrate of OATP transporter. Therefore, repaglinide metabolism in the liver may be limited by the uptake process of this transporter<sup>[104,105]</sup>. The AUC of repaglinide in patients with severe renal failure was approximately 3-fold greater than that in patients with normal renal function<sup>[106]</sup>. A physiologically-based pharmacokinetic (PBPK) model analysis by Zhao *et al.*<sup>[107]</sup> has shown that an approximately 52% reduction in the OATP1B1-mediated hepatic uptake of repaglinide was required in a virtual population of patients with severe renal impairment to obtain an AUC value comparable to that observed in humans<sup>[106]</sup>. Our results and the findings of Zhao *et al.*<sup>[107]</sup> indicate that the increased pharmacokinetic profile of drugs that are predominantly subjected to OATP1B1-mediated uptake into the liver in patients with severe renal failure is caused by reduced uptake capacity of OATP1B1. If the reduction in hepatic uptake by the direct inhibition of OATP1B1 activity with uremic toxins or by suppression of *SLCO1B* gene expression (or by both) could be quantitatively predicted, PBPK models could potentially be used to calculate appropriate doses for cancer patients with severe renal failure that would produce AUCs similar to those obtained in patients with normal kidney function.

Confirmation of these results may lead to the development of a new concepts for establishing evidence-based treatment strategies for irinotecan as well as other anticancer drugs that are substrates of OATP1B in cancer patients with severe renal dysfunction.

**Gender:** Because female-predominant expression of CYP3A4 is caused by RXR $\alpha$ -mediated sex-dependent effects of growth hormone on CYP3A expression<sup>[108]</sup>, the more rapid clearance of various drugs in women as compared with men has been reported. AUC ratios of inactive metabolites to irinotecan, an *in vivo* parameter for CYP3A4 activity, were significantly higher in females than in males<sup>[109]</sup>. Although, sex-related differences in UGT activity are relatively small and are confined to several UGTs, including UGT2B15<sup>[110]</sup>, systemic exposure to SN-38 was predicted by sex and hepatic function in a population pharmacokinetic analysis<sup>[111]</sup>. A previous study has also demonstrated that both the maximum plasma concentration and the AUC of irinotecan and SN-38 are lower in women<sup>[112]</sup>, suggesting gender-dependent irinotecan pharmacokinetics (higher clearance in female). Although early studies indicated no significant association of gender with grade 3 or 4 toxicities<sup>[8,88]</sup>, more recent findings suggest that female gender is an independent predictor of severe hematologic toxicity induced by irinotecan<sup>[65,113]</sup>. However, further confirmation is necessary.

## Environmental factors

Multiple concomitant medications were significantly associated with severe irinotecan-related toxicity in patients given monotherapy or FOLFIRI<sup>[114]</sup>. The incidence of severe irinotecan-related toxicities increased in parallel to the number of concomitant medications. Thus, polypharmacy should be effectively managed to decrease the risk of adverse drug reactions in patients with cancer who receive irinotecan-based chemotherapy.

Smoking significantly lowers both the exposure to irinotecan and SN-38, and treatment-induced neutropenia, indicating a potential risk of treatment failure<sup>[115]</sup>. Modulation of CYP3A and UGT1A1 by ingredients in smoking may partly cause these phenomena, although the underlying mechanism remains poorly understood.

## CONCLUSION

This review discusses the contribution of irinotecan to chemotherapy for metastatic CRC and the optimal dosing to achieve the personalized chemotherapy. Irinotecan became a key anticancer drug because it prolonged OS. By combining irinotecan with 5-FU, oxaliplatin and molecularly-targeted drug, OS longer than 30 mo has been achieved. Exposure to SN-38, an active metabolite of irinotecan, is characterized by large inter- and intra-patient variability and can cause irinotecan-related severe toxicities. A large number of studies have recommended the dose reduction of irinotecan for patients with *UGT1A1* polymorphisms and liver dysfunction. Studies by us suggest that the dose of irinotecan should be reduced in patients with severe renal failure, even though irinotecan is predominantly eliminated *via* the liver.

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## 2015 Advances in Gastric Cancer

# Recent aspects for disseminated carcinomatosis of the bone marrow associated with gastric cancer: What has been done for the past, and what will be needed in future?

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## Abstract

Disseminated carcinomatosis of the bone marrow is characterized by widespread bone metastasis (bone marrow infiltration) from solid tumors with hematological disorders coexisted. This disease is frequently com-

plicated with gastric cancer among solid tumors although its incidence is very rare. In recent years, technological innovations in diagnosis and treatment for cancer have remarkably improved, which made survival rates of various cancers prolonged. Prognosis of disseminated carcinomatosis of the bone marrow associated with gastric cancer, however, is still poor (less than a year), possibly because this disease has not been given attention due to low incidence. In this review, I summarize the results obtained for the past, and propose ways to improve the prognosis of this disease.

**Key words:** Disseminated carcinomatosis of the bone marrow; Gastric cancer; Patogenesis; Diagnosis; Treatment

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**Core tip:** Disseminated carcinomatosis of the bone marrow is characterized by widespread bone metastasis from solid tumors with hematological disorders coexisted. This disease is frequently complicated with gastric cancer among solid tumors although its incidence is very rare. Technological innovations in diagnosis and treatment for cancer have remarkably improved in recent years, however, prognosis of this disease associated with gastric cancer remains still very poor. In this review, I summarize the results obtained for the past, and propose ways to improve the prognosis of this disease associated with gastric cancer.

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## INTRODUCTION

Disseminated carcinomatosis of the bone marrow is characterized by widespread bone metastasis (*i.e.*, bone marrow infiltration) from solid tumors and is associated with hematological abnormalities such as disseminated intravascular coagulation (DIC) and microangiopathic hemolytic anemia (MAHA). Although this disease is regarded as a subtype of bone metastasis, its true nature remains unclear; it is thought to be a clinical entity that may differ from bone metastasis of solid tumors. This disease is frequently complicated with gastric cancer among solid tumors<sup>[1]</sup>. Its incidence seems rare, however, there has been no report about the incidence of this disease until now. Yamamura *et al*<sup>[2]</sup> reported that bone metastasis was observed in 31 (1.4%) out of 2235 cases with gastric cancer who underwent curative surgery. Disseminated carcinomatosis of the bone marrow occupies only a small part of bone metastasis, therefore, the incidence of this disease seems quite rare although the details are still unclear. Besides some case reports, a few studies have reported disseminated carcinomatosis of the bone marrow associated with gastric cancer, but no large-scale surveys have been performed; thus, the pathogenesis of this disease remains unknown. Recent technological innovations in cancer diagnosis and treatment have improved survival rates overall. However, this has not been the case for disseminated carcinomatosis of the bone marrow associated with gastric cancer. Therefore, to improve prognosis, further research on the pathogenesis, diagnosis, and treatment is urgently needed.

Here, we review the results obtained since the concept of disseminated carcinomatosis of the bone marrow associated with gastric cancer was proposed approximately 30 years ago. In addition, we discuss ways to improve the prognosis of this disease.

## MOLECULAR MECHANISM

Bone metastasis develops through the following processes: (1) cancer cells break away from the primary lesion and enter the bloodstream; (2) these cells survive and multiply in the bone marrow cavity; (3) the differentiation and activation of osteoclasts occur; and (4) the cancer cells proliferate in the bone microenvironment (Figure 1)<sup>[3,4]</sup>. The main component of these processes is the osteoclasts, as they resorb the bone following differentiation and activation by cancer cells that have infiltrated the bone marrow, thereby securing a space where the cancer cells can proliferate. Various growth factors stored in the bone matrix are subsequently released into the bone marrow cavity, promoting cancer cell proliferation.

Unlike hematopoietic tumors, solid tumors, regardless of whether they are primary or metastatic tumors, generally exhibit nodularity. In contrast, disseminated carcinomatosis of the bone marrow characteristically

exhibits mainly bone marrow infiltration with little tumorigenicity, although this disease is a kind of bone metastasis from solid tumors. Thus, the mode of metastasis in this disease is extremely unique among solid tumors. Disseminated carcinomatosis of the bone marrow is frequently complicated with gastric cancer<sup>[1]</sup>. However, its molecular mechanism remains unknown. Some aspects of this disease differ from the more common bone metastases of solid tumors and are of considerable interest. Two important questions arise: (1) why does gastric cancer, which usually has a low incidence of bone metastasis, rapidly and widely infiltrate the bone marrow (*i.e.*, bone tropism)? and (2) why does the explosive proliferation of gastric cancer cells, which results in the inhibition of normal hematopoiesis, occur?

The association between bone tropism and a chemokine (CXCR4, SDF-1) and/or integrins ( $\alpha 4\beta 1$ ,  $\alpha v\beta 3$ ) have been suggested in breast and/or prostate cancers<sup>[5-8]</sup>. However, such a relationship has been demonstrated only in experimental models; the pathogenesis associated with bone tropism has not been clarified in cancer patients. Unlike breast and prostate cancers, gastric cancer is known to have a low incidence of bone metastasis. However, in disseminated carcinomatosis of the bone marrow associated with gastric cancer, gastric cancer cells rapidly infiltrate the bone marrow. Therefore, this disease seems ideal for studying the mechanism of bone tropism in cancer cells. The future development of *in vivo* models that reflect the pathogenesis of disseminated carcinomatosis of the bone marrow associated with gastric cancer will be very useful for clarifying the pathogenesis of bone tropism.

Regarding the mechanism of gastric cancer cell proliferation in the bone marrow, osteoclasts are considered to play a central role in supplying growth factors from the bone matrix. Their explosive proliferation, which may suppress normal hematopoiesis, is thought to be due to a unique mechanism that differs from that of bone metastases from solid tumors. We previously demonstrated RANKL expression in gastric cancer cells through immunostaining by using a tissue preparation from disseminated carcinomatosis of the bone marrow associated with gastric cancer (Figure 2)<sup>[9]</sup>. This finding suggests the gastric cancer cells that infiltrate the bone marrow are directly involved in the differentiation and activation of osteoclasts and may result in the increased proliferation of gastric cancer cells in the bone marrow. In clinical practice, serum levels of bone resorption markers are elevated in patients with disseminated carcinomatosis of the bone marrow associated with gastric cancer<sup>[10-12]</sup>, suggesting the involvement of osteoclasts in the pathogenesis of this disease (Table 1). In bone metastases from solid tumors, infiltrated cancer cells in the bone marrow produce cytokines (*e.g.*, PTHrP, IL-8, PGE2, *etc.*), thereby promoting osteoclast differentiation and



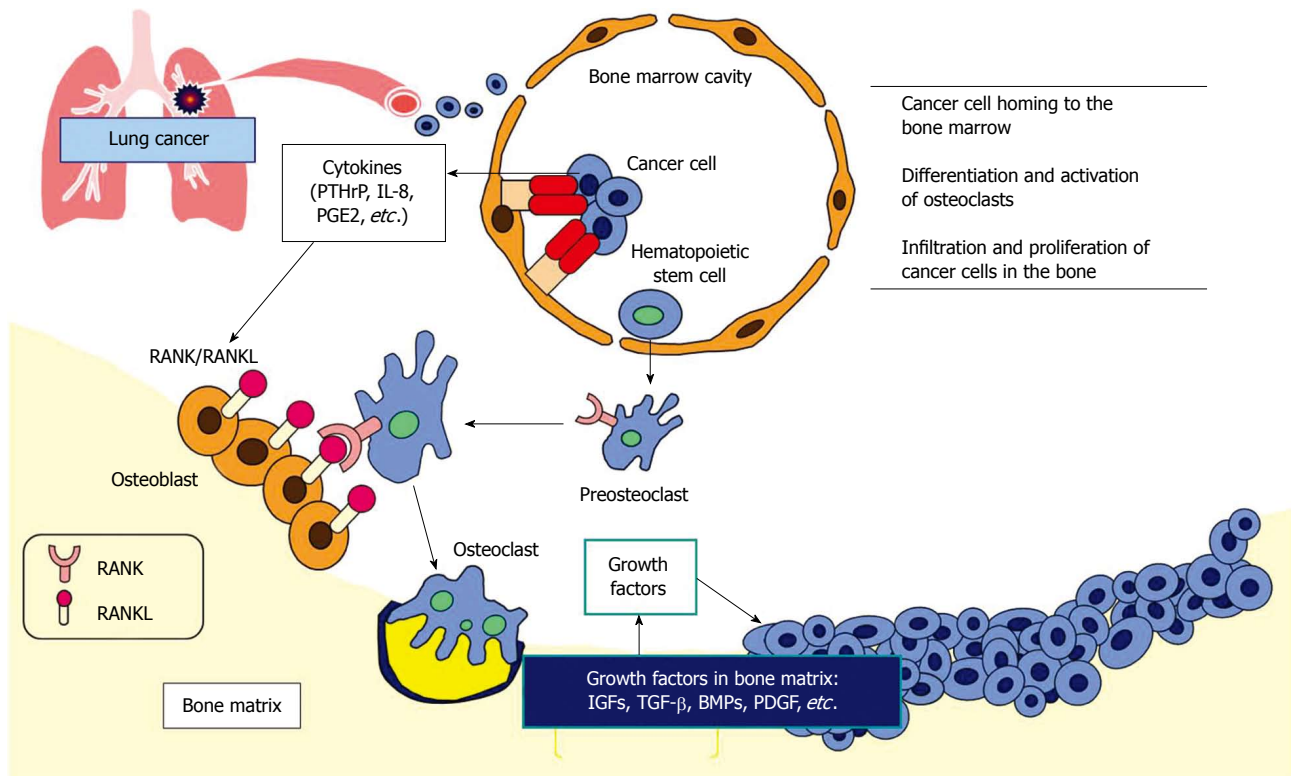
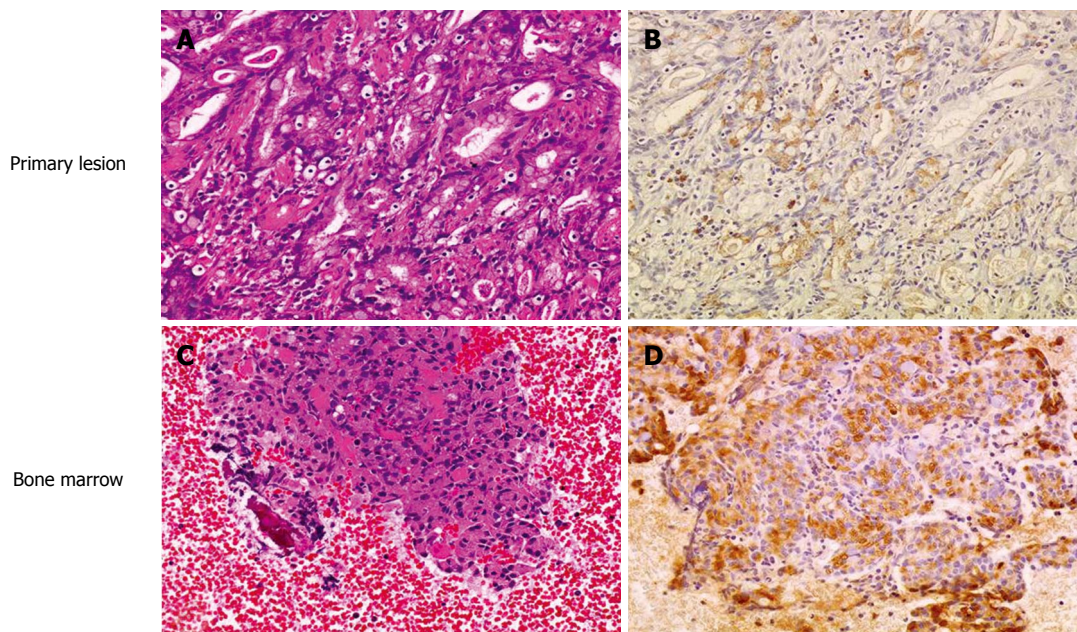


Figure 1 Molecular mechanism of osteolytic bone metastasis.



**Figure 2 RANKL expression in disseminated carcinomatosis of the bone marrow associated with gastric cancer.** Representative immunohistochemistry for RANKL in gastric cancer demonstrating disseminated carcinomatosis of the bone marrow. A: Hematoxylin and eosin staining of gastric cancer shows moderately differentiated adenocarcinoma (magnification  $\times 20$ ); B: Immunohistochemistry for RANKL in a serial section of the same specimen in (A). RANKL shows positive staining predominantly in the cytoplasm and plasma membrane of moderately differentiated adenocarcinoma cells (magnification  $\times 20$ ); C: Hematoxylin and eosin staining of a bone marrow aspiration smear shows infiltration of atypical epithelial cells, indicating metastasis from known gastric cancer (magnification  $\times 20$ ); D: Immunohistochemistry for RANKL in a serial section of the same specimen in (C). RANKL shows positive staining predominantly in the cytoplasm and plasma membrane of metastatic gastric cancer cells as is seen in the primary lesion (B) (magnification  $\times 20$ ). Adapted from Kusumoto *et al*<sup>[9]</sup>.

**Table 1 Levels of serum and/or urinary bone metabolic markers in patients with disseminated carcinomatosis of the bone marrow associated with gastric cancer**

	Age	Sex	Bone resorption markers			Bone formation markers	
			1CTP (4.5 mg/mL) <sup>2</sup>	NTx (urine) (< 89 nmol/L BCE/mmol/L Cre) <sup>2</sup>	DPD (urine) (< 7.6 nmol/L BCE/mmol/L Cre) <sup>2</sup>	BAP (< 20.9 μg/L) <sup>2</sup>	OC (< 13.0 ng/mL) <sup>2</sup>
Case report							
Takeda <i>et al</i> <sup>[10]</sup>	87	F	-	14800	59	929	32
Hasuda <i>et al</i> <sup>[11]</sup>	39	M	19.5	-	-	-	-
Mizuno <i>et al</i> <sup>[12]</sup>	44	M	-	-	59	-	37
Our cases <sup>1</sup>							
1	60	M	8.9	-	-	258	-
2	47	F	17.7	> 300	-	1260	-
3	74	F	8.0	-	-	78	-
4	54	M	9.7	-	-	55	-
5	58	M	6.7	-	-	96	-
6	47	F	-	-	-	275	-
7	58	M	13.2	-	-	159	-
8	78	F	26.5	> 300	-	429	-
9	67	F	6.7	175	-	63	-
10	71	M	14.4	> 300	-	421	-
11	53	F	9.9	235	-	468	-
12	70	M	-	20	-	62	-

<sup>1</sup>Our 12 cases have not been published; <sup>2</sup>Values in the parentheses indicate the normal range. 1CTP: C-terminal telopeptide of type I collagen; NTx: N-terminal crosslinking telopeptide of type I collagen; DPD: Deoxypyridinoline; BAP: Bone-specific alkaline phosphatase; OC: Osteocalcin.

activation<sup>[13-15]</sup>. In disseminated carcinomatosis of the bone marrow associated with gastric cancer, osteoclast differentiation and activation are also conceivably caused by cytokines secreted by gastric cancer cells. This common mechanism together with the direct action on osteoclasts through RANKL expressed in the gastric cancer cells may be associated with rapid proliferation.

Although the gastric cancer cells of disseminated carcinomatosis of the bone marrow proliferate rapidly in the bone marrow, they have little tumorigenicity. Histological types of the gastric cancer cells that cause this disease are mostly poorly differentiated adenocarcinoma or signet-ring cell carcinoma<sup>[16]</sup>. In these types of cancer cells, expression levels of adhesion molecules have been shown to be reduced<sup>[17,18]</sup>. Although these observations may explain the poor tumorigenicity, the precise mechanism remains to be clarified.

Disseminated carcinomatosis of the bone marrow associated with gastric cancer can occur over a long period ranging from several months to > 20 years even after patients have received curative resection of early gastric cancer<sup>[19,20]</sup>. Therefore, the molecular mechanism responsible for such metachronous onset of this disease warrants attention; this information is important for formulating a postoperative follow-up strategy for patients. The concept of disseminated tumor cells (DTCs) was recently introduced to explain cancer metastasis (Figure 3)<sup>[21]</sup>. The cancer cells are proposed to form a niche in the bone marrow and remain in a dormant state for a certain period until activated by an unknown trigger to form a new metastatic lesion. This mechanism might explain why

disseminated carcinomatosis of the bone marrow occurs many years after curative resection of early gastric cancer. Lu *et al*<sup>[22]</sup> showed that osteoclasts are involved in the reactivation of the cancer cell niche in the bone marrow, and bisphosphonates, which inhibit the differentiation and activation of osteoclasts, inhibit this reactivation of DTCs and exhibit anticancer activities (Figure 4). Bisphosphonates are widely used to treat bone metastasis in clinical practice. If they are proven to also inhibit DTC activation, this will lead to a new therapeutic strategy for the prevention of metastasis.

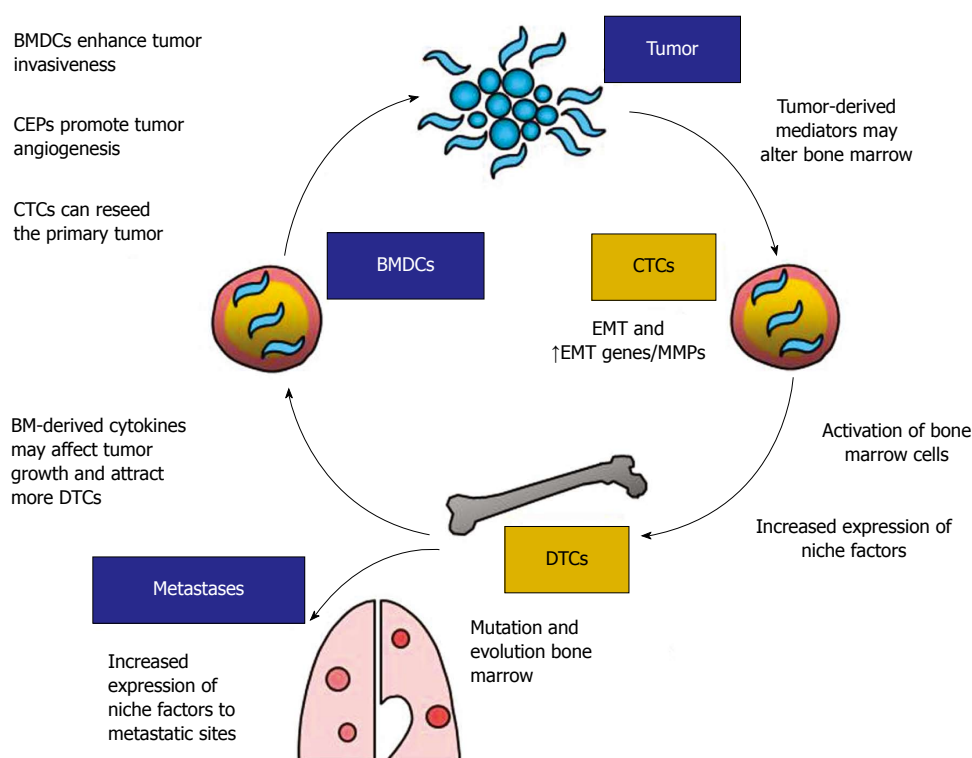
## DIAGNOSIS

### **Characteristics features of disseminated carcinomatosis of the bone marrow associated with gastric cancer**

With regard to disseminated carcinomatosis of the bone marrow associated with gastric cancer, no comprehensive articles have been published thus far, only a limited number of case reports. Therefore, we investigated the clinical characteristics of this disease in 28 cases reported in Japan during the period of 2003-2013<sup>[10,11,19,20,23-42]</sup>.

Sex and age (mean ± SD; described for 28 cases): Male (*n* = 18): 54.4 ± 12.0 years (range: 33-78 years); Female (*n* = 10): 64.5 ± 11.8 years (range: 47-87 years); There were more male than female patients, and their age tended to be younger than that with gastric cancer.

Chief complaints (*i.e.*, reasons for seeking medical attention, described for 27 cases): Pain (*n* = 19), hemorrhagic symptoms (*n* = 6), and elevation of serum ALP (*n* = 5) are frequently observed in order.



**Figure 3 Association of disseminated tumor cells in the bone marrow with cancer metastasis.** DTCs: Disseminated tumor cells; CTCs: Circulating tumor cells; CEPs: Circulating endothelial progenitors; MMP: Matrix metalloproteinase; BM: Bone marrow; BMDCs: BM-derived cells; EMT: Epithelial-mesenchymal transition. Adapted from Aft *et al*<sup>[21]</sup>.

Laboratory examination (described for 27 cases): Among hematological abnormalities, DIC was the most frequent ( $n = 23$ ), followed by anemia ( $n = 22$ ). Patients with anemia included MAHA and anemia caused by gastrointestinal hemorrhage associated with DIC. Only 4 cases refer to leukoerythroblastosis, which was found in 3 of them.

Biochemical tests showed marked elevation of ALP in all patients [ $4305 \pm 3443$  IU/L (mean  $\pm$  SD), range: 739-12600 IU/L]. Mild-to-moderate elevation of LDH was also observed in most patients ( $706 \pm 654$  IU/L, mean  $\pm$  SD, range: 141-2337 IU/L).

Time of onset (described for 28 cases): Disseminated carcinomatosis of the bone marrow was diagnosed synchronously with gastric cancer in 11 patients, and metachronously after gastric cancer surgery in 17 patients; in the latter group, the mean time (mean  $\pm$  SD) until diagnosis was  $7.2 \pm 6.7$  years (range: 18 d to 23 years). The disease was observed also in patients who had received curative resection for early gastric cancers.

Macroscopic type (described for 16 cases): Among 16 cases, 10 and 6 were advanced and early gastric cancer, respectively. Regarding the macroscopic types of advanced cancer, 8 cases had type 2 or 3, and 2 cases had type 4 according to the Borrmann classification<sup>[43]</sup>; there were no type 1 cases. Regarding the 6 cases of early gastric cancer, all were of type 0-

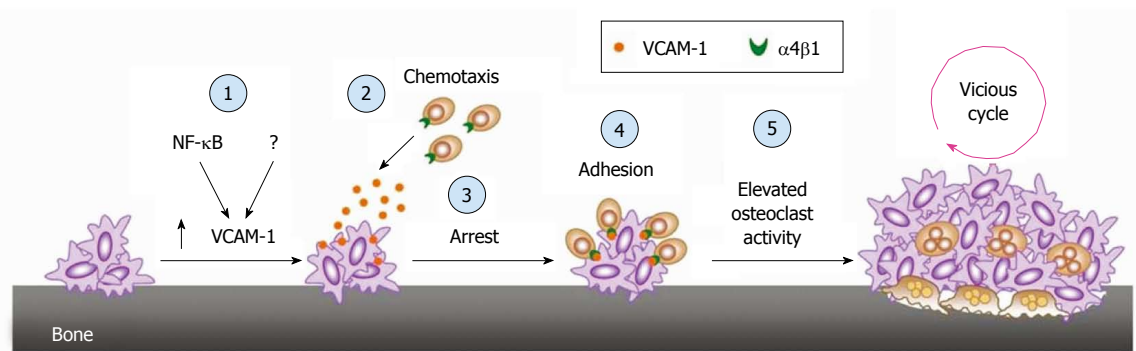
II according to the classification of early gastric cancer established by the Japanese Endoscopic Society<sup>[43]</sup>.

Histological type (described for 26 cases): The histological types of most cases were poorly differentiated adenocarcinoma ( $n = 13$ ) and signet-ring cell carcinoma ( $n = 12$ ); tubular adenocarcinoma (tub) was found in only 1 case.

### Imaging diagnosis

If serum ALP and/or LDH levels are elevated in patients complaining of low back pain and/or hemorrhagic symptoms, disseminated carcinomatosis of the bone marrow should be suspected. In order to confirm the diagnosis, imaging tests such as computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET or PET/CT) should be performed first. Among these, PET/CT can detect not only primary lesions but also early bone lesions (*i.e.*, bone marrow infiltration); therefore, it should be performed as the preferred initial imaging test. I recommend that every patient, who is suspected of disseminated carcinomatosis of the bone marrow, should have at least a PET/CT. Primary lesions of gastric cancer appear as tumor masses in the stomach and/or stomach wall thickening with  $^{18}\text{F}$ -deoxyglucose accumulation on PET/CT (Figure 5). When these findings are observed, histological diagnosis by upper gastrointestinal tract endoscopy





**Figure 4** Reactivation of disseminated tumor cells in the bone marrow: Transition from dormant micrometastasis (i.e., DTCs) to micrometastasis. Incidental activation of VCAM-1, a process possibly dependent on NF- $\kappa$ B signaling and other unidentified mechanisms (1), in micrometastasis arrests  $\alpha$ 4 $\beta$ 1-positive osteoclast progenitors through paracrine chemotaxis (2) and adhesion (3), this leads to a localized increase of the osteoclast progenitor population and increased mature osteoclast activity (4), activated osteoclasts resorb the bone and instigate the vicious cycle of bone metastasis (5). DTCs: Disseminated tumor cells. Adapted from Lu *et al*.<sup>[22]</sup>

should be performed. If primary lesions are not detected during screening, upper gastrointestinal tract endoscopy should be performed at the first instance, because gastric cancer is the most frequently observed cancer complicated with bone marrow carcinomatosis. Furthermore, a history of gastric cancer should always be acknowledged, because this disease can occur many years after gastric cancer surgery (Figure 6).

## TREATMENT

Although the survival time of patients with disseminated carcinomatosis of the bone marrow associated with gastric cancer was 2-3 mo at the time when its concept was proposed, it has improved to some degree because of recent progress in gastric cancer chemotherapy. However, this improvement has been limited, and a survival time of a few years has not been attained; thus, the prognosis for this disease remains extremely poor. The following categories are important for treatment of this disease: (1) treatment of hematological abnormalities, especially DIC; (2) treatment of gastric cancer (chemotherapy); and (3) treatment of bone lesions [with bone-modifying agents (BMAs)]. We retrospectively analyzed the treatment methods of 28 cases of disseminated carcinomatosis of the bone marrow associated with gastric cancer reported in Japan during the period of 2003-2013.

### Treatment of DIC

If a patient is diagnosed with DIC, treatment should be started promptly, because this affects prognosis. DIC coexisted in 22 of the 28 patients. Twenty-one of them received DIC treatment (gabexate mesilate + heparin) concomitantly with gastric cancer chemotherapy, and 19 fully recovered. Moreover, thrombomodulin, which was recently developed for DIC treatment, is superior to conventional treatment (i.e., heparin) for improving hemorrhagic symptoms and avoiding hemorrhage-related adverse events<sup>[44]</sup>. At present, thrombomodulin

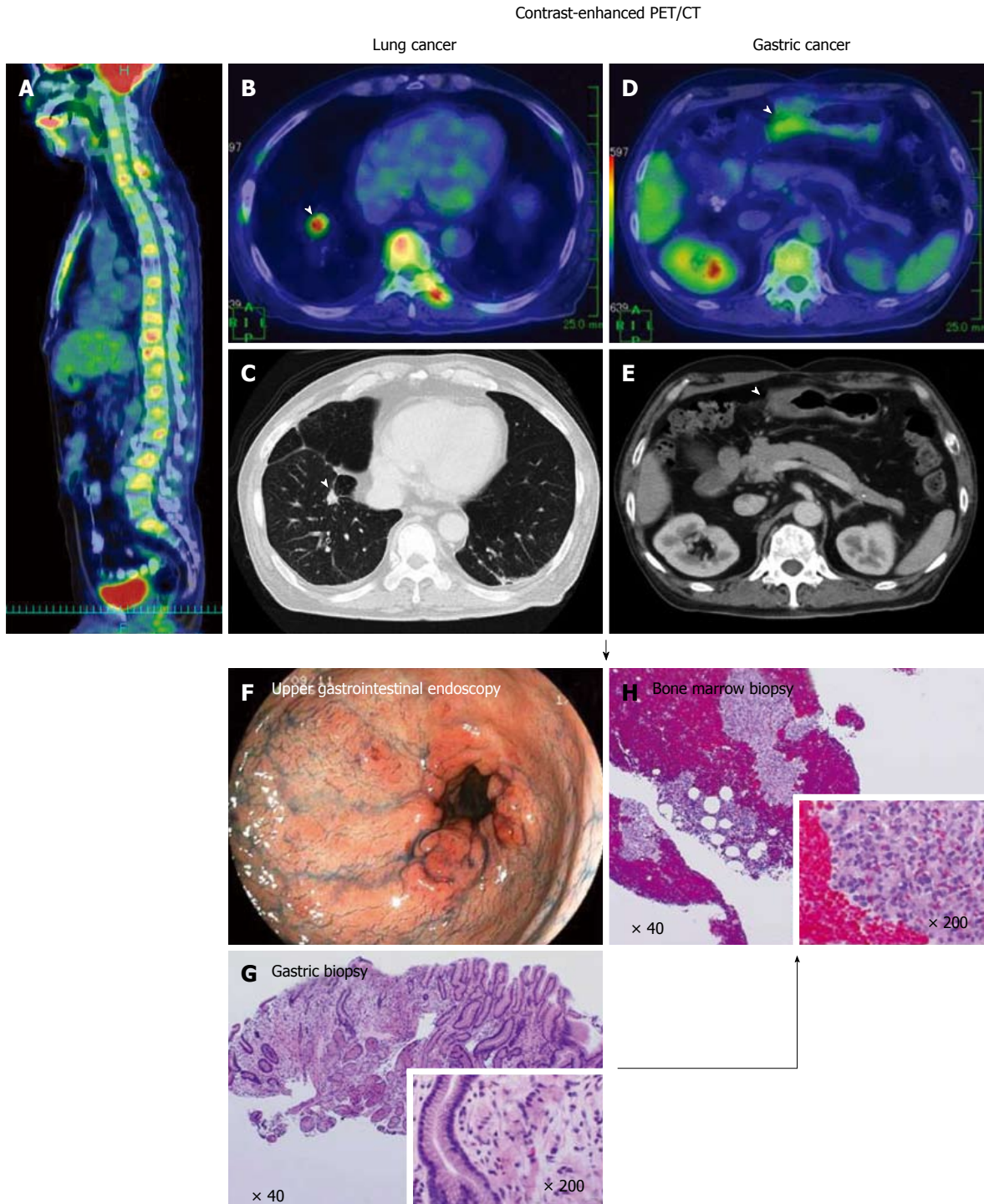
is primarily used for the treatment of DIC, but its efficacy against DIC coexisting with disseminated carcinomatosis of the bone marrow associated with gastric cancer is unknown. Thrombomodulin may increase the recovery rates of DIC patients and thereby improve the treatment outcome of disseminated carcinomatosis of the bone marrow associated with gastric cancer.

### Treatment of gastric cancer (chemotherapy)

Disseminated carcinomatosis of the bone marrow associated with gastric cancer is a rare disease, and its survival time is extremely short. There have been no prospective studies of the treatment for this disease to date. In 1980s, chemotherapy for gastric cancer was rarely administered because its options were limited and hematological disorders such as DIC and/or MAHA coexisted.

It was not until the 1990s that aggressive treatment with chemotherapy for this disease was started by Kobayashi *et al*.<sup>[45]</sup> In 1992, they reported that among 10 patients with disseminated carcinomatosis of the bone marrow associated with gastric cancer complicated with DIC treated with methotrexate and 5-fluorouracil (MF therapy), 8 successively recovered from DIC<sup>[45]</sup>. MF therapy was devised by Bertino *et al*.<sup>[46]</sup> as a biochemical modulation therapy. In Japan, its efficacy against gastric cancer was reported in 1989<sup>[47]</sup>. Because the myelosuppression of MF therapy is mild and it showed efficacy against poorly differentiated types of gastric cancer, it was the preferred chemotherapy for this disease in the 1990s. In the 2000s, the efficacy of new chemotherapeutic anticancer drugs such as S-1, CDDP, CPT-11, and PTX against gastric cancer was reported<sup>[48-50]</sup>. Furthermore, in Japan, on the basis of the results of the clinical trials (JCOG 9912 and SPIRITS), S-1 monotherapy and/or S-1 + CDDP therapy became standard therapies for gastric cancer<sup>[49,50]</sup>. Since then, S-1-based chemotherapies have primarily been used to treat disseminated carcinomatosis of the bone marrow





**Figure 5 A case of synchronous disseminated carcinomatosis of the bone marrow associated with gastric cancer in a 60-year-old man: Findings of positron emission tomography/computed tomography imaging, endoscopic examination, and bone marrow biopsy.** History: The patient visited a local doctor in August 2007 with complaints of left chest and back pain. CT and magnetic resonance imaging revealed a "lung tumor" and "bone metastasis (thoracic vertebrae)"; therefore, the patient was referred to the Shikoku Cancer Center. Laboratory findings: Marked elevation of serum ALP (2594 U/L), CRP (34.74 mg/dL), and mild elevation of serum LDH (342 U/L) were observed with hematological disorder (DIC and elevated WBC [possible leukoerythroblastosis]). Tumor marker (CA19-9 162 U/mL) and bone metabolic markers (1CTP 8.9 ng/mL; BAP 258  $\mu$ g/L) were elevated. These findings were suggestive of recurrence of gastric cancer, perhaps disseminated carcinomatosis of the bone marrow. PET/CT imaging: FDG accumulation was observed in all spinal vertebrae and the sternum in the sagittal view of the PET/CT fusion image (A). In the transaxial views of CT and PET/CT fusion images, a tumor mass with FDG accumulation was observed in the right lung (arrowheads; B, C) and thickening of the wall with FDG accumulation was observed in the gastric antrum (arrowheads; D, E). These findings on PET/CT suggested the presence of lung cancer together with gastric cancer. Endoscopic examination: Multiple erosions were observed in the gastric antrum/pyloric ring (F). In the tissue specimen obtained from the erosions, proliferation of signet-ring cell carcinoma was observed (G). Bone marrow biopsy: In order to determine the origin of the bone lesions, bone marrow biopsy was performed. Signet-ring cell carcinoma characterized by clear abundant cytoplasm and eccentrically positioned nuclei was found in the hematopoietic bone marrow (H). This histologic finding suggests the bone lesions originated from the gastric cancer. PET: Positron emission tomography; CT: Computed tomography.

PET/CT



**Figure 6** A case of metachronous disseminated carcinomatosis of the bone marrow associated with gastric cancer in a 47-year-old woman: Laboratory findings and positron emission tomography/computed tomography imaging. History: The patient underwent gastric cancer surgery at 36 years of age (histological diagnosis, poorly differentiated adenocarcinoma < signet-ring cell carcinoma), followed by chemotherapy with oral 5-FU (postoperative adjuvant therapy) for 3 years. Eleven years postoperatively, she visited a local orthopedist with a complaint of low back pain. Elevated serum ALP and multiple bone metastases were found on bone scintigraphy, and she was referred to the Shikoku Cancer Center. Laboratory findings: Marked elevation of serum ALP (11740 IU/L) and mild elevation of serum LDH (435 IU/L) were observed with hematological disorders (*i.e.*, DIC and anemia: Hb 6.9 g/dL). Tumor markers (CEA 241 ng/mL; CA19-9 212 U/mL) and bone metabolic markers (1CTP 17.7 ng/mL; Urine NTx > 300 nmol BCE/L; BAP 1260  $\mu$ g/L) were elevated. These findings were suggestive of recurrence of the gastric cancer in the bone (*i.e.*, disseminated carcinomatosis of the bone marrow). PET/CT imaging: Osteolytic changes with FDG accumulation were observed in most vertebrae. A, B: Sagittal views of CT (A) and PET/CT fusion images (B); C, D: Transaxial views of the sacrum (S1) on CT (C; arrowheads, osteolytic change) and PET/CT fusion image (D). PET: Positron emission tomography; CT: Computed tomography.

associated with gastric cancer.

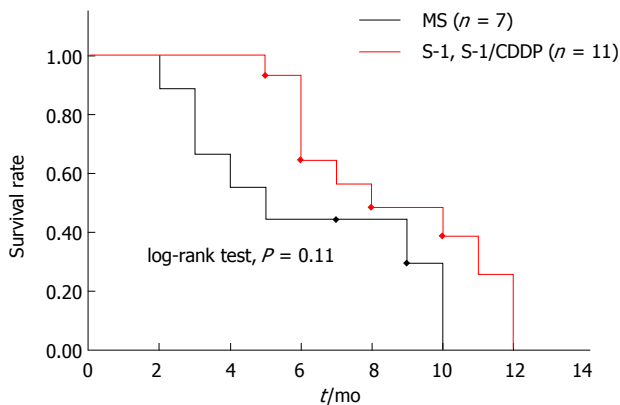
In recent years, a number of different treatments have been used to treat disseminated carcinomatosis of the bone marrow associated with gastric cancer. During the period of 2003-2013, 28 cases of this disease were reported in Japan<sup>[10,11,19,20,23-42]</sup>. The chemotherapy regimens (*i.e.*, initial treatments) used in most of these cases involved MF therapy ( $n = 9$ ) and S-1-based monotherapy or combination therapies ( $n = 14$ ). Other chemotherapies used included GEM + CDDP, 5-FU + PTX, and 5-FU + CDDP ( $n = 1$  each). One case was not treated with chemotherapy. Whether or not recovery from DIC is attained affects the prognosis of this disease. DIC-associated complications occurred in 22 of 28 patients; 21 of them were treated with DIC therapy (gabexate mesilate + heparin) and chemotherapy (MF therapy,  $n = 7$ ; S-1-based therapy,  $n = 12$ ; others,  $n = 2$ ). Nineteen patients recovered from DIC. The survival time with respect to the initial treatments (although this may have been affected by the secondary treatment performed against recurrent DIC) was examined for cases for which these data were recorded. The mean  $\pm$  SD survival times of the MF ( $n = 7$ ) and S-1-based ( $n = 11$ ) therapy groups were  $5.1 \pm 3.1$  and  $8.1 \pm 2.7$  mo, respectively (Figure 7); survival time tended to be longer in the S-1-based therapy group (log-rank test,  $P = 0.11$ ). Kikuchi *et al*<sup>[51]</sup> also reported a tendency for longer survival time with S-1-based therapy ( $n = 9$ ) than MF therapy ( $n = 21$ ), although the data used spanned the period between 1983 and 2009 when chemotherapy was not frequently administered.

Chemotherapy in addition to treatment for DIC is recommended for this disease to ensure recovery from DIC and prolonged survival. The S-1-based regimen is mainly used for gastric cancer in Japan; however, Ferrand *et al*<sup>[52]</sup> reported a case with this disease responsive to mFOLFOX6. Regimens effective for this disease should also be investigated in a prospective study.

### Treatment of bone lesions

Osteoclasts play an important role in the development of bone metastasis. The underlying, highly elaborate mechanism can be summarized as follows: the osteoclasts activated by cancer cells resorb the bone (bone destruction), securing a space for cancer cells to proliferate; concomitantly, various growth factors stored in the bone matrix are released into the bone marrow to facilitate cancer cells growth and propagation<sup>[3,4]</sup>.

Disseminated carcinomatosis of the bone marrow differs from bone metastasis of solid tumors in that



**Figure 7 Kaplan-Meier survival curves of disseminated carcinomatosis of the bone marrow associated with gastric cancer according to chemotherapy regimen.** Among 28 cases of disseminated carcinomatosis of the bone marrow associated with gastric cancer reported in Japan during 2003-2013, 27 cases were treated with chemotherapy. In these 27 cases, information about survival time was available for 18 cases. Using these data, Kaplan-Meier survival curves were calculated according to the chemotherapy regimen.

the cancer cells infiltrate the bone marrow but exhibit little tumorigenicity, characteristic clinical features are observed, and hematological abnormalities coexist. Although the mechanism for the development of disseminated carcinomatosis of the bone marrow is unknown, the disease is considered a subtype of bone metastasis. A mechanism similar to that of bone metastasis seems to occur during the infiltration and proliferation of cancer cells in the bone marrow. Marked elevation of serum ALP, which indicates increased bone formation, has been observed in this disease. Furthermore, bone resorption marker levels are reported to be elevated, although they have been measured in only a limited number of cases (Table 1)<sup>[10-12]</sup>. These other findings suggest increased bone metabolic turnover in this disease; in other words, the increased differentiation and activation of osteoclasts lead to elevated bone resorption. This mechanism may play an important role in the development of this disease similar to that for bone metastasis.

BMA s are drugs that inhibit bone metastasis by suppressing bone resorption by osteoclasts. Among them, a bisphosphonate (zoledronate) and denosumab have become reimbursable, and are used widely in clinical practice<sup>[53]</sup>. Increased bone resorption is also considered to be involved in the development of disseminated carcinomatosis of the bone marrow associated with gastric cancer; therefore, it is highly likely that BMAs are also effective for this disease. However, prospective studies are required, because there is currently no evidence for this.

## PROBLEMS TO BE ADDRESSED

### Pathogenesis

The pathogenesis of disseminated carcinomatosis of the bone marrow associated with gastric cancer is

postulated to be similar to the conventional mechanism of bone metastasis. Although osteoclasts may play a central role in this disease, few details are known. The hematological abnormalities observed in this disease are considered to be induced by the inhibition of normal hematopoiesis due to explosive proliferation of gastric cancer cells in the bone marrow; clarification of this mechanism may contribute to treatment of the disease. In addition, the development of *in vivo* animal models will be required. We previously established a lung cancer cell line with high PTHrP expression and used it to create an *in vivo* mouse model of bone metastasis<sup>[13]</sup>. This helped us elucidate the role of osteoclasts in the development of bone metastasis. *In vivo* models using gastric cancer cells with poorly differentiated adenocarcinoma and/or signet-ring cell carcinoma are required to clarify the pathogenesis of disseminated carcinomatosis of the bone marrow associated with gastric cancer.

This disease often occurs sometime after surgery; therefore, the mechanism of the metachronous development of the disease is also a topic of interest. The metachronous presentation of this disease may be explained by the presence of a cancer cell niche (*i.e.*, DTCs) in the bone marrow. Osteoclasts are involved in the activation of DTCs, and bisphosphonates, which are widely used to treat bone metastasis in clinical practice, have been shown to inhibit the reactivation of DTCs<sup>[21,22]</sup>. Therefore, it would be interesting to apply this drug in a clinical setting.

### Gastric cancer chemotherapy

The treatment outcomes of gastric cancer following chemotherapy have improved in recent years owing to the advent of molecular-targeted drugs<sup>[54-56]</sup>. On the contrary, a standard treatment for disseminated carcinomatosis of the bone marrow associated with gastric cancer has not been established. Because this disease is frequently associated with poorly differentiated adenocarcinoma and/or signet-ring cell carcinoma, regimens effective against these histological types of carcinoma are preferably administered. However, there have been no prospective studies, as the incidence rate of this disease is low. It would be beneficial to develop a system to record disease cases to allow prospective studies for the development of a treatment method specific for this disease, although conducting randomized controlled trials may prove difficult.

### Treatment of bone lesions

Osteoclasts play a central role in the pathogenic mechanism of bone metastasis; therefore, drugs targeting osteoclasts (*i.e.*, BMAs) are widely used in clinical practice. Despite circumstantial evidence such as the elevation of serum bone resorption markers, which suggests the involvement of osteoclasts in disseminated carcinomatosis of the bone marrow associated with gastric cancer, this has not been



verified by scientific evidence. The hematological abnormalities of this disease are possibly triggered by the explosive proliferation of gastric cancer cells in the bone marrow; therefore, inhibiting this process may be key to the treatment of this disease. The concomitant administration of chemotherapeutic agents and BMAs that block the supply of bone-derived growth factors may effectively prevent the rapid proliferation of gastric cancer cells. Thus, I recommend that BMAs should be administered in addition to chemotherapy especially for the patients in whom serum bone resorption markers are elevated. However, its efficacy will be verified in the prospective studies of gastric cancer chemotherapy with or without BMAs.

### Follow-up observations after gastric cancer surgery

Follow-up observations after gastric cancer surgery generally stop at 5 years. However, metachronous disseminated carcinomatosis of the bone marrow associated with gastric cancer can occur beyond 5 years postoperatively (Figure 6); this has raised questions about the optimum postoperative follow-up schedule. If a high-risk group of patients can be identified, guidelines could be established to follow this group for more than 5 years. This might facilitate early diagnosis and treatment of this disease, thereby improving treatment outcomes. The clinical and pathological characteristics of disseminated carcinomatosis of the bone marrow associated with gastric cancer are summarized in the section of "DIAGNOSIS". In addition, it is necessary not only to establish criteria to identify high-risk groups, but also develop a detection method for DTCs or determine biomarkers including those for circulating tumor cells. All of these may lead to the early diagnosis of this disease.

## CONCLUSION

More than 30 years have elapsed since the concept of disseminated carcinomatosis of the bone marrow was proposed. However, this disease has not been given much attention perhaps because of low incidence. Therefore, prognosis has not improved much during this time. In order to clarify the pathogenesis of this disease and improve its prognosis, the first step may be for researchers interested in this disease to collaborate on a global research system to study this disease.

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## 2015 Advances in Gastric Cancer

# Targeting the PI3K/Akt signaling pathway in gastric carcinoma: A reality for personalized medicine?

Shikha Satendra Singh, Wei Ney Yap, Frank Arfuso, Shreya Kar, Chao Wang, Wanpei Cai, Arunasalam M Dharmarajan, Gautam Sethi, Alan Prem Kumar

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## Abstract

Frequent activation of phosphatidylinositol-3 kinases (PI3K)/Akt/mTOR signaling pathway in gastric cancer (GC) is gaining immense popularity with identification of mutations and/or amplifications of *PIK3CA* gene or loss of function of PTEN, a tumor suppressor protein, to name a few; both playing a crucial role in regulating this pathway. These aberrations result in dysregulation of this pathway eventually leading to gastric oncogenesis, hence, there is a need for targeted therapy for more effective anticancer treatment. Several inhibitors are currently in either preclinical or clinical stages for treatment of solid tumors like GC. With so many inhibitors under development, further studies

on predictive biomarkers are needed to measure the specificity of any therapeutic intervention. Herein, we review the common dysregulation of PI3K/Akt/mTOR pathway in GC and the various types of single or dual pathway inhibitors under development that might have a superior role in GC treatment. We also summarize the recent developments in identification of predictive biomarkers and propose use of predictive biomarkers to facilitate more personalized cancer therapy with effective PI3K/Akt/mTOR pathway inhibition.

**Key words:** Epidemiology; Gastric cancer; PI3K/Akt/mTOR pathway; Predictive biomarkers; Targeted therapy

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**Core tip:** Gastric cancer (GC) is the fifth most common cancer in the world with highest incidence rate in Eastern Asia and Latin America. With increase in GC patient relapse and drug resistance, targeted therapy is gaining immense popularity for GC treatment. One of the pathways which has been reported to be dysregulated is phosphatidylinositol-3 kinases (PI3K)/Akt signaling pathway. This review focuses on how this pathway is crucial in GC oncogenesis. We also summarize the single or dual PI3K/Akt pathway inhibitors under investigation for GC treatment. Thereby, we discuss the plausible novel biomarkers under investigation for a more tailored approach for GC treatment.

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## INTRODUCTION

The phosphatidylinositol-3 kinases/Akt (PI3K/Akt) signaling pathway is activated by several cellular stimuli regulating various physiological functions such as cell growth, cell survival, cell cycle progression, protein translation, and metabolism. Dysregulation of this pathway is frequently observed in several cancers including gastric cancer (GC). Hence, a deeper understanding of this signaling pathway would help target this pathway effectively using different therapeutic approaches. In this review we will focus on how this pathway is regulated in GC and the current status of using PI3K/Akt/mammalian target of rapamycin (mTOR) targeted therapy for GC treatment.

## PI3K/AKT/MTOR PATHWAY

Several members of PI3K/Akt/mTOR pathway play a crucial role in regulating this pathway and hence, maintaining cellular homeostasis under normal physiological conditions. Some of these essential components are described below.

### PI3K

PI3K are a family of lipid kinases known to phosphorylate the inositol ring of the 3-OH group in inositol phospholipids. They are further classified into three classes: Class I, II, and III based on primary structure and regulation<sup>[1]</sup>. However, till date only Class I, assisting in tight regulation of this pathway, has been shown to be associated with cancer. Class I PI3K is a heterodimeric enzyme, with a catalytic and a regulatory subunit. The catalytic subunits for class I PI3Ks are p110 $\alpha$ , p110 $\beta$ , p110 $\gamma$ , and p110 $\delta$ . It is further subdivided into class1A, encompassing p110 $\alpha$ , p110 $\beta$ , and p110 $\delta$  with a p85 $\alpha$ , p85 $\beta$ , and p55 $\gamma$  regulatory subunit, and class1B consisting of only p110 $\gamma$  with p101, p84, and p87PIKAP regulatory subunits. A typical regulatory subunit has several protein-protein interacting domains, one of them, the inter-SH2 domain (iSH2) interacts with the p110 catalytic subunit, stabilizing p110 and its activities<sup>[2]</sup>. Reports have suggested the p110 $\alpha$  subunit encoded by the PIK3CA gene as being the only catalytic subunit associated with several cancers<sup>[3]</sup>. p110 $\alpha$  typically links with p85 $\alpha$ , which is the most highly expressed regulatory subunit. The substrate for class1 PI3K, phosphatidylinositol-4,5-bisphosphate (PI-4,5-P<sub>2</sub>), generates the second messenger phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P<sub>3</sub>).

### Akt

Serine/threonine protein kinase Akt belongs to the AGC [named after the protein kinase A, G, and C families (PKA, PKC, PKG)] family of kinases. Three highly homologous isoforms (Akt1, Akt2, and Akt3) of Akt have been identified so far. Structurally, Akt is mainly comprised of three domains: an N-terminal pleckstrin homology (PH) domain, a central kinase catalytic domain (CAT), and a C-terminal extension (EXT) containing a regulatory hydrophobic motif (HM). Phosphorylation of residues in both the catalytic and C-terminal extension domain is essential for complete activation of Akt downstream of PI3K signaling. PDK1 selectively phosphorylates Thr (308) on the CAT domain of Akt, while the kinases responsible for phosphorylation of Ser (473) on the EXT domain of Akt are still unknown<sup>[4]</sup>. Thus, phosphorylation of both Ser (473) and Thr (308) residues on Akt is required for its complete activation<sup>[5,6]</sup>. Fully activated Akt further regulates several processes downstream, along with positive regulation of mTOR and thereby mediating



mTOR activation.

### **mTOR**

The mTOR protein, a 289-kDa serine/threonine kinase, is a master regulator of cell growth. It can be distinguished into two distinct multi-protein complexes; mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The mTORC1 complex is composed of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein8 (MLST8), PRAS40, and DEPTOR. It functions downstream of Akt, integrating the Akt and mTOR pathway with 4E-BP1 and S6K, which are immediate downstream targets of the mTORC1 complex. The mTORC2 complex, on the other hand, is composed of rapamycin-insensitive companion of mTOR, MLST8 and mammalian stress-activated protein kinase interacting protein 1. This complex functions upstream of Akt/PKB and plays a role in complete activation of Akt by phosphorylating Akt at the Ser473 residue. Hence, downstream substrates of the mTORC2 complex include Akt<sup>[7]</sup> as well as PKC<sup>[8,9]</sup>. Hence, both complexes are important for effective regulation of the Akt/mTOR dual pathway, with the mTORC1 complex responsive towards growth factors, nutrients, energy, or oxidative stress indirectly while the mTORC2 complex plays an important role towards Akt activation to assist in complete activation of the PI3K/Akt/mTOR pathway.

### **ACTIVATION MECHANISM**

Several receptor tyrosine kinases regulate the activation of the PI3K/Akt/mTOR pathway upon growth factor stimulation. Growth factors such as Insulin growth factor (IGF), epidermal growth factor (EGF), and Hepatocyte growth factor activate receptor tyrosine kinases (RTKs) *via* autophosphorylation on their tyrosine residues. Lipid kinases, such as PI3K, then associate with these phosphorylated tyrosine residues to activate the catalytic subunit of PI3Ks. For PI3Ks of class1A, the p110 $\alpha$  catalytic subunit is activated upon p85 $\alpha$  associating with the RTKs. Activated PI3Ks further phosphorylate substrates like phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate (PIP3) within a few seconds. Secondary messengers such as PIP3 further recruit Akt to the membrane by interacting with the PH-domain of Akt. Upon membrane translocation, AKT gets activated by phosphorylation of its Ser473 and Thr308 residues by the PDK1 and mTORC2 complex respectively. Fully activated Akt then regulates several cellular processes by interacting with different substrates downstream of Akt. In the meanwhile, PTEN, a PIP3 phosphatase, acts a regulator of this pathway by maintaining homeostasis for this pathway activation. Activated Akt stimulates the mTORC1 complex by phosphorylating tuberous

sclerosis complex2 (TSC2) and PRAS40, which are both negative regulators of mTOR. The mTORC1 complex controls protein translation and cell growth by phosphorylating ribosomal S6 kinase and the inhibitory partner of the translation initiation factor 4E (4E-BP1), which are regulators of protein synthesis<sup>[10]</sup>. Thus, under normal physiological conditions, Akt regulates cellular dynamics such as cell growth, cytoskeletal reorganization, cell cycle progression, cell survival, cell proliferation, protein translation, and cellular metabolism by interacting with various substrates, which will now be discussed in more detail.

## **CELLULAR ROLE OF THE AKT/mTOR PATHWAY**

### ***Cell survival and cell cycle progression***

Akt acts as a central regulator of cell survival by interacting with anti-apoptotic signals both transcriptionally and post translationally. Akt phosphorylates Bad, a Bcl-2 family of anti-apoptotic proteins at Ser-136 and Caspase-9, a protease at Ser-196, thereby partially blocking cell death and supporting cell survival signals. Akt also regulates anti-apoptotic functions transcriptionally by translocating into the nucleus and regulating the transcription of the forkhead box O (FoxO) family of transcription factors. The FoxO family of transcription factors regulate cell death signals *via* expression of various members of both intrinsic and extrinsic modes of apoptosis as well as cyclin-dependent kinase inhibitors. Upon nuclear translocation, Akt represses the transcription of FoxO1, FoxO3, and FoxO4, thereby enhancing cell survival signals<sup>[11]</sup>.

Akt also plays an important role in regulating cell cycle progression in normal cells. It either directly phosphorylates or indirectly regulates the protein expression levels of several molecules of cell cycle progression at the G1/S and G2/M phase of the cell cycle. These substrates are mentioned in Table 1.

### ***Cellular metabolism and protein synthesis***

Cellular metabolism of carbohydrates into proteins, nucleotides, and lipids is a fundamental aspect of cell growth and proliferation, with nutrients acting as a fuel for cell growth. mTOR plays a crucial role in regulating this metabolism in response to nutrient availability. Of the two mTOR complexes, the mTORC1 complex plays a key role in regulating cellular metabolism<sup>[12]</sup>. It receives signals of activation from nutrients and growth factors. For example, during metabolism of carbohydrates, there is a spike in insulin levels, which activates the mTORC1 complex of the Akt/mTOR pathway *via* inhibition of the TSC1/2 complex by phosphorylation of TSC2 at multiple sites to inhibit TSC1<sup>[13]</sup>. In this process, eventually Ras homolog enriched in brain (Rheb), a small GTPase belonging to the Ras family of guanine-nucleotide

**Table 1** Role of Akt in regulating cell cycle

Cell cycle regulation by Akt upon mitogen stimulation			
Cell cycle phase	Direct regulation	Indirect regulation	Downstream effect
G1 → S phase	P-p21 at Thr145 residue <sup>[107]</sup>	↑ Transcription of c-MYC <sup>[108]</sup>	Increase in CyclinD expression Decrease in cdk inhibitors: p21 <sup>cip1</sup> , p21 <sup>kip1</sup> , p15 <sup>INK4B</sup>
G2 → M phase	P-Cdc25B at Ser353 <sup>[109]</sup>		Cdc-25B inactivation Cyclin B activation
	P-Wee1Hu at Ser642 <sup>[110]</sup>		Inactivation of Wee1Hu results in G2/M cell cycle progression
	P-Myt1 at Ser75 <sup>[111]</sup>		Activation of Cyclin B-associated Cdk1 kinase activity
S → G2 phase	P-Cdk2 at Thr39 <sup>[112]</sup>		Cytoplasmic shuttling of Cdk2

binding proteins that enhances apoptotic signalling at cellular levels<sup>[14]</sup>, is inhibited upon TSC1 complex inactivation. The mTORC1 complex is also stimulated in the presence of amino acids by promoting the conversion of Ras-related GTP-binding protein (RAG) heterodimers (RAGA or RAGB, and RAGC or RAGD) into their active conformation, which further assists in mTORC1 complex cellular localization from the cytoplasm to the surface of the lysosome where it binds to inactivated RHEB<sup>[15-17]</sup>. The activated mTORC1 complex also tightly regulates pathways such as the AMP-activated protein kinase (AMPK) pathway by preventing its activation in the presence of a high ATP/AMP ratio. However, in the absence of energy in cells, AMPK gets activated by phosphorylating TSC2 at Ser1387 and Raptor from the mTORC1 complex at Ser-792, resulting in mTORC1 inactivation<sup>[18,19]</sup>. After mTORC1 activation and subsequent complete activation of the Akt/mTOR pathway, immediate downstream substrates of mTORC1 complex such as S6K (ribosomal S6 kinase), 4E-BP1, and ULK1 (UNC-51 like kinase) are phosphorylated at different residues. Interestingly, activated S6K further phosphorylates Insulin receptor substrate-1 (IRS-1), upstream of mTORC1. Phosphorylation of IRS-1 at serine residues by S6 kinases prevents IRS-1 functions and thereby PI3K activation<sup>[20]</sup>. This negative feedback loop of the PI3K/Akt/mTOR pathway is an important aspect of maintaining homeostasis in cellular metabolism, protein synthesis, and cell growth.

## ONCOGENIC POTENTIAL OF PI3K/AKT/mTOR PATHWAY IN GC

Dysregulations caused by genetic alterations of the PI3K/Akt/mTOR pathway have been recently identified to play a crucial role in gastric oncogenesis. GC is the second most common cause of cancer-related death worldwide. *PIK3CA*, the gene encoding the catalytic subunit p110 of PI3K, is frequently mutated in gastric carcinoma cell lines and tumor tissues. Some reports identify mismatch repair deficiency as one of the factors contributing towards the *PIK3CA* mutations<sup>[21-23]</sup>. Another study suggested *PIK3CA* amplifications contributing towards the PI3K/Akt/mTOR pathway

deregulation in GC<sup>[23]</sup>. This *PIK3CA* amplification was also associated with poor prognosis of GC patients.

PTEN (Phosphatase and tensin homolog), a tumor suppressor of the PI3K/Akt/mTOR pathway is frequently mutated or abnormally expressed in GC, with eventual functional inactivation of this gene product. Its inactivation is associated with increased progression towards gastric tumorigenesis. This inactivation is attributed to many possible mechanisms. While PTEN gene mutations are a rare phenomenon of PTEN inactivation, loss of heterozygosity of PTEN is more common in GC<sup>[24,25]</sup>. Abnormal PTEN promoter hypermethylation at the CpG islands also inhibits PTEN expression in GC tissues<sup>[26]</sup>. Post-transcriptional repression of PTEN by microRNAs is another well studied mechanism of PTEN repression in GC. miR21 and miR-221/222 have been identified as PTEN targets repressing PTEN expression by complexing with its 3'-UTR region<sup>[27-29]</sup>. PTEN also undergoes post-translational modifications like phosphorylation for its regulation. A recent study indicated that increased phosphorylation of PTEN at the Ser380 residue and reduced expression of PTEN could contribute to PTEN inactivation in gastric tumor tissues<sup>[30]</sup>. Overall, PTEN inactivation has several functional consequences that fall in the category of hallmarks of cancer, such as angiogenesis and evading apoptosis. PTEN has been shown to elevate the apoptotic cascade *via* Fas/FasL or cytochrome-c mediated activation of caspase-3 under normal physiological functions. Hence, with PTEN inactivation in GC cells, bypassing apoptosis *via* dysregulation of the apoptotic cascade will result in "evading apoptosis", a hallmark of cancer<sup>[31,32]</sup>. PTEN is also known to be a negative modulator of endogenous VEGF-mediated signaling. Thus, PTEN inactivation is associated with increased VEGF expression, and thereby potentiating angiogenesis in GC cells<sup>[33]</sup>. PTEN inactivation also results in constitutive activation of Akt, a PKB kinase regulating cell growth, cell death, and cell cycle. Using gastric tumor specimens, there has been a statistically significant correlation demonstrated between increased phosphorylation of Akt with poor prognosis of GC patients<sup>[34-36]</sup>. Functionally, this constitutive expression of phosphorylated Akt further contributes towards hallmarks of cancer such as

escaping cellular death pathways, cell cycle inhibition, and promoting survival and angiogenesis. One of the immediate downstream substrates of Akt is the FoxO family of transcription factors, which promotes growth inhibitory or/and pro-apoptotic signals by either regulating cell cycle inhibitory proteins such as p21<sup>KIP1</sup> or p27<sup>WAF1/CIP1</sup> or pro-apoptotic proteins of the Bcl-2 family of proteins<sup>[37,38]</sup>. Activated Akt phosphorylates FoxO and thereby inhibits transcriptional functions of this family of proteins, resulting in increased cell survival and proliferation<sup>[37]</sup>. Constitutive phosphorylation of FoxO is also correlated with increased expression of angiogenesis-related molecules in gastric tumor tissues<sup>[37]</sup>. Akt also directly phosphorylates anti-apoptotic proteins such as Bad at Ser-136. Thus, GCs with increased Akt expression show elevated levels of P-Bad at Ser136 that are sufficient to promote cell survival<sup>[39]</sup>. Another important substrate of Akt that acts as an initiator of the mitochondrial apoptotic pathway is caspase-9. Phosphorylation of caspase-9 at Ser-196 results in its inactivation. There is a significant correlation between constitutive phosphorylation of Akt with caspase-9 phosphorylation in gastric tumor tissues, although the mechanism still remains unclear<sup>[40]</sup>. This apoptosis resistance conferred by p-Akt also occurs by regulating increased expression of survivin, an inhibitor of apoptosis protein with a significant correlation between p-Akt and survivin expression levels in gastric tumor staging<sup>[39]</sup>. Pro-survival signals by Akt can also be intensified with its interaction with components of other signaling pathways such as the NF- $\kappa$ B pathway. Akt can phosphorylate NF- $\kappa$ B kinases such as I $\kappa$ B kinase (IKK $\alpha$ ) at Thr23, resulting in a stimulatory phosphorylation and thereby NF- $\kappa$ B activation. This further augments the expression of pro-survival signals in GC cells<sup>[41,42]</sup>. Another immediate downstream target of Akt is the mTORC1 complex, which is activated by phosphorylation of TSC2 by Akt and subsequent TSC1/2 complex formation, which acts on RHEB (Ras homolog enriched in brain) to further phosphorylate mTOR at Ser2448 and thereby resulting in mTOR activation. Therefore, low expression or mutations in TSC1 are associated with a dysfunctional TSC1/2 complex and constitutively activated mTORC1 complex<sup>[43]</sup>. Recent studies have identified high p-mTOR expression to be associated with poor prognosis and with some clinicopathological characteristics in GC tumor specimens both independently and in combination with low TSC1 expression<sup>[44,45]</sup>. A preliminary epidemiological study identified functional polymorphisms of mTORC1 contributing towards GC susceptibility in Eastern Chinese population<sup>[46]</sup>. With studies focussing on mTORC1 complex dysregulation and functional consequences, further studies are important to understand mTORC2 dysregulations, the cause of which still remains unclear. Immediate downstream effectors for the activated mTORC1 complex are p70S6K and 4E-BP1. p70S6K phosphorylation and activation, which mainly occurs in

the cytoplasm, results in translation of 40s ribosomal S6 protein, while phosphorylated 4E-BP1 acts as a translational repressor. Recent studies have also shown p70S6K and mTOR regulating each other, with p70S6K also acting as a kinase for mTOR phosphorylation at Ser2448<sup>[47]</sup>. Aberrant expression of p-p70S6K is linked to GC carcinogenesis and its aggressiveness. Nuclear localization of this protein could have some inhibitory effects towards GC pathogenesis<sup>[48]</sup>.

## PRECLINICAL STUDIES AND ONGOING CLINICAL TRIALS WITH PI3K/AKT INHIBITORS

Involvement of several molecules of the PI3K/Akt/mTOR pathways in GC carcinogenesis has eventually led to development of both single, as well as recently, dual inhibitors essential for molecular targeted therapy for GC.

### PI3K inhibitors and GC

PI3K inhibitors are classified into three classes based on their selectivity for the ATP-binding cleft on PI3K and pharmacokinetic properties: pan-class I, isoform-selective, and dual PI3K/mTOR inhibitors<sup>[49,50]</sup>.

### Pan-Class I inhibitors

Pan-class I inhibitors have inhibitory effects against each isoform of p110 (PIK3CA). Several pan-class I inhibitors are under investigation for GC targeted therapy, since PIK3CA gene mutations comprise 25% of gastric tumors, resulting in PI3K dysregulation in GC<sup>[51]</sup>. The first report of a molecular agent inhibiting PI3K was quecertin, which was, however, a non-specific kinase inhibitor<sup>[52]</sup>. Eventually, more specific pan-class I inhibitors were identified, such as Wortmannin and a quecertin analogue, LY294002. Although both LY294002 and Wortmannin exhibited potent PI3K-inhibitory properties, there were considerable limitations for them to proceed towards clinical trials<sup>[52-54]</sup>. LY294002 showed non-specific targeting, a short half-life, and toxicity *in vivo*<sup>[55,56]</sup>, while Wortmannin had limitations involving biological stability and short half-life<sup>[57]</sup>.

To further improve on the pharmacological availabilities, a structural analogue of Wortmannin, PX-866 was developed.

**PX-866:** PX-866 is a semisynthetic pan-class-1, Wortmannin analogue with inhibitory concentrations in nanomolar ranges and better efficacy and a safer pharmacokinetic profile than Wortmannin. Pre-clinical *in vivo* studies have shown its anti-cancer effect against several xenograft models of various cancers<sup>[57,58]</sup>. It is currently in Phase II clinical trials for patients with glioblastoma and head and neck cancer<sup>[59,60]</sup>.

PX-866 recently also came under limelight for a multicenter trial for advanced solid tumors including gastric tumors. Data from the trial show that PX-866 can be administered with endurable toxicity for patients with advanced solid tumors<sup>[61]</sup>.

**NVP-BKM120 (Buparlisib):** NVP-BKM120 is a potent pan-class I PI3K inhibitor with its activity in nanomolar ranges for all the isoforms of Class I PI3K. Preclinical investigations have revealed its effectiveness in a diverse range of cancer cell lines, with increased sensitivity in tumors harboring PIK3CA mutations<sup>[62]</sup>. Similar results were also observed in a panel of GC cell lines<sup>[63]</sup>. Additionally, combination therapy using PI3K and STAT3 inhibitors showed better efficacy and a synergistic effect in GC cell lines harboring KRAS mutations. The STAT3 pathway is also known to be constitutively activated in GC<sup>[64]</sup>. Although preclinical studies on BKM120 in GC are still ongoing, it has reached Phase II clinical trials for other cancers such as brain, breast, colorectal, endometrial, NSCLC, and renal cell carcinoma<sup>[49,65-67]</sup>. Thus, BKM120 has a potential to progress into clinical trials for GC treatment using targeted therapy.

**ZSTK474:** ZSTK474, a pan-class 1 PI3K inhibitor inhibits all the four isoforms of PI3K and exhibits anti-tumor activity *in vivo* against human tumor xenograft models<sup>[68-70]</sup>. *In vitro* studies in GC cell lines suggest combination therapy of ZST474 and IGFR inhibitors for treating IGFR-positive cancers to overcome any intrinsic resistance to inhibition of PI3K/Akt/mTOR signaling, since over-expression of IGFR correlated with increased tyrosine phosphorylation on Insulin Receptor substrate, leading to increased PI3K activation. Hence, combination therapy with both ZST474 and IGFR inhibitors on GC cells with high IGFR expression exerted a superior therapeutic response<sup>[71]</sup>.

**BAY80-6946:** BAY80-6946 is synthesized by Bayer healthcare and is a highly potent, selective, and reversible pan-class I inhibitor working in nanomolar concentrations against all the isoforms of p110. However, it shows preferential activity against p110 $\alpha$  and  $\beta$  than p110  $\gamma$  and  $\delta$  in tumor cell lines and xenograft models<sup>[72]</sup>. BAY80-6946 demonstrated acceptable safety profiles in phase I clinical trials for advanced solid tumors, and therefore, exhibiting a potential to be progressed to phase II clinical trials for patients with advanced solid tumors.

#### ***Isoform specific PI3K inhibitors***

PI3K isoform specific inhibitors were designed with an aim to provide comparable or superior efficacy than pan-class I inhibitors. Some of them under investigation for GC treatment will now be discussed.

**BYL719:** BYL719 is an  $\alpha$ -isoform specific PI3K inhibitor

working at nanomolar concentrations with minimal activity against other PI3K isoforms<sup>[73]</sup>. With the PI3K/Akt/mTOR pathway being frequently dysregulated in GC, BYL719 exhibited its inhibitory effects in synergy with another inhibitor LJM716, a ligand dependent as well as independent HER3 inhibitor, in GC xenograft models<sup>[74]</sup>. Interestingly, the combination study was done in HER2 positive GC cell lines, suggesting the sensitivity of this drug towards HER amplifications. BYL719 also recently completed Phase1b clinical trial for advanced stage GC in a combinational study with the HSP90 inhibitor AUY922 in patients whose tumors either harbour molecular alterations of PIK3CA or HER2 amplification<sup>[75]</sup>.

**INK1117:** INK117 is another novel, selective p110 $\alpha$  inhibitor. It is particularly more effective and sensitive to tumors bearing PIK3CA mutations. With good oral bioavailability in preclinical xenograft studies, it has entered a phase- I study for advanced solid tumors including GC, to evaluate its safety, tolerability, pharmacokinetic and pharmacodynamic properties<sup>[76]</sup>.

#### ***Dual PI3K/mTOR inhibitors***

PI3K/mTOR dual inhibitors inhibit PI3K and the downstream mTOR kinase activity by binding to the ATP-binding cleft of these enzymes. Relative to the single inhibitors, these drugs have the benefit of inhibiting mTORC1 and mTORC2, as well as all the isoforms of PI3K. Evidence has suggested that the mTORC1/S6K axis has a "two-edge sword"-like function in activation of the PI3K/mTOR pathway by promoting growth signals downstream of Akt, as well as mediating a potent negative feedback loop that restrains signaling *via* the insulin/IGFR and other RTKs. Dysregulation of this negative feedback loop has been reported to contribute towards resistance in cancers subjected to single inhibitors<sup>[77]</sup>. Hence, the need of dual PI3K/mTOR inhibitors arises with an aim to discover drugs with low toxicity and good pharmacokinetic profile.

**NVP-BE2235:** NVP-BE2235 is a novel dual ATP-competitive PI3K and mTOR inhibitor for p110 $\alpha/\beta/\gamma/\delta$  and mTOR kinase, with inhibitory doses at nanomolar ranges. It first entered phase trials for breast cancer<sup>[78]</sup>. The effectiveness of BE2235 has been investigated in both PIK3CA mutated and wild type cell lines *in vitro* and in xenograft models *in vivo*. The first group reporting an effect of BE2235 on gastric xenografts showed reduced tumor growth for NCI-N87 but not MKN-45 or MKN-28 xenografts. Interestingly, the reduction in tumor growth correlated with thymidine kinase expression and not PI3K/mTOR pathway inhibition<sup>[79]</sup>. Another group demonstrated *in vitro* increased sensitivity of AGS, PIK3CA mutated cells than for NCI-N87 and MKN-45, wild type PIK3CA GC cells<sup>[63]</sup>. Clinically, the response rate for BE2235 was



highest for patients with PIK3CA mutations than those without this mutation<sup>[80]</sup>. However, another recent study showed an increased anti-tumor response with BEZ235 alone or in combination with nab-paclitaxel in NCI-N87, AGS, and SNU-16 GC cells, independent of PIK3CA mutation status *in vitro* and in a SNU16 xenograft model *in vivo*<sup>[81]</sup>. Hence, with increasing preclinical studies focusing on using NVP-BEZ235 for GC targeted therapy, and its gaining popularity in clinical trials for other cancers, NVP-BEZ235 might be a good potential candidate drug to be considered for clinical trials for solid tumors such as GC.

**VS-5884:** VS-5884 is a dual PI3K and mTOR inhibitor, inhibiting all the isoforms of PI3K and both mTOR complexes (mTORC1 and mTORC2), with nanomolar inhibitory concentrations for a panel of cancer cell lines *in vitro* and increased sensitivity towards cell lines harboring PIK3CA mutations. It also exhibits a favourable pharmacokinetic profile *in vivo*. VS-5884 shows a statistically significant inhibition of tumor growth in HER-over-expressing GC xenograft models (NCI-N87). This drug also exhibits a synergistic response in these xenograft models with gefitinib, an EGFR inhibitor (EGFRi), currently in phase II trials for GC treatment. Since this drug has proven its efficacy for monotherapy and combination therapy in GC xenograft models<sup>[82]</sup>, these data provide a rationale for testing VS-5884 in early phase clinical trials for GC patients.

**PI-103:** PI-103 is an ATP-competitive PI3K and mTOR inhibitor with variable sensitivities towards different isoforms of p110 and mTOR at nanomolar concentrations. It was recently assessed for its synergistic effect to enhance the anti-tumor response for GC both *in vitro* and *in vivo* with 5-FU treatment. This study suggested PI-103 usage for enhancing 5-FU chemotherapy for GC; with 5-FU currently being used to treat GC patients but demonstrating limitations due to inter-variability in response rate of these patients. This synergistic effect of PI-103 with 5-FU was also associated with PIK3CA mutations and reduction of downstream effectors of PI3K/Akt/mTOR pathway and thymidylate synthase, an enzyme that generates thymidylate precursors for DNA synthesis *in vitro*<sup>[83]</sup>.

### Akt inhibitors and GC

The Akt signaling cascade controls a spectrum of tumorigenesis events such as cell growth, proliferation, survival, angiogenesis, invasion, and metastasis, as well as activation of the mTOR signaling cascade. Several mutations or amplifications in the Akt/mTOR signaling cascade contribute towards constitutive activation of Akt, which includes PTEN mutations and PIK3CA mutations, as well as over-expression/amplification of Akt itself<sup>[84-87]</sup>. With activated Akt playing a crucial role in tumorigenesis, several Akt inhibitors have been designed that have entered

preclinical as well as clinical trials. Akt inhibitors that are currently being investigated for GC treatment include:

**AZD5363:** AZD5363 binds to and inhibits all the isoforms of Akt (Akt1, Akt2, and Akt3) with potency in the nanomolar range. Reports have suggested increased sensitivity towards AZD5363 in cancer cells harboring PIK3CA mutations, PTEN mutations, or HER-2 amplifications both *in vitro* and *in vivo*<sup>[88]</sup>. This sensitivity of AZD5363 towards the activating mutations was also tested in GC xenograft models, one with a PIK3CA mutation and another with PTEN loss. AZD5363 exhibited a relatively more significant anti-tumor response towards PIK3CA mutant GC xenografts than those with PTEN loss alone. Interestingly, for GC xenografts with inactivated PTEN treated with a combination of AZ5363 and taxotrene, a synergistic and potent anti-tumor response was observed rather than monotherapy with AZD5363<sup>[89]</sup>. Hence, selection of patients based on their mutational status would be beneficial for targeted therapy, which can eventually lead to more effective and tailored therapy either with single agents or in combination.

**MK-2206:** MK-2206 is a highly selective, allosteric Akt inhibitor, with higher potency for Akt1 and Akt2 isoforms than Akt3. Its efficacy was investigated both *in vitro* and *in vivo* as a single agent as well as in combination with several chemotherapeutic drugs or molecular targeted drugs (EGFRi) to overcome any potential resistance. This drug enhances the anti-tumor response in combination therapy, making it a suitable and promising agent for the second line of therapy in cancer patients receiving chemotherapy or targeted therapy<sup>[90]</sup>. This drug is currently used in phase II trials as a second-line therapy for gastric and gastroesophageal cancer<sup>[91]</sup>.

**Perifosine:** An oral anti-cancer agent and an Akt inhibitor, Perifosine has entered clinical trials for major human cancers. eIF4E is a downstream effector of the Akt/mTOR pathway, and increased levels of phosphorylated eIF4E and total-eIF4E correlate with increased GC in tumor tissues. A recent study showed that Perifosine treatment of GC cells with increased eIF4E expression (p-eIF4E) down-regulated eIF4E expression, and thereby exerting an inhibitory effect on the Akt/mTOR pathway. Also, the combination of eIF4E inhibitor with Perifosine in these GC cells further sensitized the cells towards more effective treatment<sup>[92]</sup>. Another study of combination therapy in GC revealed the effectiveness of Perifosine in combination with a miR-27a inhibitor, an oncogene that contributes to drug resistance in GC cells<sup>[93]</sup>. With more studies identifying the molecular mechanisms of Perifosine inhibition in GC, a recent study shows Perifosine inhibiting tumor growth in GC cells *via* inhibition of the Akt/GSK3 $\beta$ /C-MYC signaling pathway,

with significant down-regulation of AEG-1 (Astrocyte elevated gene), a gene reported to play an important role in cellular processes such as proliferation, apoptosis, and invasion<sup>[94]</sup>. Hence, Perifosine is a good potential therapeutic Akt inhibitor when used in combination therapies to overcome drug resistance, with scope for further progression into clinical trials.

**TCN-PM:** Triciribine Phosphate Monohydrate (TCN-PM) is a potent Akt inhibitor inhibiting all the three isoforms of Akt. In Phase I studies of patients with solid tumors, where TCN-PM was administered to patients with increased p-Akt levels (as assessed by immunohistochemical staining), a moderate reduction in p-Akt was observed after single TCN-PM therapy, which may have been possibly due to a small sample size. Further studies to confirm its availability as a single agent as well as its efficacy in combination treatments would help to promote its importance for phase II clinical trials for GC<sup>[95]</sup>.

#### **mTOR inhibitors**

mTOR is often dysregulated in GC, with several preclinical studies suggesting mTOR as a potential therapeutic target. mTOR forms two types of complexes to perform its cellular function based on its interacting partner and substrate specificity, these being the mTORC1 and mTORC2 complex. The mTORC1 complex is rapamycin sensitive<sup>[96]</sup>; rapamycin being the first mTOR inhibitor developed, while the mTORC2 complex is rapamycin insensitive. mTOR inhibitors are classified into two types based on their specificity for mTOR complexes: Rapalogs and mTORC1/2 inhibitors.

**Rapalogs:** Rapamycin and its analogs (referred as rapalogs), first form a complex with the intracellular receptor FK506 binding protein 12 (FKBP12) and then bind to a domain separate from the catalytic site of mTOR, preventing mTOR function. Rapalogs are effective against the mTORC1 complex<sup>[97]</sup>. Some of the rapalogs under preclinical and clinical studies for GC treatment are as follows:

**Temsirolimus:** Temsirolimus binds to FKBP12, and the resultant protein-drug complex prevents mTORC1 activity. A Phase I clinical study determined a favorable toxicity profile, maximum tolerated dose, pharmacokinetics, and anti-tumor efficacy in patients with advanced cancer including GC, making it a favorable drug to proceed towards phase II trials<sup>[98]</sup>. Everolimus - Everolimus is another oral mTORC1 complex inhibitor that has demonstrated good safety and clinical tolerability profile in Phase I trials for several cancers including GC. Phase II trials for patients with advanced GC treated with Everolimus exhibited a good median progression free survival (PFS). Phase III trials for previously treated GC did not show a significant overall survival benefit vs best

supportive care patients. However, the PFS for six-months and the safety profile was significant, which highlights the need for predictive biomarkers for Everolimus treatment response in order to obtain better efficacy for this drug<sup>[99]</sup>.

**Ridaforolimus:** Ridaforolimus, a rapamycin analog, is under clinical investigation with its well defined anti-tumor response in preclinical studies. It showed a synergistic anti-tumor response in patients with solid tumors including GC, in a Phase Ib trial, where ridaforolimus was given in combination with capecitabine, a prodrug that converts into FU<sup>[100]</sup>.

#### **mTORC1/2 inhibitors**

Although mTORC1 inhibition by rapamycin analogs results in substantial tumor growth inhibition, drug resistance has been reported due to a negative feedback loop in the PI3K/Akt/mTOR pathway either via RTKs upregulation with increased Akt activation or crosstalk of PI3K with Ras signaling, leading to MAPK pathway activation. Hence, mTORC1/2 complex inhibitors have gained interest owing to their ability to act as ATP-competitive inhibitors of mTOR kinase activity.

**PP242:** PP242 significantly inhibits mTOR kinase activity, inhibiting both mTORC1 and mTORC2 complex activities. It shows superior anti-proliferative and anti-angiogenesis effects in GC cell lines *in vitro* relative to rapamycin, indicating its promising potential as a therapeutic drug in future for GC<sup>[101]</sup>.

Other mTORC1/2 inhibitors that have been under investigation for solid tumor treatment include AZD2014, AZD8055, and OSI-027; however, there are currently no reports on their usage for GC treatment.

## **POTENTIAL BIOMARKERS FOR TARGETED THERAPY**

The PI3K/Akt/mTOR pathway is activated in approximately 30%-60% of GC tumors. Hence, targeted therapy using either single or dual Akt/mTOR inhibitors is under investigation in several clinical trials as summarized in Table 2, with Everolimus, an mTOR inhibitor, being the only drug to date that has progressed towards phase III trials for advanced GC patients<sup>[99]</sup>. Unfortunately, overall survival (OS) for GC patients treated with Everolimus was not significant; hence, identification of specific biomarkers for patient selection for Everolimus treatment would aid in more personalized therapy, with a potential for better efficacy and anti-tumor response. Although Everolimus failed to significantly improve the overall survival of patients with refractory advanced gastric cancer (AGC), an interesting case study showed satisfactory Everolimus monotherapy in a patient with refractory AGC harboring PIK3CA mutations and pS6 aberrations<sup>[102]</sup>, indicating the

**Table 2 Summary of phosphatidylinositol-3 kinases/Akt/mTOR inhibitors under investigation for gastric cancer treatment**

Classification of PI3K/Akt/mTOR inhibitors	Inhibitors under investigation for GC	Clinical status for GC
PI3K inhibitors: 3 classes of PI3K inhibitors		
Pan-class I inhibitors	PX-866	Phase-II study for solid tumors
	NVP-BKM120	Phase- I study for advanced solid tumors
	ZSTK474	Preclinical studies
	BAY80-6946	Phase- II study for advanced solid tumors
Isoform specific PI3K inhibitors	BYL719	Phase- I study
	INK117	Phase- I study
Dual Akt/mTOR inhibitors	NVP-BEZ235	Preclinical studies
	VS-5884	Phase- II study
	PI-103	Phase- I study
Akt inhibitors	AZD5363	Preclinical studies
	MK-2206	Phase- II study
	Perifosine	Preclinical studies
	TCN-PM	Phase- I study for solid tumors
mTOR inhibitors: 2 types		
Rapalogs	Everolimus	Phase-III study
	Ridaforolimus	Preclinical studies
	Sirolimus	Phase- I study
	Temsirolimus	Phase- II study
mTORC1/2 inhibitors	PP242	Preclinical studies

GC: Gastric cancer; PI3K: Phosphatidylinositol-3 kinases.

importance of predictive biomarkers for various subpopulations of AGC for effective treatment. With studies ongoing for biomarker discovery for better prognosis with Everolimus treatment for AGC patients, some biomarkers that have been explored to predict Everolimus sensitivity in other cancers are PIK3CA/PTEN genomic aberrations<sup>[103,104]</sup>.

With the lack of predictive biomarkers, the need arises to discover new molecules as surrogate markers to further segregate the AGC patient population for more efficient PI3K/Akt/mTOR pathway inhibition. Recent reports have also shown constitutive activation of the Akt/mTOR pathway being regulated by receptor interacting protein-1 (RIP1), a key enzyme in the activation of survival pathways such as Akt/mTOR as well as NF- $\kappa$ B<sup>[105]</sup>. RIP-1 down-regulates PTEN expression as well as suppresses the mTOR/PI3K feedback inhibitory loop, leading to potent activation of this pathway. At the same time, RIP1 also mediates activation of NF- $\kappa$ B pathway, where TAK-1 (TGF- $\beta$ ), a key regulator of the signaling cascade, is recruited to the TNF- $\alpha$  receptor complex, which is a pivotal step for IKK (I $\kappa$ B kinase) activation. The NF- $\kappa$ B pathway is aberrantly activated in several cancers including breast and GC, and recently, an oncogene DP103 (a DEAD-box RNA helicase), was identified by our group to be upregulated in breast, prostate, gastric and colon cancers, and was shown to define the metastatic potential *via* activation of the NF- $\kappa$ B pathway in two independent breast cancer cohorts<sup>[106]</sup>. DP103 regulates the NF- $\kappa$ B pathway *via* direct interaction with TAK1 and thus, the DP103-TAK1 protein complex regulates activation of NF- $\kappa$ B in breast cancer<sup>[106]</sup>. With TAK1 activation also being RIP-1 dependent, cross talk between the Akt/mTOR and NF- $\kappa$ B pathways suggests exploring the role of DP103 as a future biomarker for

GC upon aberrant PI3K/Akt/mTOR pathway activation.

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## 2015 Advances in Inflammatory Bowel Disease

# MicroRNA in inflammatory bowel disease: Translational research and clinical implication

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## Abstract

Idiopathic inflammatory bowel disease (IBD) predominantly includes ulcerative colitis and Crohn's disease. The pathogenesis of IBD is complex and not

completely understood. MicroRNAs belong to a class of noncoding small RNAs that post-transcriptionally regulate gene expression. Unique microRNA expression profiles have been explored in IBD. In this review, we focus on the unique microRNA expression pattern in both tissue and peripheral blood from IBD patients and emphasize the potential diagnostic and therapeutic applications. The discovery of microRNAs has contributed to our understanding of IBD pathogenesis and might lead to clinical advance in new therapeutics.

**Key words:** Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; MicroRNA; Pathogenesis; Gene expression

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**Core tip:** Idiopathic inflammatory bowel disease (IBD) predominantly includes ulcerative colitis and Crohn disease. The pathogenesis of IBD is complex and not completely understood. MicroRNAs belong to a class of noncoding small RNAs that post-transcriptionally regulate gene expression. Unique microRNA expression profiles have been explored in IBD. In this review, we focus on the unique microRNA expression pattern in both tissue and peripheral blood from IBD patients and emphasize the potential diagnostic and therapeutic applications. The discovery of microRNAs has contributed to our understanding of IBD pathogenesis and might lead to clinical advance in new therapeutics.

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## INTRODUCTION

Idiopathic inflammatory bowel disease (IBD) predominantly includes ulcerative colitis (UC) and Crohn's disease (CD), which is a chronic and recurrent inflammatory disorder primarily involving the gastrointestinal tract. The pathogenesis of IBD is multifactorial and not completely understood, but genetic, epigenetic, infectious, physiological, and immunological factors may all play important roles in the genesis and progression of the diseases<sup>[1-3]</sup>. So far, IBD is generally accepted as a complicated consequence attributable to inadequate immunological responses to luminal factors in genetically predisposed subjects.

MicroRNAs are encoded within the genomes of a wide variety of eukaryotes, including more than 700 different microRNA genes in the human genome<sup>[4,5]</sup>. MicroRNAs are evolutionarily conserved, single-stranded non-coding RNA molecules of 19-24 nucleotides, which represent a class of regulatory RNAs that decrease stability and suppress gene expression at a post-transcriptional level. MicroRNAs concurrently modulate the expression levels of dozens or more distinct messenger RNA (mRNA) targets. Alternatively, any given mRNA sequence may be targeted by several different microRNAs<sup>[4-6]</sup>. To date, they have been predicted to target and control the expression of at least 30% of the entire mammalian genome<sup>[7]</sup>. Since their discovery in 1933, microRNAs have been found to be involved in multiple pathophysiological networks<sup>[8,9]</sup> and in the pathogenesis of a broad-spectrum of human diseases, including cancer and inflammation<sup>[10-15]</sup>. Given their potential as therapeutic targets, microRNAs have drawn a lot of attention recently.

Knowledge of microRNA in IBD has accumulated in the past seven years and has indicated that microRNAs play critical roles in the pathogenesis of chronic inflammation and oncogenic transformation. Herein, the review focuses on the current understanding of microRNA as biomarkers of pathogenesis and potential therapeutic implication in IBD.

## DYSREGULATED MICRORNAS IN IBD

Multiple studies have demonstrated distinct microRNA expression profiles in tissue and peripheral blood of IBD patients. Many studies have been conducted on tissue and serum of patients with active or inactive IBD in an attempt to identify biomarkers and drivers of pathogenesis.

### ***Aberrant microRNA profiles in mucosal tissue of UC***

Since 2008, dysregulated microRNAs have been identified by examining inflamed or uninfamed colonic tissue in UC patients<sup>[16-25]</sup>. As listed in Table 1, comparing to normal healthy controls aberrantly elevated microRNAs have been found including miR-7, miR-16, miR-20b, miR-21, miR-23a, miR-24, miR-29a,

miR-29b, miR-31, miR-98, miR-125b-1\*, miR-126, miR-126\*, miR-127-3p, miR-135b, miR-146a, miR-150, miR-155, miR-195, miR-196a, miR-206, miR-223, miR-324-3p, miR-375, miR-422b, miR-548a-3p, miR-650, miR-663, miR-let-7e\*, and miR-let-7f. The decreased microRNAs include miR-143, miR-145, miR-188-5p, miR-192, miR-194b, miR-196b, miR-215, miR-216b, miR-320a, miR-346, miR-375, miR-489, miR-548e, miR-559, and miR-630.

Given the variable anatomic location of colonic tissue, the diverse inflammatory status (either inflamed or uninfamed with or without treatment), the different cohorts of healthy control and analytical systems, it is not surprising that the findings are not consistent among researchers. However, three microRNA candidates, miR-21<sup>[16-18,24]</sup>, miR-29a<sup>[16,19]</sup> and miR-31<sup>[19,23]</sup>, have been found aberrantly elevated by at least two independent groups.

### ***Aberrant microRNA profiles in mucosal tissue of CD***

As shown in Table 2, distinct microRNA expression profiles have also been studied in patients with CD<sup>[19,23,25-29]</sup>. MiR-9, miR-9\*, miR-16, miR-21, miR-22, miR-23b, miR-26a, miR-29b, miR-29c, miR-30a, miR-30b, miR-30c, miR-31, miR-34c-5p, miR-106a, miR-126, miR-126\*, miR-127-3p, miR-130a, miR-133b, miR-141, miR-146a, miR-146b-5p, miR-150, miR-155, miR-181c, miR-191, miR-196, miR-196a, miR-206, miR-223, miR-324-3p, miR-375, miR-594 and miR-663 have been found significantly increased comparing to the normal controls<sup>[19,23,26,28,29]</sup>. The decreased microRNAs include miR-7, miR-18a\*, miR-19b, miR-140-3p, miR-194b, miR-216b, miR-548e, miR-559, miR-629, miR-629\*, and miR-let-7b<sup>[23,27,30]</sup>.

Among them, miR-21<sup>[19,26]</sup>, miR-31<sup>[19,23,29]</sup>, miR-106a<sup>[19,26]</sup>, miR-146a<sup>[19,23]</sup>, and miR-223<sup>[19,26]</sup> have been found dysregulated by at least two independent groups.

### ***Aberrant microRNA in peripheral blood of UC***

As summarized in Table 3, microRNA expression is also altered in the peripheral blood in patients with UC<sup>[24,25,31-34]</sup>. In studies examining microRNAs in peripheral blood mononuclear cells of patients with either active or inactive UC, miR-15b, miR-16, miR-19a, miR-20b\*, miR-21, miR-22, miR-24, miR-27a, miR-27a\*, miR-28-3p, miR-28-5p, miR-29a, miR-30e, miR-31, miR-92a-1\*, miR-93, miR-103, miR-103-2, miR-103-2\*, miR-128, miR-138, miR-140-3p, miR-142-5p, miR-143\*, miR-146a-3p, miR-150\*, miR-151-5p, miR-155, miR-181b, miR-188-5p, miR-196b, miR-199a-3p, miR-199a-5p, miR-221, miR-223, miR-330-3p, miR-340\*, miR-345, miR-362-3p, miR-362-5p, miR-374b, miR-378, miR-378\*, miR-422a, miR-423-5p, miR-500, miR-501-5p, miR-532-3p, miR-532-5p, miR-550\*, miR-598, miR-720, miR-760, miR-769-3p, miR-769-5p, miR-874, miR-941, miR-1271, miR-1274b, miR-1296, miR-let-7d, miR-

**Table 1** Aberrant microRNA expression in human colonic tissue in ulcerative colitis

Status	Tissue type	Control	Aberrant microRNA expression	Ref.
Active UC	Sigmoid, <i>n</i> = 15	Healthy	Decreased: miR-192 and 375 Increased: miR-16, 21, 23a, 24, 29a, 126, 195, 422b and let-7f	Wu <i>et al</i> <sup>[16]</sup> , 2008
	Sigmoid, <i>n</i> = 12	Healthy	Increased: miR-21 and 155	Takagi <i>et al</i> <sup>[17]</sup> , 2010
	Sigmoid, <i>n</i> = 12	Healthy	Increased: miR-21 and 126	Feng <i>et al</i> <sup>[18]</sup> , 2012
	Colon, nonspecific, <i>n</i> = 10	Healthy	Decreased: miR-188-5p, 215, 320a and 346 Increased: miR-7, 31, 135b and 223	Fasseu <i>et al</i> <sup>[19]</sup> , 2010
	Colon, nonspecific, <i>n</i> = 5	Healthy	Increased: miR-150	Bian <i>et al</i> <sup>[20]</sup> , 2011
	Colon, nonspecific, <i>n</i> = 8	Healthy	Decreased: miR-143 and 145	Pekow <i>et al</i> <sup>[21]</sup> , 2012
	Colon, nonspecific, <i>n</i> = 20	Healthy	Increased: miR-20b, 98 and let-7e*	Coskun <i>et al</i> <sup>[22]</sup> , 2013
Active or inactive UC	Colon, distalmost, <i>n</i> = 10	Healthy	Decreased: miR-194b, 216b, 548e and 559 Increased: miR-31, 146a, 206 and 663	Lin <i>et al</i> <sup>[23]</sup> , 2014
Inactive UC	Sigmoid, <i>n</i> = 15	Healthy	Increased: miR-16, 23a, 24, 29a, 375 and 422b	Wu <i>et al</i> <sup>[16]</sup> , 2008
	Colon, nonspecific, <i>n</i> = 8	Healthy	Decreased: miR-188-5p, 215, 320a and 346 Increased: miR-29a, 29b, 126*, 127-3p, 196a and 324-3p	Fasseu <i>et al</i> <sup>[19]</sup> , 2010
Unknown	Colon, nonspecific, <i>n</i> = 19	Healthy	Increased: miR-20b and 125b-1*	Coskun <i>et al</i> <sup>[22]</sup> , 2013
	Colon, nonspecific, <i>n</i> = 15	Healthy	Increased: miR-21	Yang <i>et al</i> <sup>[24]</sup> , 2013
Active UC	Colon, nonspecific, <i>n</i> = 20	Inactive UC	Increased: miR-98	Coskun <i>et al</i> <sup>[22]</sup> , 2013
	Colon, left or sigmoid, <i>n</i> = 9	Inactive UC	Decreased: miR-196b, 489 and 630 Increased: miR-548a-3p and 650	Iborra <i>et al</i> <sup>[25]</sup> , 2013

UC: Ulcerative colitis.

**Table 2** Aberrant microRNA expression in human colonic tissue in Crohn's disease

Status	Tissue type	Control	Aberrant microRNA expression	Ref.
Active CD	Sigmoid, <i>n</i> = 5	Healthy	Decreased: miR-19b and 629 Increased: miR-23b, 106a and 191	Wu <i>et al</i> <sup>[26]</sup> , 2010
	Terminal ileum, <i>n</i> = 6	Healthy	Increased: miR-16, 21, 223, and 594	Wu <i>et al</i> <sup>[26]</sup> , 2010
	Colon, nonspecific, <i>n</i> = 16	Healthy	Increased: miR-9, 21, 22, 26a, 29b, 29c, 30b, 31, 34c-5p, 106a, 126, 126*, 127-3p, 130a, 133b, 146a, 146b-5p, 150, 155, 181c, 196a, 324-3p and 375	Fasseu <i>et al</i> <sup>[19]</sup> , 2010
Active and inactive CD	Colon, nonspecific, <i>n</i> = 8	Healthy	Decreased: miR-7	Nguyen <i>et al</i> <sup>[27]</sup> , 2010
	Colon, nonspecific, <i>n</i> = 120	Healthy	Increased: miR-196	Brest <i>et al</i> <sup>[28]</sup> , 2011
	Colon, nonspecific, <i>n</i> = 15	Healthy	Increased: miR-31 and 141	Huang <i>et al</i> <sup>[29]</sup> , 2015
	Colon, distalmost, <i>n</i> = 9	Healthy	Decreased: miR-194b, 216b, 548e and 559 Increased: miR-31, 146a, 206 and 663	Lin <i>et al</i> <sup>[23]</sup> , 2014
Inactive CD	Colon, nonspecific, <i>n</i> = 8	Healthy	Increased: miR-9*, 21, 22, 26a, 29b, 29c, 30a*, 30b, 30c, 31, 34c-5p, 106a, 126*, 127-3p, 133b, 146a, 146b-5p, 150, 155, 196a, 223 and 324-3p	Fasseu <i>et al</i> <sup>[19]</sup> , 2010
Active CD	Colon, left or sigmoid, <i>n</i> = 9	Inactive CD	Decreased: miR-18a*, 140-3p, 629* and let-7b Increased: miR-328, 422a and 885-5p	Iborra <i>et al</i> <sup>[25]</sup> , 2013

CD: Crohn's disease; UC: Ulcerative colitis.

let-7e, miR-let-7g, miR-let-7i\*, and miR-plus-E1271 are increasingly expressed comparing to the normal population<sup>[24,25,31-34]</sup>. The decreased profiles include miR-150 and miR-505\* comparing to the normal controls<sup>[25,31,33]</sup>.

Among them, nine microRNAs, miR-21<sup>[24,32]</sup>, miR-28-5p<sup>[31,32]</sup>, miR-151-5p<sup>[31,32]</sup>, miR-199a-5p<sup>[31,32]</sup>, miR-345<sup>[25,34]</sup>, miR-362-3p<sup>[31,33]</sup>, miR-505\*<sup>[31,33]</sup>, miR-532-3p<sup>[31,33]</sup> and miR-532-5p<sup>[25,34]</sup>, have been recognized by at least two independent groups.

#### Aberrant microRNA in peripheral blood of CD

As listed in Table 4, altered microRNA expression profiles are also found in the peripheral blood in patients with CD<sup>[25,31-33]</sup>. Compared to healthy controls, the increased microRNA profiles in the serum of patients with active CD include miR-16, miR-20a, miR-21, miR-23a, miR-27a\*, miR-29a, miR-30e,

miR-93, miR-106a, miR-107, miR-126, miR-140, miR-140-3p, miR-140-5p, miR-188-5p, miR-191, miR-192, miR-195, miR-199a-5p, miR-200c, miR-340\*, miR-362-3p, miR-484, miR-532-3p, miR-877, miR-plus-E1271, and miR-let-7b. The significantly decreased microRNAs consist of miR-18a, miR-128, miR-140-5p, miR-145, miR-149\*, miR-877, and miR-plus-F1065.

Among them, six microRNAs, including miR-16<sup>[25,32,33]</sup>, miR-106a<sup>[32,33]</sup>, miR-195<sup>[25,33]</sup>, miR-199a-5p<sup>[31,32]</sup>, miR-362-3p<sup>[31,32]</sup>, and miR-532-3p<sup>[31,32]</sup>, have been found by at least two independent groups.

#### MicroRNA as a differential biomarker to distinguish between UC and CD

As shown in Table 5, studies have shown that microRNAs are differentially expressed between UC and CD<sup>[19,31,35]</sup>. The panel of microRNAs that have been found differentially expressed in colonic tissue includes

**Table 3** Aberrant microRNA expression in human peripheral blood in ulcerative colitis

Status	Tissue type	Control	Aberrant microRNA expression	Ref.
Active UC	Peripheral blood, <i>n</i> = 13	Healthy	Decreased: miR-505* Increased: miR-28-5p, 103-2*, 151-5p, 199a-5p, 340*, 362-3p, 532-3p and plus-E1271	Wu <i>et al</i> <sup>[31]</sup> , 2011
Active and inactive UC	Peripheral blood, <i>n</i> = 88	Healthy	Increased: miR-16, 21, 28-5p, 151-5p, 155 and 199a-5p	Paraskevi <i>et al</i> <sup>[32]</sup> , 2012
	Peripheral blood, <i>n</i> = 18	Healthy	Decreased: miR-150 Increased: miR-15b, 19a, 24, 27a, 28-3p, 29a, 30e, 93, 103, 128, 142-5p, 196b, 199a-3p, 221, 223, 345, 374b, 423-5p, 532-5p, 598, 760, let-7d, let-7e and let-7g	Iborra <i>et al</i> <sup>[25]</sup> , 2013
Inactive UC	Peripheral blood, <i>n</i> = 13	Healthy	Decreased: miR-505* Increased: miR-103-2, 362-3p and 532-3p	Zahm <i>et al</i> <sup>[33]</sup> , 2011
Inactive UC	Peripheral blood, <i>n</i> = 10	Healthy	Decreased: miR-505* Increased: miR-103-2*, 362-3p and 532-3p	Wu <i>et al</i> <sup>[31]</sup> , 2011
Unknown	Peripheral blood, <i>n</i> = 20	Healthy	Increased: miR-20b*, 22, 27a*, 31, 92a-1*, 138, 140-3p, 143*, 146a-3p, 150*, 181b, 188-5p, 330-3p, 362-5p, 345, 378, 378*, 422a, 500, 501-5p, 532-5p, 550*, 720, 769-3p, 769-5p, 874, 941, 1271, 1274b, 1296 and let-7i*	Dutttagupta <i>et al</i> <sup>[34]</sup> , 2012
	Peripheral blood, <i>n</i> = 15	Healthy	Increased: miR-21	Yang <i>et al</i> <sup>[24]</sup> , 2013

UC: Ulcerative colitis.

**Table 4** Aberrant microRNA expression in human peripheral blood in Crohn's disease

Status	Tissue type	Control	Aberrant microRNA expression	Ref.
Active CD	Peripheral blood, <i>n</i> = 14	Healthy	Decreased: miR-149* and plus-F1065 Increased: miR-199a-5p, 340*, 362-3p, 532-3p and plus-E1271	Wu <i>et al</i> <sup>[31]</sup> , 2011
	Peripheral blood, <i>n</i> = 46	Healthy	Increased: miR-16, 20a, 21, 30e, 93, 106a, 140, 192, 195, 484 and let-7b	Zahm <i>et al</i> <sup>[33]</sup> , 2011
	Peripheral blood, <i>n</i> = 128	Healthy	Increased: miR-16, 23a, 29a, 106a, 107, 126, 191, 199a-5p, 200c, 362-3p and 532-3p	Paraskevi <i>et al</i> <sup>[32]</sup> , 2012
Active and inactive CD	Peripheral blood, <i>n</i> = 18	Healthy	Decreased: miR-877 Increased: miR-16, 27a*, 140-3p, 140-5p and 195	Iborra <i>et al</i> <sup>[25]</sup> , 2013
Inactive CD	Peripheral blood, <i>n</i> = 5	Healthy	Decreased: miR-149*	Wu <i>et al</i> <sup>[31]</sup> , 2011
			Increased: miR-340*	
Active CD	Peripheral blood, <i>n</i> = 9	Inactive CD	Decreased: miR-18a, 128, 140-5p and 145 Increased: miR-188-5p and 877	Iborra <i>et al</i> <sup>[25]</sup> , 2013

CD: Crohn's disease; UC: Ulcerative colitis.

**Table 5** Differential microRNA expression between ulcerative colitis and Crohn's disease

Status	Tissue type	Control	Aberrant microRNA expression	Ref.
Inactive UC	Colon, nonspecific, <i>n</i> = 8	Inactive CD	Decreased: miR-100a-3p, 100b-5p, 150, 196b, 223 and 320a	Fasseu <i>et al</i> <sup>[19]</sup> , 2010
Active or inactive UC	Colon, distalmost, <i>n</i> = 12	Active or Inactive CD	Increased: miR-19b, 23b, 106a, 191 and 629	Lin <i>et al</i> <sup>[35]</sup> , 2013
		Inactive CD		
Active UC	Peripheral blood, <i>n</i> = 13	Active CD	Increased: miR-3180-3p, plus-E1035 and plus-F1159	Wu <i>et al</i> <sup>[31]</sup> , 2011

CD: Crohn's disease; UC: Ulcerative colitis.

miR-19b, miR-23b, miR-100a-3p, miR-100b-5p, miR-106a, miR-150, miR-191, miR-196b, miR-223, miR-320a, and miR-629<sup>[19,35]</sup>. Wu and colleagues found three microRNAs (miR-3180-3p, miR-plus-E1035 and miR-plus-F1159) differentially expressed in the peripheral blood between UC and CD<sup>[31]</sup>. Although at least two groups have developed tissue microRNA panels that attempted to delineate between UC and CD, there is little overlap. Importantly, these studies vary in the activity status of IBD during sampling, which may explain the differences seen by independent groups.

### MicroRNA in indeterminate IBD

A diagnosis of idiopathic IBD requires comprehensive analysis of clinical, radiographic, endoscopic, surgical, and histologic data. While most cases of IBD can be specifically classified as either UC or CD, 5%-10% of IBD patients bear equivocal features, falling into the category of indeterminate colitis<sup>[36-38]</sup>. The ability to better classify cases of indeterminate colitis would allow for better clinical and surgical management of these patients, especially regarding the choice of pouch procedure.

In a study by Lin and colleagues, a panel of miR-

19b, miR-23b, miR-106a, miR-191 and miR-629, was evaluated in 16 patients with clinical diagnosis of indeterminate colitis. They found that 15 patients demonstrated UC-like and one CD-like microRNA expression patterns<sup>[35]</sup>. They concluded that microRNA expression pattern in indeterminate colitis are far more similar to those of UC than CD. The study of microRNA expression pattern in indeterminate colitis provides molecular evidence indicating that most indeterminate colitis are probably UC, rather than CD, which is similar to the data from long-term clinical follow-ups. Molecular testing using microRNA as promising markers to improve the classification of indeterminate IBD has the considerable advantage of being testable at the time of colectomy for improved pouch surgery selection. Before being used as a clinically validate test, clinical validation in large samples of indeterminate colitis patients, especially with correlation to pouch prognosis, is a necessity.

## MICRORNA AS A POTENTIAL DRIVER OF PATHOGENESIS

Despite the heterogeneity of microRNAs identified as deregulated in IBD, a few microRNAs confirm in multiple studies and may represent causative agents in disease development. Here we focus on the microRNA with the best evidence as driver of pathogenesis.

### *MiR-21 potentiates disease severity in IBD*

As discussed above, miR-21 has been identified as being upregulated in active UC and CD, consistent with its possible role in the pathogenesis of IBD<sup>[16-18,24]</sup>. *In vitro* experiments have shown that the genetic deletion of DNMT1 and DNMT3b caused dysregulation of approximately 10% of microRNAs, demonstrating tight regulation by DNA methylation<sup>[39]</sup>. The use of microarray and confirmatory pyrosequencing have shown the miR-21 locus is hypomethylated, and therefore overexpressed, in samples of peripheral blood in active CD in pediatric and adult patients<sup>[40]</sup>. To determine if miR-21 was a potential driver of IBD pathogenesis, a miR-21 knockout mouse model was developed and treated with dextran sodium sulphate (DSS) to induce a chronic colitis model with an elevation of tumor necrosis factor alpha (TNF- $\alpha$ ) that mimics human IBD<sup>[41]</sup>. In wild type mice, the addition of DSS caused a significant increase in miR-21 levels, a dramatic reduction in weight, and significant mortality while the miR-21 knockout mice were resistant to these negative effects, which supports a role of miR-21 in IBD pathogenesis.

The pathogenic effects of miR-21 overexpressing are thought to be mediated through at least 3 separate mechanisms. First, miR-21 is thought to cause increased intestinal permeability, a factor thought to initiate IBD. At baseline, no difference in intestinal permeability was seen between wild type and miR-21

knockout mice<sup>[41]</sup>. After treatment with DSS, intestinal permeability was greater in wild type mice than that of miR-21 knockout strain. Secondly, miR-21 is pro-apoptotic. Although the mechanism has not been elucidated, miR-21 knockout mice treated with DSS had less intestinal epithelial cell apoptosis<sup>[41]</sup>. Prevention of epithelial cell apoptosis may help maintain the epithelial cell barrier and limit inflammation and disease progression. Thirdly, interstitial fibrosis is a hallmark of IBD and miR-21 has been associated with fibrosis in multiple disease models. Mouse models of renal fibrosis have shown that cellular injury leads to increased levels of TNF- $\alpha$  and subsequent induction of miR-21<sup>[42]</sup>. Inhibition of miR-21 prevented fibrosis, presumably through preventing the recruitment of pro-fibrotic inflammatory cells<sup>[42]</sup>. Increased serum levels of miR-21 were seen in humans with idiopathic pulmonary fibrosis and may serve as a non-invasive biomarker for disease progression<sup>[43]</sup>. Analysis of serum and hepatic tissue from patients with cirrhosis has also shown that increased miR-21 levels are associated with levels of fibrosis<sup>[44]</sup>. Although miR-21 has not been experimentally linked to fibrosis in IBD yet, its role deserves further study. Interestingly, miR-21 expression was found to be high in IBD-associated dysplasia suggesting that its expression is maintained throughout the development of dysplasia and carcinogenesis, but more controlled studies are needed to define its role<sup>[45]</sup>.

## MICRORNA AS A POTENTIAL BIOMARKER FOR CARCINOGENESIS

Longstanding IBD is a well-known risk factor for colorectal cancer, although mechanisms of carcinogenesis are poorly known<sup>[46,47]</sup>. Studies have shown that the risk of IBD-associated colon cancer is related to the extent of the disease, severity of inflammation, and duration<sup>[48-50]</sup>. With chronic inflammation, colonic epithelium undergoes a transformation from inflamed, but not dysplastic to progressively dysplastic, and eventually to adenocarcinoma. Colonoscopies with surveillance biopsies for IBD-associated dysplasia are used to help guide surgical timing of colectomies. Although histologic examination can reproducibly identify dysplasia, IBD-associated dysplasia cannot be distinguished from sporadic dysplasia based on histologic appearance alone. Histologic examination of IBD-associated adenocarcinomas has characteristic features and demographics which may indicate a specific pathway to carcinogenesis<sup>[51]</sup>. Molecular alterations have been shown to lead to this histological progression<sup>[52-58]</sup>. Previous studies have demonstrated molecular abnormalities in normal-appearing non-dysplastic mucosa from patients with UC who had a remote dysplastic lesion<sup>[55-57,59-61]</sup>. Aneuploidy, chromosomal alterations, p53 mutation, loss of heterozygosity, and chromosome instability are present in



normal-appearing mucosa before the development of dysplasia<sup>[55-57,59-61]</sup>.

Studies of microRNAs may elucidate distinct pathways that may help reliably identified IBD-associated dysplasia and subsequent carcinogenesis. Recent studies demonstrate that microRNAs are largely involved in oncogenesis *via* their regulation of tumor suppressors and oncogenes<sup>[62]</sup>. In a study by Olaru *et al.*<sup>[63]</sup>, microRNA arrays were performed on tissue from eight patients with IBD-associated dysplasia. Twenty two microRNAs (miR-31, miR-31\*, miR-96, miR-135b, miR-141, miR-183, miR-192, miR-192\*, miR-194, miR-194\*, miR-200a, miR-200a\*, miR-200b, miR-200b\*, miR-200c, miR-203, miR-215, miR-224, miR-375, miR-424\*, miR-429, and miR-552) were significantly upregulated and 10 microRNAs (miR-122, miR-139-5p, miR-142-3p, miR-146b-5p, miR-155, miR-223, miR-490-2p, miR-501-5p, miR-892b, and miR-1288) were downregulated in dysplastic epithelium compared to the non-dysplastic inflamed tissue.

#### **MiR-31 identifies IBD-associated dysplasia**

MiR-31 is upregulated in UC and CD, but not in other non-IBD colitis, such as microscopic colitis, that have no association with dysplasia or malignancy<sup>[64]</sup>. As early as 2007, miR-31 was found to be upregulated in sporadic colorectal adenocarcinomas<sup>[65-67]</sup>. However, the role of miR-31 in IBD-associated dysplasia or malignancy has only recently been examined. An assessment of the baseline miR-31 expression in normal tissue regardless the different anatomic locations of the colon allows for comparison of all colon specimens equally<sup>[63]</sup>. In addition, no difference of miR-31 expression level was seen between IBD-associated dysplasia and IBD-associated carcinomas. Importantly, the levels of miR-31 were found 11-fold higher in IBD-associated dysplasia or carcinoma when compared to that of IBD tissue without dysplasia<sup>[63]</sup>. Although in a smaller study set, these findings were not replicated and a link between microRNAs and p53 dysregulation was indicated<sup>[68]</sup>. Taken together, these findings suggest that miR-31 alteration might happen early in carcinogenesis and may be used a biomarker for IBD-associated dysplasia or malignancy.

## **MICRORNA AS POTENTIAL THERAPEUTIC TARGETS FOR IBD**

Understanding the underlying mechanisms that regulate gene expression and the complex interplay of factors is essential to develop novel therapeutics in IBD. The post-transcriptional regulation of gene expression is unique and is becoming increasingly important.

The ability of microRNAs to target multiple genes and biological signaling pathways has drawn great attention in potential clinical utility as innovative

therapeutic agents in treatment. Antisense oligonucleotides complementary to microRNAs, namely anti-microRNA oligonucleotides, can target specific microRNAs abolishing their function in *in vitro* cultured cells, or *in vivo* in animal models. For example in the achievement of cancer research, recent accumulating preclinical studies have shown the feasibility of slowing tumor progression by either overexpressing tumor suppressive microRNAs, or by neutralizing the activities of oncogenic microRNAs in cell- or animal-based cancer models<sup>[69-72]</sup>. In addition, a number of clinical drugs have shown to modulate the microRNA expression as anticancer effect *in vitro*<sup>[73,74]</sup>.

Particularly in the field of IBD, the mechanisms to modify microRNAs that might activate or inactivate pathways required for the inflammation progress are worth investigating. Potential therapeutic application targeted on microRNA is to block inflammatory progression to improve sensitivity to conventional therapies. The pharmacologic targeted tissue delivery consists of two general strategies: (1) antisense oligonucleotides complementary to specific mature microRNAs to inactivate the overexpressed pro-inflammatory process; and (2) to replace the expression of suppressive microRNAs.

To date, no therapeutic manipulation of microRNAs in IBD has been published in either cell lines or animal models yet. Although recent study has shown that inhibition of miR-21, a promising pathogenetic driver in IBD, slows the proliferation and progression in a nasopharyngeal carcinoma cell line<sup>[75]</sup>. The similar approach is expected to be tested in IBD cell line or animal model. Although side effects are another essential issue to be considered before an effective drug enters the markets, we can't help speculating that a new therapeutic concept, targeted microRNA drug for IBD, maybe emerges in the near future.

## **DILEMMAS**

During the past 7 years, the identification of microRNA in IBD has broadened our knowledge. However, the lack of a standardized approach often leads to inconsistent or even conflicting results.

The nomenclature for microRNA has continued to evolve since its discovery in 1993<sup>[8,9]</sup>. MicroRNAs were named in the order they were discovered, leading to identical microRNAs being given different names by different groups. As the microRNA field continues to expand, significant efforts have been made to clarify nomenclature using a unified system. Recent data added from deep genome sequencing has pushed the number of annotated microRNAs to roughly 1900 in the most recently nomenclature database, miRBase version 21<sup>[76]</sup>. The complicated historical nomenclature of microRNA makes literature evaluation difficult and diligent effort to confirm sequence identity of each in the literature must be made.

One of the most commonly encountered problems

is when we attempt to verify microRNA's role in IBD pathogenesis. Recent developments in microarrays have led to numerous attempts to identify microRNAs associated with a diverse set of disease processes. Despite the ability of candidate microRNAs to be validated by additional RT-PCR, there has been little reproducibility between groups. Differences in samples obtained from various anatomic locations, treatment regimens, and activity level of disease may account for discrepancies seen between studies. Additionally, microRNAs with the same sequence identity are given modifiers in their name based on their location within the genome. Most techniques do not distinguish microRNAs that have the same sequence but at different locations in the genome<sup>[76]</sup>. A more clear understanding of the genetic loci associated with microRNAs can provide insight into how they are regulated and become deregulated in pathogenesis.

## CONCLUSION

In summary, the accumulating knowledge of microRNA has significantly expanded our understanding of the pathogenesis of IBD and has demonstrated the usefulness of microRNAs as biomarkers with emerging clinical utility and the potential for personalized therapies.

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## 2015 Advances in Inflammatory Bowel Disease

# Th17 plasticity and its changes associated with inflammatory bowel disease

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## Abstract

CD4 T helper (Th) cell differentiation into distinct T

cell subsets is critical to the normal function of the immune system. Until recently, the paradigm held that naïve T cells differentiated into distinct subsets under the guidance of environmental cues (*e.g.*, cytokines) and that once polarized, these cells were committed to a particular functional state. However, the existence of transdifferentiated T cell populations, which express signature transcription factors and cytokines associated with more than one Th subset, challenges the immutability of T helper subsets and suggests that plasticity is a feature of multifaceted immune responses. How this process impacts immune dysregulation in diseases such as inflammatory bowel diseases (IBD) and the machinery that underlies this process is far from fully understood. Interleukin (IL)-17 secreting helper T (Th17) cells have been heavily implicated in tissue-specific immune pathology including murine models of IBD, human Crohn's disease and ulcerative colitis. Plasticity within this subset is suggested by the existence of IL-17 secreting cells, which, can also secrete interferon- $\gamma$ , the signature cytokine for Th1 cells or, can co-express the anti-inflammatory transcription factor forkhead box p3, a signature transcription factor of regulatory T cells. In this review we mainly discuss evidence for Th17 plasticity, mechanisms, which govern it, and highlight the potential to therapeutically target this process in human IBD.

**Key words:** Th7; Regulatory T cells; T cell plasticity; Inflammatory bowel disease

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**Core tip:** Recently, two innovative clinical failures in inflammatory bowel disease which sought to manipulate T helper (Th) subsets *via* either transplantation of regulatory T cells or interleukin-17 blockade using secukinumab, suggest that altering the balance between inflammatory and regulatory subsets in inflammatory bowel diseases (IBD) may be more complex than

previously thought. One reason may be the flexible nature of T helper subset commitment, otherwise referred to as plasticity. Here we discuss plasticity between regulatory and inflammatory subsets in T helper CD4<sup>+</sup> cells, especially Th17 cell subset, and the potential to therapeutically target this process in human IBD.

Ueno A, Ghosh A, Hung D, Li J, Jijon H. Th17 plasticity and its changes associated with inflammatory bowel disease. *World J Gastroenterol* 2015; 21(43): 12283-12295 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12283.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12283>

## INTRODUCTION

The spectrum of immune-mediated disease encompasses a wide variety of chronic inflammatory conditions such as inflammatory bowel disease (IBD) comprised of Crohn's disease (CD), and ulcerative colitis (UC) as well as rheumatoid arthritis (RA), multiple sclerosis, and psoriasis. Although the underlying triggers remain poorly understood, it is now clear that T cells are essential to the development and perpetuation of these diseases<sup>[1-3]</sup>. Since the landmark description of functionally distinct T helper 1 (Th1) and Th2 CD4<sup>+</sup> effector subsets, each with unique cytokine profiles<sup>[4]</sup>, much work has focussed on dissecting their roles in both health and disease. The spotlight has recently been drawn to a novel subset, the Th17 cell, so named due to its ability to secrete interleukin (IL)-17A, which has emerged as a major player in tissue-specific immune pathology. The initial emphasis on the detrimental effects of Th17 is reflected by the plethora of early literature supporting such a role in both human and murine studies<sup>[5-10]</sup>. However, a growing body of evidence now suggests essential protective roles, particularly in the context of mucosal integrity and defence against extracellular pathogens<sup>[5,11-16]</sup>. Th17 cells and their associated cytokines have been found to interact more closely with other adaptive immune cells than previously thought, raising interesting questions about how to select and design therapeutic strategies targeting this cell population<sup>[2,17]</sup>. New technologies such as transcriptome profiling, global epigenetic mapping and computerized simulation analysis<sup>[18,19]</sup> have captured a more accurate picture of this T cell subset revealing it to be more transient, complex and perhaps more reversible than previously imagined. In addition to a well-established role in extracellular pathogen clearance, Th17 cells can also participate in intracellular pathogen clearance *via* unconventional interferon (IFN)- $\gamma$  secretion<sup>[20,21]</sup>. Human forkhead box p3 (Foxp3) + regulatory T cells (Treg) can differentiate into IL-17 promoting cells *in vitro*<sup>[22]</sup>. Flexibility within this subset may allow Th17 cells to embrace pro-

inflammatory and protective roles in mucosal immunity by secreting a spectrum of cytokines without requiring *de novo* differentiation of naïve T cells. Likewise, Th17 cells may be generated quickly from Treg in order to defend from acute invasion of pathogens. This ability to transition between functional states is defined as T cell plasticity.

This review mainly focuses on human studies and outlines the major features of Th17 plasticity including the Treg/Th17 paradigm shift in the context of IBD and in the maintenance of intestinal homeostasis.

## CHARACTERISATION OF TH17 AND TREG SUBSETS IN IBD

In the last 15 years or so, the focus of attention regarding T cell subsets has shifted from the classical Th1/Th2 paradigm to that of Th17/Treg. This has indeed been the case in IBD.

The discovery of extrathymic Treg development in the intestine has attracted enormous attention and highlights the importance of Treg cells in intestinal homeostasis. Hori *et al.*<sup>[23]</sup>, demonstrated that co-transfer of peripherally generated Foxp3 positive Treg cells could attenuate disease in the adoptive transfer model of mouse colitis. Shortly after this study, Makita *et al.*<sup>[24]</sup>, showed the intestinal prevalence of Treg in patients with IBD. Mucida *et al.*<sup>[25]</sup>, have since identified retinoic acid, derived from vitamin A and metabolized by dendritic cells, as a key signal regulating Foxp3 expression by naïve T cells in response to TGF- $\beta$ . Overall, the digestive tract requires high levels of inducible Treg cells in order to preserve tolerance to the enormous antigenic burden comprised by commensal flora and dietary antigens<sup>[26]</sup>.

At around the same time, Fujino *et al.*<sup>[27]</sup>, first reported on the prevalence of Th17 cells in patients with IBD. Patients with UC and CD show increased IL-17A levels in serum and mucosa<sup>[17]</sup> and an IL-17A gene polymorphism has been linked to UC susceptibility<sup>[28]</sup>. This cytokine, in addition to promoting barrier function, is a potent promoter of granulopoiesis and neutrophil chemotaxis and plays an important role in the clearance of extracellular bacterial and fungal infections<sup>[29]</sup>. Recently, Ciofani *et al.*<sup>[30]</sup> have described an intracellular network regulating Th17 specification. Interestingly, genome-wide association studies linked at least 24 loci within this network to single nucleotide polymorphisms (SNPs) associated with ulcerative colitis and Crohn's disease, highlighting the importance of this T cell subset towards the pathogenesis of IBD<sup>[30,31]</sup>.

### IL-17 secreting Th17 subset

Intestinal effector T cells arise from naïve lymphocytes derived from the thymus, which then undergo functional differentiation in the intestinal mucosa upon encountering their cognate antigen displayed by activated antigen-presenting cells (APCs). APCs and

potentially other cells release cytokines, which act in combination with environmental cues, including bacterial and dietary products as well as salt concentration<sup>[32,33]</sup>, thereby activating the Jak-STAT and other signalling pathways to exert their biological functions.

Differentiation of Th17 cells is exclusively dependent on signal transducer and activator of transcription 3 (STAT3)<sup>[34]</sup> and crucially requires the expression of the transcription factor retinoic acid receptor-related orphan receptor  $\gamma$  thymus in mouse (ROR $\gamma$ t)<sup>[35]</sup>. Studies in the mouse and *in vitro* human cell cultures have revealed the critical roles of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) alongside IL-6<sup>[36-38]</sup>, IL-1 $\beta$ <sup>[39]</sup> and IL-21<sup>[40]</sup> in Th17 polarization. IL-23, secreted mainly by innate myeloid cells including activated dendritic cells (DC), monocytes and macrophages, is critical for Th17 proliferation and maintenance, though dispensable for the initiation of Th17 development. Importantly, mutations within the *il23r* locus, which encodes a receptor subunit unique to IL-23, are associated with psoriasis<sup>[41]</sup>, ankylosing spondylitis<sup>[42]</sup> and IBD<sup>[43,44]</sup>.

Whilst Th17 cells are distinguished by IL-17A production, they are also capable of producing other cytokines, including but not limited to IL-17F, IL-21 and IL-22. IL-17F, a member of the IL-17 family, may also have dual roles in the context of mucosal disease. In the DSS model of IBD, IL-17F<sup>-/-</sup> mice show less severe disease than wild-types<sup>[28]</sup>. IL-17F evidently appears to have a distinct set of roles, despite structural similarity to IL-17A, and further research will be necessary to establish its specific importance. Substantial evidence exists for pathogenic roles of IL-22, particularly in the context of psoriasis<sup>[45]</sup>. However, IL-22 may play a critical role in the maintenance of the intestinal epithelial barrier and in mucosal healing<sup>[45]</sup>. It is thus important to emphasize that although Th17 cells are defined by their expression of IL-17 and RORc, human counterpart of ROR $\gamma$ t, these cells likely express heterogeneous cytokine profiles, at times simultaneously expressing both protective (IL-22) and deleterious (IL-17A, IFN- $\gamma$ ) cytokines in a tissue-temporal specific manner<sup>[5]</sup>. In addition, the unexpected failure of secukinumab<sup>[46]</sup>, a human anti-IL-17 monoclonal antibody, in the treatment of Crohn's disease reflects the complex role of IL-17 and the heterogeneous causes of the disease<sup>[47]</sup>.

### **Foxp3<sup>+</sup> Treg subset**

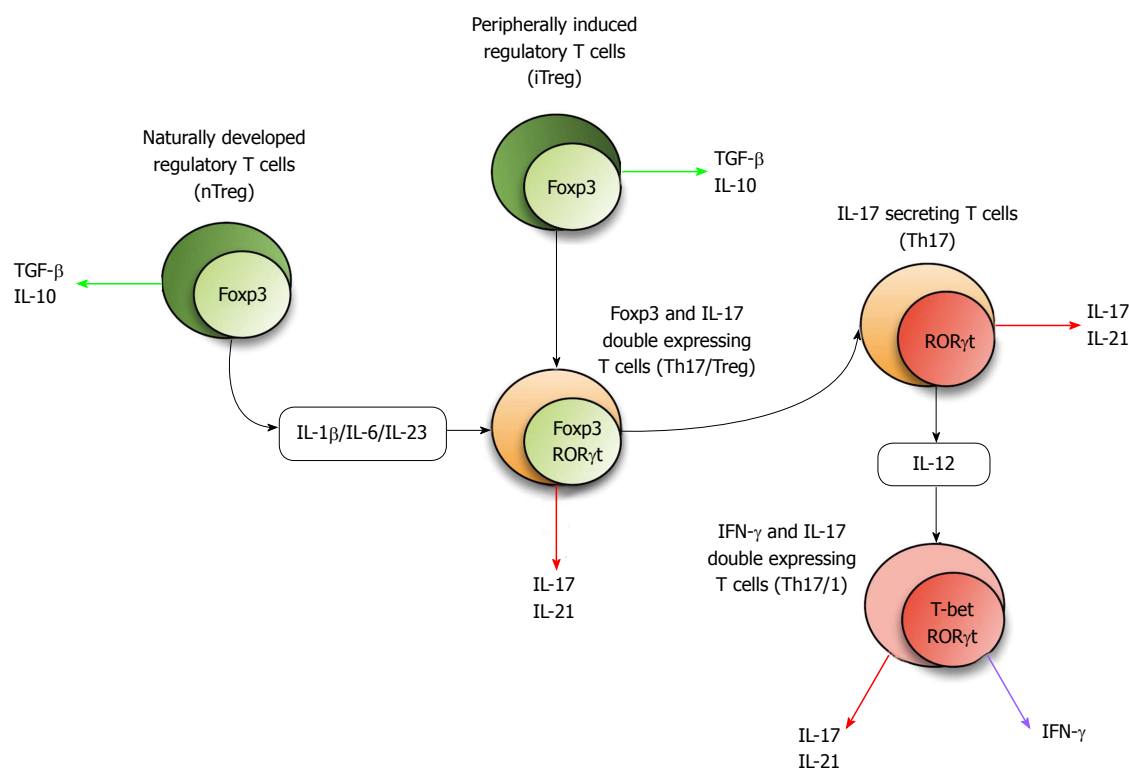
In contrast, anti-inflammatory Foxp3-expressing Treg cells play an important role in tissue homeostasis *via* controlling pro-inflammatory effector T cells. Treg cells were first described as "self"-recognising T cells which develop in the thymus. Nowadays this population of Treg cells has come to be referred to as naturally occurring Treg<sup>[48]</sup>. Initially, Treg cells were believed to differentiate along a distinct pathway to that of

conventional T cells which arise from naïve T cells in the periphery (extra-thymically). It is now apparent however that naïve progenitor T cells can give rise to a population of anti-inflammatory Foxp3 expressing T cells extra-thymically. This population is referred to as inducible Treg (iTreg)<sup>[48]</sup>. The major residential and developmental organs of iTreg cells are in fact the gut mucosa and mesenteric lymph nodes. Differentiation of iTreg cells is dependent on STAT5 and requires activation of the lineage-specifying transcription factor Foxp3. *In vitro* studies using human cells have revealed the critical roles for TGF- $\beta$ 1, together with the cofactor retinoic acid in iTreg development from naïve Th cells<sup>[49]</sup>.

### **Similarities and differences between Th17 and Treg development**

Interestingly, both Th17 and iTreg cells share essential developmental cues, and thus similar developmental pathways, as both subsets can be generated under the influence of TGF- $\beta$ 1. However additional signals specify development of each subset. Similar to Th1 and Th2 subsets, Th17 and Treg can negatively influence each other. In mice, increasing concentrations of TGF- $\beta$ 1 are associated with increased Foxp3 levels and decreased IL-23R expression, leading to decreased IL-23-dependent maintenance of Th17 cells and resulting in impaired Th17 development<sup>[50]</sup>. In addition, Foxp3 was found to directly inhibit IL-17 expression. As a result, Foxp3 and ROR $\gamma$ t double expressing T cells in the lamina propria produced lower levels of IL-17 compared to T cells expressing ROR $\gamma$ t alone<sup>[50]</sup>. Smad 2, 3, and 4, which transduce the extracellular TGF- $\beta$ 1 signal to the nucleus, are pivotal to iTreg generation whilst dispensable for Th17 development<sup>[51,52]</sup>.

Gut resident and pathogenic microbes contribute to gut homeostasis and disease in part by shaping Th subset polarization. Interestingly, Th17 cells are induced by components of the intestinal flora, as has been shown for segmented filamentous bacteria (SFB)<sup>[53]</sup>. This requires MHC class II-dependent presentation of SFB antigens by DCs. On the other hand, commensal bacteria are known to influence gut tolerance by generating Treg cells specific to themselves. Transplantation of specific *Clostridia* clusters into germ free mice induces increased colonic Treg cells<sup>[54,55]</sup>. *Lactobacillus reuteri* colonization is also associated with an increase in gut residential Treg cells<sup>[56]</sup>. Interestingly, bacterial fermentation products may also play an important role in Treg generation. The short-chain fatty acids can promote DC tolerance along with generation of colonic Treg cells<sup>[57,58]</sup>. Another bacterial product, polysaccharide A from *Bacteroides fragilis* can induce anti-inflammatory Foxp3<sup>+</sup>, IL-10 secreting Treg cells *via* TLR2 signalling<sup>[59]</sup>. Thus, intestinal dietary and commensal products can



**Figure 1 Concept of 17 secreting helper T plasticity.** The Foxp3 expressing Treg subset either develops naturally (nTreg) or is peripherally induced from naïve T cells (iTreg). Th17 generating factors including cytokines, transcription factors, and other molecular cues can induce these cells to differentiate into transitional cells with co-expression of Foxp3 and ROR $\gamma$ t. Th17 cells may be converted into Th17/1-transitional cells with co-expression of ROR $\gamma$ t and TBX21. Th17: 17 secreting helper T; Foxp3: Forkhead box p3; ROR $\gamma$ t: Related orphan receptor  $\gamma$  thymus in mouse; IL: Interleukin; TGF: Transforming growth factor; IFN: Interferon.

influence Treg and Th17 development.

## MECHANISMS OF PLASTICITY AND RECIPROCAL REGULATION BETWEEN TREG AND TH17 SUBSET

There are at least four major pathways and/or factors which contribute to Th17 plasticity. The cytokine milieu directs T cell subset development and also induces plasticity *via* the activation of different and specific STAT molecules and multiple transcription factors. Furthermore, it has emerged that immune-regulatory microRNA (miR) plays a fundamental role in controlling gene expression, thus influencing T cell fate and plasticity. There is also the unique role of aryl hydrocarbon receptor (AhR) and its environmental and physiological ligands alongside histone methylation and epigenetic modifications which may fundamentally influence T cell plasticity. Figure 1 summarizes the concept of Th17 plasticity.

### Cytokine pathways (main contributor to plasticity)

As described above, TGF- $\beta$  signalling with the additional influence of IL-1 $\beta$ , IL-6 and IL-21 are critical to Th17 lineage development as well as the role of IL-23 signalling for the maintenance and function of this subset. High concentrations of

exogenous Th17-generating cytokines are able to convert Foxp3<sup>+</sup> Treg cells into IL-17 secreting Th cells *in vitro*. Since cytokine secretion is a final step in lineage differentiation, this population is considered to possess two signature features for two different subsets; Foxp3 for Treg, and IL-17 for Th17. We found increased levels of circulating IL-17 and Foxp3 double expressing T cells in IBD patients compared to healthy controls. Furthermore, we demonstrated the conversion of CD25<sup>+</sup> CD45RO<sup>-</sup> Treg cells from peripheral blood of IBD patients into IL-17 secreting Foxp3 expressing cells as well as ROR $\gamma$ t and Foxp3 expressing cells in the presence of combinations of the above mentioned cytokines<sup>[60]</sup>. Comparing the *in vitro* generation of double expressing cells in two types of IBD patients, CD patients have a higher prevalence of double expressing cells generated in the presence of IL-1 $\beta$ /TGF- $\beta$ /IL-6 than UC while UC patients have an increased frequency of this population in response to IL-21/IL-23 than CD samples, suggesting disease-associated plasticity of Th cells in IBD<sup>[60]</sup>. A recent elegant study by Basu *et al.*<sup>[61]</sup>, revealed that IL-1 signalling represses SOCS3, an inhibitory molecule of STAT3 altering the STAT3/STAT5 balance resulting in Th17 generation. This model offers an explanation for why exogenous IL-1 $\beta$  converts Foxp3<sup>+</sup> Treg cells into IL-17<sup>+</sup> Foxp3<sup>+</sup> double expressing Th cells<sup>[61]</sup>.

Cytokines which induce Th17 development and



**Table 1 Cytokines related to 17 secreting helper T development and plasticity in human**

Cytokine	Effect	Ref.
IL-1 $\beta$	Accelerate IL-17A secretion from IL-17 secreting Innate Lymphoid cells and Th17	[69]
IL-6	Induce autocrine IL-21 and generation of Th17	[20]
IL-10	Suppress IL-17A and Th17 generation	[62]
IL-12	Induce IFN- $\gamma$ secretion from Th17 cells (Th1/Th17 plasticity)	[20]
IL-21	Potentiates pathogenic effects of Th17 cells in the gut	[64,65]
IL-22	Attenuate the development of intestinal pathology <i>via</i> Stat3-mediated effects on epithelial cells	[66,67]
IL-23	Induce IL-22 and IL-17A secretions and maintain Th17 expansion	[68]
TGF- $\beta$ 1	Initiate generation of Treg and Th17 populations dependent on the concentration	[63]

Th17: 17 secreting helper T; IL: Interleukin; TGF: Transforming growth factor; IFN: Interferon.

plasticity are summarised in Table 1<sup>[62-69]</sup>.

### Transcription factors

There is now vast literature detailing multiple transcription factors and their relationship to T cell plasticity. These transcription factors directly regulate and/or promote gene expression by binding to their promoter regions in order to contribute to the multifaceted nature of T cell subsets.

Ciofani *et al.*<sup>[30]</sup> recently identified FOS-like antigen 2 (Fosl2) as a key determinant of Th17 plasticity in their remarkable study of Th17 cell specification<sup>[30]</sup>. *in vitro* differentiation of mouse CD4 T cells deficient in Fosl2 under Th17 polarizing conditions yielded IL-17 producing cells which co-expressed Foxp3. Interestingly, Fosl2 deficiency also enabled IFN- $\gamma$  production in Th17 and Th2 cultures, particularly when Th17 cells were subsequently exposed to Th1-skewing conditions.

Interferon regulatory factor 4 (IRF4) is a transcription factor expressed in hematopoietic cells and plays pivotal roles in the immune response. IRF4 levels were augmented in patients with active inflammatory bowel disease and correlated with enhanced production of IL-17 and IL-22 mRNA<sup>[70]</sup>. On the other hand, lack of IRF4 seems to cause resistance to Th17-mediated autoimmune diseases<sup>[71]</sup>. Basic leucine zipper transcription factor, BATF, along with IRF4, was recently proposed as a "pioneer factor" in T cells<sup>[30]</sup>. After T cell receptor ligation, BATF expression is rapidly induced in naïve T cells. The BATF-JUN heterodimer and IRF4 bind to the same regulatory regions to mediate chromatin remodelling and facilitate accessibility to regulatory elements by other Th cell subset-specific transcription factors including STAT3, MAF and ROR $\gamma$ t in Th17. Interestingly, BATF and IRF4 are also necessary for Treg differentiation in visceral adipose tissue through

**Table 2 Transcription factors related to 17 secreting helper T development and plasticity**

Transcription factor	Effect	Ref.
Fosl2	A key determinant of cellular plasticity	[30]
IRF4	Th17 differentiation <i>via</i> ROR $\gamma$ t dependent and independent pathways	[115]
	Augmented in patients with IBD and correlated with enhanced production of IL-17 and IL-22 mRNA	[70]
BATF	Required for differentiation of Th17 <i>via</i> induction of ROR $\gamma$ t, and bound to IL-17, IL-21 and IL-22 promoters	[116]
	BATF+ ROR $\gamma$ - Th17 cells are found in gut tissues from UC but not CD patients.	[73]
HIF-1	A key transregulator of Th17 polarization and suppressor of Foxp3 in Treg	[33,81]
	Reciprocal regulation between HIF-1 and miR210	[81]
Jmjd3	H3K27 demethylase, important for Th1/Th17 plasticity	[101]
ROR $\gamma$ c (human)	Essential for Th17 differentiation induced by	[35]
ROR $\gamma$ t (mouse)	TGF- $\beta$ 1 and IL-6 or IL-21	
IKZF3 (Aiolos)	Promotes Th17 differentiation <i>via</i> silencing of the IL-2 locus	[74]
	Aiolos <sup>+</sup> iTreg respond to IL-1 $\beta$ and downregulate their suppressor functions	[75]

ROR $\gamma$ t: Related orphan receptor  $\gamma$  thymus in mouse; IL: Interleukin; TGF: Transforming growth factor; CD: Crohn's disease; UC: Ulcerative colitis; HIF: Hypoxia-inducible factor; IRF4: Interferon regulatory factor 4; IBD: Inflammatory bowel diseases.

direct regulation of IL-33 receptor, ST2 and PPAR- $\gamma$  expression<sup>[72]</sup>. BATF<sup>+</sup> ROR $\gamma$ - Th17 cells are found in gut tissues from UC but not CD patients<sup>[73]</sup>. Taken together BATF/IRF4 axis may direct Treg/Th17 balance and plasticity in the gut.

A member of the Ikaros family, IKZF3 (or Aiolos) is known to promote Th17 differentiation by suppressing IL-2 production<sup>[74]</sup>. Interestingly, IKZF3 is also expressed in iTreg lacking the expression IKZF2, (or Helios). These IKZF2<sup>-</sup> IKZF3<sup>+</sup> Foxp3<sup>+</sup> Th cells express IL-17 and exert reduced regulatory functions in healthy human blood samples<sup>[75]</sup>. Furthermore, polymorphism in *Ikzf3* locus shows an association with CD and UC<sup>[31]</sup>. Thus, IKZF3 may turn out to be an important regulator of Th17-Treg plasticity in IBD.

An environmental sensor, Hypoxia-inducible factor 1 (HIF-1), which is induced by Th17 cells to promote signalling in a Stat3-dependent manner, cooperates with ROR $\gamma$ t to control expression of Th17 genes, such as IL-17A, IL-17F, and IL-23R. Furthermore, HIF-1 negatively regulates Treg development by mediating Foxp3 protein degradation<sup>[33]</sup>.

On the other hand, Liu *et al.*<sup>[76]</sup> recently reported that TGF- $\beta$  and IL-6 regulate Th1 cell conversion into the Th17 subset *via* expression of Runx1. This is supported by the finding that siRNA mediated silencing of Runx1 inhibits this conversion. Furthermore, TGF- $\beta$  enhanced histone H3K9 acetylation but inhibited H3K9

**Table 3** MicroRNAs influencing 17 secreting helper T development and plasticity

MicroRNA	Inducer	Target	Effect	Ref.
miR10	TGF- $\beta$	Bcl-6	Limit Th17, convert Th cells into Treg	[78,83]
miR17-92 cluster		Creb1, TGF- $\beta$ RII, KZF4 (miR17), PTEN (miR19)	Accumulation of antigen-specific iTreg development, IL-10 production, and possibly Treg cell migration	[78,83]
miR29	NOD2	IL-12p40, IL-23p19	Inhibit IL-23R signalling	[80]
miR126		PIK3R2	Treg-mediated Immunosuppression	[83]
miR132/212 cluster	TCDD, FICZ	Bcl-6	Enhance Th17 development <i>via</i> AhR pathway	[82,83]
miR146a	TLR2-5	STAT1	Block Th1 development	[78,79]
miR155	TLR2-4, TLR9	SOC1	Unleash STAT1, 4, and 5 signals, and promote Th1, Th2, and Treg	[78,79]
miR210		HIF-1 (counter regulator)	Control Foxp3 expression	[81,83]
miR301		PIAS3	Unleash STAT3 signal, and generate Th17	[78]
miR326		EST-1	Critical for Th17 development	[78]

TGF: Transforming growth factor; Th17: 17 secreting helper T; FICZ: 6-formylindolo [3,2-b] carbazole; TLR: Toll-like receptor; TCDD: Tetrachlorodibenzo-*p*-dioxin; FICZ: Formylindolo [3,2-b] carbazole.

trimethylation of Runx1- and ROR- $\gamma$ t-binding sites on the *IL-17* or *RORc* gene in Th1 cells in this disease model<sup>[76]</sup>.

Transcription factors which are critical for Th17 development and plasticity are summarised in Table 2.

#### Micro-RNA (a cause of plasticity)

miR are small fragments of non-coding RNA (mostly 17-22 nucleotides) that act as regulators of RNA expression through binding to the 3'-UTR of complementary mRNA resulting in repression/silencing of target RNAs. Several miRs have been reported to influence the differentiation of Th cell subsets<sup>[77]</sup> as well as plasticity and reciprocal regulation among Th cell subsets<sup>[78]</sup>.

Targeting STAT1, which is required for optimal Th1 development, miR146a plays an essential role in Treg function and development<sup>[78]</sup>. Targeting suppressor of cytokine signalling 1 (SOCS1), an inhibitor of STAT1, 4, and 5, miR155 plays a unique role in the development of Th1, Th2, and Treg cells and conversely in the suppression of Th17 differentiation. Targeting of PIAS3, an antagonist of STAT3, miR301 influences Th17 expansion<sup>[78]</sup>. Interestingly, the miR146a and miR155 are induced by toll-like receptors, well-known bacterial sensors, suggesting a critical involvement of micro-organisms<sup>[79]</sup>. Moreover, NOD2, a bacterial sensor closely linked to the pathogenesis of Crohn's disease, induces miR29 resulting in downregulation of IL-23 secretion by dendritic cells through targeting of IL-12p40 and IL-23p19. This, in turn, was shown to suppress ROR- $\gamma$ t expression in the DSS mouse model of colitis<sup>[80]</sup>.

In activated T cells, miR-210, which targets HIF-1, is upregulated 100 fold. This is especially notable in Th17 cells, resulting in decreased HIF-1 $\alpha$  expression. Deletion of *Mir210* promotes Th17 differentiation under hypoxic conditions. In experimental colitis, miR210 reduced the abundance of *Hif1a* transcripts and the proportion of cells that produced inflammatory cytokines resulting in decreased disease severity<sup>[81]</sup>. Induced by natural and environmental ligands of the

aryl hydrocarbon receptor, the miR132/212 cluster promotes Th17 development<sup>[82]</sup>. The mechanisms of Th plasticity in aryl hydrocarbon receptor signalling will be described in the next section of this review.

Targeting EST1 which is a negative regulator of Th17 cells, miR326 is critical for Th17 differentiation. Furthermore, miR10, which is selectively expressed in Treg and induced by TGF- $\beta$  signalling together with retinoic acid, can limit Th17 differentiation and furthermore can convert conventional Th cells into iTreg. The contribution of this miRNA to Treg stability is highly dependent on Foxp3 expression yet not responsible for Foxp3 induction<sup>[78]</sup>. A regulator of the suppressive activity of Treg cells, miR-126 leads to enhanced Foxp3 expression by targeting PIK3R2, a regulatory component of PI3K which downregulates Foxp3 induction<sup>[83]</sup>. The cluster of miR17-92, a complex of 6 miRNAs, influences Treg function *in vitro* resulting in the generation of IL-10 secreting Foxp3<sup>+</sup> T cells. Although miR17 specifically targets TGF- $\beta$  receptor II and Creb1, the targets of other parts of this cluster are still unknown<sup>[78]</sup>.

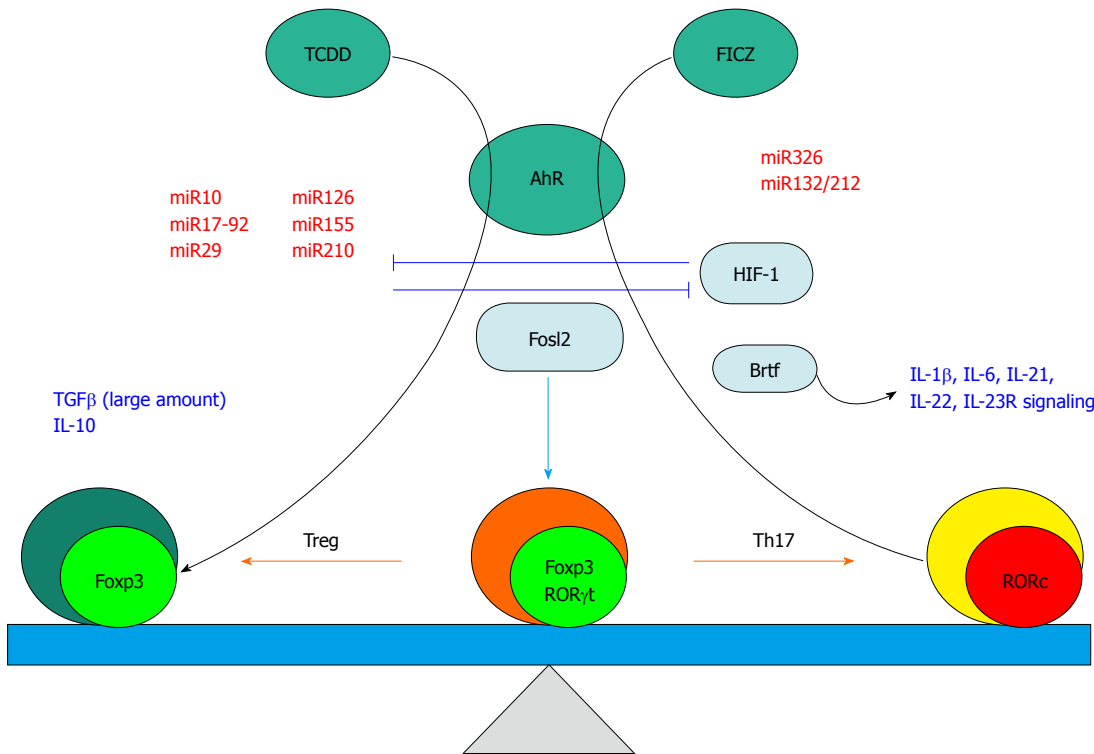
Interestingly, deletion of critical compounds for miR signalling, such as DICER or DROSHA which are found in the micro RNA biogenesis pathways, showed that Treg-specific micro RNA expression is required to suppress T effector cells and maintain tolerance, suggesting that lack of Treg-specific miR results in immune-dysregulation<sup>[78]</sup>.

Since miRs directly regulate the expression of many genes essential to Treg and Th17 subsets, it is highly likely they contribute to Th17-Treg plasticity. However, this exciting field is still in its infancy and further studies are undeniably required.

MicroRNA which influence Th17 development and plasticity are summarised in Table 3.

#### Aryl hydrocarbon receptor (unique mechanistic role of a receptor with dual functions)

The AhR first came to attention 1970-80s<sup>[84,85]</sup> as a receptor for a recognized carcinogen, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). This receptor is



**Figure 2 Factors influencing the plasticity between Treg and 17 secreting helper T subsets.** Cytokines and growth factors (shown in blue letters) may trigger transdifferentiation of the pre-committed Foxp3 expressing Treg population to RORc expressing cells. Micro RNAs (miR, shown in red letters) play a pivotal role in differentially regulating Treg/Th17 plasticity. Transcription factors (aqua ovals) direct the plasticity by positively or negatively controlling Foxp3 and/or RORc expression. The aryl hydrocarbon receptor (AhR, green circle) can promote distinct differentiation pathways in response to two pathway-specific ligands (TCDD and FICZ, Green circles) resulting in either augmentation of Foxp3 or RORc, respectively. Th17: 17 secreting helper T; FICZ: 6-formylindolo [3,2-b] carbazole; TCDD: Tetrachlorodibenzo-*p*-dioxin; FICZ: Formylindolo [3,2-b] carbazole; AhR: Aryl hydrocarbon receptor; Treg: Regulatory T cells.

a ligand-activated transcription factor, which is responsible for the regulation of several xenobiotic response genes, such as the cytochrome P450 family<sup>[86]</sup>. Soon, attention shifted to the contribution of this receptor to oncogenesis *via* the suppression of immune-surveillance in response to TCDD, suggesting a TCDD-induced immune regulatory function of AhR pathways<sup>[87]</sup>. Later, 6-formylindolo [3,2-b] carbazole (FICZ) was recognised as an endogenous ligand of AhR; however the function of the receptor bound to FICZ was not the same as that bound to TCDD, suggesting that this receptor has ligand specific functions<sup>[88]</sup>. In regard to T cell polarization, Quintana *et al*<sup>[89]</sup>, discovered that activation of the AhR with TCDD leads to the generation of Tregs, and in the same issue of Nature, Veldhoen *et al*<sup>[90]</sup>, reported alternative activation with FICZ leading to Th17 cell differentiation. The effect on Th17 generation has been confirmed by subsequent studies *in vitro* and *in vivo*<sup>[91,92]</sup>, while the influence on Treg generation has been more controversial<sup>[93]</sup>. Recently, Mezrich *et al*<sup>[94]</sup>, showed evidence for a missing link in AhR-derived Treg generation in that kynurenine, a metabolite in the indoleamine 2,3-dioxygenase (IDO) dependent tryptophan degradation pathway, is a key ligand of AhR<sup>[94,95]</sup>. IDO is an enzyme known to suppress effector T cells and is expressed in regulatory plasmacytoid

dendritic cells in response to IFN- $\gamma$ . This kynurenine bound AhR generates Treg *via* influencing the TGF- $\beta$  signalling pathway<sup>[94]</sup>. Furthermore, Moura-Alves *et al*<sup>[96]</sup>, has reported that AhR binds pathogen-associated molecular patterns (PAMPs), regulating immunity in response to bacteria. Although this observation was limited to myeloid and epithelial cells from lung in a murine infectious model, there is a possibility that bacterial activation of AhR may modulate development of Th subsets in a complex bacterial environment, such as the digestive tract.

Taken together one can speculate that a unique function of AhR may be to contribute to plasticity between the Treg and Th17 subsets *via* differential binding to environmental or physiological ligands including bacteria-derived metabolites and PAMPs.

Figure 2 illustrates the contributing factors to the plasticity between Treg and Th17.

### Histone methylation (monitoring the characteristics of plasticity)

Histone modifications including acetylation, methylation, and phosphorylation, are associated with gene expression or repression *via* alternations in chromatin structure<sup>[77]</sup>. In regard to Th1, Th2 and Th17 subset development, trimethylation of lysine 4 (H3K4me3) and lysine 27 (H3K27me3) play important roles in activation

and repression, respectively, of gene expression downstream of transcription factors specific to Th subsets. Thus, monitoring H3K4me3 and H3K27me3 status can visualize the potential for plasticity in T cell subsets.

H3K4me3 marks the signature cytokine genes (*Ifng* for Th1, *Il4* for Th2, *Il17a*) within the Th17 subset while repressive H3K27me3 is found on Th17 signature cytokine genes in Th1 (*Il4*, *Il17a*), Th2 (*Ifng*, *Il17a*) and Th17 (*Il4*, *Ifng*), suggesting lineage commitment to these particular subsets within these cells. Interestingly however, trimethylation on some transcription factor genes is bivalent (both H3K4 and H3K27) suggesting an element of reversibility in epigenetic status, particularly in Th17 cells. In these cells it is possible to find bivalent methylation in the Th1 transcription factor *Tbx21* and Th2 transcription factor *Gata3*, in addition, Foxp3 may remain unmethylated suggesting a “neutral” state. Thus, histone methylation states may help explain why Th17 cells can swing to other subsets more easily than other T cell subsets<sup>[97-99]</sup>. Cytokines regulate the trimethylation status of Th17, resulting in subset conversion and plasticity. Also, the universal bivalent status of *Tbx21* in all Th subsets except Th1, where you have only permissive H3K4me3, explains why plasticity towards the Th1 subset is dominant<sup>[99]</sup>. Thus, monitoring trimethylation status of H3K4 and H3K27 is useful in predicting the potential for, and direction of Th cell plasticity.

A histone modifying enzyme, JMJD3 is a histone H3K27 demethylase<sup>[100]</sup>. Li *et al.*<sup>[101]</sup>, reported that JMJD3 ablation promoted Th cell differentiation into Th2 and Th17 cells in the small and large intestines, and inhibited T-cell differentiation into Th1 cells *in vitro* and in a Th1-dependent colitis model. JMJD3 deficiency also restrains plasticity of Th2, Th17 and Treg cells towards Th1 cells. The skewing of T-cell differentiation is concomitant with changes in the expression of key transcription factors and cytokines *via* changes in H3K27me3 and H3K4me3 levels<sup>[101]</sup>.

## TH17 PLASTICITY IN CHRONIC INFLAMMATORY DISEASES INCLUDING IBD

IFN- $\gamma$ <sup>+</sup> IL-17<sup>+</sup> double expressing cells are considered a cross-over transition of Th17 into Th1 lymphocytes<sup>[102]</sup>. This cell population represents an efficient local host defense system which shifts host defense from extracellular pathogens to intracellular microbial infections<sup>[103]</sup> and may contribute to autoimmune pathogenesis in both mouse models and in human diseases<sup>[104,105]</sup>. Globig *et al.*<sup>[106]</sup>, suggested that this population is indeed a subpopulation of Th17 cells and may be involved in IBD pathogenesis of both CD and UC. Interestingly, Weaver and colleagues recently have provided evidence that Th17 cells can act as

precursors for IFN- $\gamma$  secreting Th1 cells in a mouse of colitis, showing the indispensability of Th17/Th1 plasticity in the pathogenesis of colitis<sup>[21]</sup>.

Likewise, IL-17<sup>+</sup> Foxp3<sup>+</sup> double expressing CD4<sup>+</sup> T cells may differentiate into Th17 cells under pro-inflammatory conditions such as IL-1 $\beta$ , IL-6, IL-21, IL-23 and TGF- $\beta$ <sup>+</sup>, having as yet largely unknown consequences for human disease initiation or progression<sup>[107]</sup>. On the other hand, plasticity from Th17 to Treg has not been frequently reported. However, a recent study from Yale University, suggests that this can in fact occur in the context of intestinal immune responses. According to their findings, Th17 cells generated during bacterial infection can be converted into IL-10<sup>high</sup>, Foxp3<sup>lo</sup> Tr1-like regulatory T cells. This was dependent on TGF- $\beta$ 1 and could be enhanced *in vitro* using an AhR ligand, FICZ<sup>[108]</sup>. This suggests that Th17 cells may alter their inflammatory status during infection, thus quenching inflammation and suggests possible avenues *via* which this mechanism could be harnessed therapeutically<sup>[108]</sup>.

The Th17-promoting cytokine, IL-23 is known to play an essential role in driving intestinal inflammation. This cytokine also plays an inhibitor role in augmentation of intestinal iTreg generation<sup>[109]</sup>. Furthermore, IL-23 together with IL-12 signalling promotes IFN- $\gamma$  secretion from Th17 in intestine, leading Th17/Th1 plasticity<sup>[110]</sup>. IL-23 signalling pathway is considered as a plasticity initiator in regard of Th17 subset *via* decreasing Foxp3 expression and assisting increased IFN- $\gamma$  secretion.

In 2011, a hallmark study of Hovhannisyan *et al.*<sup>[111]</sup> showed evidence for Treg/Th17 plasticity in IBD by showing the presence of IL-17 producing, Foxp3 expressing Th cells in inflamed intestinal mucosa from Crohn's disease patients. Importantly, this population showed suppressor activity *in vitro*. We have gone on to demonstrate the presence of this unique T cell subset in peripheral blood from IBD patients<sup>[60]</sup> and have found evidence for several types of plasticity including Treg/Th17, Th1/Th17 and Th22/Th17 within the lamina propria of lesions from IBD patients<sup>[112]</sup>.

## CONCLUSION

Plasticity between Treg and Th17 likely occurs in the context of dynamic changes in the inflammatory milieu. Thus, pro-inflammatory stimuli may promote conversion of immune-suppressive regulatory T cells into pro-inflammatory Th17 cells, while resolution of inflammation may trigger or even require the alternate shift from Th17 to Treg. This concept is just becoming appreciated and requires further study to correlate both causes and outcomes. Targeting plasticity may offer avenues to pharmacologically restore the Th17/Treg balance in the intestine for therapeutic benefit<sup>[113]</sup>. In addition, targeting plasticity may help improve upon future therapies. Clinical trials of Treg therapy of Crohn's disease failed<sup>[114]</sup> perhaps in part due to Treg



instability. Further understanding of Treg plasticity may help to augment Treg therapy in the future.

Many studies have implicitly suggested the possibility of plasticity during their experiments and observation of inflammatory processes. It is clear however that several pathways give rise to the heterogeneous populations referred to as Th17 and Treg in the intestine. The field is in part limited due to constraints inherent to the techniques used to analyse these cells. Most investigators rely upon multicolor flow cytometry, requiring the precise selection of surface and intracellular markers and setting of conditions and controls in order to provide limited functional and expression data. New technologies look to overcome these technical issues as well as to enhance the depth of analysis. Examples include deep immune-phenotyping using CYTOF 2 mass spectrometry which allows the analysis of over 100 parameters at the single cell level without dealing with spectral overlap, alone or in combination with single cell transcriptomics.

To conclude, unravelling the complexity that underlies plasticity between Th17 and Treg cells may be key to understanding the intricate pathogenesis of T cell-mediated immune disorders, such as IBD. However, novel approaches and their application to human IBD will be required to reach this objective.

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2015 Advances in Inflammatory Bowel Disease

## Genetics of inflammatory bowel disease from multifactorial to monogenic forms

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### Abstract

Inflammatory bowel disease (IBD) is a group of

chronic multifactorial disorders. According to a recent study, the number of IBD association loci is increased to 201, of which 37 and 27 loci contribute specifically to the development of Crohn’s disease and ulcerative colitis respectively. Some IBD associated genes are involved in innate immunity, in the autophagy and in the inflammatory response such as *NOD2*, *ATG16L1* and *IL23R*, while other are implicated in immune mediated disease (*STAT3*) and in susceptibility to mycobacterium infection (*IL12B*). In case of early onset of IBD (VEO-IBD) within the 6<sup>th</sup> year of age, the disease may be caused by mutations in genes responsible for severe monogenic disorders such as the primary immunodeficiency diseases. In this review we discuss how these monogenic disorders through different immune mechanisms can similarly be responsible of VEO-IBD phenotype. Moreover we would highlight how the identification of pathogenic genes by Next Generation Sequencing technologies can allow to obtain a rapid diagnosis and to apply specific therapies.

**Key words:** Inflammatory bowel disease; Primary immunodeficiency disease; Early onset; Next generation sequencing; Genome wide association studies

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**Core tip:** Genetic investigation is of fundamental importance describing inflammatory bowel disease (IBD) as a complex disease, as well as in identifying the monogenic disorders that may present with IBD-like features. Using third-generation technology should be leveraged to accelerate the screening and allow the identification of the most rare monogenic defects. Furthermore, the study of genetic variants in monogenic and in sporadic IBD could help unraveling the complex interplay between defective and compensatory immune responses, opening the way to the

identification of new targets for therapy.

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## INTRODUCTION

Inflammatory bowel disease (IBD) is the result of an unbalanced crosstalk between gut luminal content and the mucosal immune system. IBD encompasses a continuum of clinical disorders, ranging from Crohn's disease (CD) through indeterminate colitis (IC) to ulcerative colitis (UC). In fact, some patients may present significant clinical overlap between these forms and even develop one form from another. However, there are distinctive genetic, environmental and pathogenic factors that can be involved in the three forms.

In general, CD is characterized by changes in intestinal microbiota (dysbiosis), focal translocation of bacteria across the mucosal barrier, altered mucosal response to bacterial invasion, development of chronic granulomatous inflammation and activation of adaptive immunity as results of compensatory mechanisms to minor defects of innate immunity or autophagy. Genetic factors can involve variants of different groups of genes, leading to a leaky epithelial barrier and impaired mechanisms of phagocytosis and autophagy. Whatever is the particular combination of factors in each patient, the common result is a vicious circle of dysbiosis, granulomatous inflammation and activation of T cell immunity<sup>[1]</sup>.

In contrast, a major role in UC seems to be played by dysregulation of lymphocyte immunity, with increased activation of T cells and/or reduced regulatory T cell function. Risk factors implicate a number of variants in genes associated with T cell activity and with down regulation of mucosal inflammation.

Furthermore, intermediate forms of IBD can share various clinical and genetic features with both CD and UC<sup>[2,3]</sup>. Indeterminate colitis is particularly common among subjects with very early onset in the first years of life (VEO-IBD). Indeed, patients with VEO-IBD present quite distinctive clinical features and display worse clinical course and usually poorer response to treatments compared with adult onset disease.

Although genetic factors have been associated with different forms of IBD, the diagnosis in each subject is commonly based on clinical and histopathology data, rather than genetic results. Indeed, the particular profile of common genetic variants has little consequences on the prediction of the disease course and

response to treatments.

However, genetic analysis can have an important impact on clinical practice for VEO-IBD.

Monogenic disorders are believed to be very rare, but it is expected that in the severe form of earlier onset of IBD, genetic factors play a significant role in pathogenesis. In some cases, VEO-IBD can result from monogenic disorders such as primary immunodeficiency diseases (PID).

Several anecdotal reports showed that a number of PIDs can onset with a clinical presentation compatible with IBD. Taking into account this possibility can allow genetic confirmation and effective treatment with hematopoietic stem cell transplantation (HSCT), avoiding ineffective and dangerous treatments with immunosuppressant, biological inhibitors and even surgery. In most cases, the application of an integrated clinical, functional and genetic approach can allow the identification of some PID diagnosis, however clinical and functional signs can be unexpressed or overlooked and the correct genetic assay may be delayed. Thus, given the importance of the earliest detection of PID, we dedicated in this review a large space to the detailed description of monogenic forms of IBD. We also highlight the potential role of severe VEO-IBD.

In the first part of this review, we will discuss about the susceptibility to develop IBD followed by how the availability of improved genetic tools can impact on the early diagnostics of monogenic VEO-IBD.

In addition, the study of monogenic causes of IBD may provide significant information for a better understanding of sporadic adult onset disease. Indeed, the role of defective mucosal immunity in IBD is a fundamental unsolved question.

Increasing evidence support the idea that in most cases hyperactive intestinal inflammation can be the result of compensatory responses to the environment in presence of various immune defects. The study of genetic variants in monogenic and in sporadic IBD could help unraveling the complex interplay between defective and compensatory immune responses, opening the way to the identification of new targets for therapy.

In the second part of this review, we will discuss how defects in regulation of innate or adaptive immunity can be relevant to the pathogenesis of inflammation in IBD.

## GENES INVOLVED IN MULTIFACTORIAL SUSCEPTIBILITY IBD

Until the last year several genome-wide associations studies (GWAS), followed by meta analysis of both principal forms of IBD (CD and UC) identified a total of 163 IBD loci: 60 loci with heterogeneous effects while the effects of the other 50 loci were not distinguishable in CD or UC. The remaining 53 loci were specific only for CD ( $n = 30$ ) or for UC ( $n = 23$ ). A total of 113 of

the 163 IBD loci were shared with other complex traits such as immune mediated diseases and mycobacterial infection<sup>[4-6]</sup>. Recently by immunochip genotype data from both European and East Asian, Indian or Iranian cohorts implicate new 38 loci in IBD risk most of which (27 loci) contribute to both diseases (CD and UC) while of the remaining 11 loci, 7 were classified as specific to CD and 4 to UC<sup>[7]</sup>.

The innate immune receptor nucleotide oligomerization domain containing 2 (*NOD2*) was the first gene associated with inflammatory bowel disease<sup>[8,9]</sup>. Three mutations (R702W, R703C and L1007fs) in the *NOD2* coding region were demonstrated to be associated with CD in Quebec affected patients that carried at least one variant<sup>[10]</sup>.

In Hungarian CD patients, as well as in other countries, the three-mentioned *NOD2* variant are associated with early onset and the presence of one variant allele increases the risk for developing CD from 1.5 to 4.3 folds, while two variants alleles increase susceptibility to develop the disease from 20 to 40 folds compared with the general population<sup>[11]</sup>.

The mechanisms linking *NOD2* variants to the risk of CD are not fully clear. In fact, these variants lead to loss-of-function of the protein, suggesting a link between an impaired innate immune response to bacterial infections and disease development<sup>[8,9]</sup>. In contrast, *NOD2* gain-of-function mutations, in the NATCH domain of the receptor, are associated with Blau Syndrome (BS) and early onset Sarcoidosis (EOS), causing a rare autosomal dominant disease characterized by a triad of symptoms (rashes, uveitis and arthritis) and onset among 3 and 4 years of age<sup>[12,13]</sup>.

Another strong association with CD regards the autophagy related 16-like 1 (*ATG16L1*) gene<sup>[14,15]</sup>, while interleukin-23 receptor (*IL23R*) gene, results associated with both UC and CD<sup>[16]</sup>.

Few GWAS have been performed also in pediatric patients: a study identified for the first time for pediatric IBD onset the *TNFRSF6B* gene within the 20q13 and the *PSMG1* gene within the 21q22 loci<sup>[17]</sup>. In early onset cases, an association was described between *IL23R*, *STAT3*, *JAK2* and *IL12B* and CD<sup>[18,19]</sup>.

Most of these genes concern the functions of innate immunity, autophagy and inflammatory cytokines production. In addition, the associations with *HNF4A* and *GNA12* point out the role of defects of epithelial barrier function<sup>[20]</sup>. Moreover Kaser *et al.*<sup>[21]</sup> identified an association among hypomorphic *XBPI* variants with both IBD forms, reporting that the deletion in the intestinal epithelial cells induces spontaneous enteritis.

A study carried out on the Korean population proves the different genetics IBD among different populations. Several Korean children suffered from UC at the time of diagnosis showed diarrhea and hematochezia like the features in Western studies. In a particular way this study demonstrated that genetic

of the IBD between the affected populations reflects the ethnic differences. In fact the *NOD2* and *ATG16L1* variants, strongly associated with IBD in western populations, were not associated in the Korean IBD patients, who conversely displayed an association with three genes (*ATG16L2*, *DUSP5* and *TBC1D1*) that aren't associated in Western population<sup>[22]</sup>.

Recently, new technologies allow expanding the possibility of genetic analysis in IBD. Indeed, Whole Exome Sequencing studies (WES) performed the identification of further genetic association with CD, including missense mutations in the PR domain-containing 1 (*PRDM1*), that encodes a transcription factor expressed by T and B cells, and a common variation in Nuclear domain 10 protein 52 (*NDP52*), an adaptor protein that acts in selective autophagy of intracellular bacteria<sup>[23]</sup>.

In addition, Xu *et al.*<sup>[24]</sup> discovered in Chinese patients by WES novel genetic variants in the *DLG1* gene involved in cell proliferation, T cell polarity and T cell receptor signalling, as a susceptibility gene for CD.

## MONOGENIC FORMS IN EARLY ONSET IBD

EO-IBD is defined by the onset of disease within the 6<sup>th</sup> year of age. This group includes neonatal IBD (first 28 d of age), Infant and toddler onset IBD (younger than 2 years, VEO-IBD), and early childhood groups<sup>[25]</sup>.

In VEO-IBD, the disease tend to be much more severe and much more difficult to control with conventional therapies, compared with adult-onset IBD. Increasing evidence suggest a stronger genetic contribution to these forms compared with adults. Indeed some patients with VEO-IBD may have developed intestinal inflammation as part of a monogenic disease, usually a PID. In fact, these cases may account, at least in part, for the phenomenon of missing heritability in IBD, which is the inability to explain all the genetic contribution to IBD based solely on the additive effect of common risk gene variants<sup>[26]</sup>. Overall, at least 58 genes can play a role in VEO-IBD (Table 1), in addition to those associated with susceptibility to multifactorial IBD. Most of these genes are the cause of very rare monogenic disorders that can present with clinical and histopathological features similar to IBD. The different diseases associated with early onset IBD-like symptoms have been recently reviewed elsewhere<sup>[27]</sup>. Distinguishing monogenic forms among VEO-IBD is a crucial importance to allow the best treatment. A panel of candidate genes used for the analysis of VEO-IBD can allow a timing diagnosis and an effective cure in many patients, as well as an epidemiologic definition of the real impact of PIDs in this field.

However, it is worth noting that most cases of VEO-IBD can still recognize a multifactorial origin, as suggested by the evidence of the increased incidence of early onset cases of IBD in recent decades, reaching



**Table 1 Genes involved in the phenotype of monogenic very early onset of inflammatory bowel disease**

Gene	Inheritance	Chr	OMIM	Disease	Clinical Features IBD-like	Treatment	Ref.
Hyper and autoinflammatory disorders							
<i>MVK</i>	AR	12q24	#260920	Mevalonate kinase deficiency	Abdominal pain, Diarrhea, vomiting	Anakinra	[28,30-32]
<i>MEFV</i>	AR	16p13	#134610 #249100	Familial Mediterranean Fever	Diarrhea, abdominal pain, mucus in the stool, peritonitis	Colchicine	[33,34]
<i>PLCG2</i>	AD	16q23	#614878	Autoinflammation, antibody deficiency, and immune dysregulation syndrome	Bloody diarrhoea, UC, enterocolitis		[35]
<i>NLRP12</i>	AD	19q13	#611762	Familial cold autoinflammatory syndrome 2	Abdominal pain	Anakinra	[37]
<i>NLR4</i>	AD	2p22	#616050	Autoinflammation with infantile enterocolitis	Neonatal-onset enterocolitis	Anakinra	[38-40]
Immune regulation and dysregulation disorders (innate and adaptive immune responses)							
<i>XIAP</i>	XL	Xq25	#300635	X-linked lymphoproliferative syndrome 2	Perianal abscesses	HSCT	[41-44]
<i>STXBP2</i>	AR	19p13	#613101	Familial haemophagocytic lymphohistiocytosis type 5	IDB-like colitis	HSCT	[45]
<i>HPS1</i>	AR	10q23	#203300	Hermansky Pudlak syndrome (type 1, 4 and 6)	IBD, UC, Granulomatous colitis	Platelet transfusion Anakinra	[46-50]
<i>HPS4</i>		22q12	#614073		Granulomatous colitis	Infliximab	
<i>HPS6</i>		10q24	#614075		Gastrointestinal symptoms, granulomatous colitis, imperforate anus, gluteal flap repairs	Subtotal colectomy	
<i>FOXP3</i>	XL	Xp11	#304790	Immunodysregulation, polyendocrinopathy and enteropathy	Intractable diarrhea, total or subtotal intestinal villous atrophy, enteropathy	HSCT	[51]
<i>AIRE</i>	AR/AD	21q22	#240300	Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy	Malabsorption, diarrhea, chronic atrophic gastritis	No specific treatment is available	[52]
<i>IL10</i>		1q32	*124092	IL-10 Signaling defects	Severe early-onset enterocolitis		
<i>IL10RA</i>	AR	11q23	#613148	Inflammatory Bowel Disease-28, early onset	Early onset enterocolitis, enteric fistula, perianal abscesses	HSCT	[53-63]
<i>IL10RB</i>	AR	21q22	#612567	Inflammatory Bowel Disease-25, early onset	Early onset enterocolitis, perianal abscesses, enterocutaneous and rectovaginal fistula		
Defects in phagocyte bacterial killing and neutropenia							
<i>SLC37A4</i> <i>GSD-1b</i>	AR	11q23	#232220	Glycogen storage disease 1b	Perioral and perianal lesions, ileitis, colitis, CD-like, protuberant abdomen	Granulocyte colony stimulating factor, prophylactic oral iron	[64-66]
<i>G6PC3</i>	AR	17q21	#612541	Severe Congenital neutropenia 4	Diarrhea, colitis, abdominal pain, perianal fistula or abscess, CD-like, oral aphthous ulceration	Granulocyte colony stimulating factor	[67]
<i>ITGB2</i>	AR	21q22	#116920	Leucocyte adhesion deficiency 1	CD-like with discontinuous stomatitis, ileocolitis, perianal and rectal abscess, fistulas, adhesion, strictures	HSCT	[70,71]
<i>NCF1</i>	AR	7q11	#233700	Chronic granulomatous disease	Colitis, perirectal abscess	HSCT	[70-75]
<i>NCF2</i>	AR	1q25	#233710		Perirectal abscesses due to immunodeficiency		

NCF4	AR	22q12	#613960		Chronic granulomatous colitis, diarrhea perianal infections, erosions and ulceration of the gastric fundus and colonic mucosa, multiple small granulomata on colonic biopsy.		
CYBA	AR	16q24	#233690		Perirectal abscesses due to immunodeficiency		
CYBB	XL	Xp11	#306400		Gastrointestinal perirectal abscesses due to immunodeficiency, enteritis and colitis		
T and B lymphocyte selection activation defects							
WAS	XL	Xp11	#301000	Wiskott-Aldrich Syndrome	Diarrhea, hematemesis and melena IBD, UC like, colonic inflammation with crypt abscess	HSCT/transfusion of autologous genetically modified	[77-79]
DCLRE1C	AR	10p13	#603554 #602450	Omenn Syndrome; Athabaskan-type severe combined immunodeficiency	Chronic diarrhea	HSCT	[25,80-82,85,86]
RAG1	AR	11p12	4	Omenn Syndrome			
RAG2	AR		#603554				
LIG4	AR	13q33	#606593	LIG4 Syndrome	Protracted diarrhea	HSCT	
ADA	AR	20q13	#102700	Partial adenosine deaminase deficiency	Enzyme replacement therapy using frozen irradiated red blood cells/HSCT	HSCT	
IL2RG	XL	Xq13	#300400 #312863	Severe Combined Immunodeficiency; Moderate Immunodeficiency			
CD3G	AR	11q23	#615607	Immunodeficiency 17	Diarrhea autoimmune, gastroenteritis, recurrent, enteropathy	HSCT	
ZAP70	AR	2q11	#269840	Selective T-cell defect	Diarrhea	HSCT	
LCK	AR	1p35	#615758	Immunodeficiency 22	Diarrhea autoimmune, panniculite	HSCT	[83,84]
LRBA	AR	4q31	#614700	Common variable immunodeficiency 8	Colitis, IBD	Ig replacement therapy/HSCT	[88,90,91]
ICOS	AR	2q33	#607594	Common variable immunodeficiency 1	Early onset gastrointestinal tract infections, enteritis, recurrent diarrhea	Ig replacement therapy/HSCT	[92]
IL21	AR	4q27	#615767	IL-21 deficiency	Early onset IBD	Ig replacement therapy/HSCT	[93]
CTLA-4	AD	2q33	#616100	Autoimmune lymphoproliferative syndrome type V	Early onset IBD and autoimmunity	No specific treatment is available	[122]
TNFRSF13B	AR/AD	17p11	#240500	TACI deficiency	Enteritis, recurrent diarrhea	Ig replacement therapy/HSCT	[94]
COG6	AR	13q14	#614576	Congenital disorder of glycosylation, type III	Anal anteposition, recurrent diarrhea, IBD	No specific treatment is available	[123]
BTK	XL	Xq22	#300755 #307200	Agammaglobulinemia Isolates growth hormone deficiency type III	Diarrhea	Ig replacement therapy/HSCT	[99,100]
PIK3R1	AR	5q13	#615214	Agammaglobulinemia 7	Recurrent gastroenteritis		[101]
CD40LG	XL	Xq26	#308230	Immunodeficiency with hyper-IgM type I	Diarrhea		[96,97]
AICDA	AR	12p13	#605258	Immunodeficiency with hyper-IgM type II	Gastrointestinal tract infections		[98]
Disorder of apoptosis							
CASP8	AR	2q33	#607271	Caspase 8 deficiency	chronic diarrhea	No specific treatment is available	[102]
ITCH	AR	20q11	#613385	Autoimmune disease, multisystem with facial dysmorphism	Enteropathy, chronic diarrhea, malabsorption, gastrostomy tube feeding	Immunosuppressive treatment	[112]

<i>MASP2</i>	AR	1p36	#613791	MASP2 deficiency	IBD, UC-like		[113]
WELL defined syndromes associated with EO-IBD							
<i>TTC7A</i>	AR	2p21	#243150	Multiple intestinal atresia	Multiple areas of atresia along the small and large intestines, Intestinal malrotation Intraluminal calcification, bowel distention Mucous membrane ulceration	Surgery	[103-105]
<i>TTC37</i>	AR	5q15	#222470	Trico hepato enteric syndrome	Diarrhea, severe villous atrophy	Parenteral nutrition	[107,108]
<i>SKIV2L</i>	AR	6p21	#614602	Trico hepato enteric syndrome 2	Diarrhea, colitis, severe and intractable villous atrophy	Ig supplementation	[106]
<i>NEMO/IKBKG</i>	XL	Xq28	*300248	X-linked ectodermal dysplasia and immunodeficiency	CD-like colitis, villous atrophy, recurrent digestive tract infections, intractable diarrhea and recurrent ulcerations	HSCT	[109,110]
<i>GUCY2C</i>	AD	12p13	#614616	Familial Diarrhea	Early onset chronic diarrhea, IBD, CD, small-bowel obstruction, esophagitis, irritable bowel syndrome, ileal inflammation, abdominal pain	Parenteral nutrition	[111]
Defects affecting the integrity of intestinal barrier							
<i>COL7A1</i>	AR	3p21	#226600	Dystrophic epidermolysis bullosa	Gastrointestinal complications, diarrhea, colitis, esophageal blisters strictures, anal blisters, constipation	Immuno myeloablative chemotherapy and allogenic HSCT	[114]
<i>ADAM17</i>	AR	2p25	#614328	Neonatal inflammatory skin and bowel disease 1	Perioral and perianal erythemas with fissuring, diarrhea, malabsorption, plasma cell duodenitis crypt hyperplasia, villous atrophy	EGFR Ligands	[115]
<i>FERMT1/KIND1</i>	AR	20p12	#173650	Kindley syndrome	Intestinal involvement with haemorrhagic diarrhoea, UC	No specific treatment is available	[116]
<i>EGFR</i>	AR	7p11	#616069	Neonatal inflammatory skin and bowel disease 2	Diarrhea	No specific treatment is available	[117,118]
<i>TGFBR1</i>	AD	9q22	#609192	Loeys-Dietz syndrome, type 1	Gastrointestinal disorders,	Medication and preventative surgery	[119]
<i>TGFBR2</i>	AD	3p24	#610168	Loeys-Dietz syndrome, type 2			

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; HSCT: Hematopoietic stem cell transplantation; CD: Crohn's disease.

4.37 per 100000 children<sup>[25]</sup>.

Below we discuss how monogenic disorders involving different immune mechanisms can similarly be responsible for VEO-IBD.

### Hyper and autoinflammatory disorders

A chronic or episodic inflammatory disease of the intestine can occur as part of a complex clinical picture in subjects affected by several autoinflammatory disorders.

Mevalonate kinase deficiency (MKD) is an autosomal recessive disease caused by mutations in the *MVK* gene and it is characterized by febrile attacks associated with diarrhea, vomiting and abdominal pain. The occurrence of abdominal pain and diarrhea, sometimes with blood and mucus, together with

leukocytosis, chronic anemia and increased CRP could raise the suspicion of IBD<sup>[28]</sup>. In most cases intestinal inflammation occurs only during febrile flares, yet the use of glucocorticoids can hidden the typical periodicity of the disease, reducing the severity but increasing the frequency of symptoms, thus making more difficult the diagnosis<sup>[29]</sup>. In some cases, patients with MKD may present with VEO-IBD with the characteristics of indeterminate colitis. Of note, treatment with anti-IL-1 agents can allow relieving inflammatory colitis as well as other febrile and inflammatory features typical of the disease<sup>[30-32]</sup>.

IBD can be also more frequent and severe in patients with *MEFV* mutations. Identification of *MEFV* can allow diagnosis of Familial Mediterranean Fever and effective treatment with colchicine<sup>[33,34]</sup>.

Recently, a *de novo* missense mutation (S707T) in the *PLCG2* gene has been detected by the exome analysis in two patients suffering from an autosomal dominant inflammatory disorder with severe enterocolitis and mild immunodeficiency, even if it is not clear whether gut inflammation is facilitated by the hyper-inflammatory defect or by the immunodeficiency<sup>[35]</sup>.

Familial cold autoinflammatory syndrome-2, a systemic auto-inflammatory disease caused by heterozygous mutations in the *NLRP12* gene can present abdominal pain vomiting and buccal aphthous ulcers together with cold-induced fever<sup>[36]</sup> and in some cases hypogammaglobulinemia<sup>[37]</sup>.

Familial cold autoinflammatory syndrome 4 is an autosomal dominant disease caused by heterozygous mutation in the *NLR4* gene and characterized by intermittent episodes of rash, arthralgia, and fever after exposure to cold<sup>[38]</sup>. Recently a family was reported with a syndrome featuring neonatal onset enterocolitis, in which the father and two respective sons carried a missense mutation (V341A) in the *NLR4* gene. It has also been shown that this mutation, functionally associated with gain of function, cosegregates in the family with the disease<sup>[39]</sup>.

Canna *et al.*<sup>[40]</sup> report a *de novo* missense substitution (T337S) on *NLR4* NBD domain that causes early onset recurrent fever flares and macrophage activation syndrome (MAS).

#### **Defect of cytotoxicity and hyperinflammation**

The association of inflammatory enterocolitis with MAS can be found also in subjects with *XIAP* mutations, as described by Worthey<sup>[41]</sup>. Indeed, *XIAP* deficiency, already associated with X-linked lymphoproliferative syndrome-2 (XLP2), can be the cause of Crohn's like features in the absence of MAS<sup>[42]</sup>. In particular, Zeissig *et al.*<sup>[43]</sup> identified *XIAP* private variants in absence of symptoms related to XLP2 in a cohort of German boys with early onset CD. Of note, macrophage activation in subjects with *XIAP* deficiency is a compensatory phenomenon sustained by Interferon- $\gamma$  production by lymphocytes and natural killer cells with impaired antiviral capacity, thus it is not of autoinflammatory origin. Similarly, the development of Crohn's-like disease seem to be due to a deficiency rather than to an excess of *XIAP* function, leading to defective activation of NOD2 in monocytes. In contrast to lymphohistiocytosis, IBD has been reported also in female subjects heterozygous for *XIAP* mutations<sup>[44]</sup>.

Inflammatory colitis has been reported also in anecdotal cases of subjects with X-linked lymphoproliferative syndrome, although in this case the mechanisms are unknown<sup>[45]</sup>.

Hermansky-Pudlak syndrome (HPS) is a rare autosomal recessive disease characterized by typical syndromic features including albinism, hemorrhagic diathesis, and pigmented reticuloendothelial cells. The HPS patients with specific mutations in the respective genes (*HPS-1*, *HPS-4*, *HPS-6*) have major organ

involvement including also severe granulomatous colitis with pathologic features suggestive of CD<sup>[46-50]</sup>.

#### **Immune regulation and dysregulation disorders (innate and adaptive immune responses)**

Autoimmune enteropathy is part of the X-linked immune dysregulation, polyendocrinopathy, enteropathy disease and it is caused by *FOXP3* gene mutations. Although the disorder has distinctive features making it well distinguishable from IBD, mutation in *FOXP3* have been recently associated with very-early IBD phenotype<sup>[51]</sup>.

Autoimmune enteropathy has been described, albeit more rarely, also in the autoimmune polyendocrinopathy syndrome type<sup>[52]</sup>.

In recent years, several studies highlighted the causative role of the immunoregulatory cytokine *IL-10* and of its receptor in the early onset IBD<sup>[53-62]</sup> and the genetic analysis of *IL-10*, *IL10RA*, *IL10RB* has become routinely in patients who developed the first symptoms within the 3 mo of life, regardless of parents' consanguinity<sup>[63]</sup>.

#### **Defects in phagocyte bacterial killing and neutropenia**

Neutrophil defects are often associated with intestinal inflammation. In particular, subjects with glycogen storage disease-type 1b show neutrophil dysfunction and run increased risk of developing Crohn's Disease-like<sup>[64-66]</sup>.

In a similar manner, subjects with defect of *G6PC3*, often develop CD-like inflammation, which is also associated with persistent T cell lymphopenia<sup>[67]</sup>.

Other neutrophil defects associate with early onset IBD include Leukocyte Adhesion Deficiency-1 (*LAD-1*, *ITGB2* mutated), which can be associated with chronic ileocolitis<sup>[68]</sup>, early onset ulcerative colitis and a non-specific Crohn's like colitis<sup>[69]</sup> as well as with bacterial infections.

However, the best characterized defect of phagocytes associated with VEO-IBD is chronic granulomatous disease (CGD), both in its X-linked and autosomal recessive forms. Severe infections by catalase positive bacteria and fungi are usually prominent clinical features, however, cases presenting first with intestinal inflammation, often in the first months of life, are not rare, in particular among subjects with autosomal recessive CGD<sup>[70-73]</sup>. IBD in subjects with CGD reproduces the clinicopathological features of CD<sup>[74]</sup>. Recently, Dhillon *et al.*<sup>[75]</sup> found some variants in heterozygosis in NADPH complex genes not leading to appreciable immunodeficiency, yet associated with susceptibility to VEO-IBD. Actually, the pathogenesis of inflammation in CGD could also be attributed to a deficiency of autophagy, leading to autoinflammatory response dominated by IL-1 release<sup>[76]</sup>.

#### **T and B lymphocyte selection and activation defects**

Intestinal inflammation is a common feature in several



PID affecting adaptive immunity.

Wiskott Aldrich Syndrome, an X-linked PID due to mutation in WASP protein, can often present with neonatal or infantile hemorrhagic and inflammatory colitis that can occur before other typical signs such as dermatitis and infections<sup>[77,78]</sup>. Thrombocytopenia with small platelets and in some cases also hypogammaglobulinemia usually with normal/high-IgA can help addressing the correct diagnosis<sup>[79]</sup>.

Severe combined immunodeficiency (SCID) is often followed by enteropathy and failure to thrive, even before infections. In some cases a low lymphocyte count per age can raise the suspicion of SCID. However, in other cases with hypomorphic mutations in SCID associated genes, such as *DCLRE1C*, *RAG1*, *RAG2*, *LIG4*, *ADA*, *IL2RG*, *CD3G*, *ZAP70* and *LCK*, lymphocyte count can be normal due to the development of dysfunctional lymphocytes<sup>[25,80-84]</sup>. Rarely, a leaky SCID may present for years with IBD only, in the absence of severe infections. The presence of other signs such as severe eczematous rash should raise the suspicion of a SCID<sup>[81,85]</sup>.

In all these cases only the analysis of lymphocyte subsets, and in particular of recent thymic emigrants (or the molecular measure of T cell receptor excision circles) can assist the correct diagnosis<sup>[86]</sup>.

Common variable immunodeficiency (CVID) is also associated with intestinal inflammation, but the disease rarely occurs in the first years of life<sup>[87,88]</sup>. The development of IBD seems to be favored by dysregulation of T-cells derived cytokines<sup>[89]</sup>.

Although the CVID is a polygenic disease, there are a low percentage of cases due to specific genetic defects such as LPS-responsive beige-like anchor (*LRBA*), *ICOS* and *IL-21*, which may often present with earlier onset in life.

In particular, deficiency of the *LRBA* gene has been found in patients affected by CVID with early onset hypogammaglobulinemia, inflammatory bowel disease and autoimmune cytopenia<sup>[88,90]</sup>. Serwas *et al.*<sup>[91]</sup> recently identified a new missense mutation in the *LRBA* gene in a young girl with severe early IBD-like disease without other manifestations of immunodeficiency.

Other forms of CVID associated with IBD include deficiencies of *ICOS* gene, *CTLA-4*, *PD-1*, *IL-21*, *TNFRSF13B* and *COG6*<sup>[92-95]</sup>.

IBD-like phenotype was observed also in patients with hyper-IgM syndrome resulting from defects in the CD40 ligand<sup>[96,97]</sup>, AID genes<sup>[98]</sup> and in subjects with agammaglobulinemia due to defects in *BTK*<sup>[99,100]</sup> or *PIK3R1*<sup>[101]</sup>.

### Disorders of apoptosis

Several cellular mechanisms such as the embryonic development, cell differentiation and the elimination from the intestine and from other body parts are regulated by the caspases that are cysteine proteases. Caspase dysfunction has been associated with IBD, in particular CASP-8 is involved in the inflammation

of the mucosa and controls in the CD patients the necroptosis of the Paneth cells and the death of the epithelial cells<sup>[102]</sup>.

### Well defined syndromes associated with early onset IBD

The multiple intestinal atresia (MIA) combined with SCID, is caused by mutations in the *TTC7A* gene<sup>[103]</sup>. Recently MIA was reported in different families with a very early onset form of apoptotic enterocolitis<sup>[104,105]</sup>.

Another immunodeficiency associated with low numbers of B cells and immunoglobulins is the Tricho Hepato Enteric Syndrome, a syndromic diarrhea usually associated with mutations in the *TTC37* or in the *SKIV2L* gene<sup>[106-108]</sup>.

X-linked anhidrotic ectodermal dysplasia with immunodeficiency, caused by hypomorphic mutations in the nuclear factor- $\kappa$ B essential modulator (*NEMO*), is associated both with epithelial and immune defects. Except for syndromic features of ectodermal dysplasia and susceptibility to infection from various pathogens, patients often present severe chronic colitis, which in some cases has been reported to worsen after HSCT, probably depending on the engraftment of donor immune cells on the background of defective host epithelial cells<sup>[109,110]</sup>.

Other syndromes with early onset chronic diarrhea intestinal inflammation comprise defects of *GUCY2C*, an intestinal receptor for the heat stable bacterial enterotoxins<sup>[111]</sup>, *ITCH*, involved in the ubiquitin-editing protein complex<sup>[112]</sup>, and *MASP2* which is an important bactericidal factor<sup>[113]</sup>.

### Defects affecting the integrity of intestinal barrier

Mutations in the type VII collagen gene (*COL7A1*) induced the dystrophic epidermolysis bullosa, a genodermatosis. IBD can develop both from mutations in *COL7A1* and from acquired defects in this protein due to autoimmunity<sup>[27,114]</sup>.

Other molecules involved in the intestinal barrier include *ADAM17A*, which has been associated to an autosomal neonatal recessive syndrome characterized by inflammatory skin and bowel disease<sup>[115]</sup>.

Recessive mutations in *KIND1/FERMT1* gene are responsible for Kindler syndrome, characterized by skin blistering, poikiloderma, photosensitivity and sometimes UC-like gastrointestinal symptoms and haemorrhagic diarrhea<sup>[116]</sup>.

EO-IBD can be observed also in patients with EGFR deficiency, together with skin disease<sup>[117,118]</sup>.

More complex is probably the pathogenesis of IBD in Loews-Dietz syndrome, an autosomal disease caused by mutations in the *TGFBR1* and *TGFBR2* genes that encode respectively for the receptor type 1 and 2 of the transforming growth factor  $\beta$ <sup>[119]</sup>.

## CONCLUSION

When we are faced with a patient with symptoms related to chronic inflammatory bowel disease, it is

essential to consider first both the age of onset and the severity of symptoms just to find out whether the patient is suffering from classical multifactorial IBD or if we are facing a severe clinical case, potentially caused by a monogenic form, as which characterized by a more severe phenotype and resistant to traditional therapies. This first classification is critical to guide genetic investigation. The identification of monogenic forms, that are quite rare, can have high impact on the therapeutic options, with particular reference to hematopoietic stem cell transplantation. Thus, in case with early onset and severe or atypical features, the genetic research should be directed toward the identification of a mendelian form of the disease. Target sequencing of multiple candidate genes can be accomplished with technologies such as the ION TORRENT, which are now available in many laboratories. The target sequencing must be designed to analyze all those genes that up to that time have been identified associated with a particular phenotype with severe early onset. In cases with high suspicion of a mendelian cause, but with normal target sequencing results, a more complete and sensitive analysis can be performed by comparison of the patients' whole exome sequence with that of their parents and/or relatives<sup>[120]</sup>.

When a monogenic disorder underlying intestinal inflammation is detected, we should discard the diagnosis of IBD and refer to IBD-like inflammation. This is easy and correct when we are dealing with well-defined PID, such as Wiskott Aldrich disease or chronic granulomatous disease. The distinction is much more tricky when we found other immune defects, such as those involving XIAP or LRBA.

An easy distinction could be based on the functional consequences of the genetic defect. It has been proposed that cases with lack of immunity should be indicated as PID, whilst the diagnosis of IBD should be reserved to cases with hyper-response of innate and adaptive immunity. However, it has been argued that excessive or defective immune responses can lead to similar inflammatory disorders, highlighting the importance of a proper functioning of immunity for intestinal homeostasis<sup>[121]</sup>. What makes even more complicated the clinical picture, is the fact that, a continuum may exist between severe defect, leading to both infections and inflammation signs, and mild defect, presenting only inflammatory signs.

Molecular genetics can help distinguishing inborn defects with excessive or defective immunity but, even more importantly, allow to discriminate defects expressed only in hematopoietic-derived cells from defect with an important role also in intestinal epithelium. In the first case (*e.g.*, in subjects with neutropenias, Wiskott Aldrich disease and chronic granulomatous disease) HSCT is able to cure both the immunodeficiency and gut inflammation; in the second case (*e.g.*, defects of *NEMO*, *NOD2*) HSCT can

be ineffective. More difficult is to predict the success of HSCT in other disorders in which the genetic defect can be important both in hematopoietic-derived cells and epithelia.

In brief, inflammatory bowel disease can be the result of different genetic mechanisms leading to common inflammatory phenotypes and requiring similar treatments. VEO-IBD is often resistant to conventional treatment.

Finding the molecular cause in single individuals could open the way to the development of novel and more specific therapeutic approaches.

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## 2015 Advances in Liver Transplantation

# Role of liver transplantation in human immunodeficiency virus positive patients

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## Abstract

End-stage liver disease (ESLD) is a leading cause of morbidity and mortality amongst human immunodeficiency virus (HIV)-positive individuals. Chronic hepatitis B and hepatitis C virus (HCV) infection, drug-

induced hepatotoxicity related to combined anti-retro-viral therapy, alcohol related liver disease and non-alcohol related fatty liver disease appear to be the leading causes. It is therefore, anticipated that more HIV-positive patients with ESLD will present as potential transplant candidates. HIV infection is no longer a contraindication to liver transplantation. Key transplantation outcomes such as rejection and infection rates as well as medium term graft and patient survival match those seen in the non-HIV infected patients in the absence of co-existing HCV infection. HIV disease does not seem to be negatively impacted by transplantation. However, HIV-HCV co-infection transplant outcomes remain suboptimal due to recurrence. In this article, we review the key challenges faced by this patient cohort in the pre- and post-transplant period.

**Key words:** Hepatitis B virus; Hepatitis C virus; Human immunodeficiency virus; Liver transplantation

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**Core tip:** Liver disease is a major cause of mortality and morbidity in human immunodeficiency virus (HIV) positive patients. It is therefore increasingly likely that HIV positive patients with chronic liver disease are likely to present as potential liver transplant candidates. We therefore review the current data with regards to liver transplantation in HIV positive patients.

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## INTRODUCTION

The management and treatment of human immunodeficiency virus (HIV)-1 infection was revolutionized in 1996 in Western Europe and North America following the introduction of combined anti-viral therapy (cART), resulting in the reduction of acquired immune deficiency syndrome (AIDS) and AIDS-related deaths<sup>[1]</sup>. Such was the success of cART, that now more than 50% of deaths in HIV-positive patients on cART are not directly related to HIV infection or AIDS<sup>[1-3]</sup>. The increase in non-AIDS related morbidities compared to the general population appears to be multifactorial: HIV infection leads to a state of immune dysregulation and inflammation whilst cART predisposes to dyslipidaemia and diabetes<sup>[4]</sup>. The most recent D:A:D (Data collection on adverse events of anti-HIV drugs) study demonstrated that liver disease is now the second commonest cause of a non-AIDS related death, having been overtaken by non-AIDS defining cancers (Figure 1)<sup>[5]</sup>.

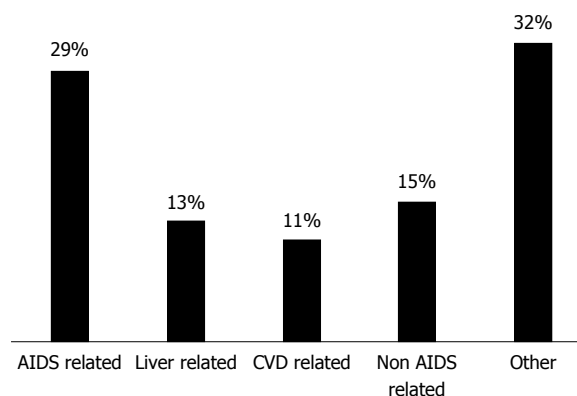
Liver related mortality however, remains high. Given the similar transmission routes, co-infection with chronic hepatitis C virus (HCV) and chronic hepatitis B virus (HBV) is common<sup>[6]</sup>. Other liver-related aetiologies amongst HIV-positive individuals include cART related liver toxicity, alcohol, non-alcohol related liver disease (NAFLD) and hepatocellular carcinoma (HCC). HIV positive patients present with the same clinical sequelae of chronic liver disease as HIV negative patients but tend to present at a younger age and a markedly reduced survival rate after the first episode of decompensation<sup>[7]</sup>. In HIV-positive patients with compensated cirrhosis an increased mortality rate is associated with age > 50 years, model for end-stage liver disease (MELD) score > 11 and poor control of HIV disease<sup>[8]</sup>.

## PRE-LIVER TRANSPLANTATION

### Viral aetiologies

One third of patients with HIV infection are co-infected with chronic HCV, and the majority of deaths in HIV-positive patients with end-stage liver disease (ESLD) can be directly attributable to HCV infection<sup>[8]</sup>. At-risk groups include intravenous drug users, haemophiliacs who were exposed to contaminated infusions of plasma derived factor VIII or X concentrate and men who have sex with men (MSM)<sup>[9]</sup>. Recent data has demonstrated an increasing incidence of acute HCV cases in MSM who are HIV-positive acquired through sexual transmission<sup>[10,11]</sup>.

HCV infection in a HIV positive individual is associated with a reduced rate of spontaneous HCV RNA clearance and therefore, more likely to result in chronic HCV infection<sup>[12]</sup>. HIV/HCV co-infection results in a more aggressive, rapid fibrosis progression rate compared HCV mono-infected patients<sup>[13-15]</sup>. In a study of 135 patients with HIV/HCV followed over a median of



**Figure 1 Causes of death amongst human immunodeficiency virus-positive patients.** Adapted from Smith *et al*<sup>[6]</sup>. AIDS: Acquired immunodeficiency syndrome; CVD: Cardio-vascular disease.

3 years, 44% had evidence of fibrosis progression whilst 13% developed cirrhosis<sup>[16]</sup>. Mechanisms for the accelerated fibrosis rates include up-regulation of pro-fibrotic pathways, enhanced microbial translocation, increased numbers of pro-fibrogenic CD8<sup>+</sup> cells, reduction in CD4<sup>+</sup> cells and reduction of interleukin-10 expression<sup>[12,17-20]</sup>. Clinical factors predictive of fibrosis progression include detectable HIV viraemia, low CD4<sup>+</sup> counts, baseline necro-inflammatory activity on liver biopsy and increased alcohol consumption (> 50 g per day)<sup>[16,21]</sup>. Survival after the first episode of hepatic decompensation is inferior amongst HIV/HCV co-infected patients compared to HCV mono-infected patients; median estimated survival of only 13 mo<sup>[22]</sup>.

Treatment of HCV in HIV-positive patients with pegylated-interferon (PEG) and ribavirin was associated with an increased side-effect profile and inferior sustained virological response rates (SVR) of 17%-35%<sup>[23-25]</sup>. The advent of the new directly acting antiviral (DAAs) agents have dramatically improved outcomes amongst HIV/HCV co-infected patients. The first generation of the new DAAs were boceprevir and telaprevir, both NS3A/4 protease inhibitors (PI). In phase II studies, triple therapy with boceprevir or telaprevir in conjunction with PEG and ribavirin resulted in SVR rates of 63% and 74% respectively in HIV-positive HCV genotype 1 infected patients<sup>[26,27]</sup>. Simeprevir, a second generation NS3/4 PI, plus PEG and ribavirin has also been evaluated in a Phase III trial demonstrating SVR12 rates of 74%<sup>[28]</sup>. Sofosbuvir, a NS5B polymerase inhibitor, has pan-genotypic activity and a high genetic barrier to resistance with no significant drug interactions with cART. Sofosbuvir plus weight-based ribavirin has been evaluated in the PHOTON-1 study in genotypes (G) 1-3<sup>[29]</sup>. Results demonstrated SVR12 rates similar to HCV mono-infected patients (G1 76%, G2 88%, G3 67%) with no evidence of HIV breakthrough<sup>[29]</sup>. SVR rates of 98% were recently published using the combination of ledipasvir and sofosbuvir for 12 wk<sup>[30]</sup>. TURQUOISE-I was a randomised, open label

**Table 1 Drug-drug interactions between directly acting anti-hepatitis C virus directly acting antiviral and combined anti-viral therapy**

Anti-HCV DAA	cART not recommended	Dose changes
Boceprevir	Atazanavir Darunavir/ritonavir Efavirenz Ritonavir	
Telaprevir	Darunavir Lopinavir/ritonavir	Efavirenz - dose increased to 1125 mg TDS
Simeprevir	Atazanavir Efavirenz Darunavir/ritonavir Nevirapine	
Daclatasvir	-	Efavirenz - dose increased to 90 mg OD Atazanavir and protease inhibitors - dose reduced to 30 mg OD
Sofosbuvir	Didanosine Zidovudine	

DAA: Directly acting antiviral; cART: Combined anti-viral therapy; HCV: Hepatitis C virus.

study assessing the all-oral 3 direct-acting antiviral (3D) regimen of ombitasvir, paritaprevir (co-dosed with ritonavir), dasabavir and ribavirin in HIV-HCV (genotype 1) co-infected patients<sup>[31]</sup>. SVR rates for 12 wk and 24 wk of this all oral treatment regimen were 94% and 91% respectively with minimal side-effects<sup>[31]</sup>. Drug-drug interactions between the anti-HCV DAAs and cART medications are concerning. Table 1 summarises possible interactions and recommended dose adjustments where appropriate.

All HIV/HCV positive patients listed for liver transplantation should be considered for anti-viral therapy. HIV/HCV co-infected patients with decompensated cirrhosis, where the treatment aim is viral suppression prior to liver transplantation, should be treated with sofosbuvir and weight based ribavirin for up to 48 wk<sup>[32]</sup>.

Ten percent of the HIV-positive population have evidence of chronic HBV infection<sup>[33]</sup>. Vertical transmission of HBV remains the most common route of infection worldwide, whilst sexual transmission and percutaneous transmission is more common in Europe and North America<sup>[34]</sup>. Chronic HBV infection is more common in HIV-positive patients, especially individuals with low CD4<sup>+</sup> cell counts<sup>[35]</sup>. In addition, HIV/HBV co-infected patients have higher HBV DNA titres compared to HBV mono-infected patients, which translates into accelerated fibrosis rates and an increased risk of developing HCC<sup>[36]</sup>. Anti-viral therapy is considerably easier in HIV/HBV co-infected patients compared to HIV/HCV co-infected patients due to nucleos(t)ide reverse-transcriptase inhibitors (lamivudine, emtricitabine and tenofovir) that have both anti-HIV and anti-HBV activity.

### Non-viral aetiologies

Abnormal liver enzymes are common in HIV-positive patients occurring in up to two thirds of patients on cART<sup>[37]</sup>. Identifying the culprit drug can be difficult due to the use combination therapies. Patterns of liver injury include hypersensitivity, idiosyncratic hepatotoxicity, mitochondrial toxicity, immune reconstitution syndrome and hepatic steatosis<sup>[38-40]</sup>. Non-cirrhotic portal hypertension (NCPH) is an increasingly recognised clinical entity amongst HIV-positive patients especially those with previous didanosine exposure<sup>[41]</sup>. The pathogenesis of NCPH has been linked to a pro-thrombotic state. The histological spectrum includes nodular regenerative hyperplasia, hepatoportal sclerosis, peri-portal fibrosis and sclerosing portal venopathy<sup>[42-44]</sup>.

Steatosis and steatohepatitis are common in diabetics, alcohol users and those with features of the metabolic syndrome<sup>[45,46]</sup>. Compared to the general population (14%-31%), NAFLD is more common in HIV-positive patients (30%-40%)<sup>[47,48]</sup>. The D:A:D study highlighted that the prevalence of the metabolic syndrome has increased over the last decade from 19% in 2000-2001 to 42% in 2006-2007<sup>[49]</sup>. This is likely to result in an increase in the prevalence of NAFLD, given that NAFLD is the hepatic manifestation of the metabolic syndrome. There is limited data available on the risk factors for NAFLD in HIV-positive patients but attention has focused on the role of cART because of its negative effects of insulin resistance, glucose metabolism and lipid metabolism. Central adiposity, male sex, low serum high-density lipoprotein levels, raised triglycerides levels and an increased ratio of alanine aminotransferase to aspartate aminotransferase have been suggested as risk factors for the development of NAFLD<sup>[46]</sup>. Raised gamma glutamyl transpeptidases and HOMA-IR > 2.5 are associated with NASH independent of cART and abdominal fat<sup>[50]</sup>.

Hepatocellular carcinoma (HCC) cases in HIV-positive patients is expected to increase predominately due to the co-infection in chronic HBV and HCV infection. Data from the MORTAVIC study and the French Mortalite study has certainly demonstrated an increased number of deaths related to HCC<sup>[8,51]</sup>. HIV-positive patients with HCC tend to present at a younger age with symptomatic and multiple or invasive disease<sup>[36]</sup>. HIV-positive patients with cirrhosis should undergo 6-monthly surveillance with a liver ultrasound and serum alpha-feto protein. Standard treatment therapies (radio-frequency ablation, transarterial chemo-embolisation, liver resection and liver transplantation) should be considered on an individual patient basis.

### Selection criteria for liver transplantation in HIV-positive individuals

HIV-positive patients with liver disease should be

**Table 2** Criteria for liver transplantation in human immunodeficiency virus-positive individuals**The criteria for liver transplantation are met**

CD4<sup>+</sup> cell count > 100 cells/ $\mu$ L (> 200 cells/ $\mu$ L with a previous history of opportunistic complications)  
 HIV viral load < 50 copies/mL (using ultrasensitive Amplicor Monitor PCR assay)  
 Absence of AIDS-defining illness<sup>1</sup>  
 Absence progressive multi-focal leukoencephalopathy, chronic intestinal cryptosporidiosis (> 1 mo) or primary CNS lymphoma

<sup>1</sup>None after combined anti-viral therapy-induced immune reconstitution.  
 CNS: Central nervous system; AIDS: Acquired immune deficiency syndrome; HIV: Human immunodeficiency virus.

managed in a multi-disciplinary environment with an experienced transplant hepatologist and HIV physician. Given their rapid disease kinetic, HIV-positive patients with end stage liver disease (ESLD) need to be identified early. The MELD score appears to be a sensitive predictor of patient outcome amongst HIV-positive patients pre-liver transplantation<sup>[52,53]</sup>. Even after adjusting for CD4<sup>+</sup> counts and HIV RNA titres, the MELD score remains the only significant predictor of mortality<sup>[52]</sup>.

Guidelines for liver transplantation in HIV-positive patients have evolved as our experience with this cohort has increased. The current United States National Institutes of Health multicenter trial guidelines for liver transplantation in HIV-positive patients with chronic liver disease are listed in Table 2.

Optimal control of HIV disease is required for all HIV-positive patients undergoing consideration for liver transplantation. In patients with portal hypertension, splenic sequestration of T lymphocytes can lead to a fall in the CD4<sup>+</sup> T cell count. In such cases a CD4<sup>+</sup> cell count > 100 cells/ $\mu$ L is acceptable. Historically, a fall in the CD4<sup>+</sup> cell count could also be precipitated by the use of PEG. In our opinion, CD4<sup>+</sup> T cell percentages may represent a more sensitive indicator of immune reconstitution in HIV-positive patients with portal hypertension. HIV-positive patients undergoing evaluation for liver transplantation also require an undetectable HIV viral load (< 50 copies/mL) except for those that presently acutely. The inability to achieve an undetectable HIV RNA viral load before liver transplantation has been associated with an increased mortality rate (HR = 3.5,  $P < 0.001$ )<sup>[53]</sup>. In addition to good therapeutic options available in the pre-transplant period, HIV-positive patients require future cART options based upon their previous regimens and genotype resistance testing.

Certain HIV-positive patients may not be able to tolerate cART medications pre-liver transplantation due to poor liver synthetic function. This group should not be automatically excluded from liver transplantation as long as control of their HIV is deemed possible post-liver transplantation. cART intolerance post-

liver transplantation, however has been identified as an important predictor of survival<sup>[54]</sup>. A thorough knowledge of past opportunistic infections is also required. A distant history of an opportunistic infection in a patient that was not taking cART is not a contraindication to liver transplantation unless there is no effective treatment available for possible recurrence post-liver transplantation. Absolute contra-indications include multidrug resistant HIV infection, resistant fungal infections, chronic intestinal cryptosporidiosis, progressive multi-focal leukoencephalopathy and central nervous system lymphoma.

## POST-LIVER TRANSPLANTATION

Standard surgical techniques with conventional arterial, venous and biliary anastomoses are recommended. Previous concerns regarding the possible transmission of HIV to the surgical team appear to be unfounded. The risk of HIV transmission is low and substantially lower than the risk of transmission of HBV and HCV<sup>[55]</sup>. In the event of HIV exposure, current regimens provide effective prophylaxis<sup>[55]</sup>. HIV infection is associated with a pro-thrombotic state and therefore concerns have been raised regarding an increased risk of vascular complications post transplantation<sup>[56]</sup>. Data appears to be conflicting regarding an increased incidence of hepatic artery thrombosis and at present no firm conclusions can be drawn<sup>[57,58]</sup>. In our institution, we introduce prophylactic subcutaneous heparin (5000 units every 8 h) once the international normalised ration is below 1.5 and the platelet count is greater than  $50 \times 10^9$  cells/L.

### Historical experiences

Initial case series of HIV-positive patients undergoing liver transplantation reported poor outcomes<sup>[59,60]</sup>. It is important to note that this was before the introduction of cART regimens. Retrospective data since has demonstrated an increasing understanding of the complexities faced by this unique patient cohort. One of the largest studies performed analysed data provided by the US United Network for Organ Sharing (UNOS) liver transplant database (1997-2006) and identified 138 HIV-positive patients<sup>[61]</sup>. Overall survival rates were inferior in the HIV-positive cohort compared to a comparative HIV negative cohort ( $n = 30520$ ) at 2- and 3-years post transplant (70% and 60% vs 81% and 77%,  $P < 0.047$ ). Considerable data however, was missing from the HIV cohort raising the possibility that HIV infection may not have been optimally treated prior to liver transplantation.

### Outcomes amongst HCV/HIV co-infected patients

Outcomes in HIV/HCV co-infected patients is clearly suboptimal when compared to other aetiologies; survival rates ranging between 64%-88% at 1 year and 33%-51% at 5 years<sup>[54,62-64]</sup>. To date, two



**Table 3** Prospective studies conducted in human immunodeficiency virus/hepatitis C virus co-infected patients undergoing liver transplantation

	<i>n</i>	Patient survival	Incidence of ACR	Risk factors for death amongst HIV/HCV recipients <sup>1</sup>
Miro <i>et al</i> <sup>[65]</sup> , 2012	HIV/HCV - 84 HCV - 252	1, 3, 5 yr: 88%, 62%, 54% 1, 3, 5 yr: 90%, 76%, 71% <sup>1</sup>	38% 20% <sup>1</sup>	HCV G1 Donor risk index MELD score Center < 1 transplant/yr <sup>2</sup> Combined liver-kidney transplant BMI < 21 Anti-HCV positive donor Older donor
Terrault <i>et al</i> <sup>[66]</sup> , 2012	HIV/HCV - 89 HCV - 235	1 and 3 yr: 92% and 79% 1 and 3 yr: 76% and 60% <sup>1</sup>	39% 24% <sup>1</sup>	

<sup>1</sup>Statistically significant ( $P < 0.05$ ); <sup>2</sup>Less than one liver transplant per year in a human immunodeficiency virus (HIV)-infected patient. HCV: Hepatitis C virus; BMI: Body mass index; ACR: Acute cellular rejection.

prospective studies have been performed in HIV/HCV co-infected patients undergoing liver transplantation (Table 3), one conducted in the United States and the other in Spain<sup>[65,66]</sup>. The United States study reported outcomes in 89 HIV/HCV co-infected patients and 235 HCV mono-infected controls performed at 17 United States centers<sup>[66]</sup>. Compared to HCV controls, HIV/HCV co-infected patients were younger (49 years vs 54 years,  $P < 0.0001$ ), had lower body mass index (BMI) at listing (25 kg/m<sup>2</sup> vs 28 kg/m<sup>2</sup>,  $P < 0.0001$ ), more likely to have HBV co-infection (6% vs 1%,  $P = 0.02$ ), were more likely to receive a non-heart beating graft (17% vs 4%,  $P = 0.0002$ ), longer median warm ischaemia time (41 min vs 21 min,  $P = 0.001$ ) and were less likely to be given tacrolimus-based (vs cyclosporine) initial immunosuppression (58% vs 80%,  $P < 0.0001$ ). 1- and 3-year patient survival rates were 76% and 60% in HIV/HCV cohort compared to 92% and 79% in the HCV mono-infected cohort ( $P < 0.001$ ). Graft loss was also significantly higher in the HIV/HCV co-infected cohort ( $P < 0.001$ ). Multivariate analysis identified HIV infection as the only baseline factor associated with increased risk of death (HR = 2.3,  $P = 0.002$ ) and graft loss (HR = 1.9,  $P = 0.01$ ). Analysis of the HIV/HCV co-infected cohort only identified that receipt of a combined kidney-liver transplant (HR = 3.8,  $P = 0.003$ ), BMI < 21 kg/m<sup>2</sup> at enrolment (HR = 3.2,  $P = 0.01$ ), receipt of an anti-HCV positive donor (HR = 2.5,  $P = 0.03$ ), and older donor age (HR = 1.3 per decade,  $P = 0.04$ ) were significant predictors of reduced graft survival. The cumulative incidence of acute cellular rejection (ACR) requiring treatment was significantly higher in HIV/HCV patients compared to HCV-mono-infected patients (39% vs 24% at year 3, HR = 2.1,  $P = 0.01$ ). 50% of the cases of ACR occurred within the first 21 d following LT. Reasons for the increased incidence of ACR remain unclear but the immunosuppression protocol post transplantation was not standardized.

The Spanish study enrolled 84 HIV/HCV co-infected patients and were matched with 252 HCV mono-infected patients<sup>[65]</sup>. This study reported a higher overall mortality amongst the HIV/HCV co-

infected recipients (43%,  $n = 36$  vs 30%,  $n = 75$ ,  $P = 0.03$ ) during a median of 3.6 years. Unsurprisingly, the leading cause of death was HCV recurrence in both patient cohorts but significantly increased in the HIV/HCV co-infected cohort (21% vs 12%,  $P = 0.049$ ). 1-year patient survival was similar between the two cohorts (88% vs 90%) but inferior patient survival outcomes were observed at 3 and 5 years in HIV/HCV co-infected recipients (62% vs 76% and 54% vs 71% respectively,  $P = 0.008$ ). Independent risk factors for death amongst the HIV/HCV recipients included HCV genotype 1 (HR = 3.0,  $P = 0.008$ ), donor risk index (HR = 9.5,  $P < 0.01$ ) and a negative HCV RNA viral load before or after liver transplantation (HR = 0.14,  $P = 0.009$ ). Important pre-transplant variables predictive of death included the MELD score (HR = 1.06,  $P = 0.023$ ) and a transplant center with less than one liver transplant per year in a HIV-infected patient (HR = 2.3,  $P = 0.03$ ). A higher incidence of ACR was once again reported amongst the HIV/HCV co-infected recipients (38% vs 20%,  $P < 0.001$ ).

Recurrence of HCV post liver transplantation is universal in all patients with detectable HCV viraemia at the time of liver transplantation. The rate of HCV recurrence and therefore fibrosis is influenced by a variety of recipient, donor, viral, infectious and immunosuppression related factors<sup>[64,67,68]</sup>. An accelerated disease course is well recognised in HIV/HCV co-infected patient especially the aggressive severe fibrosing cholestatic variant of recurrent hepatitis C (FCH)<sup>[69]</sup>. FCH and sepsis appear to be the leading causes of death post liver transplantation amongst HIV/HCV co-infected patients<sup>[62,64,70]</sup>. No reliable markers are available to identify patients who will develop FCH but higher HCV viral loads immediately after liver transplantation at week 1 and week 2 may be an indicator for those at risk<sup>[71]</sup>. FCH usually occurs between 2 and 6 mo after liver transplantation and is associated with a high mortality (50% at 12 mo) and is invariably refractory to pegylated interferon (PEG-IFN) and ribavirin (RBV) antiviral therapy<sup>[72,73]</sup>. However, case reports are now emerging on the use of the new directly acting

antiviral (DAA) agents in patients with FCH resulting in a sustained viral response (SVR). Long-term fibrosis progression rates are also accelerated in HIV/HCV co-infected patients compared HCV mono-infected patients with an increased likelihood of progression to a fibrosis score  $\geq 2$ <sup>[64]</sup>.

Prior to the advent of DAAs, antiviral therapy post liver transplantation consisted of PEG-IFN and RBV for a minimum of 48 wk irrespective of HCV viral genotype<sup>[74]</sup>. Most institutions, as in our own, instigated treatment when histological disease was demonstrable ( $F \geq 2$ ). Reported SVR rates (38%) for the treatment of recurrent HCV post-liver transplantation in HCV mono-infected patients were inferior to patients pre-liver transplantation (60%)<sup>[75,76]</sup>. Data from a prospective study in HIV/HCV co-infected patients after liver transplantation demonstrated a SVR rate of only 21% compared to 36% in HCV mono-infected patients ( $P = 0.013$ )<sup>[77]</sup>. These poor SVR rates are explained by the combination of high discontinuation rates (40%), dose reductions (75%) and haematological toxicity commonly anaemia.

Data is now emerging on the use of the new DAAs in HIV/HCV co-infected patients following liver transplantation which have demonstrated improved SVR rates<sup>[78,79]</sup>. Certain caveats should be taken into account when considering anti-viral regimens: anti- HIV protease inhibitors should be avoided with boceprevir, a higher telaprevir dose is required in patients receiving efavirenz based cART. Sofosbuvir and ribavirin have been used on a compassionate-use basis patients with severe HCV recurrence and in patients with FCH post liver transplantation<sup>[80]</sup>. This study included both HCV mono-infected and HIV/HCV co-infected patients. Overall SVR rates were 59% with higher SVR rates (73%) reported in patients with early severe recurrence<sup>[80]</sup>. In addition, the use of sofosbuvir and ribavirin appeared safe and also resulted in an improvement in liver biochemistry, MELD and ascites. The SOLAR-1 study was a large, multicenter, randomized controlled trial that included liver transplant recipients with HCV recurrence with genotype 1 and 4 HCV<sup>[81]</sup>. Patients received ledipasvir, sofosbuvir and ribavirin for either 12 or 24 wk. The results demonstrated only 2% of patients discontinued treatment due to adverse events and encouraging SVR12 rates that varied with Child-Pugh class (A, 96%; B, 85%; C, 60%). Although HIV-HCV co-infected patients were not included in this study, these results are encouraging and are applicable to the HIV-HCV co-infected patients in the post-liver transplant period. Further studies are awaited with DAAs for recurrent HCV in HIV/HCV co-infected patients post liver transplantation.

#### **Outcomes amongst patients without HCV co-infection**

Patients co-infected with HBV and non-viral aetiologies including those that present with acute liver failure,

have excellent short and long-term outcomes post-liver transplantation. Reported median survival at 1-year ranges between 75%-100% and 100% at 5 years<sup>[82-84]</sup>. The largest prospective study to date in HIV/HBV co-infected patients was conducted in 21 patients for a median of 42 mo with no patient suffering graft loss<sup>[85]</sup>.

The key difference between HIV/HBV and HIV/HCV co-infected patients is the presence of highly potent anti-viral agents against HBV in the therapeutic armamentarium. Patients co-infected with HBV receiving cART will undoubtedly be receiving an oral nucleo(s)tide analogue that will have anti-viral actions against both HIV and HBV. Tenofovir in conjunction with emtricitabine (Truvada) is recommended<sup>[85,86]</sup>. The use of these highly efficacious, potent oral agents results in the majority of patients undergoing liver transplantation with an undetectable HBV viral load. Immuno-prophylaxis with Hepatitis B Immunoglobulin (HBIG) is recommended in the post-liver transplant period indefinitely. Reported data on the use of HBIG and oral anti-viral agents has demonstrated that this combination is highly effective at preventing HBV recurrence even in those who have a detectable HBV viral load at the time of liver transplantation<sup>[87]</sup>. Data on patients with HIV and non-viral liver disease undergoing liver transplantation is limited but evidence suggests that these patients have similar survival rates as HIV-negative patients<sup>[82,84]</sup>.

Liver transplantation for HCC in HIV positive patients has been performed although the experience remains limited. A single center experience compared outcomes in 21 HIV-positive patients against 65 HIV-negative patients<sup>[88]</sup>. The authors reported a trend towards a higher dropout rate amongst the HIV positive patients but overall survival following liver transplantation at 1 and 3 years (81% and 74%, HIV positive group vs 93% and 85%, HIV negative group) and recurrence free survival at 1 and 3 years were similar between the two groups (69% and 69%, HIV positive group vs 89% and 84%, HIV negative group)<sup>[88]</sup>.

Another study, this time across 3 centers, compared 30 HIV positive patients with 125 HIV negative patients<sup>[89]</sup>. Once again similar survival rates between the two groups were reported (77% and 65%, HIV positive group vs 85% and 70%, HIV negative group)<sup>[89]</sup>. The time to HCC recurrence was longer in the HIV positive group (27 mo) compared to the HIV negative group (10 mo,  $P < 0.01$ ) but mortality remained similar suggesting the possible positive role of cART in attenuating hepato-carcinogenic progression.

#### **Immunosuppression**

Immunosuppression should be tailored to the individual taking into account aetiology of liver disease, renal function, risk factors for the metabolic syndrome and specifically to HIV patients the possible interactions with cART. Dual immunosuppression with calcineurin

**Table 4 Antiretroviral medications and their effect on calcineurin inhibitors**

Drug	Effect on CNI level
Protease inhibitor	
Darunavir	↑↑
Fosamprenavir	↑↑
Lopinavir	↑↑
Ritonavir	↑↑↑
Saquinavir	↑↑
Non nucleoside reverse transcriptase inhibitors	
Efavirenz	↓↓
Nevirapine	↓↓
Integrase inhibitors	
Raltegravir	No effect

CNI: Calcineurin inhibitors.

inhibitors and cortico-steroids is recommended post-liver transplantation. Target trough levels in the first 3 mo should be the same as HIV negative patients (cyclosporin, 100-250 ng/mL; tacrolimus, 8-10 ng/mL). Utilising data from HCV mono-infected patients post-LT, rapid withdrawal of cortico-steroids should be avoided due to the association of a more severe recurrence of HCV<sup>[90]</sup>. We therefore recommend that prednisolone, which is usually commenced at 20 mg/d, be withdrawn by a slow taper at 3 mo. Anti-fungal prophylaxis (fluconazole 50 mg/d) should be given for a minimum of 3 mo post-LT. Episodes of acute cellular rejection (ACR) should also be managed as one would in HIV negative patients namely moderate-severe episodes be treated with a 3-d course of intravenous methylprednisolone (1 g/d). Consideration of the introduction of mycophenolate mofetil after the 2<sup>nd</sup> episode of ACR is recommended.

Tacrolimus and cyclosporin are metabolised *via* the P450 cytochrome. In addition, non-nucleoside reverse-transcriptase inhibitors (NNRTIs) and protease inhibitors, which are commonly part of cART regimens, are also metabolised by the same pathway, therefore increasing the risk of drug - drug interactions (see Table 4). NNRTIs (e.g., efavirenz) decrease serum CNI concentrations by induction of the P450 cytochrome whilst PIs (e.g., ritonavir and lopinavir) are inducers resulting in increased CNI concentrations<sup>[91]</sup>. We have used tacrolimus doses as low as 1 mg per week in certain individuals. Raltegravir, a novel HIV-1 integrase inhibitor, is not metabolised *via* the P450 cytochrome and has been used successfully post transplantation in combination with nucleoside reverse-transcriptase inhibitors and standard CNI doses<sup>[92]</sup>. Meticulous monitoring and surveillance is required to reduce the possibility drug-drug interactions and toxicity.

### **HIV disease following liver transplantation**

At present no standardised cART regimen is utilised,

but instead is tailored to the individual patient reflecting known resistance and mutations. The re-introduction of cART post-LT also varies between individual centers, with some continuing cART throughout the transplant period whilst others re-introduce the medication between 4-14 d. Our practice is to re-introduce cART medication once liver graft function has normalised thereby avoiding the possibility of confusion with the other causes of graft dysfunction immediately post-LT.

In a recent study of HIV/HCV patients, bacterial infections were identified as the principal aetiological agents of post-liver transplantation infections<sup>[93]</sup>. Risk factors associated with severe infections included a pre-liver transplantation MELD score > 15 (HR = 3.5, 95%CI: 1.7-7.1,  $P = 0.001$ ), history of category C AIDS-defining events (HR = 4.0, 1.9-8.6,  $P < 0.001$ ) and non-tacrolimus based immunosuppression (HR = 2.5, 1.3-4.8,  $P = 0.006$ ). The same study also suggested that opportunistic infections namely CMV disease, disseminated HSV, invasive fungal infections and tuberculosis were increased in HIV positive patients, affecting 11% of their cohort. These "opportunistic" infections however can occur in HIV negative patients post-liver transplantation. This study also did not have a HIV negative comparative group therefore not allowing the authors to demonstrate that these deemed opportunistic infections were associated with HIV infection only. Reassuringly reported data from other studies have failed to demonstrate a higher incidence of opportunistic infections in comparison to HIV negative patients<sup>[94]</sup>.

## **RE-TRANSPLANTATION**

There is very little experience with re-transplantation in HIV positive patients and this appears to be limited to HIV/HCV co-infected patients<sup>[95]</sup>. In one study 14 HIV positive patients (13 with HIV/HCV co-infection) were compared to 157 HIV negative patients undergoing re-liver transplantation. The authors reported an inferior survival rate in the HIV positive group (42% vs 64%) at 3 years although this did not reach statistical significance ( $P = 0.2$ ). HIV positive patients with a detectable HCV viral load at the time of re-transplantation do not appear to be appropriate re-transplant candidates due to poor 3 year survival rates (22% vs 65% in HIV negative patients,  $P = 0.008$ )<sup>[95]</sup>. A recent multinational study re-emphasised the poor outcomes in HIV positive patients with active HCV infection undergoing re-transplantation<sup>[96]</sup>.

## **CONCLUSION**

HIV infection is now regarded as a long-term illness with improving survival rates in patients maintained on cART. Subsequently, liver disease remains a common

cause of morbidity and mortality in this cohort. HIV-positive patients with liver disease should be managed in a multi-disciplinary environment. Current data would suggest HIV-positive patients not co-infected with HCV have excellent outcomes following liver transplantation, similar to HIV negative patients. In stark contrast, published data has demonstrated inferior graft and patient survival rates in HIV/HCV co-infected patients predominately due to HCV recurrence. Emerging data with the use of DAAs in HIV/HCV co-infected patients would suggest that treatment of HCV in the pre- and post-transplant period will be more efficacious resulting in improved outcomes in this patient cohort. HIV/HCV co-infected patients would therefore be treated as HCV mono-infected patients with better outcomes post liver transplantation.

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## Effect of rifaximin on gut microbiota composition in advanced liver disease and its complications

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### Abstract

Liver cirrhosis is a paradigm of intestinal dysbiosis. The

qualitative and quantitative derangement of intestinal microbial community reported in cirrhotic patients seems to be strictly related with the impairment of liver function. A kind of gut microbial "fingerprint", characterized by the reduced ratio of "good" to "potentially pathogenic" bacteria has recently been outlined, and is associated with the increase in Model for End-Stage Liver Disease and Child Pugh scores. Moreover, in patients presenting with cirrhosis complications such as spontaneous bacterial peritonitis (SBP), hepatic encephalopathy (HE), and, portal hypertension intestinal microbiota modifications or the isolation of bacteria deriving from the gut are commonly reported. Rifaximin is a non-absorbable antibiotic used in the management of several gastrointestinal diseases. Beyond bactericidal/bacteriostatic, immune-modulating and anti-inflammatory activity, a little is known about its interaction with gut microbial environment. Rifaximin has been demonstrated to exert beneficial effects on cognitive function in patients with HE, and also to prevent the development of SBP, to reduce endotoxemia and to improve hemodynamics in cirrhotics. These results are linked to a shift in gut microbes functionality, triggering the production of favorable metabolites. The low incidence of drug-related adverse events due to the small amount of circulating drug makes rifaximin a relatively safe antibiotic for the modulation of gut microbiota in advanced liver disease.

**Key words:** Liver cirrhosis; Gut microbiota; Rifaximin; Hepatic encephalopathy; Spontaneous bacterial peritonitis; Ascites; Endotoxemia; Thrombocytopenia

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**Core tip:** Advanced liver disease is characterized by intestinal dysbiosis, which has been involved in the pathogenesis of complications. Rifaximin is able to improve cognitive tests and practical abilities, to reduce



the risk of hepatic encephalopathy (HE) recurrence and the number of HE-related hospitalizations. Rifaximin efficacy seems not associated with major changes in gut bacteria composition but rather with a shift in the microbiome functionality. Rifaximin is useful in the prevention of spontaneous bacterial peritonitis in patients with ascites. Rifaximin reduces endotoxemia and has beneficial effects on cirrhotic patients hemodynamics, reducing the incidence of complications related to portal hypertension.

Ponziani FR, Gerardi V, Pecere S, D'Aversa F, Lopetuso L, Zocco MA, Pompili M, Gasbarrini A. Effect of rifaximin on gut microbiota composition in advanced liver disease and its complications. *World J Gastroenterol* 2015; 21(43): 12322-12333 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12322.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12322>

## INTRODUCTION

Rifaximin is a non-systemic antibiotic approved for the treatment of traveler's diarrhea, irritable bowel syndrome (IBS) with diarrhea and overt hepatic encephalopathy (HE)<sup>[1]</sup>. It has *in vitro* bactericidal and bacteriostatic activity against aerobic and anaerobic Gram-positive and Gram-negative species, being also able to reduce bacterial virulence and translocation, and to inhibit bacterial adherence to gut mucosa<sup>[2-7]</sup>.

Due to the low systemic absorption (only 0.4% of the oral administered dose), rifaximin has an optimal tolerability profile, and side effects as well as the induction of bacterial resistance are nearly lacking<sup>[1,8,9]</sup>.

Beyond that, rifaximin has particular features which are not typical of a common antibiotic molecule. *In vitro* and *in vivo* models and preliminary experiences in humans<sup>[10-14]</sup> have demonstrated that rifaximin does not change the overall composition of the gut microbiota while it is able to provide minimal changes, such as promoting the growth of bacteria beneficial to the gut. Nevertheless, rifaximin modulates the release of inflammatory cytokines<sup>[15,16]</sup> and increases NF- $\kappa$ B expression, exerting anti-inflammatory effects that could counteract the pro-inflammatory response observed in conditions of gut microbiota derangement<sup>[17]</sup>.

Based on these evidences, rifaximin use has been extended to the management of pathologies associated with gut microbiota deregulation such as irritable bowel syndrome<sup>[11,18-21]</sup>, inflammatory bowel diseases<sup>[10,13,22-30]</sup>, diverticular disease<sup>[31-36]</sup> and liver cirrhosis and its complications.

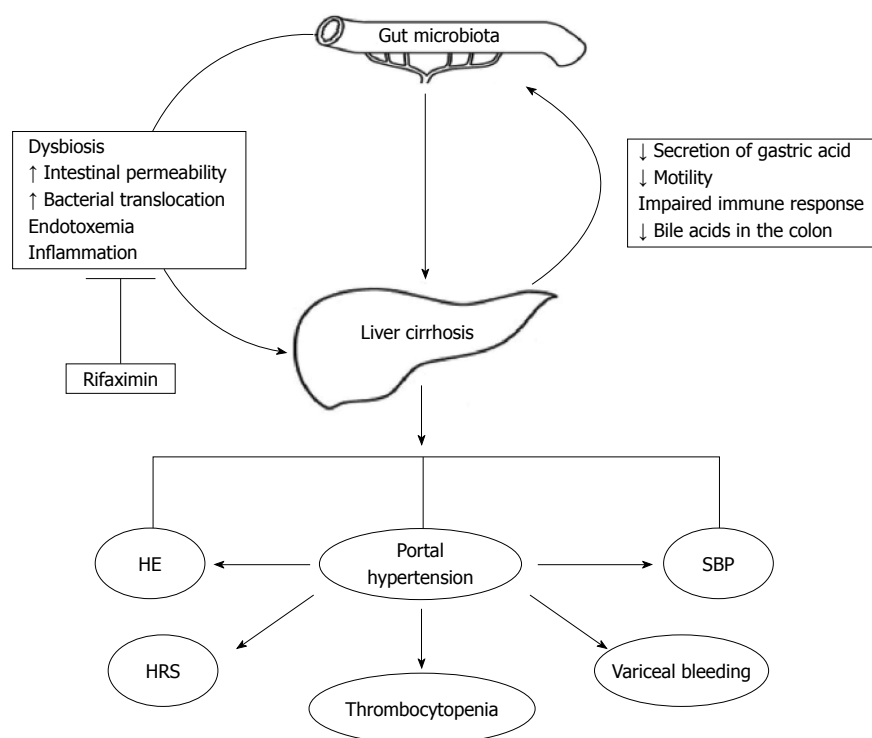
Liver cirrhosis is a paradigm of intestinal dysbiosis. Indeed, the physiological partitioning of the gastrointestinal tract is deranged in cirrhotic patients, due to the decreased secretion of gastric acid (often favored by medications<sup>[37]</sup>), to the reduced gastrointestinal

motility, to the impaired systemic and mucosal immune response and to the low concentration of bile acids in the colon<sup>[38]</sup>. The epiphenomenon of this chronic dysfunction is a profound alteration of the gut microbiota composition, which is both quantitative (Small Intestinal Bacterial Overgrowth, SIBO) and more pronounced in the advanced stages of the disease and in case of decompensation (Figure 1).

This is the rationale for gut microbiota modulation in patients with liver cirrhosis, especially in those with severe impairment of liver function presenting with complications.

## THE "FINGERPRINT" OF GUT MICROBIOTA IN LIVER CIRRHOSIS

The introduction of metagenomic techniques such as 16S rRNA-based pyrosequencing has recently allowed to identify which modifications of the gut microenvironment are the most frequently observed in liver cirrhotic patients<sup>[39]</sup>. The human gut hosts a bacterial core community involved in maintaining gastrointestinal health and mainly composed of the phyla Bacteroidetes and Firmicutes, which include the genera *Bacteroides*, *Clostridium clusters XIVa* and *IVa*, *Eubacterium*, *Faecalibacterium*, *Lactobacillus*, and *Roseburia*. In patients affected by liver cirrhosis, at the phylum level, Bacteroidetes are decreased in favor of Fusobacteria and Proteobacteria, such as Enterobacteriaceae and Pasteurellaceae<sup>[40-42]</sup>. Looking at family, genus and species division, the increase in Enterobacteriaceae, Streptococcaceae and Veillonellaceae abundance has been reported in cirrhotic patients compared with healthy controls, whereas Lachnospiraceae, Ruminococcaceae, *Clostridium clusters XI* and *XIVab*, lactic acid bacteria, Bifidobacteria and *Faecalibacterium prausnitzii* seem to be reduced<sup>[40-46]</sup>. Notably, Enterobacteriaceae family includes *Escherichia coli* and *Klebsiella spp.*, key bacteria in the pathogenesis of spontaneous bacterial peritonitis (SBP). In addition to the unbalance between potentially pathogenic and beneficial bacteria, the major part of the metagenomic species enriched in cirrhotics' fecal samples belong to *Veillonella* or *Streptococcus* taxa, which usually derive from the mouth or the small intestine<sup>[47]</sup>. Although this may apparently confirm the subversion of the gastrointestinal physiology occurring during the course of liver disease, when cirrhotics' salivary microbiota is specifically analyzed and compared with the fecal one, they seem significantly different rather than similar<sup>[46]</sup>. More in detail, Streptococcaceae are prevalent in the saliva, whereas stools are characterized by a reduction in the autochthonous taxa Lachnospiraceae, Ruminococcaceae, and Clostridiales XIV. However, about half of samples analyzed in this study belonged to patients who have had previous episodes of HE and were on lactulose, with the addition of rifaximin in



**Figure 1 Effects of Rifaximin on gut-liver axis.** Rifaximin decreases endotoxemia and inflammation both directly and indirectly, by reducing bacterial translocation, counteracting bacterial overgrowth and modulating gut microbiota composition and function. Due to these peculiar effects, rifaximin is used for the treatment of advanced liver disease complications. HE: Hepatic encephalopathy; SIBO: Small intestinal bacterial overgrowth; SBP: Spontaneous bacterial peritonitis; HRS: Hepatorenal syndrome.

two cases. Further analyses to discriminate conditions predisposing to the “mixing-up” of bacteria from different sites of the gastrointestinal tract are needed to quell this debate.

Interestingly, the alteration of gut microbiota composition seems to have a prognostic significance, or at least to follow the evolution of liver disease. Generally speaking, Qin *et al.*<sup>[47]</sup> demonstrated that metagenomic species enriched in cirrhotic patients correlate with the severity of the disease, in a proportion dependent on bacterial load. In other studies, the reduction in Clostridiaceae as well as in Veillonellaceae and in Porphyromonadaceae has been associated with inflammation and with the progression of liver disease and Streptococcaceae have been reported to correlate positively with Child Pugh score in contrast to Lachnospiraceae which correlated negatively<sup>[41,43,48]</sup>.

Taking together these findings, cirrhotic patients’ microbiota is characterized by a higher proportion of potentially pathogenic bacteria, lacking of those species recognized as beneficial to intestinal health and homeostasis. Notwithstanding, the reduction in the ratio between “good” (e.g., Lachnospiraceae, Ruminococcaceae and Clostridia cluster XIV) and potentially “bad” bacteria (e.g., Staphylococcaceae, Enterobacteriaceae and Enterococcaceae) - namely “cirrhosis dysbiosis ratio” or CDR - is characteristic of the individuals with a more severe disease, such as cirrhotic outpatients and inpatients<sup>[48]</sup>.

Given the evidence that the progression of liver disease is associated with a change in the gut microenvironment, liver cirrhosis complications consequently grow in the soil of intestinal dysbiosis.

## RIFAXIMIN AND GUT MICROBIOTA MODULATION IN ADVANCED LIVER DISEASE AND ITS COMPLICATIONS

### *Rifaximin and gut microbiota modulation in HE*

Several differences have been reported in the gut microbiota of cirrhotic patients with or without HE. In patients with minimal HE, Streptococcaceae represent the prevalent bacterial family, and the abundance of *Streptococcus salivarius*, which is involved in the production of ammonia, is increased<sup>[45]</sup>. Alcaligenaceae, Porphyromonadaceae and Enterobacteriaceae have also been associated with HE in cirrhotics; in particular, Alcaligenaceae and Porphyromonadaceae are significantly linked with poor cognitive performance, and Enterobacteriaceae with a worse MELD score<sup>[49]</sup>. In addition, a decreased CDR has been reported in cirrhotic patients with HE<sup>[48]</sup>. Similar results have been obtained by the analysis of mucosal microbiome from sigmoid biopsies: *Enterococcus*, *Veillonella*, *Megasphaera*, *Bifidobacterium*, and *Burkholderia* were predominant in patients with HE, whereas cirrhotics without HE presented an increased abundance of the “good” genus *Roseburia*, and the healthy controls

an increased abundance of *Dorea*, *Subdoligranulum*, *Incertae Sedis XIV*, *Blautia*, *Roseburia*, *Faecalibacterium* and a few pathogenic genera<sup>[50]</sup>. Since the intestinal microenvironment of cirrhotics without HE has been demonstrated to be closer to healthy people's one<sup>[46]</sup>, it is not surprising that, in patients with HE, the more the mucosal microbiota resembled that of controls, the better was the cognitive performance and the lower were the serum markers of inflammation<sup>[50]</sup>.

Studies focused on clinical outcomes reported a high efficacy of rifaximin in cirrhotics with HE and a mild/moderate stage of disease. A randomized, double-blind, placebo-controlled trial including 299 patients has proved that rifaximin with or without lactulose is able to reduce the risk of HE recurrence and the rate of HE-related hospitalization, especially in patients with MELD score < 18<sup>[51]</sup>. Similar results were also obtained in other studies including patients in different stages of liver disease, receiving various treatment schedules (Table 1)<sup>[52-58]</sup>.

In addition to the roughly evident benefits on overt HE, rifaximin has also been reported to improve operational abilities and input integration capacity in patients with minimal HE, as demonstrated by the amelioration of driving simulator performance<sup>[59]</sup>. This positive shift in cognitive tests and practical abilities is undoubtedly accompanied by a significant improvement in health-related quality of life<sup>[60,61]</sup>.

At the microscopic level, rifaximin does not seem to change stool microbiota composition in patients with minimal HE, and only a reduction in Veillonellaceae and an increase in Eubacteriaceae have been observed<sup>[62]</sup>. Reasonably, the improvement in cognitive function and the reduced endotoxemia associated with rifaximin treatment derive from a beneficial modulation of gut microbiota metabolic profile rather than from a major rearrangement of the intestinal microbial community. Indeed, the Authors reported an increase in saturated and unsaturated fatty acids and in serum fructose, succinic acid and citramalic acid production after rifaximin treatment, but the most relevant finding was the modification of correlation networks involving several bacteria (Enterobacteriaceae, Bacteroidaceae, Veillonellaceae, Porphyromonadaceae and Rikenellaceae), metabolites and clinical outcomes, suggesting a functional change in the gut microbiome. Although only patients with minimal HE have been included and some selection biases could be identified, the study by Bajaj *et al.*<sup>[62]</sup> is to date the only published experience reporting the metagenomic and metabolomic changes produced by rifaximin treatment in cirrhotics with minimal HE. Nevertheless, despite the good results in terms of efficacy, rifaximin role in the treatment of cirrhotics at high risk of developing HE, such as patients with high MELD scores or with transjugular intrahepatic portosystemic shunts or surgical portosystemic shunts or those with a recent episode of acute variceal bleeding, needs to be further

investigated<sup>[63-65]</sup>.

### **Rifaximin and gut microbiota modulation in SBP**

Ascites and SBP are typical manifestations of decompensated liver disease. SIBO and bacterial translocation are the mainstay of SBP. Indeed, SIBO prevalence among cirrhotics is high, ranging between 30% and 70%<sup>[38]</sup> and it has been associated with the development of SBP due to the translocation of intestinal bacteria to the systemic circulation and the ascitic fluid<sup>[66]</sup>. Gram-negative bacteria such as *Escherichia coli* and *Klebsiella spp.* as well as Pneumococci, Streptococci and other Gram-positive and Gram-negative bacteria have been identified in 50% of cases by culture-based analysis of ascitic fluid<sup>[67]</sup>. However, bacterial DNA can be recognized in the ascites of half of cirrhotics even in absence of SBP and with negative cultures<sup>[44]</sup>, and several studies identified microbes usually present within the gut<sup>[41,43,68]</sup>. Ascites microbial composition is linked with the stage of liver disease; indeed, Child-Pugh score is correlated with ascitic bacteria similarity and ascitic neutrophil count, further strengthening the connection between gut microbiota and liver cirrhosis progression<sup>[68]</sup>.

Therefore, it has been hypothesized that rifaximin, being effective on SIBO, could be useful in preventing SBP. In the retrospective study by Hanouneh *et al.*<sup>[66]</sup> a 72% reduction in SBP occurrence and a transplant free survival of 72% were observed in the 49 cirrhotic patients with ascites who received rifaximin (Table 2).

Another prospective observational study reported that different bacterial species could be identified in the ascitic fluid of patients receiving rifaximin compared to those who did not receive SBP prophylaxis<sup>[69]</sup>. Indeed, Enterococci and *Escherichia coli* were isolated from the ascites of patients without prophylaxis and *Klebsiella spp.* were isolated in those on rifaximin. However, this finding had no predictive value, since the incidence of SBP was similar between the two groups.

### **Rifaximin, gut microbiota modulation and liver hemodynamics**

Intestinal decontamination improves hemodynamics in animal models of cirrhosis by reducing endotoxemia related to bacterial translocation<sup>[70]</sup>. Similar results have also been obtained in humans<sup>[71]</sup>, and have been associated with a lower incidence of complications (Table 3).

Twenty-three patients with decompensated alcoholic cirrhosis who achieved a reduction of hepatic venous pressure gradient (HVPG) after 28 d of rifaximin treatment were then followed-up for 5 years<sup>[72]</sup>. Compared to matched controls, rifaximin group showed a lower incidence of complications related to portal hypertension, such as variceal bleeding, HE, SBP and hepatorenal syndrome, and a better survival compared to controls. Other studies confirmed a reduction in

Table 1 Major studies describing the changes in gut microbiota composition and the effects of rifaximin treatment in patients with advanced liver disease and hepatic encephalopathy

Study	Study design	No. patients	Disease severity	HE type	Treatment schedule	Results	Safety
Mas <i>et al</i> <sup>[56]</sup> 2003	Prospective randomized, double-blind, double-dummy, controlled trial	103	Not reported	Overt HE	50 pts rifaximin 1200 mg/d for 5-10 d 53 pts lactitol 60 g/d for 5-10 d	Improved neuropsychiatric and psychometric parameters in both groups Reduced blood ammonia levels in both groups No significant differences in efficacy (resolution/improvement 81.6% rifaximin <i>vs</i> 80.4% lactitol; unchanged/failure 18.4% rifaximin <i>vs</i> 19.6% lactitol) HE complete resolution: 53.1% rifaximin <i>vs</i> 37.2% lactitol	Abdominal pain: 4% rifaximin Mild diarrhea: 2% lactitol Vomiting: 2% lactitol
Paik <i>et al</i> <sup>[57]</sup> 2005	Prospective randomized		CTP: rifaximin A: 0%, B: 50%, C: 50% lactulose A: 0%, B: 64%, C: 36%	Overt HE	32 pts rifaximin 400 mg TID for 7 d 22 pts lactulose 90 mL/d for 7 d	Reduction in blood ammonia levels similar in both groups Improvement in HE grade and index similar in both groups Improvement in HE grade similar in both groups	Abdominal pain: 3% rifaximin Severe diarrhea: 4.5% lactulose
Leevy <i>et al</i> <sup>[58]</sup> 2007	Retrospective	145	Not reported	Overt HE	Lactulose 30 cc BID for $\geq$ 6 mo followed by rifaximin 400 mg TID for $\geq$ 6 mo	HE grade III or IV: 6% after rifaximin 25% after lactulose ( $P < 0.001$ ) Asterixis: 63% after rifaximin <i>vs</i> 93% after lactulose ( $P < 0.001$ )	Hospitalizations (mean number): 0.5 rifaximin <i>vs</i> 1.6 lactulose period ( $P = 0.001$ ) Hospitalizations days (mean): 2.5 rifaximin <i>vs</i> 7.3 lactulose period ( $P = 0.001$ ) Diarrhea: 89% during lactulose, 99% during rifaximin Flatulence: 100% during lactulose, 100% during rifaximin Abdominal pain: 100% during lactulose, 100% during rifaximin Headache: 100% during lactulose, 99% during rifaximin However, severe adverse events were more common in the lactulose period ( $P < 0.001$ ) Incidence of adverse events was similar in the two groups; most frequently reported: nausea, diarrhea, fatigue Bacterial peritonitis: 1.4% rifaximin <i>vs</i> 2.5% placebo Bacteremia: 0.7% rifaximin <i>vs</i> 1.3% placebo <i>C. difficile</i> infection: 1.4% rifaximin <i>vs</i> 0% placebo Sepsis: 0% rifaximin <i>vs</i> 1.3% placebo
Bass <i>et al</i> <sup>[51]</sup> 2010	Prospective, randomized, double-blind, placebo-controlled	299	MELD score (%): rifaximin $\leq$ 10: 24.3% 11-18: 67.1% 19-24: 8.6% placebo: $\leq$ 10: 30.2% 11-18: 60.4% 19-24: 8.8%	Overt HE	140 pts 550 mg BID for 6 mo 159 pts placebo 90% of pts also received lactulose	Rifaximin is more effective than placebo in maintaining HE remission ( $P < 0.001$ ) Breakthrough episodes rate: 22.1% rifaximin <i>vs</i> 45.9% placebo Risk of HE-related hospitalization: 13.6% rifaximin <i>vs</i> 22.6% placebo ( $P = 0.01$ )	



Bajaj <i>et al</i> <sup>[50]</sup> 2011	Prospective, randomized, double-blind, placebo-controlled	42	MELD score (mean) rifaximin: 9 placebo: 9	Minimal HE	21 pts rifaximin 550 mg BID 21 pts placebo for 8-wk	Total driving errors improvement: 76% rifaximin <i>vs</i> 31% placebo ( $P = 0.013$ ), with a significant reduction of speeding tickets ( $P = 0.005$ ) and illegal turns on navigation ( $P = 0.01$ ) Cognitive performance improvement: 91% rifaximin <i>vs</i> 61% placebo ( $P = 0.01$ ) Improved psycho-social dimension (quality of life assessment by Sickness Impact Profile questionnaire) in the rifaximin group compared with the placebo group ( $P = 0.04$ )	Infections rate: 0% Hospitalization rate: 0% Nausea: 14% rifaximin <i>vs</i> 14% placebo Self-limited vomiting: 5% rifaximin <i>vs</i> 5% placebo Abdominal pain: 24% rifaximin <i>vs</i> 24% placebo Flatulence: 19% rifaximin <i>vs</i> 43% placebo Headache: 19% rifaximin <i>vs</i> 33% placebo Flu-like symptoms: 5% rifaximin Constipation: 5% rifaximin Self-limited diarrhea: 5% rifaximin <i>vs</i> 5% placebo Hitching: 5% placebo Anorexia and dry mouth: 5% placebo Incidence of gastrointestinal bleeding, infection, hospitalization for dehydration/ overt HE similar in both groups
Neff <i>et al</i> <sup>[51]</sup> 2012	Retrospective	203	MELD score (mean, range): rifaximin 12 (8-27) rifaximin + lactulose 13 (11-26)	Overt HE	149 pts rifaximin monotherapy (400-1600 mg/d) 54 pts rifaximin (600-1200 mg/d) + lactulose (90 mL/d) dual therapy	1-yr HE remission rate: 81% rifaximin <i>vs</i> 67% rifaximin + lactulose Lower incidence of overt HE episodes in pts with mean MELD score $\leq 20$	
Bajaj <i>et al</i> <sup>[63]</sup> 2013	Prospective	20	MELD score (mean $\pm$ SD): 9.8 $\pm$ 3.3	Minimal HE	550 mg BID for 8 wk	Significant improvement in cognitive performance on all tests apart from the block design test Significant improvement in serum bilirubin but not the other MELD score components No significant microbial change (modest reduction in Veillonellaceae and increase in Eubacteriaceae) Significant increase in serum saturated (myristic, caprylic, palmitic, palmitoleic, oleic and eicosanoic) and unsaturated (linoleic, linolenic, gamma-linolenic and arachidonic) fatty acids, serum fructose, succinic acid and citramalic acid Change in correlation networks involving several bacteria (Enterobacteriaceae, Bacteroidaceae, Veillonellaceae, Porphyromonadaceae and Rikenellaceae) reflecting a functional shift in the gut microbiome	Not reported
Sharma <i>et al</i> <sup>[53]</sup> 2013	Prospective, randomized, double-blind, placebo-controlled	120	CTP score (mean $\pm$ SD): group A 9.9 $\pm$ 2.8 group B 9.4 $\pm$ 2.5 MELD score (mean $\pm$ SD): group A 24.9 $\pm$ 6.6 group B 23.8 $\pm$ 5.18	Overt HE	group A (63 pts): lactulose + rifaximin 1200 mg/d group B (57 pts): lactulose + placebo	HE remission rate: 76% group A <i>vs</i> 50.8% group B ( $P < 0.004$ ) Mortality: 23.8% group A <i>vs</i> 49.1% group B ( $P < 0.05$ ). Death was mainly due to sepsis Hospital stay (mean $\pm$ SD): 5.8 $\pm$ 3.4 in group A <i>vs</i> 8.2 $\pm$ 4.6 group B ( $P = 0.001$ )	Diarrhea: 13% group A <i>vs</i> 10% group B ( $P > 0.05$ ) Abdominal pain: 6% group A <i>vs</i> 7% group B ( $P > 0.05$ )

Maharshi <i>et al</i> <sup>[50]</sup> 2014	Prospective, randomized, controlled	120 pts with acute variceal bleeding and no HE	CTP and MELD scores comparable between groups but not reported	Overt HE	60 pts lactulose 30 mL QID  60 pts rifaximin 400 mg TID for 5 d	Incidence of HE: 15% rifaximin <i>vs</i> 17% lactulose ( <i>P</i> = 1) Mortality: 17% rifaximin <i>vs</i> 13% lactulose ( <i>P</i> = 1)  Hospital stay (mean ± SD): 10.6 ± 3.1 d rifaximin <i>vs</i> 12.4 ± 3.5 lactulose (pts with HE, <i>P</i> = 0.35); 6.3 ± 1.6 rifaximin <i>vs</i> 6.9 ± 1.9 lactulose (pts without HE, <i>P</i> = 0.18)  LOLA, rifaximin, and probiotics are superior to placebo in improving critical flicker frequency score  LOLA, rifaximin, and probiotics are superior to placebo in improving neuropsychometric tests	Lactulose group: 26.6% diarrhea, 15% abdominal bloating
Sharma <i>et al</i> <sup>[55]</sup> 2014	Prospective, randomized, controlled	124	CTP  LOLA  A: 22.5%, B: 42%, C: 35.5% rifaximin A: 39%, B: 32%, C: 29% probiotics A: 19%, B: 66%, C: 16% placebo A: 33%, B: 27%, C: 40%	Minimal HE	31 pts LOLA 3 g TID for 2 mo  31 pts rifaximin 400 mg TID for 2 mo 32 pts probiotics BID for 2 mo 30 pts placebo		Not reported

MELD: Model for end stage liver disease; HE: Hepatic encephalopathy; CTP: Child turcotte pugh; LOLA: L-ornithine L-aspartate.

Table 2 Major studies describing the efficacy of rifaximin in preventing episodes of spontaneous bacterial peritonitis in patients with advanced liver disease							
Study	Study design	No. patients	Disease severity	Disease complication	Treatment schedule	Results	Safety
Hanounch <i>et al</i> <sup>[60]</sup> 2012	Retrospective	404	MELD score (mean ± SD): rifaximin: 17.6 ± 7.7 no rifaximin 17.7 ± 7.5  CTP score rifaximin B: 6.1%, C: 93.9% no rifaximin B: 33%, C: 67%	SBP	49 pts received rifaximin 400 mg TID mainly for HE (recurrent HE or intolerance to lactulose)	SBP incidence: 11% in pts on rifaximin <i>vs</i> 32% in controls ( <i>P</i> = 0.002)  72% SBP reduction rate in rifaximin group after adjusting for MELD score, CTP score, serum sodium, and ascitic fluid total proteins ( <i>P</i> = 0.007)  72% transplant-free survival for pts on rifaximin <i>vs</i> 57% for controls ( <i>P</i> = 0.045)	Not reported
Lutz <i>et al</i> <sup>[60]</sup> 2014	Prospective, observational	152	CTP score: no prophylaxis: A: 1%, B: 57%, C: 43% rifaximin: A: 0%, B: 33%, C: 67% systemically absorbed antibiotics: A: 12%, B: 47%, C: 41%	SBP	Group 1 (108 pts): no prophylaxis Group 2 (27 pts): rifaximin 400 mg TID Group 3 (17 pts): systemically absorbed antibiotic prophylaxis	SBP occurrence rate: 32/152 (21%) overall, 22.2% group 1, 29.6% group 2 and 0% group 3 ( <i>P</i> = 0.02 group 2 <i>vs</i> group 3 and <i>P</i> = 0.04 group 1 <i>vs</i> group 3)  Data available for SBP pts only Nosocomial infections: 38% rifaximin <i>vs</i> 54% no rifaximin ( <i>P</i> = 0.690) Isolation of bacteria resistant to III generation cephalosporin: 25% rifaximin <i>vs</i> 46% no rifaximin  Isolation of multidrug resistant bacteria: 25% rifaximin <i>vs</i> 9% no rifaximin	

SBP: Spontaneous bacterial peritonitis; CTP: Child turcotte pugh.

Table 3 Available studies describing the effects of rifaximin on endotoxemia in patients with advanced liver disease

Study	Study design	No. patients	Disease severity	Treatment schedule	Results	Safety
Vlachogiannakos <i>et al.</i> <sup>[71]</sup> 2009	Prospective	30	welve patients (40%) were Child-Pugh B and 18 (60%) Child-Pugh C CTP score: A: 0%, B: 40%, C: 60% MELD score (mean, range): 17 (11-27) B: 40%, C: 60%	Rifaximin 1200 mg/d for 28 d 8-wk course of rifaximin (1200 mg/d) Rifaximin 1200 mg/d for 8 wk	Median (range) plasma endotoxin levels decreased significantly after rifaximin administration both in systemic [1.45 (0-3.1) <i>vs</i> 0.7 (0-2.7), <i>P</i> < 0.0001] and splanchnic circulation [1.8 (0-3.4) <i>vs</i> 0.8 (0-2.1), <i>P</i> < 0.0001]. Meanwhile, the difference seen in endotoxin levels between the splanchnic and systemic circulation at day 0 ( <i>P</i> = 0.001) was not noted at day 29 ( <i>P</i> = 0.137) Reduction in endotoxin levels in both systemic and splanchnic circulation compared to baseline ( <i>P</i> < 0.0001) Reduction in HVPG compared to baseline ( <i>P</i> < 0.0001) Reduction in HVPG correlated with hepatic vein endotoxin values ( <i>P</i> = 0.023) Rifaximin significantly reduced plasma endotoxin levels Reduction in plasma endotoxin levels compared to baseline ( <i>P</i> < 0.01)	Abdominal pain: 3% Self-limited diarrhea: 3%
Kalambokis <i>et al.</i> <sup>[73]</sup> 2012	Prospective	9	CTP score: B: 56%, C: 44%	8-wk course of rifaximin (1200 mg/d) Rifaximin 1200 mg/d for 8 wk	Reduction in plasma endotoxin levels compared to baseline ( <i>P</i> < 0.0001) Median (range) plasma endotoxin levels decreased significantly after rifaximin administration both in systemic [1.45 (0-3.1) <i>vs</i> 0.7 (0-2.7), <i>P</i> < 0.0001] and splanchnic circulation [1.8 (0-3.4) <i>vs</i> 0.8 (0-2.1), <i>P</i> < 0.0001]. Meanwhile, the difference seen in endotoxin levels between the splanchnic and systemic circulation at day 0 ( <i>P</i> = 0.001) was not noted at day 29 ( <i>P</i> = 0.137) Reduction in plasma endotoxin levels in both systemic and splanchnic circulation compared to baseline ( <i>P</i> < 0.0001) Risk of developing variceal bleeding: 35% rifaximin <i>vs</i> 59.5% controls ( <i>P</i> = 0.011) Incidence of HE: 31.5% rifaximin <i>vs</i> 47% controls ( <i>P</i> = 0.034) Incidence of SBP: 4.5% rifaximin <i>vs</i> 46% controls ( <i>P</i> = 0.027) Incidence of HRS: 4.5% rifaximin <i>vs</i> 51% controls ( <i>P</i> = 0.037)	Not reported
Vlachogiannakos <i>et al.</i> <sup>[72]</sup> 2012	Prospective	69	welve patients (40%) were Child-Pugh B and 18 (60%) Child-Pugh C CTP score rifaximin A: 0%, B: 48%, C: 52% controls: A: 0%, B: 48%, C: 52% MELD score (mean $\pm$ SD) rifaximin: 17.2 $\pm$ 3.6 controls: 16.6 $\pm$ 3.5	23 pts who achieved a decrease in HVPG after 28-d rifaximin treatment <sup>[71]</sup> 46 cirrhotic controls	Median (range) plasma endotoxin levels decreased significantly after rifaximin administration both in systemic [1.45 (0-3.1) <i>vs</i> 0.7 (0-2.7), <i>P</i> < 0.0001] and splanchnic circulation [1.8 (0-3.4) <i>vs</i> 0.8 (0-2.1), <i>P</i> < 0.0001]. Meanwhile, the difference seen in endotoxin levels between the splanchnic and systemic circulation at day 0 ( <i>P</i> = 0.001) was not noted at day 29 ( <i>P</i> = 0.137) Reduction in plasma endotoxin levels in both systemic and splanchnic circulation compared to baseline ( <i>P</i> < 0.0001) Risk of developing variceal bleeding: 35% rifaximin <i>vs</i> 59.5% controls ( <i>P</i> = 0.011) Incidence of HE: 31.5% rifaximin <i>vs</i> 47% controls ( <i>P</i> = 0.034) Incidence of SBP: 4.5% rifaximin <i>vs</i> 46% controls ( <i>P</i> = 0.027) Incidence of HRS: 4.5% rifaximin <i>vs</i> 51% controls ( <i>P</i> = 0.037)	Nausea: 9% Self-limited rash in the extremities: 4% Persistent diarrhea: 4%
Kalambokis <i>et al.</i> <sup>[75]</sup> 2012	Prospective, randomized, placebo-controlled	23	CTP score rifaximin: A: 0%, B: 46%, C: 54% placebo: A: 0%, B: 40%, C: 60% MELD score (mean $\pm$ SD): 9.8 $\pm$ 3.3	13 pts: rifaximin 1200 mg/d for 4 wk 10 cirrhotic pts: placebo	Reduction in endotoxin levels compared to control group ( <i>P</i> = 0.005) Increase in mean platelets count in rifaximin group compared to controls ( <i>P</i> = 0.006)	Not reported
Bajaj <i>et al.</i> <sup>[62]</sup> 2013	Prospective	20	MELD score (mean $\pm$ SD): 9.8 $\pm$ 3.3	Rifaximin 550 mg BID for 8 wk	Reduction in plasma endotoxin levels compared to baseline ( <i>P</i> = 0.02)	Not reported

MELD: Model for end stage liver disease; CTP: Child turcotte pugh; HVPG: Hepatic venous pressure gradient.

endotoxemia, serum bilirubin, Child-Pugh and MELD scores, together with an increase in serum albumin levels after rifaximin treatment<sup>[62,73]</sup>.

Rifaximin has also been demonstrated to have beneficial effects in the treatment of thrombocytopenia, the pathogenesis of which has not been completely clarified yet in cirrhotics. Endotoxemia has been advocated to contribute, together with portal hypertension, in the development of thrombocytopenia in these patients<sup>[74]</sup>; indeed, a small preliminary study demonstrated an increase in platelets count and a decrease in endotoxin levels in 13 patients with alcoholic cirrhosis receiving rifaximin for a 4-wk course, compared to 10 controls<sup>[75]</sup>. Even if these results may encourage the use of rifaximin to minimize the complications of endotoxemia due to portal hypertension, larger, randomized, controlled studies extended also to non alcoholic liver disease are required to confirm any clinical efficacy.

## RIFAXIMIN SAFETY IN ADVANCED LIVER DISEASE

Rifaximin benefits are generally paralleled by a good safety profile, since the reported rate of adverse events between treated cirrhotics and those who did not receive the drug is similar, and toxicity mainly involves the gastrointestinal tract (e.g., abdominal pain or diarrhea) (Tables 1-3). In particular, nor increase in the rate of infections neither development of antibiotic resistance are common in cirrhotics treated with rifaximin<sup>[76,77]</sup>. Although some cases of *Clostridium difficile* infection have been reported<sup>[51,78]</sup>, the incidence is comparable to that observed in patients with advanced liver disease and is affected by confounding factors, such as age, repeated hospitalizations, ongoing therapy with proton pump inhibitors and previous courses of antibiotics<sup>[78]</sup>. *Candida albicans* has also been isolated in fecal samples of about 20% of cirrhotics during rifaximin treatment<sup>[65]</sup>. Probably, this finding should not be considered unequivocally harmful, since *Candida* organisms commonly colonize the human gastrointestinal tract as a component of the resident mycobiota<sup>[79]</sup>.

Even if the limited incidence of adverse events has to be attributed to the small amount of rifaximin reaching the systemic circulation, a special consideration regarding its absorption in patients with advanced liver disease is mandatory. Indeed, due to the increased intestinal permeability, higher systemic rifaximin concentrations have been observed in cirrhotics compared to healthy subjects<sup>[80]</sup>. For this reason, although it may not represent a major problem in the short-term drug administration, the effects of a possible increase in systemic absorption should be cautiously taken into account in cases of prolonged rifaximin administration.

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## Stem cell-based regenerative opportunities for the liver: State of the art and beyond

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### Abstract

The existing mismatch between the great demand for liver transplants and the number of available donor organs highlights the urgent need for alternative therapeutic strategies in patients with acute or chronic liver failure. The rapidly growing knowledge on stem cell biology and the intrinsic repair processes of the liver has opened new avenues for using stem cells as a cell therapy platform in regenerative medicine for hepatic diseases. An impressive number of cell types have been investigated as sources of liver regeneration: adult and fetal liver hepatocytes, intra-hepatic stem cell populations, annex stem cells, adult bone marrow-derived hematopoietic stem cells, endothelial progenitor cells, mesenchymal stromal cells, embryonic stem cells, and induced pluripotent stem cells. All these highly different cell types, used either as cell suspensions or, in combination with biomaterials as implantable liver tissue constructs, have generated great promise for liver regeneration. However, fundamental questions still need to be addressed and critical hurdles to be overcome before liver cell therapy emerges. In this review, we summarize the state-of-the-art in the field of stem cell-based therapies for the liver along with existing challenges and future perspectives towards a successful liver cell therapy that will ultimately deliver its demanding goals.

**Key words:** Stem cells; Liver regeneration; Liver cirrhosis; Acute liver injury; Stem cell based therapy

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**Core tip:** Liver transplantation is the only effective treatment for end-stage liver diseases, but its appli-



cation is limited mainly due to donor shortage. In order to fulfil the unmet medical needs in the field, alternative, cell-based therapies for the treatment of end-stage hepatic diseases are under investigation. This review aims to summarize the state of the art on stem cell-based approaches towards liver regeneration as well as to critically discuss and highlight new perspectives and challenges.

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## INTRODUCTION

The liver possesses a remarkable capacity to regenerate in response to injury; however, in severe cases its regenerative capacity prove insufficient and hepatic injury may progress to end-stage disease and subsequent liver failure. Orthotopic liver transplantation is currently the only effective treatment for patients with end-stage liver diseases, including acute liver failure and hepatic cirrhosis. Nevertheless, donor shortage and waiting list mortality, postoperative morbidity and mortality, high costs and long-term side effects severely limit its application<sup>[1,2]</sup>. Hepatocyte transplantation has been suggested as an alternative approach to liver transplantation because mature hepatocytes have been traditionally recognized as the major contributors to liver repair and are functionally the most robust cell type for liver cell therapy<sup>[3,4]</sup>. Indeed, many preclinical and clinical studies have been conducted using this approach to cure metabolic and end-stage liver diseases<sup>[5]</sup>. However, the widespread application of hepatocyte transplantation is limited by organ unavailability, the negative impact of cell culture on hepatocyte viability, function and engraftment<sup>[5,6]</sup>, as well as hepatocyte susceptibility to cryopreservation damage inducing cell rupture, necrosis, and apoptosis after thawing<sup>[7,8]</sup>. Therefore, alternative therapies are needed to supplement organ transplantation and bridge the gap between the need for liver transplantation and the lack of a timely available cadaveric graft.

## ADULT LIVER STEM/PROGENITOR CELLS

When hepatocyte proliferation is impaired, deficient, or overwhelmed by severe liver injury, bipotent intrahepatic stem cell (SC) populations, known as resident liver progenitor cells (LPC) in humans or oval cells (OCs) in rodents, emerge and become activated, expand, and actively contribute to the regenerative process by giving rise to hepatocytes and biliary epithelial cells<sup>[9-12]</sup>.

The term "oval" cell is used to describe small, rounded proliferating cells with a large nuclear to cytoplasmic ratio which reside in the terminal branches of the intrahepatic biliary tree, the Canals of Hering, considered along with the space of Disse as the putative hepatic SC niches. OC/LPC coexpress biliary and hepatocytic markers and also hematopoietic progenitor cell antigens<sup>[13,14]</sup>.

Regarding the mechanism controlling OC fate in response to liver injury both in humans and in murine models, it has been proposed that during LPC/OC-mediated liver regeneration, an "inductive" niche is formed around OCs, constituting the ductular inflammatory reaction. This niche is populated by recruited macrophages and myofibroblasts and requires new synthesis or remodeling of extracellular matrix to facilitate appropriate OC/LPC expansion and ultimately biliary and hepatocyte regeneration<sup>[15]</sup>. The role of Wnt and Notch signaling in hepatic cell fate has been recently recognized through the proliferation and differentiation of human LPCs into hepatocytes or cholangiocytes respectively, providing potential targets for future targeted-therapies<sup>[15,16]</sup> for the liver.

The precise identification of endogenous liver SCs and of the mechanisms that govern their proliferation and differentiation into mature hepatocytes in the case of severe parenchymal extinction could facilitate their *in vitro* and *in vivo* maturation to hepatocytes and their application in clinical practice. This process was histologically identified by the description of regenerative nodules, the so called "buds" composed of small clusters of hepatocytes admixed with ductules<sup>[17]</sup>. These "buds" were suggested to be composed of new hepatocytes derived from SCs located in the small bile ducts and the canals of Hering, thus appearing to be the structures that contain SC-derived hepatocytes<sup>[18]</sup>. The progressive evolution of buds from stem/progenitor cells to integrated mature liver parenchyma was described in a recent study using various anatomic and immunohistochemical markers including epithelial cell adhesion molecule (EpCAM), K19, CD34, glutamine synthetase, and Ki-67<sup>[19]</sup>.

Interestingly, hepatic stellate cells (HSTCs), considered as liver-resident mesenchymal cells<sup>[20]</sup>, have recently been shown to represent a source of liver progenitor cells. Indeed, an isolated population of retinoid-storing hepatic stellate cells were able to contribute to liver regeneration through differentiation. HSTCs gave rise to parenchymal and bile duct cells and ameliorated the glucuronidation defect in GUNN rats, thus providing functional hepatocytes<sup>[21]</sup>.

## FETAL LIVER STEM CELLS

Fetal liver SCs appear during embryogenesis, after the establishment of the hepatic endoderm and when the liver bud is growing. Hepatoblasts, resident cells in the developing liver bud, express the signature marker  $\alpha$ -fetoprotein and are considered bipotential,

being able to give rise to both mature hepatocytes and bile duct epithelial cells (cholangiocytes)<sup>[22]</sup>. Many experimental studies have focused on the regenerative capacity of fetal hepatic progenitor cells (HPCs) as, in contrast to adult hepatocytes, fetal liver SCs can be readily isolated while they are highly proliferative, less immunogenic, and more resistant to cryopreservation<sup>[22-25]</sup>, and as such, could be of clinical benefit in the treatment of liver diseases.

Indeed, their capacity to repopulate the liver upon transplantation has been demonstrated in animal models<sup>[26-28]</sup> and clinical trials (Table 1)<sup>[29,30]</sup>. In a clinical study, 25 patients with liver cirrhosis of different etiologies, were infused with human fetal liver-derived SCs. The procedure proved safe and efficient, offering a potentially supportive modality to organ transplantation in the management of liver diseases<sup>[29]</sup>. In another study, immune-sorted, human fetal biliary tree cells were safely administered to two patients with advanced liver cirrhosis who were monitored through a 12-mo follow-up period. Immunosuppressants were not required, and the patients did not experience any adverse event or immunological complications. Both patients showed biochemical and clinical improvement within the first 6 mo and one maintained the benefits for 12 mo<sup>[30]</sup>.

The ability of fetal liver SCs to expand clonogenically *in vitro*, their pluripotency, and the evidence that they yield mature liver cells, encourage their clinical utility for transplantation and generation of bioartificial livers. However, ethical issues and the possibility of teratoma/teratocarcinoma formation in the recipients, justify their reserved use mainly in preclinical or pilot studies.

## EXTRAHEPATIC STEM/PROGENITOR CELLS

Apart from endogenous liver SCs, several populations of exogenous stem/progenitor cells have shown potential to contribute to the liver healing process and are discussed below.

### Embryonic stem cells

Human embryonic SCs (ESCs) are pluripotent cells, derived from the inner cell mass of blastocyst stage embryos, having the ability to self-renew indefinitely while maintaining the potential to give rise to all cell types in the human body when provided with the appropriate differentiation signals<sup>[31]</sup>. Because of this plasticity and the unlimited capacity for self-renewal, ESC regenerative therapies have been proposed for tissue replacement after injury or disease.

ESCs are able to differentiate efficiently into hepatocyte-like cells *in vitro*, producing cells which possess some of the properties of mature hepatocytes<sup>[32-34]</sup>. ESC-derived hepatocyte-like cells contribute to the recovery of injured liver tissue in

mice, not only by cell replacement but also by delivering trophic factors that support endogenous liver regeneration<sup>[32,35]</sup>. *In vitro* ESC-derived hepatocytes, bearing the typical mature hepatocyte morphology and expressing hepatocyte-specific genes, colonized liver tissue upon transplantation and rescued liver-injured mice from death<sup>[36]</sup>.

ESCs provide a valuable tool for studying the molecular basis of hepatocyte differentiation and form the basis for cell therapies. However, despite remarkable progress and the development of sophisticated differentiation protocols mimicking the normal embryonic development, ESC-derived "hepatocyte-like" cells usually fail to fully function as "true" hepatocytes. In addition, the risk for immunological rejection of the transplanted cells as well as ethical and legal concerns, hamper their use as cell replacement therapy<sup>[37,38]</sup>.

### Induced pluripotent stem cells

Induced pluripotent SCs (iPSCs) are embryonic-like SCs produced *in vitro* via reprogramming of somatic cells through the transient, forced expression, of key transcription factors such as OCT4 (O), SOX2 (S), KLF4 (K), and c-MYC (M) (so called OSKM cocktail) or O, S, NANOG (N) and LIN28 (L) (so called OSNL), traditionally by using, permanently integrated, retroviral vectors<sup>[39,40]</sup>.

As factor expression is not required beyond the end of the reprogramming process and the semi-random integration of retroviral vectors has been associated with insertional mutagenesis<sup>[41]</sup>, several investigators have explored techniques for iPSC generation using more clinically relevant methodologies of reprogramming, such as excisable vector systems<sup>[42]</sup>, non-integrating DNA vectors<sup>[43]</sup>, DNA-free methods<sup>[44,45]</sup>, and small molecules<sup>[46]</sup>.

iPSCs possess unique characteristics of pluripotency that render them extraordinary tools for cell and gene therapies, such as (1) unlimited self-renewal capacity *in vitro*, a feature that allows their indefinite maintenance in culture as cell lines; and (2) potential for directed differentiation to any cell type. In addition to their potential for regeneration, iPSCs provide a novel platform for *in vitro* disease-modeling<sup>[47]</sup> and drug-screening<sup>[48]</sup>.

It has been shown that iPSCs can be efficiently induced to differentiate into hepatocyte-like cells (HLCs)<sup>[49-52]</sup>, whereas transplantation of iPSC-derived HLCs reversed lethal fulminant hepatic failure, enhanced liver regeneration, and improved the performance status of NOD-SCID<sup>[52]</sup>, fumarylacetoacetate hydrolase-deficient<sup>[53]</sup>, or CCl<sub>4</sub>-injured<sup>[54]</sup> mice. In an acute hepatic failure model, iPSCs were reprogrammed from human dental pulp-derived fibroblasts into iPSCs (DP-iPSCs) capable of differentiating into HLCs (iPSC-HLCs). An injectable carboxymethyl-hexanoyl chitosan hydrogel (CHC) with sustained hepatocyte growth factor (HGF) release (HGF-CHC) was developed to

**Table 1** Clinical trials using stem cells for the treatment of liver diseases

Ref.	Cell Source	No. of patients/ administration route	Disease cause	No. of cells infused	Follow-up period	Outcomes
29	Fetal liver-SCs (EpCAM+)	25: hepatic artery	End-stage liver cirrhosis	$80 \times 10^6$	6 mo	Improved liver function and MELD score
30	Fetal liver-SCs (EpCAM+)	2: hepatic artery	Advanced cirrhosis	$42 \times 10^6$ and $60 \times 10^6$	12 mo	Biochemical and clinical improvement
133	BM-MSCs	4: peripheral vein	Decompensated liver cirrhosis	$31.73 \times 10^6$	12 mo	Well tolerated and safe procedure; improved liver function
134	MSCs from iliac crest	8: peripheral or portal vein	End-stage liver disease	$30 \times 10^6$ - $50 \times 10^6$	24 wk	No adverse effects; improved MELD and liver function
135	BM-MSCs stimulated to hepatic lineage	20: control 10: intrasplenic 10: intrahepatic	post-HCV end- stage liver disease	$2 \times 10^7$ in a total of $2 \times 10^8$ MNCs	6 mo	Improved ascites, MELD and CP score; no difference between intrahepatic and intrasplenic groups
136	BM-MSCs	105: control 53: treated/hepatic artery	post-HBV liver failure	$3.4 \times 10^8$ - $3.8 \times 10^8$	192 wk	No serious side effects or complications; improved ALB, TBIL, PT and MELD score
137	Differentiated BM-MSCs <i>vs</i> undifferentiated	10: control 15: treated/intravenous	post-HCV liver cirrhosis	$1 \times 10^6$ /kg body weight	6 mo	Improved MELD score, BIL, ALB and PC
138	BM-MSCs	20: intrasplenic	post-HCV liver cirrhosis	$10 \times 10^6$	6 mo	Decreased TBIL, AST, ALT, PT; improved ALB, PC, PT, INR
139	BM-MSCs	11: hepatic artery	Alcoholic cirrhosis	$5 \times 10^7$ injected twice	12 mo	No significant side effects; histological improvement; improved CP score
140	UC-MSC	15: control 30: treated/intravenous	post-HBV decompensated liver cirrhosis	$0.5 \times 10^6$ /kg body weight	1 yr	No significant side effects; improved liver function and MELD score; reduced ascites
141	UC-MSC	19: control 24: treated/intravenous	post-HBV acute- on-chronic liver failure	$0.5 \times 10^6$ /kg body weight	72 wk	No significant side effects; improved liver function and MELD score; increased survival
142	UC-MSC	7: peripheral vein	Primary biliary cirrhosis	$0.5 \times 10^6$ /kg	48 wk	No obvious side-effects; decreased serum ALP and GGT
143	Autologous MSCs	12: control 15: treated/peripheral vein	Decompensated cirrhosis	$195 \times 10^3$	12 mo	No beneficial effect
166	BM-MNCs	9: peripheral vein	Liver cirrhosis	$5.20 \pm 0.63 \times 10^9$ MNCs	24 wk	No major adverse effects; improved ALB, CP scores
175	G-CSF mobilization	40: controls 8: treated/subcutaneous	Severe liver cirrhosis	G-CSF: $5 \mu\text{g}/\text{kg}$ every 12 h for 3 d	8 mo	No adverse events; improved MELD score
176	Autologous G-CSF mobilized CD34 <sup>+</sup> cells	2: peripheral vein	End-stage liver disease	G-CSF: $10 \mu\text{g}/\text{kg}$ per day: 4-5 d/CD34 <sup>+</sup> cells: $2.31 \times 10^6/\text{kg}$ and $4 \times 10^6/\text{kg}$	30 to 34 mo	Safe and well tolerated procedure; improved CP and MELD scores
177	Autologous G-CSF-mobilized CD34 <sup>+</sup> cells	3: portal vein 2: hepatic artery	Liver insufficiency	CD34 <sup>+</sup> cells: $1 \times 10^6$ to $2 \times 10^8$	60 d	No complications or specific side effects; improved ALB
178	G-CSF mobilization	11: control 13: treated/ subcutaneous	Alcoholic cirrhosis	G-CSF: $10 \mu\text{g}/\text{kg}$ per day 2 times daily for 5 d	12 wk	Effective CD34 <sup>+</sup> cells mobilization; increased HGF; induced HPC proliferation
179	G-CSF mobilization	24: control 23: treated/ subcutaneous	Acute-on-chronic liver failure	G-CSF: $5 \mu\text{g}/\text{kg}$ for 12 doses	60 d	Increased survival; reduced CTP, MELD and SOFA scores
180	G-CSF mobilization	23: control 23: treated/ subcutaneous	Severe alcoholic hepatitis	G-CSF: $5 \mu\text{g}/\text{kg}$ every 12 h for 5 d	3 mo	Safe and effective HSCs mobilization; improved liver function and survival
181	Experimental PA-PE, combined with G-CSF	1: subcutaneous	Acute-on-chronic liver failure	$10 \mu\text{g}/\text{kg}$ per day for 5 d	2 mo	Rapid and long lasting clinical improvement; HSCs mobilization and a ductular reaction
182	G-CSF mobilization	24: subcutaneous	Acute on chronic liver failure	G-CSF: 5 and $15 \mu\text{g}/\text{kg}$ per day for 6 d		Safety and feasibility of G-CSF mobilization; no clinical/biochemical improvement
183	G-CSF mobilization	18: subcutaneous	Liver cirrhosis	increasing doses of G-CSF daily for 7 d	3 wk	No severe adverse events; no liver function significant modification
184	Autologous G-CSF mobilized CD34 <sup>+</sup> cells	1: portal vein	Drug-induced hepatitis	G-CSF: $15 \mu\text{g}/\text{kg}$ /for 5 d CD34 <sup>+</sup> cells: $5 \times 10^6$	30 d	Improved liver function; wide areas of regeneration in liver biopsy

185	Autologous G-CSF-mobilized CD34 <sup>+</sup> SCs	2: hepatic artery 3: portal vein	Chronic liver disease	G-CSF: 526 µg/d; 5 d, CD34 <sup>+</sup> cells: $1 \times 10^6$ - $2 \times 10^8$	6-18 mo	No side effects; improved BIL and ALB
186	Autologous G-CSF-mobilized cultured CD34 <sup>+</sup> SCs	9: hepatic artery	Alcoholic liver cirrhosis	520 µg/d; 5 d/mean TNCC: $229.7 \times 10^6$	12 wk	No side effects; improved BIL, ALT, AST, CP score and ascites
187	PBMCs from G-CSF mobilized PB	20: control 20: treated	Decompensated liver cirrhosis	5-10 µg/kg per day for 4 d. PBMC: $10^7$ - $10^8$ /kg	6 mo	No major adverse effects; improved liver function

G-CSF: Granulocyte-colony-stimulating factor; TBIL: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; CP: Child-Pugh; BM: Bone marrow; UC: Umbilical cord; HSC: Hematopoietic stem cell; HGF: Hepatocyte growth factor; EpCAM: Epithelial cell adhesion molecule; MSCs: Mesenchymal stromal cells; HCV: Hepatitis C virus; PT: Prothrombin time; ALB: Albumin; PC: Platelet count; INR: International normalized ratio; PA-PE: Experimental plasmapheresis with plasma-exchange; MELD: Model for End-stage Liver Diseases; ALP: Alkaline phosphatase; GGT:  $\gamma$ -glutamyl transferase; UC-MSC: Umbilical cord blood-mesenchymal stromal cells; BM-MSCs: Bone marrow-mesenchymal stromal cells.

improve iPSC-HLC engraftment. Intrahepatic delivery of HGF-CHC-iPSC-HLCs rescued liver function and the recipients through high anti-oxidant and anti-apoptotic activity that shrank hepatic necrotic areas<sup>[55]</sup>. Engineered donor grafts derived from iPSCs, including re-cellularized biomatrix<sup>[56]</sup>, and liver buds produced from iPSCs<sup>[57]</sup> may someday provide “autologous” organs for liver transplantation, thus highlighting their enormous potential for treating liver failure.

In addition to acquired liver diseases, HLC differentiation from iPSCs isolated from patient somatic tissues could provide patient-specific hepatocyte sources for treatment of inherited liver diseases, combining *ex vivo* gene correction and cell transplantation<sup>[58]</sup>.

iPSCs have renewed hopes for regenerative medicine because they could deliver personalized therapies, and their production from somatic, patient-specific cells, without the use of embryonic tissues or oocytes, may overcome ethical concerns and the risk of rejection. Despite these hopes for iPSCs, the issues that still need to be addressed before moving this exciting new technology from proof of concept to the clinic are: (1) the optimal reprogramming method, using clinically relevant methodologies; (2) the avoidance of teratoma formation and tumorigenicity; (3) the development of novel and rapid differentiation protocols for the generation of mature cell types from iPSCs by cost-efficient manufacturing procedures; and (4) the long-term safety, tolerability, and efficacy of the iPSC-based treatments.

### Annex stem cells

Annex SCs derived from umbilical cord, umbilical cord blood, placenta, and amniotic fluid (AF) are an easily accessible source of pluripotent SCs capable of giving rise to hematopoietic, epithelial, endothelial, and neural cells both *in vitro* and *in vivo*<sup>[59]</sup>, thus constituting an attractive target for cell-based therapy. Human umbilical cord blood SCs, when infused into NOD-SCID mice with induced liver damage, can differentiate into HLCs in the absence of fusion events<sup>[60]</sup>, boost regeneration and reduce mortality<sup>[61]</sup>. *In vitro* expanded and differentiated umbilical cord

SCs exhibited hepatocyte-like morphology, expressed upregulated levels of markers of hepatic lineage, and were capable of *in vivo* liver repopulation and expression of hepatic markers upon transplantation into mice<sup>[62,63]</sup>.

Placenta-derived multipotent cells have also been shown to differentiate into multilineage cells including HLCs. These cells not only expressed characteristics of human liver cells, but also demonstrated several functions of typical hepatocytes<sup>[64,65]</sup>.

## EXTRAHEPATIC ADULT BONE MARROW STEM CELLS

As already mentioned, liver regeneration is mainly an endogenous process, driven by mature hepatocytes<sup>[3,4]</sup> and resident intrahepatic SC populations<sup>[9,10]</sup>. Bone marrow (BM) is the largest reservoir of pluripotent SCs in adults and traditionally considered as giving rise to only hematopoietic cell lineages. This concept was challenged by reports demonstrating that BM-derived SCs (hematopoietic, mesenchymal and endothelial cells) can generate a variety of adult cell types that express non-hematopoietic cell markers and contribute to the liver healing process after tissue injury<sup>[66-72]</sup>.

### Endothelial progenitor cells

Endothelial progenitor cells (EPCs) may contribute to the repair and regeneration of the damaged liver mainly by promoting the secretion of factors supportive of the host's endogenous repair mechanisms. EPC transplantation halted established liver fibrosis in rats by suppressing activated hepatic stellate cells, increasing matrix metalloproteinase activity, and regulating hepatocyte proliferation<sup>[73]</sup>. BM-derived liver sinusoidal EPCs recruited to the injured rat liver, promoted hepatocyte proliferation and contributed to organ recovery<sup>[74]</sup>. Antifibrogenic and regenerative effects of engrafted EPCs, in transplanted rats, were mediated by increased expression of endogenous and exogenous growth factors, such as HGF, transforming growth factor (TGF)- $\alpha$ , epidermal growth factor, and vascular endothelial growth factor which triggered the generation of a new vascular network and promoted



hepatocyte proliferation, ultimately resulting in liver regeneration<sup>[75-77]</sup>.

### **Mesenchymal stromal cells**

Bone marrow stroma contains a subset of mesodermal progenitor cells, named mesenchymal stromal cells (MSCs) which are fibroblast-like, plastic-adherent, multipotent cells rapidly expanding *in vitro* under standard culture conditions. MSCs are most frequently isolated from bone marrow (BM-MSCs)<sup>[78]</sup>, but can also be obtained from a variety of tissues including umbilical cord blood (UC-MSCs)<sup>[79]</sup>, trabecular bone<sup>[80]</sup>, synovial membrane<sup>[81]</sup>, adipose tissue (AT-MSCs)<sup>[82]</sup>, placenta<sup>[83]</sup>, AF-MSCs<sup>[84]</sup>, fetal lung (FL-MSCs), and blood<sup>[85]</sup>. MSCs have the capacity to differentiate into tissues of mesodermal origin (bone, cartilage, fat) but also to give rise to cells from unrelated embryonic layers such as nerve cells and hepatocytes. In addition, they have low immunogenicity and possess immunomodulatory properties which allow them to evade the host immune surveillance<sup>[86]</sup>. Because of these features, MSCs have been proposed as a cell therapy source with increased therapeutic potential for a wide range of diseases<sup>[87-91]</sup>, including acute and chronic liver diseases. Studies conducted both in rodents<sup>[92-94]</sup> and humans<sup>[95-100]</sup> have shown that MSCs derived from BM, AT, AF, dental pulp, UC, and FL under specific culture conditions, are able to transdifferentiate *in vitro* into HLCs which express genes and fulfill some metabolic functions typical of hepatocytes.

BM-MSCs, the first and the best characterized source reported to contain MSCs, AT-MSCs, an abundant and easily accessible source of MSCs, and UC-MSCs, obtainable by the least invasive method, have been tested comparatively in terms of morphology, enrichment in MSCs following isolation and expansion, colony formation, multilineage differentiation capacity, and immune phenotype. While there were no distinct morphological or immune phenotypic features among the three sources of MSCs, AT provided a 100% success rate in MSC isolation and the highest colony frequency, while UC-derived MSCs had the highest rates of proliferation in culture, suggesting UC and AT as attractive alternatives to BM for obtaining MSCs<sup>[101]</sup>.

### **MSCs and acute liver failure**

The therapeutic effect of MSCs in models of acute liver failure has been elucidated in various studies. MSCs derived from BM, placenta, and AT showed potential for differentiation into hepatocytes *in vitro* and *in vivo*, ameliorated liver damage, reduced mortality, and exerted immunoregulation by suppressing intrahepatic natural killer T cells and inhibiting inflammatory signaling, in animal models of induced acute liver failure<sup>[102-106]</sup>.

When AT-, UC blood-, and human BM-derived MSCs, either as undifferentiated MSCs or as MSC-derived HLCs (DHLCs), were compared for their

capacity to reverse acute fulminant hepatitis in an animal model, it was demonstrated that undifferentiated MSCs and DHLCs from AT and BM sources equivalently regenerated the damaged liver, suggesting that hematopoietic pre-differentiation of MSCs may not be necessary for liver repopulation. In addition, because of the abundance and accessibility of AT-MSCs as well as their consistent hepatocyte expression profile upon differentiation, AT may be an excellent SC source for liver-regenerative procedures<sup>[107]</sup>.

The conversion of MSCs into HLCs has been repeatedly demonstrated<sup>[108,109]</sup>, and effort has been made to characterize hBMSC-derived hepatocytes *in vitro* and *in vivo*. Towards this end, tissue inhibitor of metalloproteinases 4 and follistatin expression have been associated with transdifferentiation events and suggested as two potential novel biomarkers for the characterization of hBMSC-derived hepatocytes<sup>[110]</sup>. However, accumulating evidence supports the notion that the therapeutic effects of MSCs in acute liver injury are mediated to a large degree *via* paracrine mechanisms releasing trophic and immunomodulatory factors, rather than true transdifferentiating events. This is reinforced from experiments with MSC-conditioned medium where soluble factors contained in MSC-conditioned medium (interleukin-6, VEGF, HGF, and insulin-like growth factor binding proteins) seem responsible for reduced hepatocyte apoptosis<sup>[111]</sup>, downregulation of proinflammatory cytokines, increased hepatocyte proliferation<sup>[112]</sup> and decreased mononuclear cell infiltration in the liver<sup>[113]</sup>. Indeed, secreted molecules in culture supernatant from both hFL-MSCs and hepatocyte progenitor-like cells derived from hFL-MSCs had a therapeutic effect in a CCl<sub>4</sub>-induced acute liver injury model<sup>[114]</sup>. In addition, transplantation of different origin MSCs rescued acute liver failure and repopulated mouse liver through paracrine effects that reduced the inflammatory response, inhibited apoptosis in the liver, and stimulated endogenous regeneration mechanisms<sup>[115,116]</sup>.

### **MSC-based therapy for liver cirrhosis**

The beneficial effect of MSCs in liver cirrhosis has been extensively demonstrated both in animal and clinical studies. Infused BM-MSCs have been shown to engraft into host liver and ameliorate fibrosis in a time-dependent manner by decreasing  $\alpha$ -smooth muscle actin expression, reducing collagen deposition, and improving recovery of damaged hepatocytes in animal models of experimental liver fibrosis<sup>[117-119]</sup>. Recently, AT-MSCs have attracted much interest as liver repopulating cells in different models of cirrhosis. AT-MSCs, transplanted intraportally, rather than through the tail vein, inhibited the proliferation and activation of hepatic stellate cells *in vitro* and ameliorated liver fibrosis in CCl<sub>4</sub>-treated rats by improving the microcirculation of the fibrotic liver<sup>[120,121]</sup>. In a murine steatohepatitis cirrhosis model, injected AT-MSCs

resided in the liver and expressed albumin, ultimately restoring albumin expression in hepatic parenchymal cells. Gene expression profiling of AT-MSCs revealed that the amelioration of hepatic fibrosis in this model correlated with induction of anti-inflammatory and regeneration/repair pathways as well as suppression of pathogenic helper T-cell activation<sup>[122]</sup>.

In contrast to the similar hepatic integration between undifferentiated AT-MSCs and AT-MSCs pre-differentiated to HLCs shown in acute liver injury models<sup>[107]</sup>, other liver injury models suggest that pre-differentiation of AT-MSCs to HLCs may facilitate liver engraftment. In a xenogenic transplantation model of liver regeneration, long-term engraftment of human AT-MSC-derived HLCs was demonstrated and was significantly improved when *in vitro* pre-differentiated AT-MSCs, instead of undifferentiated MSCs were used, reaching repopulation rates of more than 10% along with functional hepatic regeneration<sup>[123]</sup>.

Fibroblast growth factor (FGF)-pretreatment of AT-MSCs facilitated their transdifferentiation towards hepatic lineage *in vitro*, and the infused FGF-pretreated AT-MSCs reduced hepatic fibrosis in mice<sup>[124]</sup>. In chronic liver injury models, FGF-treated AT-MSCs led to enhanced hepatocyte proliferation and induction of hepatic stellate cell apoptosis through activation of JNK-p53 signaling in hepatic stellate cells<sup>[125]</sup>, while BM-MSCs pretreated with hepatocyte growth factor (HGF) and FGF4 or with injured liver tissue showed increased homing and hepatic differentiation ability providing therapeutic benefit in injured mice<sup>[126,127]</sup>.

It seems that MSCs exert their therapeutic effects predominantly by releasing trophic and immunomodulatory factors rather than trans-differentiating into parenchymal hepatocytes. MSCs modulate the function of activated stellate cells *via* paracrine secretion of IL-10, HGF and Nerve Growth Factor, providing a plausible explanation for the protective role of MSCs in liver inflammation and fibrosis<sup>[128-130]</sup>. Additionally, MSCs may alleviate hepatic cirrhosis through the expression of matrix metalloproteinases (MMP-9, MMP-13), enzymes capable of degrading the extracellular matrix, thus exerting a direct antifibrotic effect in the injured liver<sup>[131,132]</sup>.

Several clinical trials (Table 1) have investigated the therapeutic potential of MSCs derived from BM or UC blood in liver cirrhosis, providing however, conflicting results. In two pilot, phase I and I - II, studies, autologous BM-MSCs were injected into peripheral or portal vein of a small number of patients with end-stage liver disease. Liver function and clinical features were improved while the procedure was safe and well tolerated<sup>[133,134]</sup>. Safety and short-term efficacy of autologous BM-MSCs stimulated towards hepatic lineage and injected *via* intrasplenic or intrahepatic route was evidenced in two groups of 20 patients with post-HCV end-stage liver cell failure. Patients significantly improved their Child and

MELD score, fatigue scale and performance status over the control group who received conventional supportive treatment<sup>[135]</sup>. In 53 patients with post-HBV liver failure, autologous transplantation of BM-MSCs through the hepatic artery provided short-term efficacy in respect to several clinical and biochemical parameters, but long-term outcomes were not markedly improved<sup>[136]</sup>. Similarly, in a phase II trial with autologous transplantation of BM-derived, undifferentiated and differentiated, MSCs in 15 post-HCV cirrhotic patients, follow up at 3 and 6 mo postinfusion, revealed partial improvement of liver function tests and decline of elevated bilirubin and MELD score<sup>[137]</sup>. Another study in post-HCV cirrhotic patients, suggested the safety, feasibility, and efficacy of intrasplenically administered autologous BM-MSCs in improving liver function<sup>[138]</sup>. Eleven patients with alcoholic cirrhosis safely received autologous BM-MSCs through the hepatic artery in a phase II clinical trial; histological and clinical (by Child-Pugh score) improvement was observed in 54.5% and 90.9% of patients respectively, while the levels of TGF- $\beta$ 1, type 1 collagen, and  $\alpha$ -smooth muscle actin were significantly decreased<sup>[139]</sup>. Similarly, UC-MSC infusion was well tolerated in patients with decompensated cirrhosis, acute in chronic liver failure and in patients with primary biliary cirrhosis, resulting in significant improvement of liver function and increased survival rates<sup>[140-142]</sup>.

In contrast to the above mentioned studies, a randomized, placebo-controlled trial using peripheral administration of autologous MSCs to cirrhotic patients, failed to show a beneficial effect of MSCs in cirrhotic patients. Indeed, 3 of 15 patients who received MSCs died in the first 5 mo following cell administration while the absolute changes in Child and MELD scores, serum albumin, INR, serum transaminases and liver volumes did not differ significantly between the MSC and placebo group at 12 mo-follow-up, indicating that further studies with higher number of patients are warranted to clarify the true impact of systematic or liver-directed MSC infusion in cirrhosis<sup>[143]</sup>.

### Considerations on the clinical application of MSCs

The unique properties of MSCs including easy access and expansion, engraftment capacity, paracrine secretion, trans-differentiation and immunomodulation render them ideally suited for cell therapies. Importantly, compared to embryonic SCs, MSCs do not raise ethical issues and presumably have a safer profile in terms of tumorigenesis. Up to date, a considerable amount of preclinical and clinical evidence is currently available as regards the promise of MSCs as a relatively safe and effective approach in improving liver disease. However, several issues still need to be addressed before MSCs-based liver therapy passes to the clinical practice and these are discussed below.

There is a lack of uniformity in the design of

clinical trials, characterized by different MSC sources, doses and routes of administration, all of which may influence the outcome of MSC infusion on the basis also of the underlying disease; MSCs engrafted into injured or regenerating livers only after intrahepatic but not intrasplenic injection<sup>[144]</sup> whereas intravenously injected BM-MSCs migrated and engrafted into normal and injured liver parenchyma, under conditions of chronic but not acute injury<sup>[145]</sup>. On the contrary, the systematic administration of MSCs in a randomized trial with cirrhotic patients failed to provide efficacy over placebo<sup>[143]</sup>.

In terms of safety, and despite the absence of severe adverse events in the clinical trials conducted thus far, a pro-fibrogenic potential of MSCs and unwanted differentiation into myofibroblasts has been described in several studies<sup>[144-146]</sup>. To avoid this unwanted differentiation, some groups have suggested that BM-MSCs should be induced to differentiate into HLCs before their infusion<sup>[123]</sup>. Alternatively, others have proposed the microencapsulation of MSCs in alginate-polyethylene glycol microspheres as a means to prevent scar formation through the artificial interruption of the cell-to-cell interactions but still the enablement of release of soluble molecules<sup>[147]</sup>.

Although MSCs are at low risk of malignant transformation, concern exists on their potential to promote tumor growth *in vivo*<sup>[148-150]</sup>. Thus, screening of MSCs for a gene expression signature before administration, could serve as a safety measure<sup>[151]</sup>. *In vitro*, the spontaneous transformation of MSCs resulting in tumorigenesis was a rather rare event and occurred only after extended (beyond five weeks) culture. On the contrary, because of their immunomodulatory properties, MSCs may exert an antitumor effect by modulating the inflammatory environment that characterizes many tumors and by inhibiting signaling pathways associated with tumor growth and cell division<sup>[152-156]</sup>.

### Hematopoietic stem cells

Bone marrow has been considered as a source of liver-repopulating cells that contributes to the liver healing process after tissue injury, thus challenging the dogma of BM as giving rise to only hematopoietic cell lineages. It has been reported that BM-derived SCs can differentiate into a variety of adult cell types that express non-hematopoietic cell markers<sup>[69-72]</sup>, including hepatocytes<sup>[157]</sup>. The group of Grompe first suggested that functional hepatocytes may arise from hematopoietic SCs (HSCs)<sup>[66]</sup>, and in the early 2000s, several groups demonstrated that SCs originating in the BM or circulating outside the liver participated in liver regeneration, not only in experimental animal models<sup>[67]</sup> but also in human liver<sup>[157,158]</sup>. Numerous studies followed, highlighting the contribution of HSCs in ameliorating liver damage.

Hepatic injury caused by surgical liver resection or

cirrhosis in humans, triggered BM CD34<sup>+</sup> or CD133<sup>+</sup>/c-kit<sup>+</sup>/bcrp-1<sup>+</sup> cell trafficking towards the liver and putatively the differentiation of various populations of hematopoietic progenitor cells into HLCs<sup>[159-161]</sup>. BM cell transplantation or infusion of macrophages in a mouse model of liver fibrosis indicated that the migrated to the liver cells, reduced liver fibrosis and significantly improved survival rate compared with control injured mice<sup>[162,163]</sup>, while BM-derived hepatocytes were identified in lethally irradiated mice transplanted with HSCs<sup>[164]</sup>. In patients with malignant liver lesions, a combination of portal vein embolization (PVE) and administration of CD133<sup>+</sup> BMSCs substantially increased hepatic regeneration compared with PVE alone<sup>[165]</sup>, while cirrhotic patients safely underwent autologous BM cell infusion and improved their Child-Pugh score and albumin levels (Table 1)<sup>[166]</sup>.

### G-CSF mobilization as a source of large numbers of putatively liver-repopulating cells

HSCs can easily be forced to leave the BM and circulate into the peripheral blood from where they can be apheresed and subsequently enriched by their surface expression of CD34 or/and CD133. Mobilization of BM-resident HSCs occurs at a low magnitude under specific stimuli such as tissue injury<sup>[159,167]</sup> or in high amounts after pharmacological priming with cytostatic drugs, chemokines, or hematopoietic cytokines<sup>[168,169]</sup>. Granulocyte-colony stimulating factor (G-CSF) is a hematopoietic growth factor and the most widely used mobilizing agent<sup>[170]</sup>. G-CSF, as a means of forced circulation of large numbers of HSCs, has been extensively investigated for its hepatic regenerative effect, both in animal models of liver injury<sup>[171-174]</sup> as well as in clinical trials<sup>[175-177]</sup>. In general, two approaches have been explored for liver population with mobilized HSCs, both in animal models and clinical trials; G-CSF-mobilization alone or G-CSF-mobilization followed by infusion of autologous mobilized HSCs.

As seen with BM transplantation in liver injury models and despite the higher numbers of HSCs potentially accessing the liver by G-CSF mobilization, the true contribution of mobilized HSCs to liver repopulation is low. We and others<sup>[171,173]</sup> have shown that G-CSF mobilization of BM chimeras in induced acute and chronic liver injury models results in liver regeneration and improves survival, but the vast majority of cells repopulating the liver originate *in situ*. In a comparative study of all currently available mobilizing agents (G-CSF, Plerixafor, Plerifaxor + G-CSF) with regard to their liver repopulating potential, we have shown that all mobilizing modalities ameliorate liver fibrosis, by acting differentially during the healing process. In all cases, liver recovery was not ultimately mediated by the HSCs but either from a paracrine or "bystander" signaling effect of the mobilized HSCs that triggered endogenous repair

mechanisms and stimulated tissue progenitor cells and/or a direct “trophic” effect of the mobilizing agents in the liver. These effects, however, are difficult to be experimentally dissected to definitively address this question<sup>[174]</sup>.

Clinical studies that evaluated G-CSF mobilization in patients with advanced liver disease provided conflicting results (Table 1). In trials in end-stage liver cirrhosis or alcoholic steatohepatitis patients, G-CSF was well tolerated<sup>[175,178]</sup>, and the mobilized HSCs were shown to coexpress epithelial and SC markers<sup>[175]</sup> and to induce HPCs to proliferate within 7 d of administration<sup>[178]</sup>. In acute-on-chronic liver failure (ACLF) patients, mobilization of HSCs with G-CSF promoted hepatic regeneration, and more than doubled the percentage of ACLF patients who survived for 2 mo; it also significantly reduced CTP, MELD, and SOFA scores and prevented the development of sepsis, hepatorenal syndrome, and hepatic encephalopathy<sup>[179]</sup>. Similarly, a recent randomized open study showed that the administration of G-CSF was safe and improved liver function as well as survival in patients with severe alcoholic hepatitis<sup>[180]</sup>. In an interesting case report, experimental plasmapheresis with plasma-exchange (PA-PE), as a process to eliminate circulating toxic factors, was combined with G-CSF in a patient with ACLF<sup>[181]</sup>. This regimen induced mobilization of HSCs and a rapid and long lasting clinical improvement associated with a ductular reaction, in which HPCs expressing G-CSF receptor (G-CSFR) were observed. PA-PE might have modulated the liver microenvironment thus providing a conducive milieu to G-CSF-mediated amplification of endogenous HPCs that promoted liver regeneration. Given that G-CSFR was expressed by HPCs, G-CSF might also be directly involved in modifying the HPC niche exerting a “hepatotrophic effect”<sup>[181]</sup>. In contrast, other clinical studies reported on the safety and tolerability of G-CSF mobilization but could not demonstrate significant clinical improvement, despite effective mobilization<sup>[182,183]</sup>.

The relatively easy access to large quantities of HSCs by mobilization followed by cytapheeresis, renders them ideally suited as liver repopulating cells. Thus, several groups have investigated G-CSF mobilization followed by infusion of autologous mobilized HSCs, an approach that forces a maximum SC dose to circulate at a given time, thus increasing the number of SCs that potentially home to the liver and initiate the recovery process.

We previously assessed the safety and efficacy of boost *iv* infusions of mobilized peripheral blood SCs (mPBSCs) in two patients with end-stage alcoholic liver cirrhosis. The patients tolerated well three mobilization rounds and infusions of mPBSCs that resulted in lasting amelioration in the clinical course of a previously decompensated disease, during a 30 mo follow-up<sup>[176]</sup>. In another study, a significant

biochemical and histopathological improvement was achieved in a patient with drug-induced acute liver failure after intraportal administration of mobilized CD34<sup>+</sup> BMSCs<sup>[184]</sup>.

A phase I study was performed to determine the safety and tolerability of G-CSF administration, followed by collection and intraportal or intrahepatic reinfusion of circulating CD34<sup>+</sup> cells into patients with liver failure. An improvement of the hepatic function without significant side effects in short and long term follow-up was observed in more than 50% of the subjects<sup>[177,185]</sup>. In another trial, following G-CSF mobilization and leukapheresis, the autologous CD34<sup>+</sup> cells were expanded *in vitro* and injected into the hepatic artery of nine patients with alcoholic liver cirrhosis (ALC). The clinical and biochemical improvement in the study group was encouraging while it proved safe to mobilize, expand, and reinfuse autologous CD34<sup>+</sup> cells in ALC patients<sup>[186]</sup>. In one of the largest trials, 40 patients with decompensated, hepatitis B virus-related liver cirrhosis were randomized to receive G-CSF alone or in combination with leukapheresis and reinfusion of peripheral blood monocytes (PBMC). A significant biochemical and clinical improvement was observed in both groups, but the subjects receiving G-CSF plus PBMC infusion experienced greater and longer-lasting clinical benefits during the follow-up period<sup>[187]</sup>.

### Considerations on the use of HSCs as liver-repopulating cells

The concept of BM-derived liver regeneration has been strongly questioned. Despite an improvement in several parameters of liver function, both in preclinical and clinical studies, it has become clear that, in the absence of selective pressure, the true contribution of BM to liver regeneration is extremely low in effectively supporting *per se* liver recovery<sup>[188-190]</sup>. The current belief is that the clinical benefit observed in the injured liver after HSC therapy is produced by the activation of endogenous progenitor cells through paracrine signaling interaction between donor and host cells providing cytokines and growth factors<sup>[190-192]</sup>, rather than by transdifferentiation of BMSCs into parenchymal liver cells<sup>[158]</sup> or cell fusion with resident target cells in the host tissue<sup>[193,194]</sup>.

Overall, from the various published studies on the use of HSCs as a cell therapy source for liver diseases, it seems that mobilization of HSCs, apheresis, and re-infusion is safe, while improving quality of life and disease parameters. As such, this approach may help to “bridge” patients to liver transplantation or reverse a decompensated cirrhosis to a compensated stage. In addition, the use of autologous mobilized HSCs as a cell source for liver regeneration is not associated with ethical concerns and can provide easy access to, and high yields of, SCs without the risk of rejection or need for immunosuppression. However, efficacy still needs to be confirmed, and the route of delivery, the amount



of infused cells, and the timing of infusions need to be clarified, standardized, and validated in well-designed large clinical trials.

### Liver tissue engineering

Liver tissue engineering endeavours to provide novel tools for end-stage liver diseases which will, ideally, replace organ transplantation. Therapeutic approaches towards this goal include implantable hepatic tissue engineered constructs and bioartificial liver (BAL) devices.

Implantable engineered cellular tissues provide an alternative method of cell delivery and are gaining ground in the field of regenerative medicine. They are generated mainly by immobilizing or encapsulating cells using biomaterial scaffolds. Biomaterial scaffolds provide 3-dimensional (3D) structures resembling the extracellular matrix environment *in vivo*, and have been used in association with an appropriate induction medium to promote BM-derived MSC differentiation into HLCs<sup>[195]</sup>. Apart from alginate scaffolds<sup>[195]</sup>, derived from natural polysaccharide-based biomaterials, 3D nanofibrous scaffolds of synthetic polymer-based biomaterials, allowing easy control of the quality and reproducibility of the product, have been used to investigate the hepatic differentiation potential of human BM-MSCs. The nanofibrous scaffolds enhanced SC differentiation into functional HLCs expressing liver specific markers compared with 2D culture systems<sup>[196]</sup>.

Similarly, the topographic properties of ultraweb nanofibers enhanced the differentiation of MSCs to HLCs which maintained functionality in long-term cultures. Differentiated HLCs homed to and engrafted into the injured liver of fibrotic mice, enhanced serum albumin, and rescued recipients from liver failure<sup>[197]</sup>. In another study, collagen-coated poly 3D scaffolds, supplemented with hepatocyte differentiation medium, provided a suitable environment for differentiation of BM-MSCs into mature hepatocytes over the control, monolayer culture system<sup>[198]</sup>. Recently, poly 3-hydroxybutyrate-co-3-hydroxyvalerate-co-3-hydroxyhexanoate scaffolds, made up by biodegradable polyester produced by bacteria, provided higher viability and attachment of human UC Wharton's jelly-MSCs than other polymers tested, ultimately promoting the recovery of the injured liver after transplantation in mice<sup>[199]</sup>.

BAL devices contain functional hepatocytes that supply important molecules to support hepatic function and to remove circulating toxins. This technology, however, is limited by the complexity of liver function and the shortage of human livers to provide adequate numbers of hepatocytes. Thus, *ex vivo* differentiated hepatocytes from alternative sources have been investigated. A BAL device seeded with ESC-derived hepatocytes or primary hepatocytes which was subcutaneously implanted in 90% hepatectomized

mice, improved liver function and prolonged survival over control mice, while ESC-derived hepatocytes in BAL developed characteristics nearly identical to those of primary hepatocytes<sup>[200]</sup>.

Very recently, 3D printing technologies, by fabricating complex 3D tissue engineering scaffolds and providing patient-specific tissue models showed promise in revolutionizing liver regenerative medicine towards customized transplantation approaches<sup>[201]</sup>.

For all the above technologies however, challenges still remain and dictate an in depth, understanding of the specific molecular, mechanisms and signaling pathways in the hepatic microenvironment that affect hepatic cell lineages and regulate efficient differentiation of SCs<sup>[202]</sup>.

## CONCLUSION

SC-based liver regeneration is an exciting and dynamic area of research showing remarkable advancement in liver medicine, both in basic science and in the translational field. The clinical translation for liver cell therapies however, from only a promise for cure to a treatment reality for end stage liver diseases, requires deeper understanding of SC and liver biology, and the remaining unsolved aspects to be addressed.

Up to date there has been a lack of uniformity in preclinical and clinical studies, as regards the type and the extent of injury of the liver parenchyma, the source and dose of SCs, the therapeutic timing and route of administration of SCs, and the primary endpoints. In addition, positive results in animal models have not always been translated to successful clinical trials, as clear evidence of therapeutic benefit has usually been lacking from clinical trials. As such, carefully designed clinical trials will help to elucidate the most appropriate SC therapy for different liver diseases by considering the background and severity of the target disease as well as the putative functional roles of different SCs and the intended biological action by their infusion.

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## Basic Study

# N-acetylcysteine modulates angiogenesis and vasodilation in stomach such as DNA damage in blood of portal hypertensive rats

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## Abstract

**AIM:** To evaluate the antioxidant effect of N-acetylcysteine (NAC) on the stomach of rats with portal hypertension.

**METHODS:** Twenty-four male Wistar rats weighing  $\pm$  250 g were divided into four experimental groups ( $n$  =

6 each): Sham-operated (SO), SO + NAC, partial portal vein ligation (PPVL), and PPVL + NAC. Treatment with NAC in a dose of 10 mg/kg (i.p.) diluted in 0.6 mL of saline solution was administered daily for 7 d starting 8 d after the surgery. Animals from the PPVL and SO group received saline solution (0.6 mL) for the same period of time as the PPVL + NAC and SO + NAC group. On the 15<sup>th</sup> day the animals were anesthetized and we evaluated portal pressure by cannulating mesenteric artery. After, we removed the stomach for further analysis. We performed immunohistochemical analysis for endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), and nitro-tirosine (NTT) proteins in stomach. We also evaluated eNOS and VEGF by Western blot analysis and assessed DNA damage in blood samples by the comet assay.

**RESULTS:** The portal hypertension group exhibited increases in portal pressure when compared to SO group ( $29.8 \pm 1.8$  vs  $12.0 \pm 0.3$  mmHg) ( $P < 0.001$ ). The same was observed when we compared the eNOS ( $56.8 \pm 3.7$  vs  $13.46 \pm 2.8$  pixels) ( $P < 0.001$ ), VEGF ( $34.9 \pm 4.7$  vs  $17.46 \pm 2.6$  pixels) ( $P < 0.05$ ), and NTT ( $39.01 \pm 4.0$  vs  $12.77 \pm 2.3$  pixels) ( $P < 0.05$ ) expression by immunohistochemistry of the PPVL animals with the SO group. The expression of eNOS ( $0.39 \pm 0.03$  vs  $0.25 \pm 0.03$  a.u.) ( $P < 0.01$ ) and VEGF ( $0.38 \pm 0.04$  vs  $0.26 \pm 0.04$  a.u.) ( $P < 0.01$ ) were also evaluated by *Western blot* analysis, and we observed an increase of both proteins on PPVL animals. We also evaluated the DNA damage by comet assay, and observed an increase on damage index and damage frequency on those animals. NAC decreased portal pressure values in PPVL + NAC animals ( $16.46 \pm 2$  vs  $29.8 \pm 1.8$  mmHg) ( $P < 0.001$ ) when compared to PPVL. The expression of eNOS ( $14.60 \pm 4.1$  vs  $56.8 \pm 3.7$  pixels) ( $P < 0.001$ ), VEGF ( $19.53 \pm 3.2$  vs  $34.9 \pm 4.7$  pixels) ( $P < 0.05$ ) and NTT ( $21.84 \pm 0.7$  vs  $39.01 \pm 4.0$  pixels) ( $P < 0.05$ ) evaluated by immunohistochemistry were also reduced in PPVL + NAC animals. Also, when evaluated by *Western blot* eNOS expression ( $0.32 \pm 0.03$  vs  $0.39 \pm 0.03$  a.u.) ( $P < 0.05$ ) and VEGF expression ( $0.31 \pm 0.09$  vs  $0.38 \pm 0.04$  a.u.) ( $P < 0.01$ ). Furthermore, NAC modulated DNA damage in PPVL + NAC animals.

**CONCLUSION:** In view of these results, we believe NAC is able to protect the stomach from the alterations induced by the PPVL procedure.

**Key words:** N-Acetylcysteine; Portal hypertension; Gastropathy; Oxidative stress; Antioxidant

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**Core tip:** Portal hypertension (PH) is a syndrome with serious manifestations as ascites, hepatic encephalopathy and development of collateral circulation, characterized by vasodilation and angiogenesis. This mechanism, intended to divert blood from the site

of obstruction, is the leading cause of death among these patients, since it leads to upper gastrointestinal bleeding. Therapies that may contribute to control the development of collateral circulation have been investigated in an attempt to improve the quality of life of PH patients. This paper proposes a novel therapy, using an antioxidant effective in reducing this collateral circulation in an animal model of portal hypertension.

Licks F, Hartmann RM, Marques C, Schemitt E, Colares JR, Soares MC, Reys J, Fisher C, da Silva J, Marroni NP. N-acetylcysteine modulates angiogenesis and vasodilation in stomach such as DNA damage in blood of portal hypertensive rats. *World J Gastroenterol* 2015; 21(43): 12351-12360 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12351.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12351>

## INTRODUCTION

Portal hypertension (PH) is characterized by a progressive increase in pressure within the hepatic portal system, with development of hyperdynamic circulation and a pressure gradient in the portal system (the difference between the pressure in the portal vein and the hepatic veins) exceeding 5 mmHg<sup>[1]</sup>.

Conditions in which portal blood flow is reduced or obstructed by an anatomic obstacle are the determinants of PH pathogenesis. When an anatomic obstacle arises, vascular resistance to flow increases, contributing to the development of PH<sup>[2]</sup>. Depending on the location of this obstacle to blood flow, PH may be classified as pre-hepatic (portal or splenic vein thrombosis), intra-hepatic (cirrhosis), or post-hepatic (inferior vena cava thrombosis), among other examples<sup>[3]</sup>.

The result of increased vascular resistance in the splanchnic circulation is increased pressure within the portal system. This leads to the development of a collateral circulation in an attempt to decompress the system, shunting blood directly into the systemic circulation, and increasing local venous blood flow through vasodilation<sup>[4]</sup>. Vasodilation in the splanchnic territory leads to the development of varices, particularly in the stomach. Gastric varices can be located from the fundus to the upper third of the stomach, and are caused by exacerbated dilation of blood vessels secondary to PH<sup>[5]</sup>.

Among the various vasoactive substances implicated in the development of a splanchnic collateral circulation, nitric oxide is the key mediator involved in this process. Vasodilation and the formation of portosystemic collaterals contribute to increased blood flow and worsen PH, increasing the risk of upper gastrointestinal bleeding<sup>[6]</sup>. Nitric oxide derived from endothelial nitric oxide synthase (eNOS) plays a major role in the pathophysiology of PH. Nitric oxide is a potent vasodilator, and acts not only on the splanchnic

circulation but also on the arterial circulation. It diffuses into smooth muscle cells and activates guanylate cyclase, producing cyclic guanosine monophosphate and thus contributing to the excessive vasodilation observed in PH<sup>[7]</sup>.

The pathways of eNOS activation include endothelial stimuli such as shear stress, proinflammatory cytokines, and vascular endothelial growth factor (VEGF)<sup>[8,9]</sup>.

The angiogenesis process, characterized by the formation of new blood vessels from preexisting ones, also contributes to the development and persistence of the collateral circulation. The role of VEGF in this process has been established by experimental studies in which it was shown to be the main angiogenic mediator in rats with PH. VEGF appears to contribute to the deterioration of PH by stimulating the development of portosystemic collaterals and increasing the permeability of the mesenteric microvasculature<sup>[10,11]</sup>.

The role of oxidative stress in the vascular dysfunction of PH has been well established in the literature<sup>[12,13]</sup>. The overproduction of nitric oxide caused by the vascular abnormalities present in portal hypertension facilitates the reaction of NO to superoxide anion radical ( $O_2^{\cdot-}$ ) and forms peroxynitrite ( $ONOO^{\cdot-}$ ) contributing to an increase in oxidative phenomena<sup>[14]</sup>. The reactive oxygen species that characterize oxidative stress have the potential to bind to proteins, break DNA, and induce cell damage by interactions with various cell components. This phenomenon is associated with a series of disorders, including PH<sup>[15]</sup>.

N-Acetylcysteine (NAC) is a compound that has been widely used in clinical practice for decades: as a mucolytic agent; in the management of ischemia-reperfusion injury; in acute respiratory distress syndrome and bronchitis; in the treatment of paracetamol toxicity and heavy metal poisoning; in HIV; and in psychiatric disorders. Its broad indications are due to its extensive antioxidant effects, which are attributable to its ability to react rapidly with  $\cdot OH$ ,  $\cdot NO_2$ ,  $CO^{\cdot-}$ , and thiyl radicals, as well as to replenish vital cell components depleted by injury<sup>[16]</sup>.

Within this context, the aim of the present study was to assess the effects of the antioxidant NAC in a rat model of PH, while evaluating the involvement of oxidative stress, vascular damage, and DNA damage in PH.

## MATERIALS AND METHODS

### Ethics

All animal-related procedures were performed in compliance with the guidelines of the Research Ethics Committee of Hospital de Clínicas de Porto Alegre (HCPA), state of Rio Grande do Sul, Brazil, and with the United States National Academy of Sciences Principles for Research Involving Animals<sup>[17]</sup>.

### Animals

We used 24 male Wistar rats, weighing 250 g each, obtained from the HCPA vivarium. All were kept in plastic bin cages, measuring 47 cm × 34 cm × 18 cm and lined with wood chips, under a 12-h dark/light cycle (lights on from 7 a.m. to 7 p.m.), at a controlled temperature of  $22 \pm 4^\circ C$ . The rats were fed commercially available chow (Purina® - Nutripal, Porto Alegre, RS, Brazil), 16 g/d, and had access to water *ad libitum*.

### Groups and treatment protocols

Animals were divided into four experimental groups ( $n = 6$  each): sham-operated (SO), SO + NAC, partial portal vein ligation (PPVL) and PPVL + NAC. NAC (Sigma Chemical Co., St. Louis, MO, United States; CAS registry number 616-91-1) was administered at a dose of 10 mg/kg, intraperitoneally, dissolved in 0.6 mL of normal saline solution (0.9% NaCl). This dose was based on previous studies performed by our research group<sup>[18]</sup>. Treatment was administered once daily for 7 d, starting on day 8 after surgery. Animals in the PPVL and SO groups received the same volume of saline solution, for the same period, instead of NAC.

### Induction of portal hypertension

The animals were initially anesthetized with ketamine hydrochloride (100 mg/kg i.p.) and xylazine hydrochloride (10 mg/kg i.p.). After induction of anesthesia, a midline laparotomy was performed and the bowels were gently retracted with a gauze pad soaked in saline. Briefly, the portal vein was isolated using 3-0 silk and a 20G needle was placed in front of it to establish PPVL. Both the portal vein and the needle were tied using the silk suture and the needle was withdrawn. The SO group underwent a sham version of the same procedure, in which the portal vein was not ligated<sup>[19]</sup>.

### Euthanasia

On day 15 after surgery, animals were anaesthetized with ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg i.p.). Blood was collected from the retro-orbital plexus using a heparinized capillary tube and stored in heparinized Eppendorf microtubes for later assessment of DNA damage<sup>[20,21]</sup>. After blood sampling, the abdomen was shaved and a laparotomy performed. Portal pressure was measured by cannulation of the mesenteric vein with a catheter coupled to a polygraph (Poligraph 2006, Letica Scientific Instruments, Barcelona, Spain)<sup>[19]</sup>. The animals were then killed by exsanguination under deep anesthesia<sup>[22]</sup> and we collected the stomach for the posterior analyzes. A piece of the stomach sample was frozen and stored at  $-80^\circ C$  and another piece was cut and fixed in 10% buffered formalin for 24 h. Paraffin blocks were cut with a rotatory microtome to create 3-mm sections.

### Immunohistochemistry

Expression of eNOS, VEGF, and nitrotyrosine (NTT) antibody in stomach tissue was determined by immunohistochemical analysis. Antigen retrieval was performed using buffer at 100 °C, and endogenous peroxidase activity was blocked by incubation with absolute methanol. Slides were incubated with rabbit polyclonal antibody (NOS3 C-20 (sc-654), 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, United States), mouse monoclonal antibody (VEGF C-1 (sc-7269), 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, United States), and rabbit polyclonal antibody (Nitro-Tyrosine Antibody (#9691), 1:200, Cell Signaling Technology) overnight at 4 °C, then washed with buffer and incubated with secondary antibodies for eNOS (goat anti-rabbit IgG-HRP 2004), VEGF (goat anti-mouse IgG-HRP 2005), and NTT (anti-rabbit IgG-HRP 7074) for 30 min at room temperature. The slides were analyzed by a pathologist without prior knowledge of group allocation using a microscope coupled to a digital camera. Images were captured using Image-Plus software (Media Cybernetics, Bethesda, MD, United States). Quantification of eNOS, VEGF, and NTT antibody expression was performed via digital analysis in Adobe Photoshop® CS3 Extended 10.0 and involved counting pixels of areas stained by the immunohistochemical reagents. The level of expression was determined by multiplying the average density of the image by the percentage of positively stained areas (areas of brown staining)<sup>[23]</sup>.

### Western blot analysis

Western blot analysis was performed in cytosolic extract prepared from stomach homogenates. The supernatant fraction was collected and stored at 80 °C in aliquots until use. Protein concentration was measured as described by Bradford (1976). Lysate proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes<sup>[24,25]</sup>. The membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TTBS) for 1 h at room temperature and probed overnight at 4 °C with rabbit polyclonal antibody [NOS3 C-20 (sc-654), Santa Cruz Biotechnology, Santa Cruz, CA, United States], mouse monoclonal antibody [VEGF C-1 (sc-7269), Santa Cruz Biotechnology, Santa Cruz, CA, United States], at 1:200-1000 dilution with TTBS in 5% nonfat dry milk, and anti-β-actin (42 kDa) antibody (Sigma Aldrich, St. Louis, MO, United States) at 1:1000 dilution with TTBS in 5% nonfat dry milk. After washing with TTBS, the membranes were incubated for 1 h at room temperature with secondary HRP-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States, 1:4,000). Protein detection was performed via chemiluminescence using a commercial ECL kit (Amersham Pharmacia Biotech, Little Chalfont, Great

Britain)<sup>[26]</sup>. The density of the specific bands was quantified with imaging densitometry software (Scion Image, Maryland, MA).

### Comet assay

The alkaline comet assay was carried out as described elsewhere<sup>[20]</sup>, with minor modifications<sup>[21]</sup>. Blood samples (50 µL) were placed in 5 µL of anticoagulant (heparin sodium, 25000 IU, Liquaemin®). Blood cell suspensions (5 µL) were embedded in 95 µL of 0.75% low melting point agarose (Gibco BRL) and spread on agarose-precoated microscope slides. After solidification, slides were placed in lysis buffer (2.5 mol/L NaCl, 100 mmol/L EDTA, and 10 mmol/L Tris, pH 10.0), with freshly added 1% Triton X-100 (Sigma) and 10% DMSO for 48 h at 4 °C. The slides were subsequently incubated in freshly prepared alkaline buffer (300 mmol/L NaOH and 1 mmol/L EDTA, pH > 13) for 20 min at 4 °C. An electric current of 300 mA and 25 V (0.90 V/cm) was applied for 15 min to perform DNA electrophoresis. The slides were then neutralized (0.4 mol/L Tris, pH 7.5), stained with silver, and viewed under a microscope. Images of 100 randomly selected cells (50 cells from each of two replicate slides) from each animal were analyzed. Cells were also visually scored according to tail size into five classes, ranging from undamaged (0) to maximally damaged (4), resulting in a single DNA damage score for each animal and, consequently, for each studied group. The damage index (DI) could thus range from 0 (completely undamaged, 100 cells × 0) to 400 (maximum damaged, 100 cells × 4). The damage frequency (%) was calculated on the basis of the number of tailed versus tailless cells.

### Statistical analysis

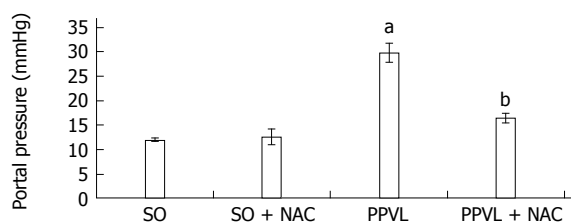
All data are presented as mean ± SE. Statistical significance was calculated using GraphPad InStat, version 3.0 for Windows. Analysis of variance and the Student-Newman-Keuls method were used for multiple analysis. For the comet assay, the normality of variables was evaluated using the Kolmogorov-Smirnov test, and statistical differences between groups were analyzed using the nonparametric two-tailed Kruskal-Wallis test with Dunn's correction for multiple comparisons. Student's *t*-test was used to compare damage between negative and positive controls. The critical level for rejection of the null hypothesis was considered to be a *P* value of < 0.05 (*i.e.*, a significance level of 5%).

## RESULTS

### Portal pressure measurement

We observed a statistically significant increase in portal pressure values in the PPVL group as compared to SO animals (*P* < 0.001). In the NAC-treated group, these values were significantly decreased (*P* < 0.001) (Figure





**Figure 1 Portal pressure.** Effects of partial portal vein ligation (PPVL) and N-acetylcysteine (NAC) administration on portal pressure. <sup>a</sup> $P < 0.001$  against SO, <sup>b</sup> $P < 0.001$  against PPVL ( $n = 6$ ). SO: Sham-operated group; SO + NAC: Sham-operated animals treated with NAC; PPVL: Partial portal vein ligation group; PPVL + NAC: Partial portal vein ligation group treated with NAC.

1).

### Immunohistochemistry

Immunohistochemical analyses for eNOS (Figure 2A;  $P < 0.001$ ), VEGF (Figure 2B;  $P < 0.05$ ) and NTT (Figure 2C;  $P < 0.05$ ) showed that animals in the PPVL group had samples markedly positive for all three proteins. NAC treatment reduced expression of eNOS, VEGF, and NTT in stomach tissue.

### Western blot analysis

Expression of eNOS (Figure 3A;  $P < 0.05$ ) and VEGF (Figure 3B;  $P < 0.01$ ) was increased in PPVL animals as compared with the SO group. Conversely, expression of these proteins was reduced in the PPVL + NAC group when compared with the PPVL group.

### Comet assay

The comet assay revealed an increased DNA damage index (Figure 4A) and increased frequency of damage in blood samples (Figure 4B) in PPVL group animals. NAC treatment reduced both of these parameters, thus demonstrating the ability of NAC to modulate DNA damage in this experimental model.

## DISCUSSION

PH is the main complication of cirrhosis, and is one of the leading causes of mortality in patients with chronic liver disease. The increased vascular resistance and blood flow present in PH are determining causes that elevate the portal pressure gradient, shunting blood from the liver into the systemic circulation. The subsequent formation of collateral vessels due to the NO overproduction is implicated in gastroesophageal bleeding, hepatic encephalopathy, and sepsis<sup>[27]</sup>.

The search for medications that can prevent or mitigate this increase in portal pressure is extremely relevant. In the present study, we sought an alternative based on the close relationship between PH and oxidative stress and on the involvement of nitric oxide in this setting.

NAC is an antioxidant with a well-established mechanism of action, based on its ability to restore glutathione levels and act as a free radical

scavenger<sup>[28]</sup>. Furthermore, NAC modulates nitric oxide through interactions of its thiol component with NO to form nitrosothiol<sup>[29]</sup>.

The increased portal pressure observed in rats subjected to PPVL corroborates the findings of previous studies<sup>[30,31]</sup> and demonstrates the efficacy of the experimental model used in this study. NAC treatment effectively reduced pressure in the portal system in PPVL + NAC animals (Figure 1). Similar results were obtained in a prior study by our research group, in which NAC was found to reduce portal system pressures as a result of its ability to modulate nitric oxide and its antioxidant properties<sup>[32]</sup>.

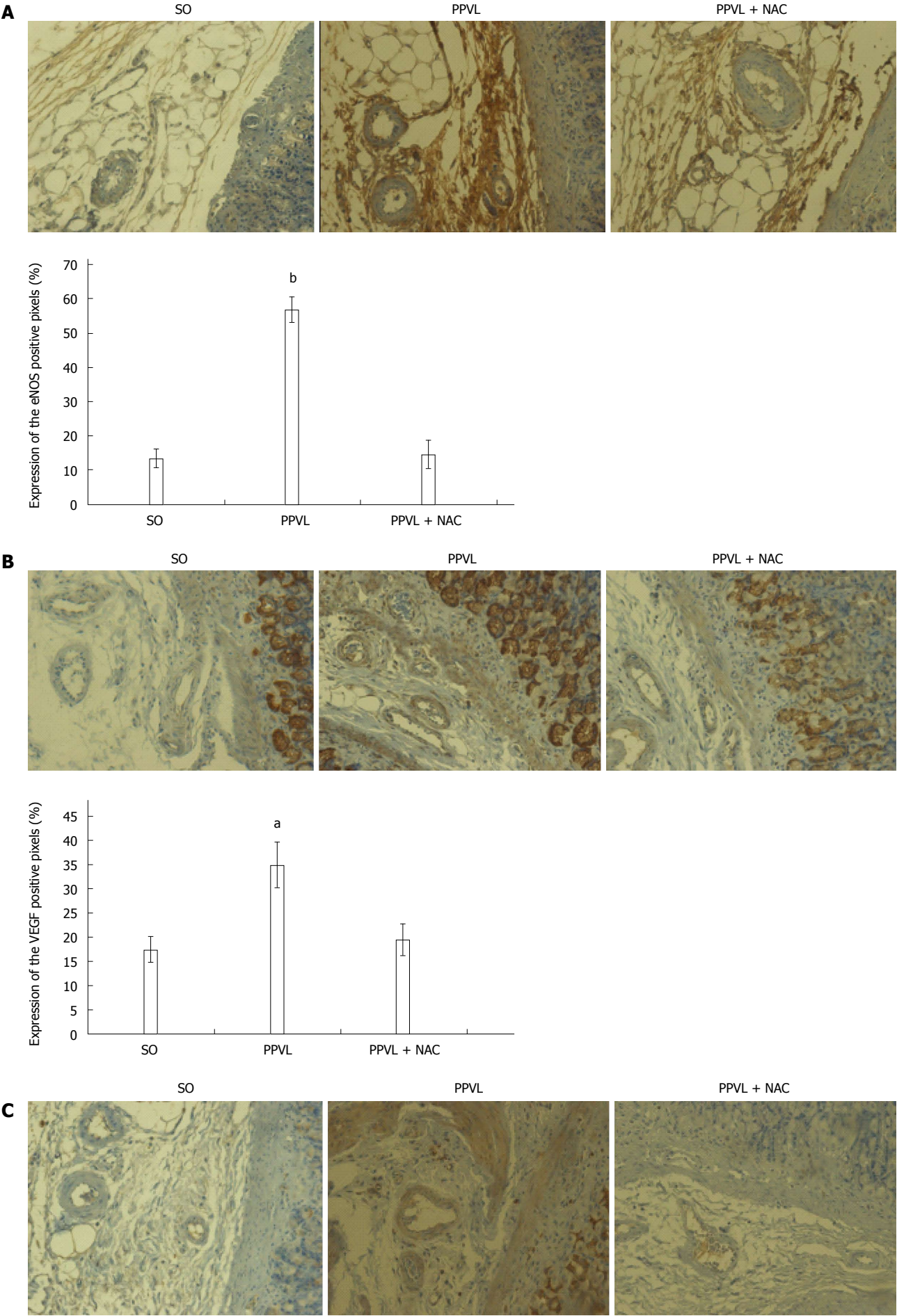
The ability of NAC to modulate nitric oxide bioavailability led to a reduction in eNOS expression in the gastric mucosa of portal hypertensive rats treated with NAC (PPVL + NAC), as assessed by immunohistochemistry (Figures 2A-C) and Western blot analysis (Figures 3A and B).

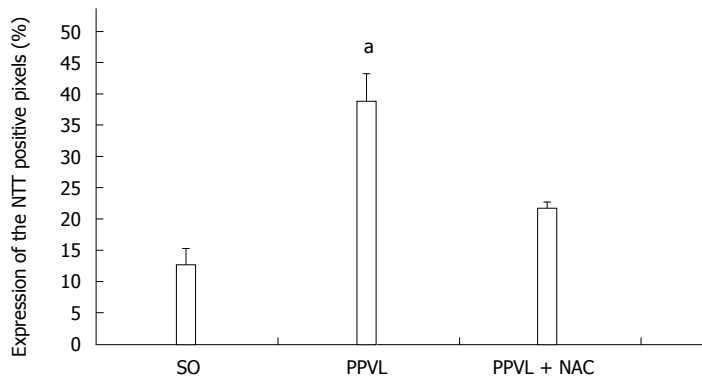
NO synthases are enzymes that play an essential role in the control of nitric oxide biosynthesis. The three main isoforms are inducible NO (iNOS) and two constitutive forms, nNOS and eNOS<sup>[33]</sup>. Nitric oxide produced by endothelial cells (eNOS) plays a major role in vascular smooth muscle relaxation. Under physiologic conditions, NO release is stimulated by acetylcholine, bradykinin, and adenosine triphosphate, among other mediators. Furthermore, the friction of circulating blood cells against vessel walls triggers eNOS-mediated nitric oxide synthesis *via* a shear stress mechanism to increase nitric oxide<sup>[34]</sup>.

In PH, development of a hyperdynamic circulation is associated with nitric oxide overproduction, and portal hypertensive gastropathy is associated with the hemodynamic changes caused by NO triggering<sup>[35]</sup>. In the present study, the PPVL model caused an increase in portal system pressure and, consequently, increased eNOS immunoreactivity and expression in the gastric mucosa of rats in the PPVL group (Figures 2A and 3A). In a previous study, NAC reduced splanchnic vasodilation by reducing NO levels in an experimental model of cirrhosis induced by common bile duct ligation<sup>[36]</sup>. Our research group found a similar effect of NAC in rats with hepatopulmonary syndrome<sup>[18]</sup>.

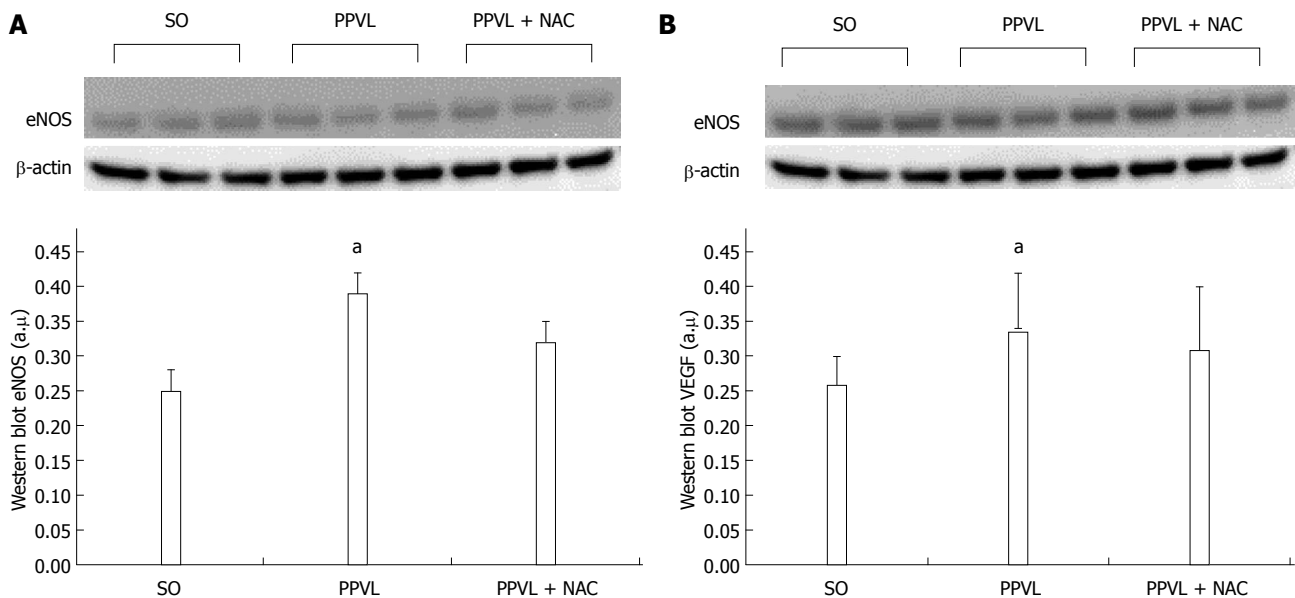
Nitric oxide toxicity is particularly prevalent in oxidative stress settings. The high reactivity of NO with molecules such as the superoxide anion ( $O_2^{\cdot-}$ ) produces highly injurious compounds, such as peroxynitrite ( $ONOO^-$ ), which may trigger nitrosative stress<sup>[37]</sup>. As PH is directly associated with oxidative stress and nitric oxide overproduction, it is to be expected that synthesis of reactive metabolites will occur, potentiating the pathophysiological changes observed in this syndrome.

This finding was proven by analysis of immunoreactivity to NTT in the gastric mucosa of study animals. We observed increased NTT reactivity in PPVL group animals (Figure 2C). The peroxynitrite generated during interactions of nitric oxide with the





**Figure 2 Immunohistochemistry for endothelial nitric oxide synthase, vascular endothelial growth factor and nitrotyrosine.** A: Effects of partial portal vein ligation (PPVL) and N-acetylcysteine (NAC) administration on eNOS immunoreactivity in gastric mucosa, <sup>b</sup> $P < 0.001$  ( $n = 6$ ); B: Effects of partial PPVL and NAC administration on VEGF immunoreactivity in gastric mucosa, <sup>a</sup> $P < 0.05$  ( $n = 6$ ); C: Effects of PPVL and NAC administration on NTT immunoreactivity in gastric mucosa, <sup>a</sup> $P < 0.05$  ( $n = 6$ ). SO: Sham-operated group; PPVL: Partial portal vein ligation group; PPVL + NAC: Partial portal vein ligation group treated with NAC. Original magnification  $\times 400$ .



**Figure 3 Western blot analysis.** A: Effects of partial portal vein ligation (PPVL) and N-acetylcysteine (NAC) administration on eNOS expression, <sup>a</sup> $P < 0.05$  ( $n = 6$ ); B: Effects of PPVL and NAC administration on VEGF expression. SO: Sham-operated group; PPVL: Partial portal vein ligation group; PPVL + NAC: Partial portal vein ligation group treated with NAC, <sup>a</sup> $P < 0.01$  ( $n = 6$ ).

superoxide anion reacts with tyrosine residues and free tyrosine to produce NTT. In addition, the tyrosyl radical generated by ROS-mediated activation of tyrosine oxidizes nitric oxide, generating NTT<sup>[37-39]</sup>. The importance of NTT as a marker is based on the hypothesis that nitric oxide production will have been great enough to yield observable products such as NTT, as in the experimental model used in the present study<sup>[40]</sup>.

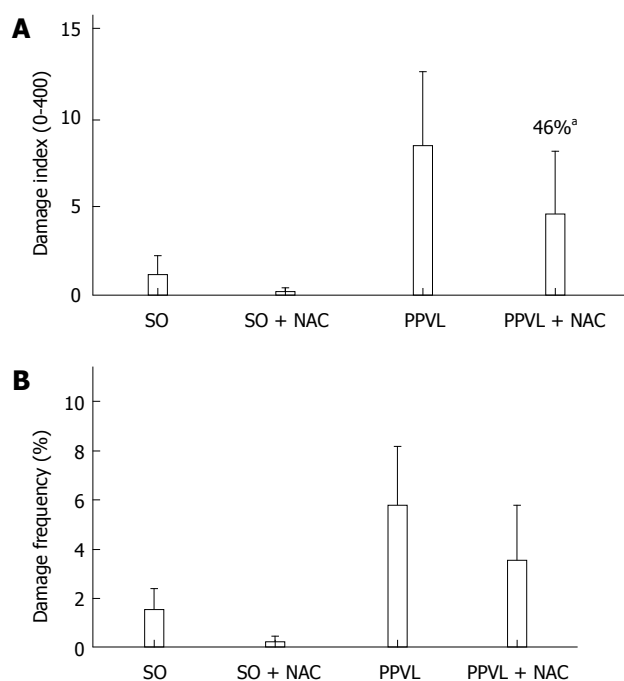
NAC treatment effectively reduced NTT immunoreactivity in PPVL animals as compared with controls (Figure 2C), which demonstrates not only the antioxidant effect of NAC as a free radical scavenger but also its effects on NO production. Fernando *et al.*<sup>[41]</sup> found that NAC administration was effective in reducing oxidative stress in PPVL animals as assessed by measurement of F2-isoprostanes and NO levels. In

the present study, reductions in NO bioavailability and free radical generation may have enabled reduction of the peroxynitrite levels observed in the gastric mucosa of experimental animals, and may explain the protection afforded by NAC therapy.

The collateral vasculature characteristic of hyperdynamic circulation is formed by dilatation of preexisting vessels and by the angiogenesis process, which is modulated by vascular growth factors such as VEGF<sup>[42]</sup>. This process is also an alternative pathway of nitric oxide production, mediated by VEGF activation<sup>[38]</sup>.

We assessed VEGF production in this experimental model by means of immunohistochemistry techniques and Western blot analysis. Rats in the PPVL group exhibited increased VEGF expression and immunoreactivity (Figures 2B and 3B) as compared





**Figure 4 Comet assay.** Damage index (A) and damage frequency (B). SO: Sham-operated group; SO + NAC: Sham-operated animals treated with NAC; PPVL: Partial portal vein ligation group; PPVL + NAC: Partial portal vein ligation group treated with NAC. <sup>a</sup>Modulation of DNA damage index induced by NAC in PPVL animals.

with control animals. This demonstrates the presence of a stimulus for development of a collateral circulation in rats subjected to PPVL. NAC effectively reduced VEGF expression in the PPVL + NAC group, as demonstrated by both techniques employed.

Inhibition of VEGF receptor-2 has been shown to reduce hyperdynamic circulation and development of collaterals in rats with PH<sup>[10]</sup>, and NAC has been shown to effectively reduce VEGF and p-VEGFR2 expression in the mesenteric vasculature of cirrhotic rats<sup>[36]</sup>. The results of the present study corroborate these findings.

Oxidative stress damages membrane lipids, proteins, and DNA<sup>[43]</sup>. The present study demonstrated an increase in DNA damage index (Figure 4A) and damage frequency (Figure 4B), as assessed by the comet assay, in PPVL animals as compared with controls. NAC reduced DNA damage when administered to PPVL animals.

NAC has antigenotoxic effects and detoxifies free radicals, which cause cellular DNA damage and has been reported to reduce cyclophosphamide-induced genotoxicity as assessed by the micronucleus assay<sup>[44]</sup>. The present study demonstrated a DNA-protective effect of NAC as shown by the comet assay. A previous investigation conducted by our research group evaluated the actions of NAC in rats with CCl<sub>4</sub>-induced cirrhosis and found antigenotoxic effects associated with its antioxidant properties<sup>[18]</sup>.

In view of these findings, we conclude that NAC protects the gastric mucosa from the oxidative damage and hemodynamic changes related to nitric oxide

overproduction in rats subjected to PPVL. NAC was able to reduce nitric oxide and thus decrease portal system pressure. This effect, added to its antioxidant and antiangiogenic properties, led to a reduction of the hyperdynamic collateral circulation and had a protective action on the gastric mucosa.

## COMMENTS

### Background

Portal hypertension is the leading cause of bleeding and death in cirrhotic patients due to recurrent cases of upper gastrointestinal bleeding. This high rate of bleeding is due to the development of collateral circulation, which despite being a physiological mechanism for decompressing the system, leads to progressive dilatation of the vessels and their likely breakdown especially in stomach. Therefore, is very large number of studies trying to seek a way to reduce this vasodilation. In the case of this study, we elected the antioxidant N-acetylcysteine (NAC).

### Research frontiers

NAC is a drug already used in the clinic, easily accessed, inexpensive and well - tolerated. The importance of the NAC is due to its direct and indirect antioxidant action, so important in this disease.

### Innovations and breakthroughs

Previous work published by the group showed that NAC was effective in reducing oxidative stress and portal pressure in an experimental model of partial ligation of portal vein. In this present study, the authors aimed for better understanding the pathways involved in this effectiveness, pointing out the mechanisms used by this medicine to improve the gastropathy of portal hypertension.

### Applications

The elucidation of the mechanisms of NAC action is of paramount importance for understanding the pathophysiology of portal hypertension. Therefore, this is indispensable to study a future alternative treatment for this disease. NAC is effective in reducing gastric damage and oxidative stress in an experimental model of portal hypertensive gastropathy that demonstrates all the physiological alterations present in patients affected by this disease.

### Terminology

Gastropathy is the non-inflammatory macroscopic lesions present in patients with portal hypertension. The portal hypertension is characterized by progressive increase of pressure in the portal system, which leads to a risk of vascular rupture and upper gastrointestinal bleeding.

### Peer-review

This is a well-performed study in which the authors analyzed the protective effect of NAC on portal hypertensive gastropathy in rats. The results are interesting and suggest that this antioxidant is a potential therapeutic substance that could be used for preventing the upper gastrointestinal bleeding of portal hypertension patients.

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## Basic Study

# Hepatitis C virus NS5A promotes insulin resistance through IRS-1 serine phosphorylation and increased gluconeogenesis

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## Abstract

**AIM:** To investigate the mechanisms of insulin resistance in human hepatoma cells expressing hepatitis C virus (HCV) nonstructural protein 5A (NS5A).

**METHODS:** The human hepatoma cell lines, Huh7 and Huh7.5, were infected with HCV or transiently-transfected with a vector expressing HCV NS5A. The effect of HCV NS5A on the status of the critical players involved in insulin signaling was analyzed using real-time quantitative polymerase chain reaction and Western blot assays. Data were analyzed using Graph Pad Prism version 5.0.

**RESULTS:** To investigate the effect of insulin treatment on the players involved in insulin signaling pathway, we analyzed the status of insulin receptor substrate-1 (IRS-1) phosphorylation in HCV infected cells or Huh7.5 cells transfected with an HCV NS5A expression vector. Our results indicated that there was an increased phosphorylation of IRS-1 (Ser<sup>307</sup>) in HCV infected or NS5A transfected Huh7.5 cells compared to their respective controls. Furthermore, an increased phosphorylation of Akt (Ser<sup>473</sup>) was observed in HCV infected and NS5A transfected cells compared to their mock infected cells. In contrast, we observed decreased phosphorylation of Akt Thr308 phosphorylation in HCV NS5A transfected cells. These results suggest that Huh7.5 cells either infected with HCV or ectopically expressing HCV NS5A alone have the potential to induce insulin resistance by the phosphorylation of IRS-1 at serine residue (Ser<sup>307</sup>) followed by decreased phosphorylation of Akt Thr<sup>308</sup>, FoxO1 Ser<sup>256</sup> and GSK3β Ser<sup>9</sup>, the downstream players of the insulin signaling

pathway. Furthermore, increased expression of PECK and glucose-6-phosphatase, the molecules involved in gluconeogenesis, in HCV NS5A transfected cells was observed.

**CONCLUSION:** Taken together, our results suggest the role of HCV NS5A in the induction of insulin resistance by modulating various cellular targets involved in the insulin signaling pathway.

**Key words:** Hepatitis C virus nonstructural protein 5A; Insulin resistance; Forkhead box protein O1; Glycogen synthase kinase-beta; Gluconeogenesis

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**Core tip:** The underlying molecular mechanisms of insulin resistance in response to hepatitis C virus (HCV) infection are poorly understood. Previous studies have demonstrated the effect of HCV core and envelop proteins on insulin signaling in human hepatocytes. However, the role of HCV nonstructural protein 5A (NS5A) in insulin resistance is not known. Our data clearly indicate the role of NS5A in insulin resistance through increased phosphorylation of IRS-1<sup>Ser307</sup> and decreased phosphorylation of AktThr<sup>308</sup>, FoxO1Ser<sup>256</sup>, and GSK3βSer<sup>9</sup>.

Parvaiz F, Manzoor S, Iqbal J, Sarkar-Dutta M, Imran M, Waris G. Hepatitis C virus NS5A promotes insulin resistance through IRS-1 serine phosphorylation and increased gluconeogenesis. *World J Gastroenterol* 2015; 21(43): 12361-12369 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12361.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12361>

## INTRODUCTION

Hepatitis C virus (HCV) is a blood-borne pathogen, belonging to the family *Flaviviridae*. The HCV genome is a positive sense single stranded RNA molecule, which encodes a polyprotein that is cleaved by viral proteases and host cell signal peptidases into mature structural and non-structural proteins<sup>[1,2]</sup>. Chronic infection with HCV progresses into a number of pathological conditions including insulin resistance, fibrosis, steatosis, cirrhosis and ultimately hepatocellular carcinoma<sup>[3,4]</sup>.

Insulin is the major anabolic hormone that has to utilize excessive glucose and maintain energy needs of the body. Insulin primarily performs this function by downregulating gluconeogenesis and glycogenolysis<sup>[4]</sup>. The primary targets of insulin action are skeletal muscles, cardiac muscles and the liver<sup>[5]</sup>. In certain pathological conditions, insulin is vulnerable to perform its function and results in abnormal metabolic condition known as insulin resistance that refers to the complex array of metabolic disorders involving lipid deposition, enhanced

fatty acids release and unfolded protein response<sup>[6-11]</sup>. One of the key downstream insulin signaling molecules is Akt that has been implicated in insulin resistance with the accumulation of diacylglycerols and ceramides<sup>[12]</sup>. In addition to this, ceramides may acts through an atypical isoform of protein kinase C (PKC), *i.e.*, PKC- $\zeta$  that sequesters Akt and inhibits its function in normal insulin signaling<sup>[13,14]</sup>.

In normal insulin signaling, insulin receptor substrate 1 (IRS-1) undergoes tyrosine phosphorylation and initiates a cascade of downstream signaling. However, there is impaired phosphorylation of IRS-1 in obese patients with type 2 diabetes mellitus<sup>[15]</sup>. Studies also reveal that pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 are involved in insulin resistance *via* serine phosphorylation of IRS-1<sup>[16]</sup>. During insulin signaling, the insulin receptor has to bind with the adaptor protein, IRS, and triggers the metabolic pathway effectively. In contrast, serine phosphorylation of IRS-1 leads to the degradation of IRS protein and hampers the insulin signaling pathway<sup>[4]</sup>. Akt is the downstream insulin signaling molecule which phosphorylates forkhead box protein O1 (FoxO1) and glycogen synthase kinase-beta (GSK3 $\beta$ ) and modulates insulin signaling<sup>[17,18]</sup>. FoxO1 is the first direct downstream target of Akt and is involved in mediating hepatic glucose production *via* peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ )<sup>[19]</sup>. FoxO1 regulates the expression of genes involved in glucose and lipid metabolism<sup>[20]</sup>. FoxO1 undergoes posttranslational modifications including acetylation, ubiquitination and importantly phosphorylation to perform its stimulatory effect<sup>[18]</sup>. FoxO1 has multiple phosphorylation sites but phosphorylation at serine 256 modulates its DNA binding activity and hampers normal metabolic pathways<sup>[21]</sup>. Downstream to the Akt/protein kinase B signaling pathway also lies another important protein GSK3 $\beta$  that gets phosphorylated and inactivated in response to insulin, and suppresses key gluconeogenic genes, *i.e.*, glucose-6-phosphatase (G6P) and phosphoenol pyruvate carboxykinase (PEPCK)<sup>[22,23]</sup>. Insulin regulates homeostasis by regulating two opposite pathways, *i.e.*, glycolysis and gluconeogenesis. Gluconeogenesis involves the conversion of pyruvate back to glucose with the aid of PEPCK, G6P and several other enzymes<sup>[22-28]</sup>. In the cyclic adenosine monophosphate (cAMP) axis, there is a cascade of genes that get activated and inactivated in response to the insulin mediated actions. Previous studies have shown that PGC-1 $\alpha$  strongly upregulates gluconeogenic genes like cAMP response element binding protein (CREB), PEPCK and G6P<sup>[29-31]</sup>. Akt inactivates PGC-1 $\alpha$  and inhibits gluconeogenesis under normal metabolic conditions<sup>[32]</sup>.

During the course of chronic HCV infection, the insulin signaling pathway is altered and the glucose cannot be metabolized properly with the concomitant increased transcriptional and translational expression of gluconeogenic genes/proteins<sup>[33,34]</sup>. So far little is



**Table 1** Primers used in the study

Gene	Forward primer	Reverse primer
CREB	GATCTTAGTGCCAGCAACC	GACGGACCTCTCTTTTCGT
PEPCK	GGCTACAACCTCGGCAAATACC	GGAAGATCTTGGGCAGTTTGG
G6P	CATTGACACCACACCCTTTGC	CCCTGTACATGCTGGAGTTGAG
TNF- $\alpha$	AGGCGCTCCCCAAGAAGACA	TCCTTGGCAAACTGCACCT

TNF- $\alpha$ : Tumor necrosis factor-alpha; PEPCK: Phosphoenol pyruvate carboxykinase; G6P: Glucose-6-phosphatase; CREB: CRE-binding protein.

known about the molecular mechanism behind the role of HCV in insulin resistance. Previously, HCV core protein has been shown to promote insulin resistance through serine phosphorylation of IRS-1 and modulating the Akt signaling pathway<sup>[33,35]</sup>.

In this study, we investigated the mechanism by which NS5A modulates key insulin signaling molecules such as IRS-1, Akt, FoxO1 and GSK3 $\beta$  at the posttranslational level and their target genes.

## MATERIALS AND METHODS

### Antibodies and reagents

Antibodies against IRS-1 (Ser<sup>307</sup>) and phospho-Akt (Ser<sup>473</sup>) were purchased from Calbiochem. Anti-phospho-FoxO1 (Ser<sup>256</sup>) and anti-phospho-GSK3 $\beta$  ( $\alpha/\beta$ ) were purchased from Cell Signaling. Anti-phospho Akt (Thr<sup>308</sup>) and anti-actin were purchased from Santa Cruz Biotechnology and Sigma, respectively. Human recombinant insulin was purchased from Invitrogen. SYBR green master mix was purchased from Applied Biosystems.

### Cell lines

Huh7 and Huh7.5 cell lines were grown in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, 100 U of penicillin/mL and 100  $\mu$ g/mL of streptomycin and cultured at 37 °C under 5% CO<sub>2</sub>. Huh7.5 cell line was kindly provided by Dr. Charles Rice (Rockefeller University, NY) to Dr. Gulam Waris at Rosalind Franklin University of Medicine and Science (RFUMS), United States.

### HCV cell culture infection system

HCV JFH-1 genomic RNA was *in vitro* transcribed and delivered into Huh7.5 cells by electroporation or liposome mediated transfection. These cells were then plated and passaged after 3 d. HCV infection in the cells and the corresponding cell culture supernatants was determined by quantitative real-time polymerase chain reaction (Applied Biosystems). The HCV cell culture supernatant was used to infect naive Huh7.5 cells at appropriate dilutions (*moi* of 1). Cells were then incubated at 37 °C for about 5-6 h in 5% CO<sub>2</sub> as previously described<sup>[36]</sup>. The infectious JFH-1 construct was provided by Dr. Takaji Wakita to Dr. Gulam Waris at RFUMS, United States.

### Transient-transfection assays

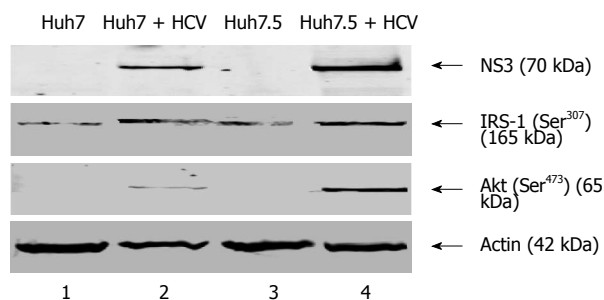
Cells at about 70% confluence in 60 mm petri dishes were transfected with an HCV NS5A expressing plasmid using lipofectamine 2000 (Invitrogen, CA). The confluent cells were washed thrice with phosphate buffered saline to remove cell debris followed by treating with 100 nmol/L insulin for three hours before harvesting the transfected cells.

### Reverse transcription and quantitative real-time polymerase chain reaction

Total cellular RNA was extracted from mock infected, HCV infected and HCV NS5A transfected Huh7.5 cells using Trizol (Invitrogen, CA). The extracted RNA was treated with DNase using RQ1 RNase-free DNase prior to cDNA synthesis. The cDNA was reverse-transcribed from 1  $\mu$ g of total RNA using oligo(dT) primers according to the manufacturer's protocol (Applied Biosystems, CA). Quantitative RT-PCR was carried out using SYBR green master mix and specific primer sets in triplicate. The 18S ribosomal RNA (18S rRNA) was used as an internal control. Amplification reactions were performed in a 25  $\mu$ L reaction mix using a real-time polymerase chain reaction (RT-PCR) reagent kit and the template RNA. Reactions were performed in a 96-well spectrofluorometric thermal cycler under the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative transcript levels were calculated using  $\Delta\Delta$ Ct method as specified by the manufacturer. Primers were used as described previously (Table 1)<sup>[37,38]</sup>.

### Western blot analysis

Mock, HCV infected and NS5A transfected cells were harvested and cellular lysates were prepared by incubating the cells with RIPA buffer (50 mmol/L Tris Base pH 7.5, 150 mmol/L NaCl, 1% NP-40, 0.50% sodium deoxycholate, 0.10% SDS, 1 mmol/L orthovanadate, 1 mmol/L sodium formate and 10  $\mu$ L/mL of protease inhibitor cocktail) on ice for 30 min. The lysates were subjected to SDS-PAGE followed by transfer to the nitrocellulose membrane in a transfer buffer (25 mmol/L Tris, 192 mmol/L glycine and 20% methanol). The membranes were then incubated for 1 h in a blocking buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5% Tween-20, and 5% non-fat dry milk). The membranes were then probed with respective



**Figure 1** Hepatitis C virus infection modulates insulin signaling in hepatitis C virus infected human hepatoma cells. Cellular lysates were made from mock and hepatitis C virus (HCV) infected Huh7 and Huh7.5 cells. An equal amount of cellular lysates were subjected to Western blot assay using p-IRS-1 Ser<sup>307</sup> and p-Akt Ser<sup>473</sup>. HCV NS3 protein expression represents the level of HCV infection. Cellular actin was used as an internal control to verify protein loading in each lane. IRS: Insulin receptor substrate; NS: Nonstructural protein.

primary antibody for 1 h at room temperature followed by washing thrice with blocking buffer without milk and then probed with respective secondary antibody for 1 h at room temperature. After doing an additional washing step, the membranes were visualized using the Odyssey Infrared imaging system (Li-Cor Biosciences, Lincoln, NE). The expression of actin protein was used to determine protein loading control in each lane.

### Statistical analysis

Data were analyzed using Graph Pad Prism version 5.0 and 2-tail error bars represent standard error of mean  $\pm$  SE of the data from three individual trials. A *P* value < 0.05 was regarded as statistically significant.

## RESULTS

### Modulation of insulin signaling pathway in HCV infected hepatoma cells

To investigate the effect of HCV upon the insulin signaling pathway, we infected human hepatoma cells (Huh7 and Huh7.5) with HCV (JFH1) and confirmed the HCV infection by Western blot analysis of HCV NS3 as shown in Figure 1. Our results also showed that Huh7.5 cells were more susceptible to JFH-1 HCV infection than Huh7 cells. To examine the effect of HCV infection on the status of IRS-1 and Akt, the above cellular lysates were subjected to Western blot analysis. The results showed an increased phosphorylation of IRS-1 (Ser<sup>307</sup>) and Akt (Ser<sup>473</sup>) in HCV infected Huh7 and Huh7.5 cells compared to their respective controls (Figure 1). Furthermore, the phosphorylation of IRS-1 and Akt was more in HCV infected Huh7.5 cells compared to HCV infected Huh7 cells (Figure 1, lane 4).

### HCV infected Huh7.5 cells show resistance towards insulin signaling

Since we observed a pronounced effect in Huh7.5

cells compared to Huh7 cells, we selected Huh7.5 cells for further experiments. To examine the effect of HCV infection on the insulin signaling pathway in HCV infected Huh7.5 cells, uninfected and HCV infected Huh7.5 cells were incubated with insulin. The results showed increased phosphorylation of IRS-1 (Ser<sup>307</sup>) in HCV infected cells which was reduced in HCV infected cells treated with insulin (Figure 2, lane 4). Furthermore, the phosphorylation of Akt (Ser<sup>473</sup>) was slightly enhanced in HCV infected cells treated with insulin. Collectively, these results suggest that HCV impairs insulin signaling *via* phosphorylation of IRS-1 (Ser<sup>307</sup>).

### HCV-NS5A alters phosphorylation level of IRS-1 (Ser<sup>307</sup>)

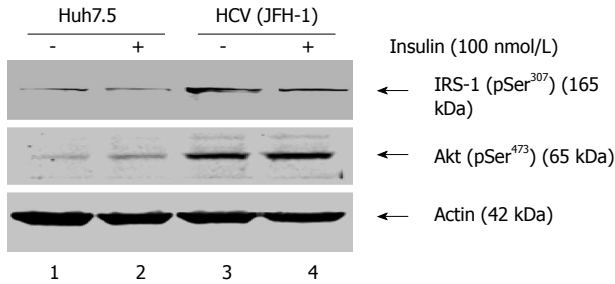
To investigate the role of HCV NS5A in the induction of insulin resistance, Huh7.5 cells were transfected with an HCV NS5A expression plasmid and the status of various cellular proteins involved in insulin signaling was examined. We observed an increased phosphorylation of IRS-1 (Ser<sup>307</sup>) in NS5A transfected cells but not in cells treated with insulin (Figure 3). In addition, NS5A transfected cells showed an increased phosphorylation of Akt Ser<sup>473</sup>, compared to untransfected cells (Figure 3). In contrast, we observed a decreased phosphorylation of AktThr<sup>308</sup> in HCV NS5A transfected cells and it was not further affected upon insulin treatment (Figure 3). These results indicate the potential role of HCV NS5A in the modulation of the insulin signaling pathway by the increased serine phosphorylation of IRS-1 and decreased phosphorylation of Akt Thr<sup>308</sup>.

### HCV-NS5A decreases the phosphorylation levels of FoxO1 (Ser<sup>256</sup>) and GSK-3 $\beta$ (Ser<sup>9</sup>)

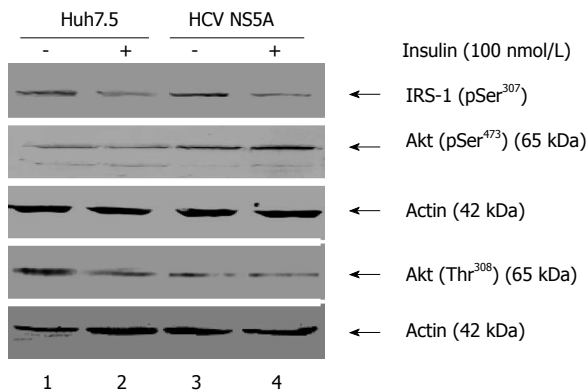
FoxO1 is an important downstream target of the insulin signaling pathway. The two important residues of FoxO1, *i.e.*, Ser<sup>256</sup> and Ser<sup>319</sup>, are known to be involved in nuclear exclusion of FoxO1 and the regulation of the normal insulin mediated signaling<sup>[33]</sup>. We observed that HCV NS5A expressing cells showed a decreased phosphorylation of FoxO1Ser<sup>256</sup>, and insulin treatment did not change the phosphorylation of FoxO1, indicating that FoxO1 may be involved in the modulation of subsequent downstream targets. In previous studies, insulin resistance has been linked with GSK3 $\beta$  signaling<sup>[17]</sup>. Similar to the FoxO1Ser<sup>256</sup>, our results also showed a decreased phosphorylation of GSK3 $\beta$ Ser<sup>9</sup> in NS5A expressing cells, indicating that the active form of GSK3 $\beta$  favors gluconeogenesis in NS5A expressing cells (Figure 4).

### HCV infection promotes hepatic gluconeogenesis

Downstream to the GSK3 $\beta$  are several gluconeogenic genes and transcription factors that are involved in gluconeogenesis. The rate limiting step of gluconeogenesis is controlled by *PEPCK* gene. Therefore, we first examined the transcriptional level of *PEPCK* in HCV infected cells. The results showed sign-



**Figure 2** Status of p-Ser<sup>307</sup> insulin receptor substrate-1 and p-Ser<sup>473</sup> Akt phosphorylation in hepatitis C virus infected hepatoma cells upon insulin treatment. Total cellular lysates were prepared from hepatitis C virus (HCV) infected and mock infected Huh7.5 cells that were treated or untreated with insulin (100 nmol/L). An equal amount of cellular lysates were subjected to Western blot assay using anti-p-IRS-1 Ser<sup>307</sup> and anti-p-Akt Ser<sup>473</sup>. Cellular actin was used as a protein loading control in each lane. IRS: Insulin receptor substrate.

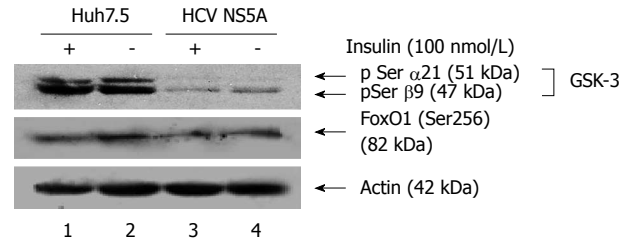


**Figure 3** Hepatitis C virus nonstructural protein 5A modulates phosphorylation levels of key insulin signaling molecules. Untransfected and nonstructural protein 5A (NS5A) transfected cells were incubated with insulin (100 nmol/L) for 3 h. An equal amount of cellular lysates were subjected to Western blot assay using anti-p-Akt Ser<sup>473</sup>, anti-p-Akt Thr<sup>308</sup> and anti-p-IRS-1 Ser<sup>307</sup>. Cellular actin was used as an internal control to verify protein loading in each lane. IRS: Insulin receptor substrate.

ificantly increased expression of PEPCK, indicating that HCV infected cells have potentially enhanced gluconeogenesis (Figure 5A). In addition, CREB is another important transcription factor that regulates the transcriptional activity of PEPCK. Our results showed significantly increased expression of CREB in the HCV infected cells (Figure 5A). Furthermore, TNF- $\alpha$  has also been linked to various HCV induced metabolic disorders. This prompted us to examine the transcriptional level of TNF- $\alpha$  in HCV infected cells. The results showed a significantly increased expression of TNF- $\alpha$  (Figure 5A), indicating the fact that this proinflammatory cytokine might have an important role in the insulin resistance mechanism.

#### NS5A promotes gluconeogenesis through transcriptional upregulation of PEPCK and G6P

In order to investigate the role of HCV-NS5A in the upregulation of gluconeogenesis, we transfected



**Figure 4** Effect of hepatitis C virus nonstructural protein 5A on the phosphorylation levels of FoxO1 Ser<sup>256</sup> and GSK-3 $\beta$  Ser<sup>9</sup>. Using the cellular lysates from nonstructural protein 5A (NS5A) transfected cell line and the controlled treated hepatoma cell line, Western blot assay was performed using anti-p-GSK3 Ser ( $\alpha^{21}/\beta^9$ ) and anti-p-FoxO1 Ser<sup>256</sup>. HCV: Hepatitis C virus.

Huh7.5 cells with NS5A and observed that there was significantly increased expression of PEPCK. The ultimate downstream step of gluconeogenesis, *i.e.*, conversion of glucose-6-phosphate to glucose, is governed by G6P. Similarly, the results indicated significantly increased transcriptional expression of G6P in HCV-NS5A transfected cells (as shown in Figure 5B). These results suggest that the gluconeogenic pathway is increased in NS5A expressing cells.

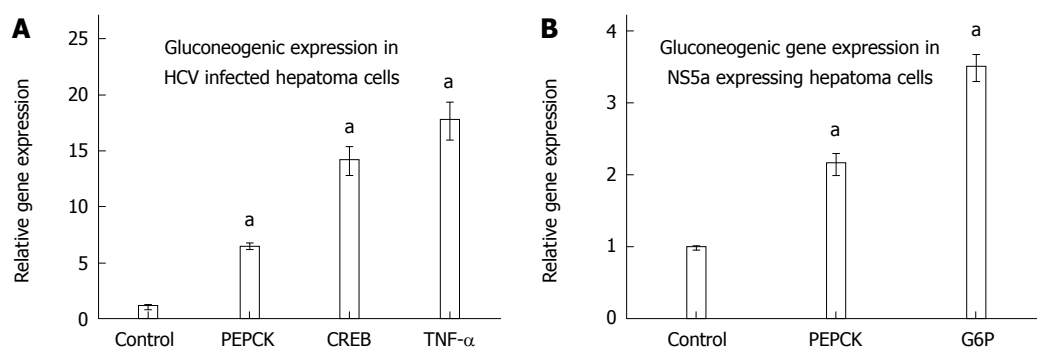
#### Model of HCV NS5A induced insulin resistance

The model of HCV-NS5A induced insulin resistance is shown in Figure 6.

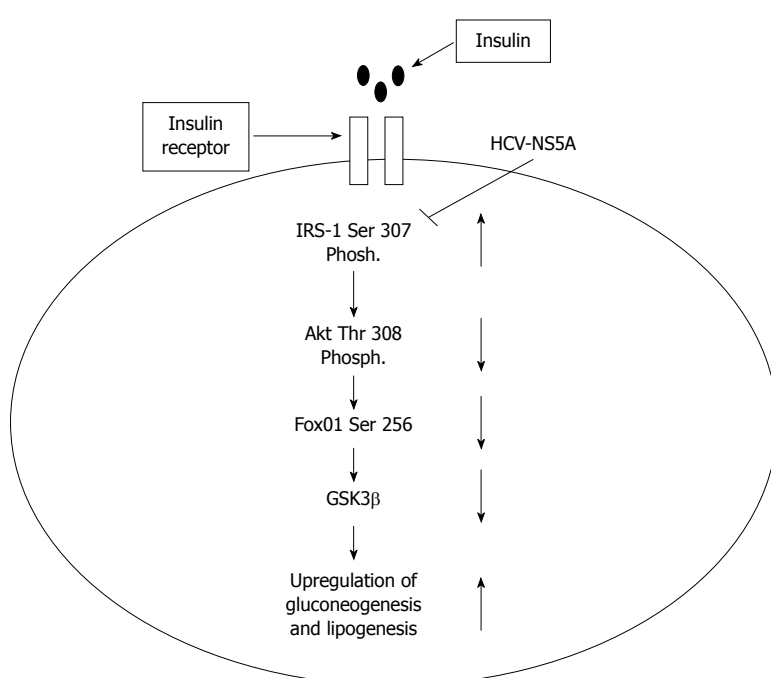
## DISCUSSION

Insulin resistance is a multifaceted disorder that involves modulation of various genes at transcriptional and translational levels. Hepatic gluconeogenesis is the core phenomenon that provokes insulin resistance. Our data suggest that HCV infection or ectopic expression of HCV NS5A increases hepatic gluconeogenesis as well as modulates phosphorylation status of various cellular proteins required for the induction of insulin resistance. Normal insulin signaling involves the binding of the insulin to insulin receptor and promotes tyrosine phosphorylation of IRS. Downstream to the IRS-1 are the Akt, FoxO1 and GSK3 $\beta$  that get differentially phosphorylated, metabolize glucose and favor homeostasis<sup>[5,17,18]</sup>. Our results suggest that HCV infection in the hepatoma cell lines favors serine phosphorylation of IRS-1 (Ser<sup>307</sup>) that is required for the insulin resistance. Furthermore, we observed an increased phosphorylation of Akt Ser<sup>473</sup> and a decreased phosphorylation of Akt Thr<sup>308</sup> in HCV NS5A transfected cells. These results are consistent with the previous studies where Akt Thr<sup>308</sup> but not Akt Ser<sup>473</sup> phosphorylation plays an important role in insulin resistance process<sup>[33,35,39]</sup>.

FoxO1 is an important insulin signaling molecule downstream of Akt and has been implicated in the modulation of transcriptional regulation of various genes (including PEPCK, G6P, *etc.*) involved in gluco-



**Figure 5 Hepatitis C virus nonstructural protein 5A favors gluconeogenic gene expression.** Total cellular RNA was extracted from hepatitis C virus (HCV, A) nonstructural protein 5A (NS5A, B) transfected cells and control cells. The quantitative real-time polymerase chain reaction was performed for the targeted genes as described in Materials and Methods. 18S rRNA was used as a housekeeping gene. Data represent mean of three independent experiments. <sup>a</sup> $P < 0.05$  vs control group. Data were analyzed with Graph Pad Prism, and 2-tail error bars represent SE of the data. TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ ; PEPCK: Phosphoenol pyruvate carboxykinase; G6P: Glucose-6-phosphatase; CREB: CRE-binding protein.



**Figure 6 Schematic representation of hepatitis C virus nonstructural protein 5A induced insulin resistance.** Based on our findings, a model has been proposed that depicts various check points in the insulin signaling pathway that gets modulated by nonstructural protein 5A (NS5A) protein. The up and down arrows represent upregulation and downregulation of proteins involved in insulin signaling cascades. The blunt headed line represents the check point that gets blocked by NS5A as this protein favors serine phosphorylation of insulin receptor substrate-1 (IRS-1) while under normal conditions tyrosine phosphorylation is known to take place. HCV: Hepatitis C virus.

neogenesis<sup>[40]</sup>. Fox01 Ser<sup>256</sup> is an important phosphorylation site that modulates its DNA binding ability, inhibits Fox01 nuclear translocation to the cytoplasm and modulates the metabolic gene expression<sup>[33]</sup>. Furthermore, GSK3 $\beta$  is another important downstream insulin signaling molecule that gets hypophosphorylated and becomes activated which, in turns, selectively upregulates the gluconeogenic gene expression, and inhibitors of GSK3 $\beta$  can selectively downregulate the transcriptional expression of these gluconeogenic genes<sup>[22,41]</sup>. Previous studies have shown that decreased phosphorylation of GSK3 $\beta$  leads to its activation and favors gluconeogenesis<sup>[18,39]</sup>. To further discern the effect of HCV-NS5A on down-

stream targets of the insulin signaling pathway, phosphorylation status of Fox01 Ser<sup>256</sup> was examined in this study. Our results suggested that there was a decreased phosphorylation level of Fox01 Ser<sup>256</sup> in the HCV NS5A transfected cells compared to control cells. Furthermore, the same pattern of decreased phosphorylation of GSK3 $\beta$  was observed in the transfected cell line.

Taken together, our data reveal that HCV NS5A is potentially able to modulate the normal insulin signaling pathway at various cellular points and favors the gluconeogenic pathway.

PEPCK is the rate limiting step of gluconeogenesis as it dictates the fate of gluconeogenesis by converting



oxaloacetate to phosphoenol pyruvate and is greatly linked with the phenomenon of insulin resistance. CREB is the main transcription factor that governs the transcriptional level of PEPCK and favors gluconeogenesis. In addition, some recent data have linked obesity and insulin resistance with the upregulation of  $\text{TNF-}\alpha$ <sup>[42-45]</sup>. Our data elucidate that HCV NS5A has a strong role in the enhancement of gluconeogenesis by the way of increased expression of key gluconeogenic genes, *i.e.*, PEPCK and G6P, with the concomitant increased expression of related transcription factors and inflammatory cytokine CREB and  $\text{TNF-}\alpha$ , respectively.

In this study, we have demonstrated that HCV NS5A favors serine phosphorylation of IRS-1, which is critically involved in the modulation of downstream insulin signaling pathway (Figure 6). Furthermore, downstream targets of the insulin signaling pathway (Akt Thr<sup>308</sup>, FoxO1 and GSK3 $\beta$ ) undergo a decreased level of serine phosphorylation that is involved in the inhibition of glycogen synthesis and favors gluconeogenesis, thereby imparting its role in the induction of hepatic insulin resistance. Hence, we characterized a mechanism through which HCV NS5A can modulate various cellular check points and leads toward hepatic insulin resistance.

## ACKNOWLEDGMENTS

We are thankful to Dr. Takaji Wakita (NIID, Tokyo, Japan) and Dr. Charles Rice (Rockefeller University, United States) for the generous gift of HCV genotype 2a (JFH1) and Huh7.5 cell line to Dr. Gulam Waris at RFUMS. We are thankful to Dr. Lance Presser and Steven McRae (RFUMS) for providing technical support. We are also thankful to Dr. Gulam Waris (RFUMS, United States) as this work was conducted in his lab by Fahed Parvaiz during the fellowship program.

## COMMENTS

### Background

Hepatitis C virus (HCV) is a lethal blood borne pathogen targeting hepatocytes and causes chronic infection in the majority of the infected individuals. Some studies reveal that chronic HCV infection attenuates the insulin signaling pathway, which can lead to glucose intolerance and the development of insulin resistance. So far, HCV core protein has been clearly shown to induce insulin resistance through the modulation of signaling pathways and upregulation of gluconeogenesis. Up till now, there is no conclusive study that reveals the potential of HCV non structural protein 5A (NS5A) in the induction of insulin resistance through the modulation of insulin receptor substrate-1 (IRS-1) protein.

### Research frontiers

HCV targets hepatocytes where various HCV proteins get replicated and favor various pathological conditions like insulin resistance, a step towards type 2 diabetes mellitus. The hotspot of this research article is the identification of HCV NS5A as a potential candidate for the development of insulin resistance. This study reveals that HCV NS5A modulates the insulin signaling pathway, thereby leading to increase gluconeogenesis.

## Innovations and breakthroughs

The underlying molecular mechanisms of insulin resistance in response to HCV infection are poorly understood. Previous studies have demonstrated the effect of HCV core and envelop proteins in insulin signaling in human hepatocytes. However, the role of HCV NS5A in insulin resistance is not known. Their data clearly indicate the role of NS5A in insulin resistance through increased phosphorylation of IRS-1Ser<sup>307</sup> and decreased phosphorylation of AktThr<sup>308</sup>, FoxO1Ser<sup>256</sup>, and GSK3 $\beta$ Ser<sup>9</sup>.

## Applications

This study suggests that HCV NS5A has multiple cellular targets that should be prevented in order to reduce disease pathogenesis. Furthermore, it highlights the fact that HCV NS5A specific inhibitors should be synthesized that can reduce the chance of disease progression and morbidity rate.

## Terminology

**Insulin resistance:** A complicated metabolic disorder that refers to the pre-diabetic phase with the modulation of insulin signaling at various cellular checkpoints like insulin receptors, IRS and impairment of homeostasis; **Gluconeogenesis:** Synthesis of glucose from non-glucose moieties; **Fibrosis:** A pathological condition in which excessive fats are deposited over liver; **Cirrhosis:** A pathological condition in which liver shrinks.

## Peer-review

This paper describes the influence of HCV NS5A on serine phosphorylation of insulin receptor substrate-1, FoxO1 and GSK-3 $\beta$ , and the mRNA levels of key gluconeogenic enzyme genes. The paper reports a potentially interesting and an important study.

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## Basic Study

# Magnesium isoglycyrrhizinate inhibits inflammatory response through STAT3 pathway to protect remnant liver function

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## Abstract

**AIM:** To investigate the protective effect of magnesium isoglycyrrhizinate (MgIG) on excessive hepatectomy animal model and its possible mechanism.

**METHODS:** We used the standard 90% hepatectomy model in Sprague-Dawley rats developed using the modified Emond's method, in which the left, middle, right upper, and right lower lobes of the liver were removed. Rats with 90% liver resection were divided into three groups, and were injected intraperitoneally with 3 mL saline (control group), 30 mg/kg (low-dose group) and 60 mg/kg (high-dose group) of MgIG, respectively. Animals were sacrificed at various time points and blood was drawn from the vena cava. Biochemical tests were performed with an automatic biochemical analyzer for the following items: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamyl endopeptidase, total bilirubin (TBil), direct bilirubin (DBil), total protein, albumin, blood glucose (Glu), hyper-sensitivity C-reactive protein, prothrombin time (PT), and thrombin time (TT). Postoperative survival time was observed hourly until death. Hepatocyte regeneration was analyzed by immunohistochemistry. Serum inflammatory cytokines (IL-1, IL-6, IL-10, and iNOS) was analyzed by ELISA. STAT3 protein and mRNA



were analyzed by Western blot and quantitative reverse-transcription PCR, respectively.

**RESULTS:** The high-dose group demonstrated a significantly prolonged survival time, compared with both the control and the low-dose groups ( $22.0 \pm 4.7$  h *vs*  $8.9 \pm 2.0$  *vs*  $10.3 \pm 3.3$  h,  $P = 0.018$ ). There were significant differences among the groups in ALT, Glu and PT levels starting from 6 h after surgery. The ALT levels were significantly lower in the MgIG treated groups than in the control group. Both Glu and PT levels were significantly higher in the MgIG treated groups than in the control group. At 12 h, ALT, AST, TBil, DBil and TT levels showed significant differences between the MgIG treated groups and the control group. No significant differences in hepatocyte regeneration were found. Compared to the control group, the high-dose group showed a significantly increase in serum inflammatory cytokines IL-1 and IL-10, and a decrease in IL-6. Both STAT3 protein and mRNA levels were significantly lower in the MgIG treated groups than in the control group at 6 h, 12 h, and 18 h after surgery.

**CONCLUSION:** High-dose MgIG can extend survival time in rats after excessive hepatectomy. This hepatoprotective effect is mediated by inhibiting the inflammatory response through inhibition of the STAT3 pathway.

**Key words:** Magnesium isoglycyrrhizinate; Excessive liver resection; Inflammation; Liver regeneration; STAT3

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**Core tip:** Magnesium isoglycyrrhizinate (MgIG), a hepatocyte protective agent, has been shown to have the effects of anti-inflammation, liver cell membrane protection, and liver function improvement. We designed this study, by using the standard 90% hepatectomy model in rats, to clarify the liver protecting function of MgIG and its mechanism. We have researched postoperative survival time, hepatocyte regeneration, liver function, serum inflammatory cytokines, STAT3 protein and mRNA expression. The protective effect of MgIG in standard 90% hepatectomy is limited, which can prolong the survival time. This hepatoprotective effect was not mediated by increasing hepatocyte regeneration but rather by inhibiting the inflammatory response through inhibition of the STAT3 pathway.

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## INTRODUCTION

Excessive liver resection is the only hope of cure for extra-large occupying liver lesions. Most patients with liver occupying lesions often have other liver abnormalities such as cirrhosis. High surgical risk could lead to fulminant hepatic failure that has a mortality rate up to 70%-90%<sup>[1,2]</sup>. Protection of the remnant liver function in the first 48 h after surgery remains a great challenge to the surgeons<sup>[3]</sup>. The efficacy of various existing measures, including the artificial liver and other supporting methods, is limited. Our previous study in rats found that liver regeneration reaches the highest level at 72 h after resection. Animals will most likely survive if the regeneration could compensate for the first 48 h<sup>[4]</sup>. Therefore, to identify appropriate and effective measures to help the remnant liver to sustain through this risky period is particularly important.

Magnesium isoglycyrrhizinate (MgIG), a hepatocyte protective agent, has been shown to have the effects of anti-inflammation, liver cell membrane protection, and liver function improvement. Efficacy studies showed that protective function of MgIG against acute liver damage is induced by D-galactosamine. MgIG can decrease the serum level of transaminases, prevent liver cell degeneration, and reduce the incidence of necrosis and inflammatory cell infiltration. MgIG is especially effective in the treatment of chronic liver injury induced by carbon tetrachloride in rats, by reducing inflammation and fibrosis, lowering the nitro-monoxide levels, and improving liver function.

Most of previous studies of MgIG have focused on chronic hepatitis, alcoholic cirrhosis, and drug-induced liver injury<sup>[5]</sup>. A few studies mainly involved surgical ischemia-reperfusion injury<sup>[6]</sup> and liver regeneration<sup>[7]</sup>. No publication was found for the anti-inflammatory effect of MgIG after liver resection. In this study, we verified the liver protective effect of MgIG in an animal model of hepatectomy and further investigated its mechanism.

## MATERIALS AND METHODS

### Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institute of Animal Care and Use Committee of Beijing Union Medical College (Permit No.: 2013050125). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize animal suffering.

### Experimental animals

Male Sprague-Dawley (SD) rats aged 8 wk (220-260 g) were provided by the Experimental Animal Center of Beijing Union Medical College Hospital.

### Reagents

MgIG was provided by JCTT Pharmaceutical Ltd. (Jiangsu Province, China), and 3% (w/v) MgIG in saline was prepared.

### Establishment of a rat model with 90% liver resection

The rats were weighted, and anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). After routine skin preparation and disinfection, an upper abdominal incision was made. Sequential isolation and resection were performed on the middle lobe, exterior lobe, right lower lobe, and right upper lobe. After confirming that there was no congestion on the caudate lobe, the abdominal cavity was closed.

### Experimental design

SD rats were randomly divided into three groups: control (C), low-dose (LD), and high-dose (HD) groups. Hepatectomy was performed by resection of 90% liver tissue, followed by intraperitoneal injection of 3 mL saline (C group), 30 mg/kg (LD group), or 60 mg/kg (HD group) MgIG on the same day of surgery, as well as once daily afterwards.

### Observation of survival time

Starting from the closing of the abdomen as 0 h, animal survival time was measured hourly until death. Meanwhile, the general condition of each animal was recorded. We used proper humane endpoints when the rats were in the moribund state or the signs of severe organ system dysfunction non-responsive to treatment appeared and euthanized rats prior to the end of experiments.

### Liver function evaluation

Animals were sacrificed at various time points and blood was drawn from the vena cava. Biochemical tests were performed with an automatic biochemical analyzer (HITACHI, Japan) for the following items: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamyl endopeptidase (GGT), total bilirubin (TBil), direct bilirubin (DBil), total protein (TP), albumin (Alb), blood glucose (Glu), hyper-sensitivity C-reactive protein (hsCRP), prothrombin time (PT), and thrombin time (TT).

### Measurement of liver regeneration

The average proportion of liver weight against the body weight was established from 5 rats of same age. It was found that the liver weight was  $3.96\% \pm 0.01\%$  of the body weight. The remnant caudate lobe was removed from the animal model at various time points after surgery and weighed. Liver regeneration rate

was calculated by the Okano T formula: Regeneration rate (R, %) =  $[C-(A-B)]/(A-B) \times 100\%$ , where A is preoperative estimation of the rat liver weight, B is the weight of resected liver tissue, and C is the weight of the remnant caudate lobe<sup>[8]</sup>.

### Observation of liver damage and regeneration by HE and BrdU staining

BrdU (Sigma, United States) (100  $\mu$ g/g body weight) was administered intraperitoneally to animals 2 h prior to the removal of remnant caudate lobe. The remaining caudate lobe was removed at 0, 6, 12 and 18 h after surgery, fixed and sectioned, and underwent immunohistochemistry procedure or HE staining. BrdU positive cells were counted under a microscope. At least 3 rats from each group, 3 slides from each rat, and 3 fields from each slide were counted, and the results were presented as the number of positive cells in every 500 total cells being counted.

### Expression of proliferating cell nuclear antigen

Paraffin embedded sections of remnant liver tissue were studied by immunohistochemistry for proliferating cell nuclear antigen (PCNA) expression. PCNA antibody was obtained from Abcam, United States. PCNA-positive cells were counted under a light microscope. At least 3 rats from each group, 3 slides from each rat, and 3 fields from each slide were counted, and the results were presented as the number of positive cells in every 500 total cells being counted.

### Western blot

Remnant liver tissue was also homogenized and treated with RIPA lysis buffer (Dingguo, China); the extracted proteins were resolved on 4%-12% acrylamide gradient gels. After electrophoresis, proteins were transferred to a PVDF membrane using iBlot fast transfer electric transfer (Invitrogen, United States). The membrane was blocked with 5% milk at room temperature for 1 h, and incubated with primary antibody against PCNA or STAT3 (1:1000, Abcam, United States) 4 °C overnight, followed by TBST washing three times, secondary antibody (1:8000, Abcam United States) incubation at room temperature for 2 h, TBST washing 3 times, and exposure to film with ECL kit (Pierce, United States). Densitometry analysis was performed with BandScan software.

### ELISA assay for inflammatory factor detection in serum

Blood was drawn from the vena cava at 0, 6, 12 and 18 h after surgery, and serum was separated and stored -80 °C. ELISA assays to determine IL-1, IL-6, IL-10, and iNOS expression levels were performed according to manufacturer's instructions (Cusabio, Wuhan, China).

### Quantitative real-time PCR

Total RNA was extracted from the remnant liver tissue

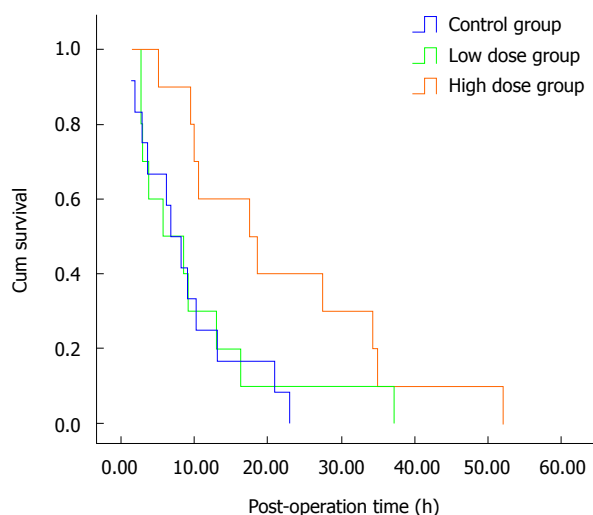


Figure 1 Kaplan-Meier survival curves of the three experimental animal groups.

with Trizol. TAKARA retroviral reverse transcriptase kit (TAKARA, Japan) was used to synthesize cDNA with the reaction condition of 37 °C for 60 min and 95 °C for 3 min. Primers were designed as forward, 5'-CACAACCTGCGAAGAATCAAG-3' and reverse, 5'-GCTGCTTCTCCGTCCTACTAC-3' for STAT3 gene; and forward, 5'-AACGGCTCCGGCATGTGCAA-3' and reverse, 5'-CTTCTGACCCATGCCACCA-3' for  $\beta$ -actin.

Real-time PCR was performed with Applied Biosystems 7500 real-time quantitative PCR instrument (Applied Biosystems 7500, United States) under the following condition: 95 °C for 20 s, 60 °C for 30 s, 72 °C for 30 s for 40 cycles<sup>[8]</sup>.

### Statistical analysis

Statistical analyses were performed using SPSS13.0 software (Chicago, IL). Data are expressed as mean  $\pm$  SD. The difference between groups was compared using one-way ANOVA, and survival analysis was performed using the Kaplan-Meier method. Two-tailed  $P < 0.05$  was considered statistically significant.

## RESULTS

### Comparison of postoperative survival

Seven out of fifteen (46.7%) rats in the control group did not recover from the anesthesia and died. The remaining rats in the control group exhibited poor condition even though they became awake from anesthesia. No active movement was observed; the hair was dry, and the breathing was slow and laborious. The response to external stimuli was weak, and there was no uptake of water. No animal from the control group survived more than 24 h after surgery. Forty percent (6/15) of the rats in the low-dose MgIG treatment group died before waking up from anesthesia. The remaining rats showed better sign of life than the control group, in that the response

to external stimuli was stronger, and some rats could uptake small volume of water. One of the animals survived longer than 24 h. In the high-dose MgIG treatment group, 26.7% (4/15) of the rats died shortly after surgery without waking up from anesthesia. The remaining animals showed slow active movement, uptake of water, and clean hair. Four rats survived longer than 24 h but none exceeded 60 h.

Survival time of the three groups was plotted using Kaplan-Meier survival curves, and the results are shown in Figure 1. Survival time of the control group was  $8.9 \pm 2.0$  h with a median of 6.8 h, low-dose group was  $10.3 \pm 3.3$  h with a median of 5.8 h, and high-dose group  $22.0 \pm 4.7$  h with a median of 17.6 h. There were significant differences in survival time among the three groups ( $P = 0.018$ ).

### Liver function assessment

Liver function of the animals at various time points after hepatectomy was assessed by studying a variety of serum biomarkers including ALT, AST, GGT, TBIL, DBIL, TP, ALB, Glu, hsCRP, PT and TT.

As shown in Table 1, there were significant differences among the groups in ALT, Glu and PT levels starting from 6 h after surgery. The ALT levels were significantly lower in the MgIG treated groups than in the control group. Both Glu and PT levels were significantly higher in the MgIG treated groups than in the control group. At 12 h, ALT, AST, TBil, DBil and TT levels showed significant differences between the MgIG treated groups and the control group ( $P < 0.05$  for TBIL and  $P < 0.01$  for all the rest). We also tested serum ALB and hsCRP at various time points after hepatectomy and found no significant difference.

### Liver regeneration

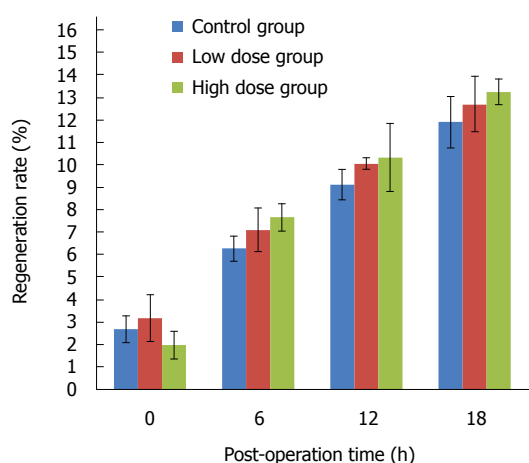
Figure 2 shows the liver regeneration status at various time points after surgery. Proportion of weight of the remnant liver was found to be higher immediately after hepatectomy surgery compared with the normal proportion of liver to the body weight in all the three groups, and the weight of remnant liver tissue increased over time. In the control group, a  $2.71\% \pm 0.58\%$  increase was found at 0 h, and  $11.95\% \pm 1.14\%$  at the time of 18 h. However, there were no significant differences among the three groups (Figure 2).

Liver cell degeneration and necrosis were observed after HE staining of the remnant liver sections (magnification,  $\times 100$ ) in all animals. Fatty degeneration was found in animals of both the low-dose and high-dose MgIG treatment groups, but was more prominent in the high-dose group. The representative HE staining of liver sections of the animals in the high-dose group at 0 h, 6 h, 12 h and 18 h is shown in Figure 3. Hepatocytes had normal size and shape, and no obvious damage was found in the nucleus at 0 h (Figure 3A). At 6 h, cells showed little change in size or shape. Cell number increased, and the arrangement

**Table 1** Serological test results of experimental animals after excessive liver resection

Item	Group	Time point (mean $\pm$ SD)				P value <sup>1</sup>
		0 h	6 h	12 h	18 h	
ALT (U/L)	Control	165.8 $\pm$ 118.0	864.0 $\pm$ 471.7	1927.5 $\pm$ 1079.0	1969.6 $\pm$ 1201.6	0.000
	Low dose	86.4 $\pm$ 23.4	357.2 $\pm$ 119.8	705.1 $\pm$ 341.5	1597.7 $\pm$ 998.6	0.000
	High dose	145.6 $\pm$ 106.8	524.7 $\pm$ 182.5	1234.8 $\pm$ 784.2	1321.2 $\pm$ 797.4	0.000
	P value <sup>2</sup>	0.133	0.002	0.003	0.3	
AST (U/L)	Control	228.4 $\pm$ 110.4	929.3 $\pm$ 477.7	1779.5 $\pm$ 495.9	1673.2 $\pm$ 668.8	0.000
	Low dose	192.6 $\pm$ 37.5	664.7 $\pm$ 171.5	1027.5 $\pm$ 396.9	1904.3 $\pm$ 794.7	0.000
	High dose	193.1 $\pm$ 99.5	694.6 $\pm$ 278.7	1370.6 $\pm$ 502.7	1355.2 $\pm$ 671.5	0.000
	P value	0.556	0.158	0.001	0.192	
GGT (U/L)	Control	1.11 $\pm$ 0.93	1.50 $\pm$ 0.85	2.14 $\pm$ 1.51	1.55 $\pm$ 0.93	0.197
	Low dose	0.70 $\pm$ 0.48	0.88 $\pm$ 0.35	1.27 $\pm$ 1.27	2.89 $\pm$ 2.42	0.008
	High dose	0.72 $\pm$ 0.47	1.00 $\pm$ 0.71	2.36 $\pm$ 1.21	2.00 $\pm$ 1.08	0.001
	P value	0.316	0.154	0.147	0.158	
TP (g/L)	Control	50.7 $\pm$ 4.9	47.3 $\pm$ 3.6	46.3 $\pm$ 4.8	45.5 $\pm$ 3.6	0.031
	Low dose	48.3 $\pm$ 2.9	45.0 $\pm$ 2.9	44.9 $\pm$ 3.2	44.6 $\pm$ 3.8	0.039
	High dose	51.6 $\pm$ 3.3	45.5 $\pm$ 2.4	48.5 $\pm$ 3.6	45.6 $\pm$ 5.8	0.001
	P value	0.101	0.212	0.094	0.849	
TBil (U/L)	Control	1.95 $\pm$ 0.62	6.68 $\pm$ 1.32	16.59 $\pm$ 2.16	20.13 $\pm$ 4.19	0.000
	Low dose	1.20 $\pm$ 0.30	4.35 $\pm$ 0.77	7.45 $\pm$ 4.66	14.82 $\pm$ 2.06	0.000
	High dose	1.67 $\pm$ 0.43	6.23 $\pm$ 2.22	14.31 $\pm$ 2.16	12.81 $\pm$ 2.29	0.000
	P value	0.539	0.532	0.011	0.206	
DBil (U/L)	Control	1.22 $\pm$ 0.37	4.27 $\pm$ 0.64	13.56 $\pm$ 1.83	16.33 $\pm$ 3.46	0.000
	Low dose	0.65 $\pm$ 0.15	2.88 $\pm$ 0.47	5.25 $\pm$ 1.01	11.15 $\pm$ 1.72	0.000
	High dose	0.99 $\pm$ 0.26	4.62 $\pm$ 1.82	10.95 $\pm$ 1.59	9.72 $\pm$ 1.80	0.000
	P value	0.353	0.527	0.004	0.14	
Glu (mmol/L)	Control	7.41 $\pm$ 1.27	4.92 $\pm$ 2.54	6.83 $\pm$ 2.33	4.21 $\pm$ 1.40	0.001
	Low dose	6.50 $\pm$ 1.21	6.30 $\pm$ 1.62	6.09 $\pm$ 1.84	4.11 $\pm$ 2.13	0.012
	High dose	7.12 $\pm$ 2.05	8.54 $\pm$ 3.69	5.25 $\pm$ 2.19	5.21 $\pm$ 1.02	0.002
	P value	0.395	0.016	0.162	0.149	
TT (s)	Control	42.7 $\pm$ 6.1	42.0 $\pm$ 4.6	47.8 $\pm$ 10.1	41.2 $\pm$ 10.0	0.754
	Low dose	48.9 $\pm$ 3.5	49.7 $\pm$ 6.6	49.5 $\pm$ 5.3	48.4 $\pm$ 0.4	0.984
	High dose	38.2 $\pm$ 6.1	46.2 $\pm$ 2.0	11.5 $\pm$ 1.1	43.2 $\pm$ 1.1	0.000
	P value	0.127	0.226	0.001	0.36	
PT (s)	Control	8.9 $\pm$ 0.6	10.3 $\pm$ 0.3	11.7 $\pm$ 1.1	12.4 $\pm$ 0.4	0.001
	Low dose	9.9 $\pm$ 0.6	10.9 $\pm$ 0.5	12.6 $\pm$ 1.9	14.2 $\pm$ 1.6	0.016
	High dose	9.1 $\pm$ 0.1	12.6 $\pm$ 0.7	11.5 $\pm$ 1.1	14.8 $\pm$ 0.8	0.000
	P value	0.077	0.003	0.618	0.076	

<sup>1</sup>Comparison among time points of same group; <sup>2</sup>Comparison among groups at same time point. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PT: Prothrombin time; TT: Thrombin time; TBil: Total bilirubin; Glu: Glucose; DBil: Direct bilirubin.

**Figure 2** Regeneration rates at various time points in three groups.

was slightly disordered (Figure 3B). At 12 h, a more increase in cell number as well as more disordered arrangements was observed. There was a small number of cells with fatty degeneration, but no obvious liver cell necrosis was visible (Figure 3C). More fatty degeneration and necrosis were observed at 18 h. The number of cells decreased, and the cell arrangement became irregular (Figure 3D). No obvious generative nodule was observed under a microscope. Obvious fatty degeneration and necrosis were observed in all three groups at 12 h and 18 h.

As a thymidine analogue, BrdU can substitute thymidine to incorporate into the double-stranded DNA during DNA synthesis. Therefore, cells that have gone through S phase (DNA synthesis phase) would become BrdU positive, an indication of cell



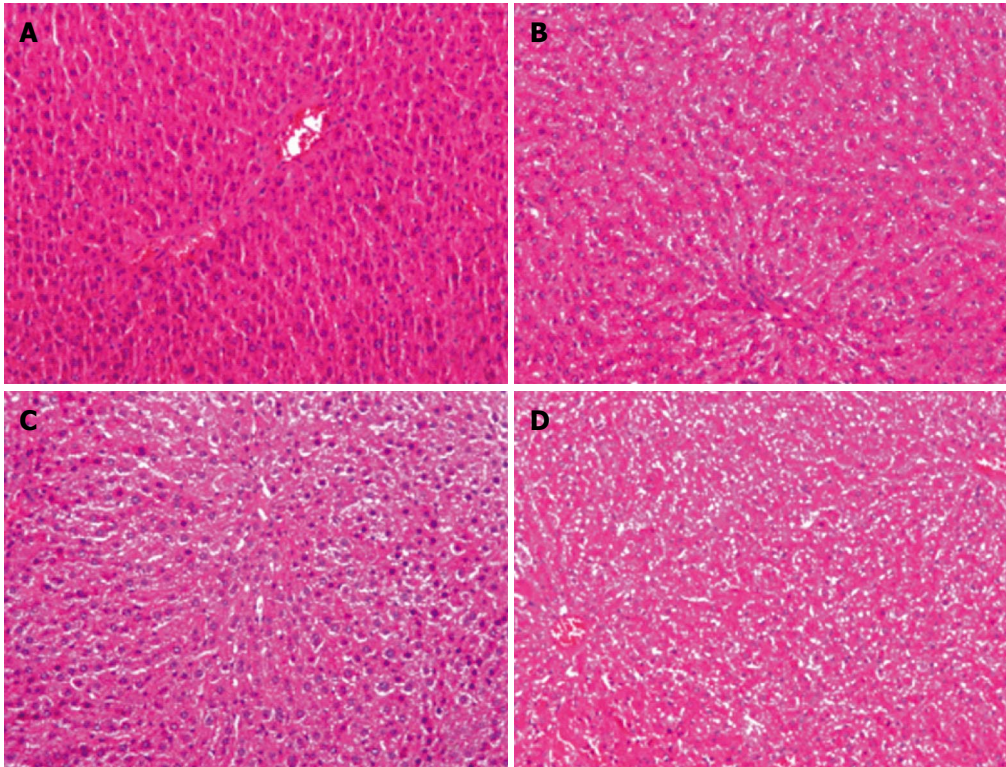


Figure 3 Hematoxylin-eosin staining in the high-dose group at 0 h (A), 6 h (B), 12 h (C) and 18 h (D) (magnification  $\times 100$ ).

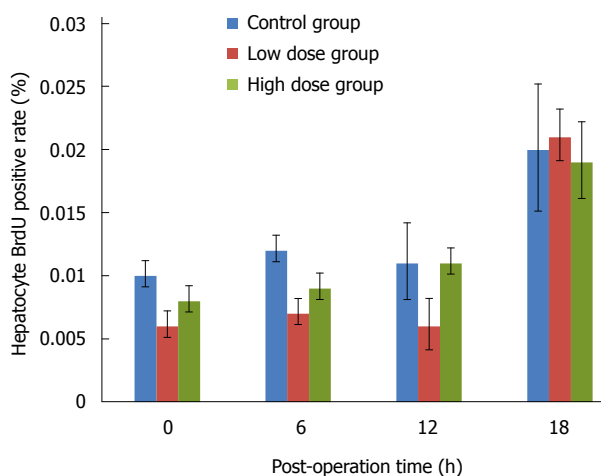


Figure 4 BrdU positive rates of the caudate lobe hepatocytes at various time points in three groups.

proliferation. As shown in Figure 4, the number of BrdU-positive cells among the three groups showed no significant difference. No obvious change was noted in BrdU-positive cells in the control group at 0 h, 6 h and 12 h, although there was a slight but not statistically significant increase in BrdU-positive cells at 18 h. Similar patterns were observed in both the high-dose and low-dose MgIG treated groups.

PCNA is an auxiliary protein for DNA synthase  $\delta$  whose expression is cell cycle dependent. Its expression starts at late G1 phase and reaches the peak level at S phase. PCNA has been used as an

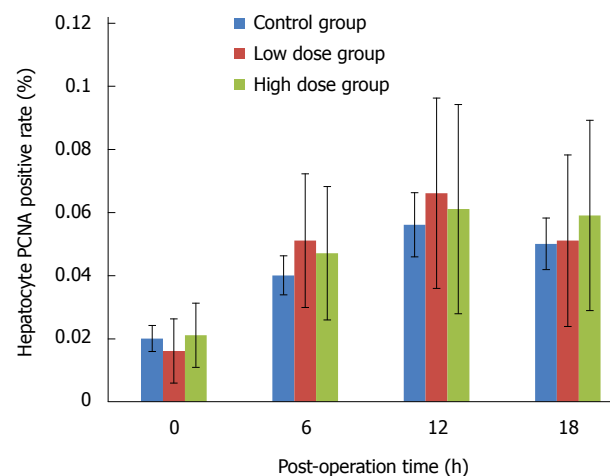
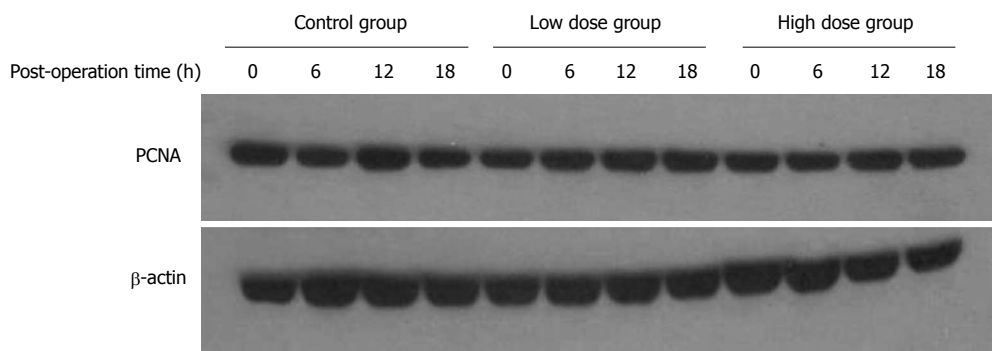
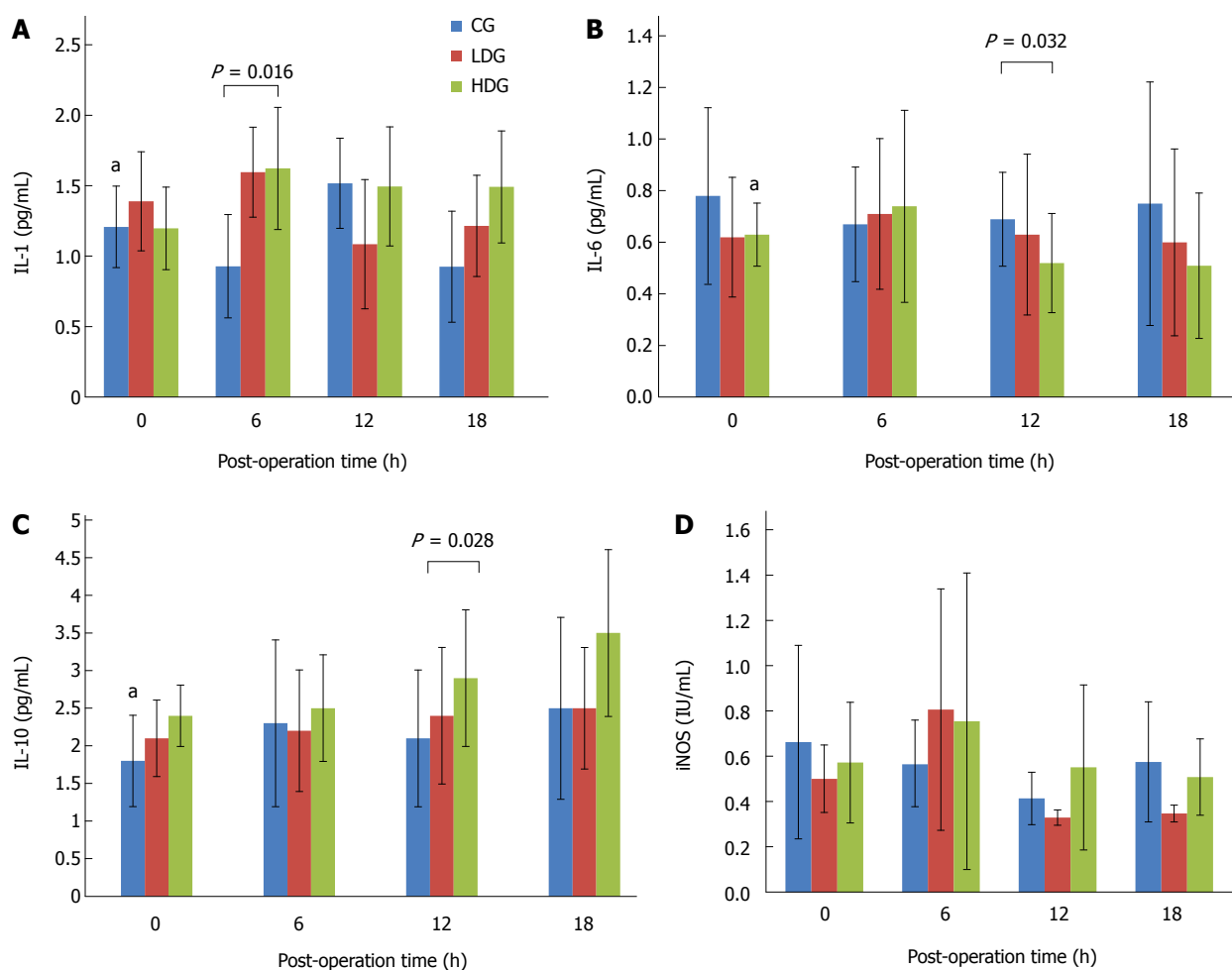


Figure 5 Proliferating cell nuclear antigen positive rates of the caudate lobe hepatocytes at various time points in three groups. PCNA: Proliferating cell nuclear antigen.

indicator for cell proliferation. In this study, we used both immunohistochemistry to detect the percentage of PCNA-positive cells in the remnant liver tissue, as well as Western blot to assess the overall expression levels of PCNA in the liver. Figure 5 shows that there was no significant difference in the percentage of PCNA-positive cells among the three groups. Over the course of 18 h, the number of PCNA-positive cells in all groups remained at a relative low level. As shown in Figure 6, there were no significant differences in the overall expression levels of PCNA among three



**Figure 6** Expression of proliferating cell nuclear antigen in the caudate lobe detected by Western blot at various time points in three groups. PCNA: Proliferating cell nuclear antigen.



**Figure 7** Expression of cytokines IL-1, IL-6, IL-10 and iNOS at various time points in three groups. \* $P < 0.05$ .

groups as indicated by Western blot analysis.

### Inflammatory cytokines

Inflammatory cytokines IL-1, IL-6, IL-10 and iNOS in serum were detected by ELISA. As shown in Figure 7, IL-1 level at 6 h after surgery was significantly higher in the serum of rats in the MgIG treated groups than in the control group ( $P < 0.05$ ; Figure 7A). IL-6 level at 12 h was significantly lower in the low-dose group than in the control group, and in the high-dose group

than in the low-dose group ( $P < 0.05$ ; Figure 7B). The level of IL-10 was found to be significantly higher in the high-dose group at 18 h than in the other groups ( $P < 0.05$ ; Figure 7C). No significant difference was found among the groups in iNOS expression at any time (Figure 7D).

### STAT3 mRNA and protein levels

STAT3 is an important transcription factor that can be activated in response to a variety of cytokines and

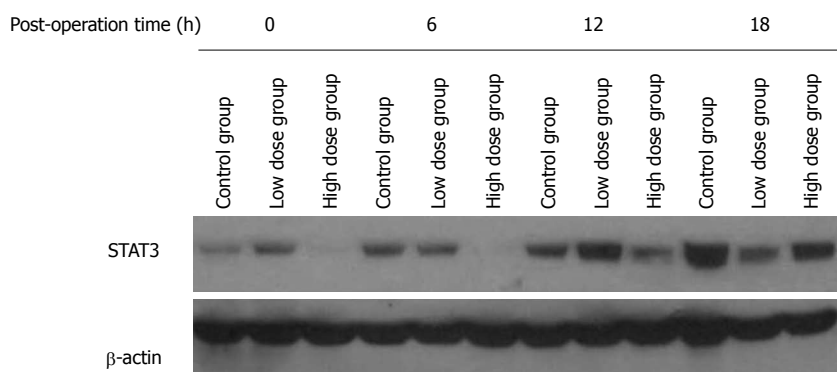


Figure 8 Expression of STAT3 in caudate lobe detected by Western blot at various time points in three groups.

Table 2 STAT3 mRNA levels at various time points after excessive liver resection in rats

Group	STAT3 mRNA expression (mean $\pm$ SD)								<i>P</i> value <sup>1</sup>
	0 h	<i>n</i>	6 h	<i>n</i>	12 h	<i>n</i>	18 h	<i>n</i>	
Control	0.33 $\pm$ 0.11	3	0.43 $\pm$ 0.21	3	0.57 $\pm$ 0.24	3	1.07 $\pm$ 0.47	3	0.021
Low dose	0.41 $\pm$ 0.15	3	0.44 $\pm$ 0.19	3	0.78 $\pm$ 0.34	3	0.89 $\pm$ 0.45	3	0.043
High dose	0.38 $\pm$ 0.24	3	0.11 $\pm$ 0.07	3	0.30 $\pm$ 0.14	3	0.87 $\pm$ 0.36	3	0.025
<i>P</i> value <sup>2</sup>	0.401		0.012		0.044		0.849		

<sup>1</sup>Comparison among time points of same group; <sup>2</sup>Comparison among groups at same time point.

growth factors. Our finding of the elevated serum levels of IL-1 and IL-10 as well as reduced level of IL-6 in the rats of the high-dose group suggests that MgIG may modulate the inflammation response. To further clarify the mechanism, we studied the STAT3 protein expression in the remnant liver tissue by Western blot. At least 6 rats from each group were studied, and Figure 8 shows one representative Western blot. Densitometry analysis was performed to calculate relative protein expression levels based on the level of  $\beta$ -actin. There was no significant difference in STAT3 levels among all three groups at 0 h. The expression level of STAT3 in the high-dose group at 6 h was found to be significantly lower than those of the control group and low-dose group ( $P < 0.05$ ). At 12 h, the high-dose group still exhibited lower STAT3 level compared with the control and low-dose groups, but there was no statistically significant difference. At 18h, STAT3 expression in the low-dose and high-dose groups was lower than that in the control group. The expression of STAT3 in the control group was found to be increasing gradually over the course of 18 h after hepatectomy, and significant differences existed between each time points ( $P < 0.01$ ). No statistically significant differences in STAT3 levels, however, were found among different time points in the low-dose group. In the high-dose group, STAT3 protein was inhibited initially, followed by a gradual increase, and at 18 h reached 4.5 times of the level at 0 h. This may explain the suppression of inflammatory reaction in the high-dose group.

Quantitative real-time PCR was used to detect the relative levels of STAT3 mRNA, and results are shown in Table 2. At 0 h, no significant differences in STAT3

mRNA levels were seen among three groups. At both 6 h and 12 h, the levels of STAT3 mRNA in the high-dose group were significantly lower than those in the control and low-dose groups ( $P < 0.05$ ). At 18 h, STAT3 mRNA levels in rat livers of the low-dose and high-dose groups were lower than that of the control group, but there was no statistically significant difference.

## DISCUSSION

Glycyrrhizin is commonly used clinically as a liver protective medicine. MgIG is the fourth generation of glycyrrhizin preparations. It has better affinity with target cell receptors, and stronger anti-inflammatory and anti-oxidation effects. It has been shown to stabilize hepatocyte membrane and improve liver function<sup>[7,9]</sup>. MgIG has shown the effects of lowering the liver toxicity of free fatty acid by preventing mitochondrial damage<sup>[10,11]</sup> and protecting hepatocytes from ischemia and reperfusion induced injury<sup>[12,13]</sup>.

Employing an improved version of Emond's method<sup>[14]</sup>, we generated an animal model of 90% hepatectomy with 100% mortality within 24 h<sup>[15]</sup>, for the purpose of evaluating appropriate treatments.

Our results showed significantly longer survival time of rats in the treatment group than in the control group. And the survival time seemed to be MgIG dose-dependent. Also, general appearance in the treated groups was also superior to that in the control group. By examining liver functions, it was found that MgIG demonstrated a liver protective effect, resulting in low ALT, AST, TBil, and DBil levels in the treated groups.

This study found that postoperative ALT, AST, TBil, and TBil gradually increased to reach the peak at 18 h, which may be due to direct physical injury and surgery factors such as ischemia and reperfusion injury. At the postoperative 0 h, the liver function and coagulation parameters had no significant differences among the three groups, implying that MgIG protects the liver function, but cannot have an immediate impact. It was found that the magnitude of increase in transaminases in the treatment group was significantly lower than that in the control group at postoperative time points of 6 h and 12 h, with the high-dose group being more obvious. These findings demonstrated a protective effect of MgIG on the remnant liver after hepatectomy. Comparing serum biochemical markers of normal rats and early death rats, we found that a rapid liver cell deterioration and significant raise of liver enzymes. This reflects an excessive inflammatory response and a severe necrosis of the residual liver cells after 90% hepatectomy, and confirms the correspondence between early death and excessive inflammation in rats.

Liver regeneration after hepatectomy is one of the major mechanisms for compensating liver volume loss, and maintaining sufficient liver function<sup>[16-18]</sup>. In this study, however, we did not find obvious regeneration, even though the weight of remnant liver tissue did increase over time, which could be caused by physical response towards surgery, such as edema and congestion, and short time period of the study. It thus implies that MgIG improves the survival time of the rats with 90% hepatectomy mainly through the decreased inflammatory response rather than regeneration.

Excessive inflammatory reaction plays an important role in remaining liver damage in the postoperative outcome<sup>[19-21]</sup>. Inhibition of inflammatory reaction would be beneficial to the hepatocyte regeneration<sup>[22-25]</sup>. MgIG may prolong the survival time after hepatectomy by enhancing liver regeneration and/or suppressing excessive inflammatory response<sup>[21,26,27]</sup>. We found that compared to the control group, serum IL-10 levels were significantly increased in the MgIG treated groups, while IL-6 levels were significantly decreased. This indicates that MgIG may modulate inflammatory response in rats after hepatectomy, in which the inflammatory response in the MgIG treated groups was inhibited. This may explain the reason that the MgIG treated groups had a prolonged survival time after hepatectomy.

The JAK/STAT3 pathway, which plays a critical role in the inflammation response, can be activated by various cytokines including IL-6<sup>[28-30]</sup>. We found that the expression levels of STAT3 in remnant livers of the animals treated with MgIG were decreased compared to the ones treated with saline. This could be one of the possible mechanisms for the inflammation inhibition. Further study is required to assess STAT3 function, as well as the expression and function of

other related proteins.

More fatty degeneration was found in the high-dose MgIG treatment group with visible necrosis, indicating that MgIG might affect the fatty acid metabolism in severely injured liver. It is not known whether high doses of MgIG could have any side effect, since this is not observed in the low-dose MgIG treatment group. The clinical significance of the fatty degeneration remains unclear. The prolonged survival time that the high-dose group demonstrated is a collective result of the administration of the MgIG; however, since no animals survived longer than 60 h in this study, it is not clear whether there is any drawback of the use of the drug in the long run.

There were certain limitations in this study. The animal model was generated to an extreme of 90% resection, which probably will never occur in human beings. Therefore, it would be hard to relate our results to clinical practice. Excessive hepatectomy might also limit the effect of MgIG. In addition, the dose-effect relationship is not clear, besides the high dose resulted in longer survival time. This creates more questions than answers such as: whether MgIG functions through receptor binding on cell surface or directly on cellular proteins; what is the mechanisms for the decreased levels of ALT, AST, TBil, and DBil resulting from MgIG treatment.

In conclusion, MgIG application in excessive hepatectomy animals resulted in prolonged survival time, reduced transaminases, total bilirubin, as well as the inflammation response. The STAT3 pathway was inhibited in a way that the expression of STAT3 protein was decreased. The prolonged survival time could be potentially critical and lifesaving, because it creates a valuable time window for other treatment applications.

## COMMENTS

### Background

Excessive liver resection is the only hope of cure for large occupying liver lesions. Protection of the remnant liver function in first 48 h after surgery remains a great challenge to the liver surgeons. The efficacy of various existing measures, including the artificial liver and other supporting methods, is often limited. Anti-inflammation in the remnant liver after hepatectomy may be of benefit for the patients.

### Research frontiers

A previous study in rats found that liver regeneration reached the highest level at 72 h after resection. Animals will most likely survive if the regeneration could compensate for the first 48 h. Therefore, to identify appropriate and effective measures to help the remnant liver to sustain through this risky period is particularly important. Magnesium isoglycyrrhizinate (MgIG), a hepatocyte protective agent, has been shown to have the effects of anti-inflammation, liver cell membrane protection, and liver function improvement. Most of the previous studies of MgIG have focused on chronic hepatitis, alcoholic cirrhosis, and drug-induced liver injury. A few studies mainly involved surgical ischemia-reperfusion injury and liver regeneration. This study was designed to investigate the protective effect of MgIG on excessive hepatectomy animal model and its possible mechanism.

### Innovations and breakthroughs

This is the first to report the anti-inflammatory effect of MgIG after liver



resection. The protective effect of MgIG has been shown to prolong the survival time following standard 90% hepatectomy. This hepatoprotective effect was not via an increase in hepatocyte regeneration, rather through inhibition in the inflammatory response through via STAT3 pathway.

## Applications

The protective effect of MgIG in standard 90% hepatectomy can prolong the survival time. This study provides experimental evidence with potential benefits for the further mechanism research or clinical studies.

## Terminology

Liver regeneration rate was calculated by the Okano T formula: Regeneration rate (R, %) =  $[C-(A-B)]/(A-B) \times 100\%$ , where A is preoperative estimation of the rat liver weight, B is the weight of resected liver tissue, and C is the weight of the remnant caudate lobe.

## Peer-review

This is an interesting study which was designed to investigate whether magnesium isoglycyrrhizinate inhibits inflammatory response through STAT3 pathway to protect remnant liver function. In this study, Sprague-Dawley rats with 90% liver resection were divided into three groups. The postoperative survival time, hepatocyte regeneration, liver function, serum inflammatory cytokines and STAT3 protein were analyzed. They found that high-dose MgIG can extend survival time in rats after excessive hepatectomy. And the hepatoprotective effect was not by increasing hepatocyte regeneration but rather by inhibiting the inflammatory response through inhibition of the STAT3 pathway. The study is well designed and conducted, and the results are reliable and interesting.

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## Basic Study

# Retinoic acid receptor $\alpha$ promotes autophagy to alleviate liver ischemia and reperfusion injury

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## Abstract

**AIM:** To study the role of autophagy and the relationship between retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) and autophagy in liver ischemia and reperfusion (IR) injury.

**METHODS:** All-trans retinoic acid (ATRA) was administered to mice for two weeks before operation. Reverse transcription-polymerase chain reaction and Western blot were used to detect the expression levels of related factors. To demonstrate the role of RAR $\alpha$ , LE540, a RAR $\alpha$  inhibitor, was used to treat hepatocytes injured by H<sub>2</sub>O<sub>2</sub> *in vitro*.

**RESULTS:** ATRA pretreatment noticeably diminished levels of serum alanine aminotransferase and as-

partate aminotransferase as well as the degree of histopathological changes. Apoptosis was also inhibited, whereas autophagy was promoted. *In vitro*, RAR $\alpha$  was inhibited by LE540, which resulted in decreased autophagy and increased apoptosis. Similarly, the expression of Foxo3a and p-Akt was downregulated, but Foxo1 expression was upregulated.

**CONCLUSION:** This research provides evidence that ATRA can protect the liver from IR injury by promoting autophagy, which is dependent on Foxo3/p-Akt/Foxo1 signaling.

**Key words:** Ischemia/reperfusion; Retinoic acid receptor  $\alpha$ ; Foxo3a; Foxo1; Autophagy

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**Core tip:** To investigate the role of autophagy and the relationship between all-trans retinoic acid (ATRA) and autophagy in liver ischemia and reperfusion (IR) injury. We found that ATRA pretreatment alleviated liver IR injury by inducing autophagy and it may involves retinoic acid receptor  $\alpha$  (RAR $\alpha$ ) activity. To clarify the mechanism of RAR $\alpha$ , we used LE540 to inhibit RAR $\alpha$  during reactive oxygen species-inducing cell damage *in vitro*. Our data showed that RAR $\alpha$  activation enhanced Foxo3a and p-Akt expression except Foxo1. The Foxo3a/p-Akt/Foxo1 pathway has previously been proven to promote autophagy. Hence, we conclude that ATRA activates RAR $\alpha$  to reduce liver IR injury by regulating the Foxo3a/p-Akt/Foxo1 pathway to promote autophagy.

Zhong C, Pu LY, Fang MM, Gu Z, Rao JH, Wang XH. Retinoic acid receptor  $\alpha$  promotes autophagy to alleviate liver ischemia and reperfusion injury. *World J Gastroenterol* 2015; 21(43): 12381-12391 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12381.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12381>

## INTRODUCTION

Ischemia and reperfusion (IR) lead to damage in aerobically metabolizing tissues or organs<sup>[1]</sup>. It is an important injury mechanism in many clinical settings, e.g., liver surgical resection and liver transplantation<sup>[2,3]</sup>. Hepatocyte death due to IR insults results in the release reactive oxygen species (ROS) that initiate a sterile immune response. This activates Kupffer cells first and then recruits neutrophils to infiltrate<sup>[4]</sup>; hence, liver IR injury has a biphasic pattern: the acute phase, which involves activation of Kupffer cells after 4-6 h of reperfusion, and the subacute phase, which results from neutrophil infiltration at 18-24 h<sup>[5-7]</sup>.

Autophagy, including macroautophagy, micro-

autophagy and chaperone-mediated autophagy, is a process that degrades macromolecules and organelles *via* lysosomes<sup>[8]</sup>. It is very important for cell differentiation, survival during nutrient depletion and maintenance of homeostasis<sup>[9]</sup>. When hepatocytes suffer from nutrient starvation and anoxia during IR insults, autophagy can ameliorate liver damage<sup>[8,10]</sup>.

All-trans retinoic acid (ATRA) has been shown to protect the liver from IR injury. The protective mechanism of ATRA relies on inhibiting ROS generation and the nuclear factor-kappa B (NF- $\kappa$ B) pathway<sup>[11,12]</sup>. In addition, ATRA has been shown to mediate autophagy in acute promyelocytic leukemia cells<sup>[13]</sup>. Similarly, ATRA promotes autophagy by redistributing the cation-independent mannose-6-phosphate/IGF II receptor<sup>[14]</sup>. ATRA increases the transcription of Forkhead box O (Foxo) 3a, which is a strong inducer of Foxo1<sup>[15,16]</sup>. The Foxo3a/p-Akt/Foxo1 signaling pathway also augments autophagy<sup>[17]</sup>. To date, there has been little research into how ATRA alleviates liver IR injury through autophagy. This report is the first to demonstrate the effects of the retinoic acid receptor  $\alpha$  (RAR $\alpha$ )/Foxo3a/p-Akt/Foxo1 pathway on liver IR injury.

## MATERIALS AND METHODS

### Animals

Male wild-type C57BL/6 mice (8-12-wk-old) were obtained from Vital River Experimental Animal Co., P.R. China. Mice were housed in special pathogen-free conditions with a 12-h light-dark cycle and free access to standard laboratory diet and water, and they received humane care in accordance with the guidelines of the Chinese Association of Laboratory Animal Care. The standards for animal use and care were approved by the Institutional Animal Care Committee of Nanjing Medical University (Protocol Number: NJMU08-092).

### Operative procedure

Before the experiment, the mice received ATRA (Sigma-Aldrich, Shanghai, China) by gavage at 15 mg/kg per day for two weeks. We induced 70% hepatic ischemia by occluding the hepatic artery, portal vein and bile duct to the cephalad hepatic lobes for 90 min. During the ischemic process, mice were anesthetized using isoflurane, and the environmental temperature was maintained at 25 °C. After liver reperfusion for 6 h or 20 h, the mice were sacrificed, and then blood and liver tissue samples were harvested for analysis.

### Hepatocellular function assay

Serum alanine aminotransferase (sALT) and serum aspartate aminotransferase (sAST) levels were measured to assess hepatocyte injury using an automated chemical analyzer (Olympus Automated Chemistry Analyzer AU5400, Tokyo, Japan).



**Histopathological study**

Liver samples were fixed with 10% neutral formaldehyde and embedded in paraffin. Paraffin sections at 5  $\mu$ m were stained with hematoxylin and eosin and then blindly analyzed and scored from 0 to 4, as described by Suzuki *et al.*<sup>[18]</sup>.

**Western blot analysis**

Proteins were extracted from liver samples and cell lysates, and the concentration was detected using a BCA Protein Assay Kit (Thermo Fisher Scientific, Shanghai, China). The nuclear proteins were extracted to detect the level of Foxo1. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transblotted onto polyvinylidene fluoride membranes (Millipore, United States). These membranes were blocked in non-fat dry milk (5% wt/vol) with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at 4 °C overnight and then incubated with primary antibodies against RAR $\alpha$  (Santa Cruz Biotechnology, Santa Cruz, CA, United States), Bcl-2, Beclin-1, LC3B,  $\beta$ -actin (Cell Signaling Technology, Danvers, MA, United States), cleaved caspase 3, p-Akt, Foxo1, Foxo3a, and p62 (Abcam, Shanghai, China). Following three washes with TBS-T, the membranes were incubated with a peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, United States) for 1 h at room temperature. Bands were quantified by densitometry using the Quantity One software for image analysis.

**Quantitative real-time reverse transcriptase-polymerase chain reaction**

RNA was isolated using TRIzol reagent (Invitrogen, Shanghai, China). cDNA was synthesized according to the manufacturer's instructions using the PrimeScript<sup>TM</sup> RT Reagent Kit with gDNA Eraser (Takara, Japan). Polymerase chain reaction (PCR) was performed using SYBR Premix Ex Taq<sup>TM</sup> (Takara, Japan). The primer sets were as follows:  $\beta$ -actin: forward, 5'-CTA CAA TGA GCT GCG TGT GG-3' and reverse, 5'-AAG GAA GGC TGG AAG AGT GC-3'; IL-6: forward, 5'-GAC TTC CAT CCA GTT GCC TTC T-3' and reverse, 5'-TTT CTC ATT TCC ACG ATT TCC CA-3'; IL-1 $\beta$ : forward, 5'-GTG TTT TCC TCC TTG CCT CTG AT-3' and reverse, 5'-GCT GCC TAA TGT CCC CTT GAA T-3'; tumor necrosis factor (TNF)- $\alpha$ : forward, 5'-CTC TGT GAA GGG AAT GGG TGT-3' and reverse, 5'-TCT TGT GTT TCT GAG TAG TTG TTG A-3'.

**Cell culture and treatment**

FL83B mouse hepatocytes were cultured in Ham's F-12K medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. Before being co-cultured with 200  $\mu$ mol/L H<sub>2</sub>O<sub>2</sub> for 6 h, cells were preincubated with ATRA (10  $\mu$ mol/L) or LE540 (10  $\mu$ mol/L, Wako Pure Chemical Industries Ltd., Osaka, Japan) for 24 h.

**Annexin V and propidium iodide labeling**

The cell samples were stained using an Annexin V (AV)-fluorescein isothiocyanate Apoptosis Detection Kit (Sigma-Aldrich, St Louis, United States) following the manufacturer's instructions. For confocal microscopy, both probes were activated by a 488 nm diode laser, and the fluorescence emission was detected at 510 and 560 nm for AV and propidium iodide (PI), respectively. The fluorescence intensity was obtained for 20000 events, and the data were analyzed using Cell-Quest<sup>TM</sup> (Becton-Dickinson, San Jose). The data are presented as the percentage of cells in four different population phenotypes-unstained, stained only with AV, stained only with PI or stained with both markers-relative to the total number of cells analyzed.

**Statistical analysis**

Data are presented as mean  $\pm$  SD from at least three independent experiments. SPSS software (Chicago, IL, United States) was used to calculate the statistical significance by performing one-way analysis of variance. All the *P* values were two-sided, and *P* < 0.05 was considered statistically significant.

**RESULTS****ATRA pretreatment ameliorates liver injury after IR**

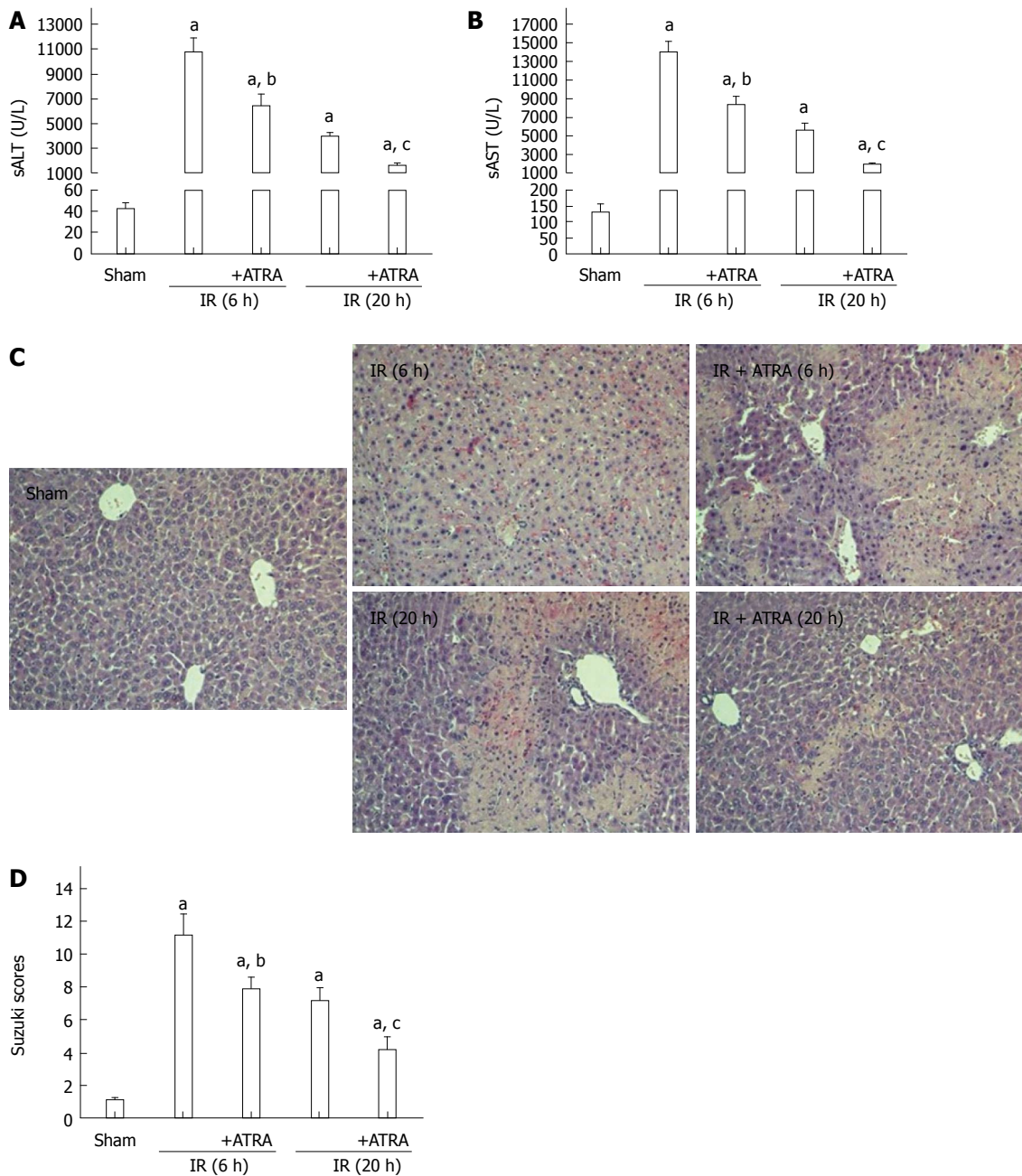
We used an established model of hepatic damage in mice, inducing 90 min of ischemia and then sacrificing the mice at 6 h or 20 h after reperfusion. Before the operation, ATRA was administered by gavage once a day for two weeks. The levels of sALT and sAST, the area of necrosis on liver histopathology and the samples' Suzuki scores were obviously decreased in the ATRA group compared with the IR control group (Figure 1).

**ATRA pretreatment reduces apoptosis and inflammation in liver IR injury**

When IR injury occurs, hepatocellular apoptosis is induced, and this condition results in liver injury<sup>[19]</sup>. According to liver function and histological score, liver damage was more serious at 6 h after reperfusion. So, we just chose the liver samples at 6 h after reperfusion to analyze by Western blot. ATRA enhanced the expression of the anti-apoptotic protein Bcl-2 while inhibited the expression of the pro-apoptotic protein cleaved caspase 3 in liver IR injury (Figure 2A). This mechanism explains the downregulation of hepatocellular apoptosis. Besides, the role of IR injury in inflammation was weakened, because the mRNA levels of TNF- $\alpha$ , interleukin (IL)-1 $\beta$  and IL-6 in the ATRA group were less than those in the IR group (Figure 2B).

**Autophagy is promoted by ATRA**

RAR $\alpha$  was activated by ATRA. Similarly, the Foxo3a/

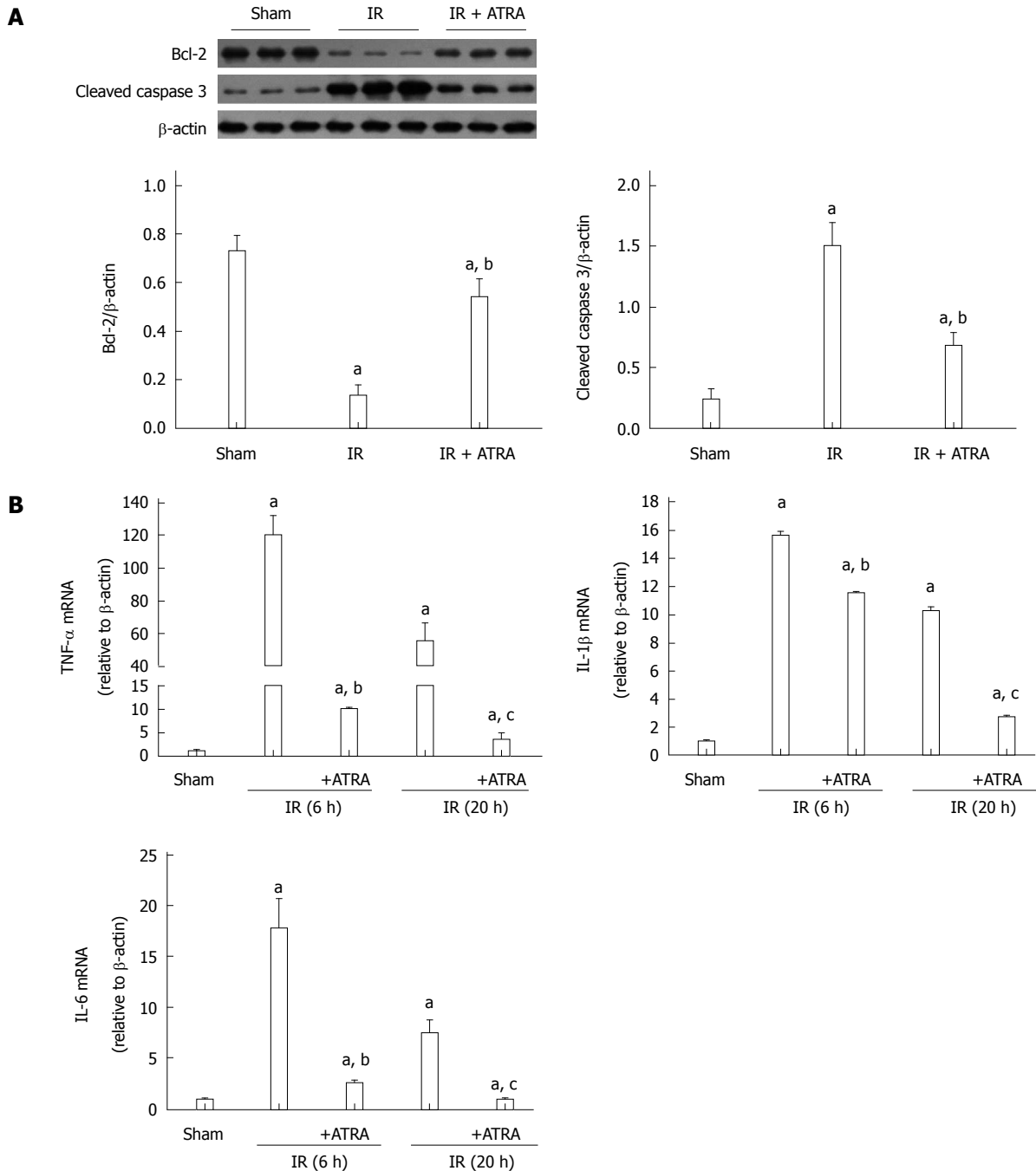


**Figure 1 All-trans retinoic acid pretreatment ameliorates liver ischemia and reperfusion injury.** Hepatic ischemia was induced in mice for 90 min, and then the mice were sacrificed at 6 h or 20 h after reperfusion. A, B: Hepatocellular function as assessed by sALT and sAST levels; C: Liver samples were stained with hematoxylin and eosin (magnification  $\times 200$ ); D: Liver histological grade by the Suzuki criteria. Mean  $\pm$  SD,  $n = 3-5/\text{group}$ ,  $^aP < 0.05$  vs the sham group;  $^bP < 0.05$  vs the IR 6 h group;  $^cP < 0.05$  vs the IR 20 h group. ATRA: All-trans retinoic acid; IR: Ischemia and reperfusion; sALT: Serum alanine aminotransferase; sAST: Serum aspartate aminotransferase.

p-Akt/Foxo1 pathway was induced by RAR $\alpha$  activation (Figure 3A). Foxo3a/p-Akt/Foxo1 has been shown to promote autophagy<sup>[17]</sup>. The proteins Beclin1, LC3 II and p62 are involved in the process of autophagy. As a substrate, p62 is degraded by autolysosome and inversely correlates with the autophagic activity<sup>[20-22]</sup>. ATRA pretreatment enhanced the levels of Beclin1 and LC3 II but decreased the level of p62 (Figure 3B). These results suggest that ATRA promotes autophagy in liver IR injury.

#### RAR $\alpha$ inhibits ROS-induced apoptosis in vitro

To further analyze the effect of RAR $\alpha$ , LE540 was used to inhibit RAR $\alpha$  activity. Following H<sub>2</sub>O<sub>2</sub> stimulation, ATRA, the RAR $\alpha$  agonist, upregulated the expression of Bcl-2, downregulated cleaved caspase 3 expression and ultimately decreased the number of apoptotic cells detected by Annexin V staining (Figure 4). However, LE540, an antagonist of RAR $\alpha$ , aggravated the hepatocyte damage in comparison with the H<sub>2</sub>O<sub>2</sub> group.

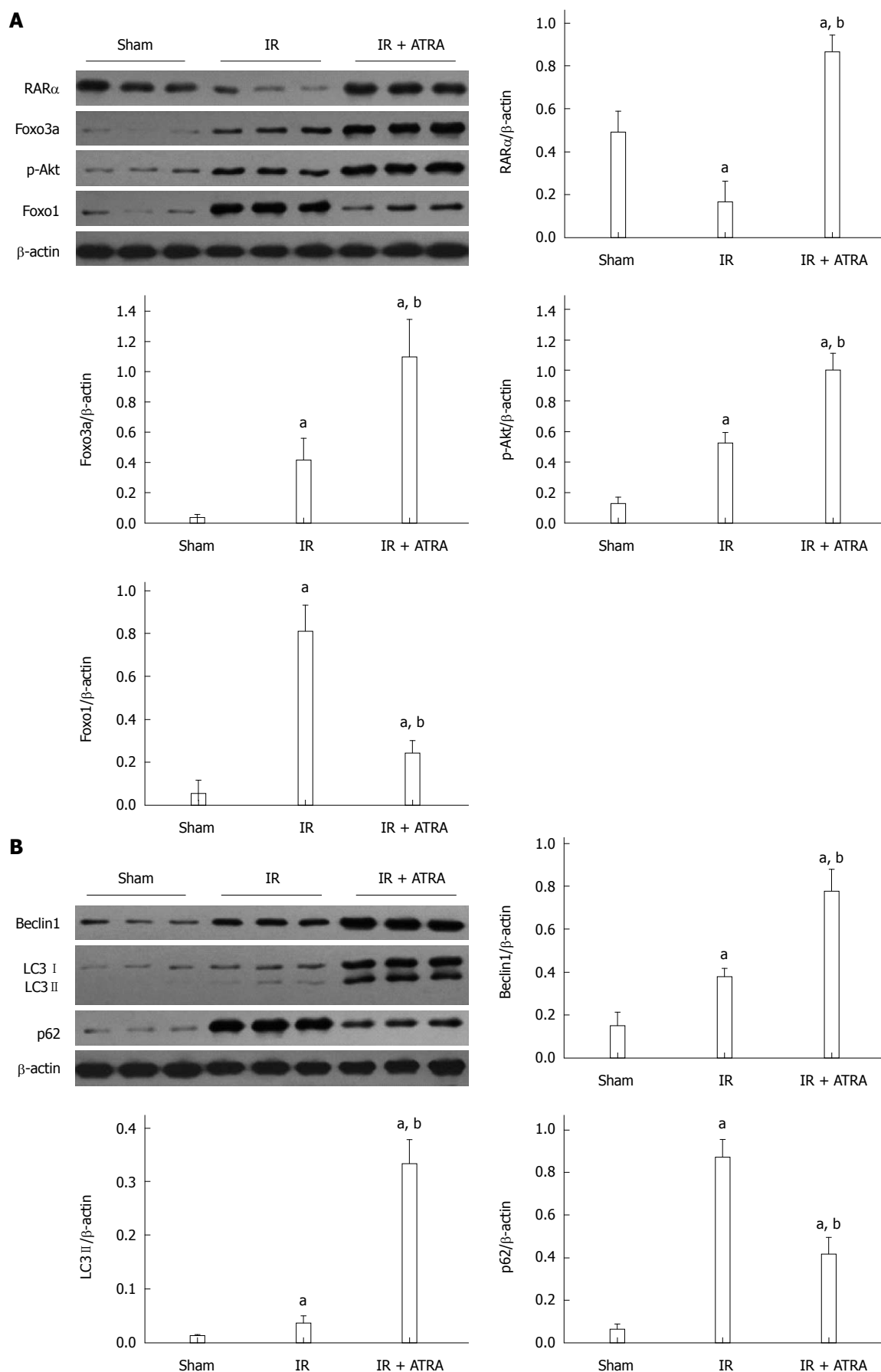


**Figure 2 All-trans retinoic acid pretreatment reduces apoptosis and inflammation in liver ischemia and reperfusion injury.** A: Apoptosis-related proteins Bcl-2 and cleaved caspase 3 expression was detected by Western blot to demonstrate relative quantities in liver tissues; B: The levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 mRNAs were analyzed. Mean  $\pm$  SD,  $n = 3$ -5/group, <sup>a</sup> $P < 0.05$  vs the sham group; <sup>b</sup> $P < 0.05$  vs the IR 6h group; <sup>c</sup> $P < 0.05$  vs the IR 20h group. TNF: Tumor necrosis factor; IL: Interleukin; IR: Ischemia and reperfusion; ATRA: All-trans retinoic acid.

### RAR $\alpha$ mediates autophagy through the Foxo3a/p-Akt/Foxo1 pathway

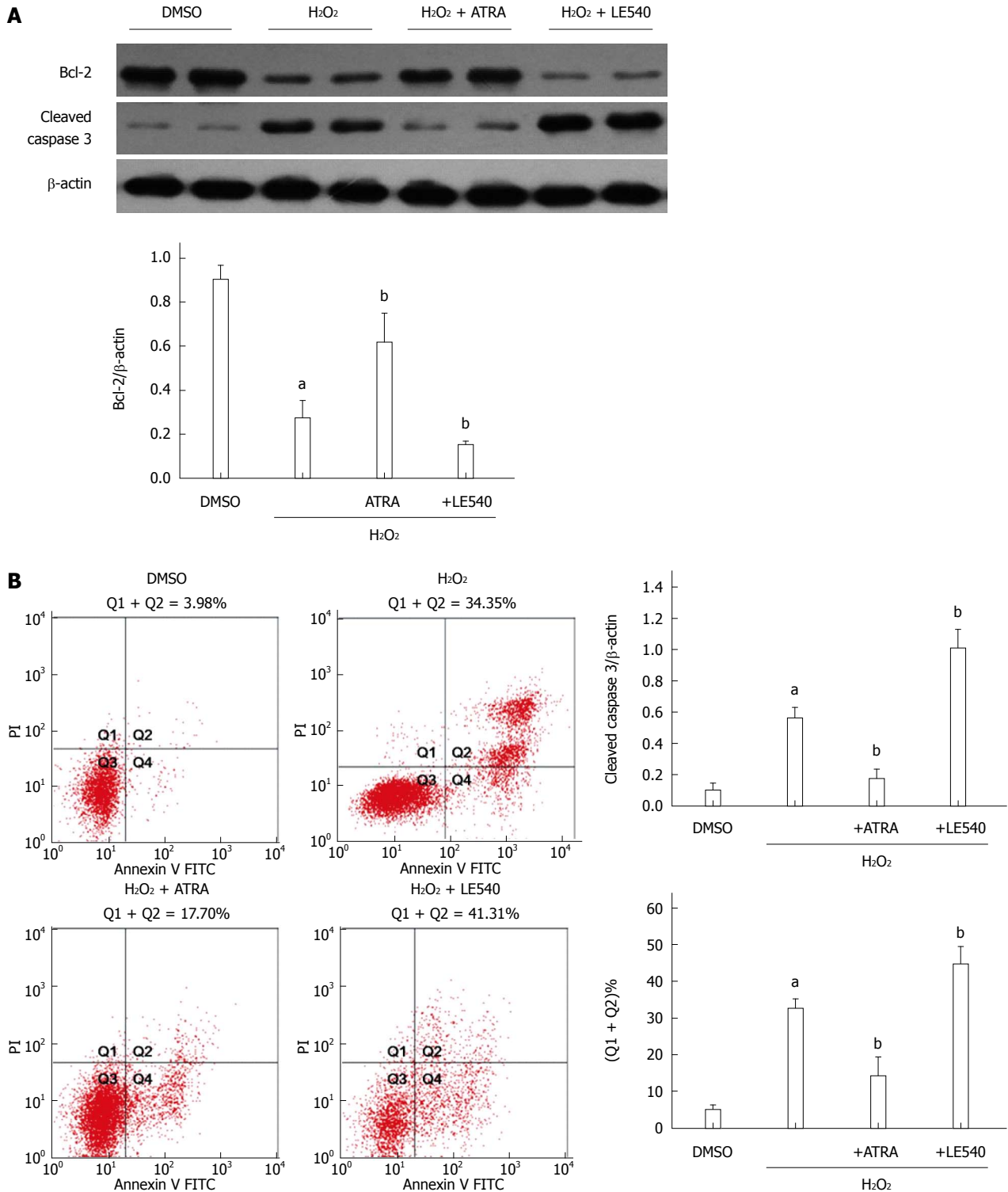
As mentioned above, RAR $\alpha$  activates the Foxo3a/p-Akt/Foxo1 pathway to mediate autophagy. Although Foxo3a and Foxo1 are both members of the Foxo family, they have opposing roles in autophagy. To enhance the level of autophagy, Foxo3a needs phosphorylated Akt to decrease Foxo1 accumulation in the nucleus<sup>[17]</sup>. In our H<sub>2</sub>O<sub>2</sub>-induced damage model, RAR $\alpha$  promoted the transcription of Foxo3a, which

resulted in an autophagy-related chain reaction including increased Beclin1 and LC3 II levels and decreased p62 levels (Figure 5B). This finding was confirmed in another manner by LE540. Because the level of Foxo3a expression was consistent with RAR $\alpha$  activation, the levels of RAR $\alpha$ , Foxo3a, p-Akt and Foxo1 were reversed in the LE540 group (Figure 5A). Ultimately, the degree of ROS-induced cell damage was strengthened by LE540 induced inhibition of autophagy.

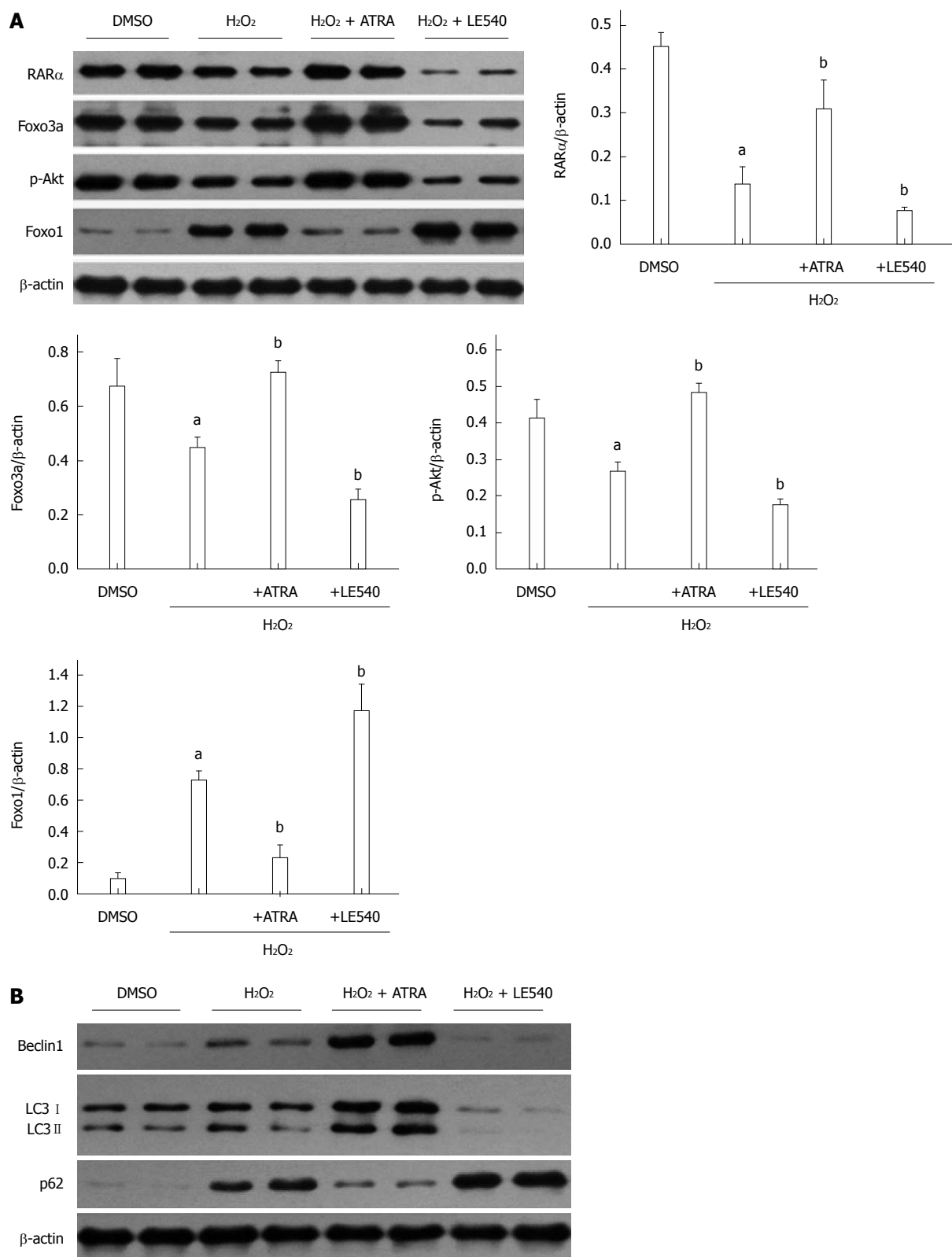


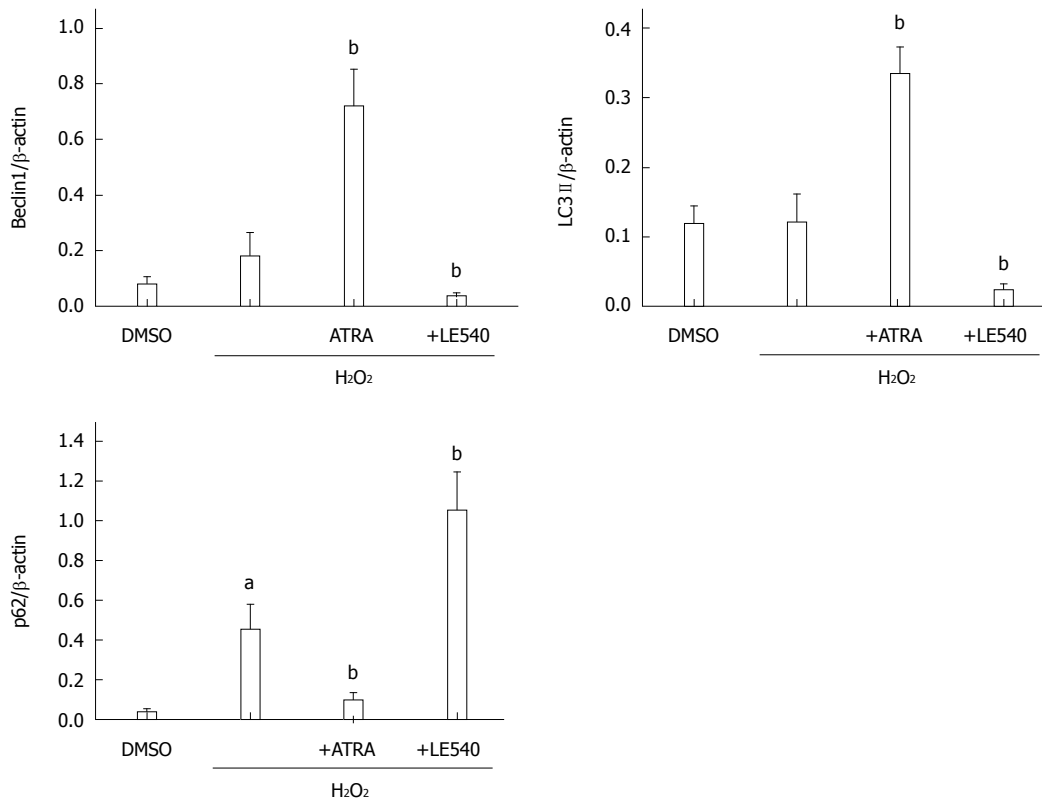
**Figure 3 Autophagy is promoted by all-trans retinoic acid.** A: Western blot assessment of RAR $\alpha$ , Foxo3a, p-Akt, Foxo1, and  $\beta$ -actin expression levels; B: Western blot analysis of Beclin1, LC3 I / II, p62 and  $\beta$ -actin expression levels. Mean  $\pm$  SD,  $n = 3-5$ /group, <sup>a</sup> $P < 0.05$  vs the sham group; <sup>b</sup> $P < 0.05$  vs the IR 6 h group. IR: Ischemia and reperfusion; ATRA: All-trans retinoic acid.





**Figure 4** Retinoic acid receptor  $\alpha$  inhibits reactive oxygen species-induced apoptosis *in vitro*. LE540, an inhibitor of RAR $\alpha$ , was cocultured with FL83B cells for 24 h, and then the cells were harvested 6 h after H<sub>2</sub>O<sub>2</sub> treatment. A: Expression levels of Bcl-2, cleaved caspase 3 and  $\beta$ -actin were measured by Western blot; B: The number of apoptotic cells was assessed by AV and PI labeling. Mean  $\pm$  SD,  $n = 3-5$ /group; <sup>a</sup> $P < 0.05$  vs the DMSO group; <sup>b</sup> $P < 0.05$  vs the H<sub>2</sub>O<sub>2</sub> group. RAR $\alpha$ : Retinoic acid receptor  $\alpha$ ; AV: Annexin V; PI: Propidium iodide; FITC: Fluorescein isothiocyanate; ATRA: All-trans retinoic acid.





**Figure 5** Retinoic acid receptor  $\alpha$  mediates autophagy through the Foxo3a/p-Akt/Foxo1 pathway. A: Western blot assessment of RAR $\alpha$ , Foxo3a, p-Akt, Foxo1, and  $\beta$ -actin expression levels. B: Western blot analysis of Beclin1, LC3 I / II, p62 and  $\beta$ -actin expression levels. Mean  $\pm$  SD,  $n = 3$ -5/group, <sup>a</sup> $P < 0.05$  vs the DMSO group; <sup>b</sup> $P < 0.05$  vs the H<sub>2</sub>O<sub>2</sub> group. RAR $\alpha$ : Retinoic acid receptor  $\alpha$ ; ATRA: All-trans retinoic acid.

## DISCUSSION

ATRA is the activated form of vitamin A, and its biosynthesis process is controlled by cytosolic alcohol dehydrogenases, retinol dehydrogenases and retinaldehyde dehydrogenases<sup>[23-25]</sup>. ATRA can also attenuate liver IR injury through suppressing NF- $\kappa$ B p65 expression and phosphorylating p38 mitogen-activated protein kinase and Akt to increase the expression of manganese superoxide dismutase<sup>[11,12]</sup>. Moreover, it can regulate the expression of many genes to mediate its biological effects by binding with RAR<sup>[26-28]</sup>. In this study, ATRA increased the transcription of Foxo3a, which had a protective role in hepatic tissue after IR injury; in turn, this effect could be blocked by the RAR $\alpha$  inhibitor LE540 *in vivo*.

Autophagy, which has an important role in cellular development, differentiation and survival, is necessary to maintain homeostasis and provide energy by degrading cytosolic components *in vivo*<sup>[8,29]</sup>. During IR insults, autophagy can degrade abnormal or dysfunctional mitochondria to decrease ROS generation. In addition, the formation of autophagic vesicles and autophagic flux is decreased in IR injury<sup>[29]</sup>. The process of autophagy is divided into three sections including the formation of autophagosomes and autolysosomes, and degradation<sup>[30-32]</sup>. Beclin1 and LC3II are involved in the initiation of autophagosome<sup>[33-35]</sup>, but they cannot absolutely reflect the

function of autophagy. Because autophagy is a degradation system. Despite the enhancement of Beclin1 and LC3II in the IR group, it does not mean that autophagy was increasing. In addition, p62, which is the substrate of autophagy and used to evaluate the degree of autophagy<sup>[36-38]</sup>, was increasing in the IR group. Hence, the autophagy was inhibited and this was associated with the exacerbation of apoptotic cells and inflammation response in the IR group. We also found the similar results in the H<sub>2</sub>O<sub>2</sub> treatment group. However, these findings were reversed by ATRA. Thus, ATRA can reduce apoptosis and inflammation by autophagy partially.

The Foxo family, which includes Foxo1, Foxo3a, Foxo4 and Foxo6 in mammals, can control many cellular processes<sup>[39,40]</sup>. Except for Foxo6, the Foxo proteins are widely expressed in most tissues. Foxo1 and Foxo3a have been shown to be important for normal and abnormal liver function<sup>[41]</sup>. Foxo3a is a strong inducer of the transcription factor Foxo1 because it positively regulates the promoter of Foxo1<sup>[15]</sup>. Foxo1 plays a different role, depending on locating in the cytoplasm or nucleus. This process is controlled by Akt kinase, which phosphorylates Foxo1 at Thr24, Ser256 and Ser319, and makes phosphorylated Foxo1 translocate from the nucleus to cytoplasm. Moreover, cytoplasmic Foxo1 promotes autophagy<sup>[42-44]</sup>. We also found that the increased level of Akt phosphorylation diminished nuclear Foxo1 accumulation. Foxo3a is

involved in mediating autophagy through the Akt/Foxo1 pathway<sup>[17]</sup>. This finding is consistent with our results.

In addition, ATRA can influence the expression of Foxo3a<sup>[16,45,46]</sup>. In the ATRA group, the expression of Foxo3a was increased. This effect is related to RAR $\alpha$  activity, which was completely inhibited by LE540 treatment. Additionally, ATRA can regulate phosphoinositide 3-kinase (PI3K) activity and the expression of PI3K catalytic subunit p110 $\beta$ <sup>[47]</sup>. ATRA can also redistribute the cation-independent mannose-6-phosphate/IGFII receptor to mediate autophagy<sup>[14]</sup>. Whether this effect is dependent on Foxo3a requires further investigation.

In conclusion, our study is the first to demonstrate that ATRA enhances autophagy to protect the liver from IR injury. This effect depends on RAR $\alpha$  activation of the Foxo3a/p-Akt/Foxo1 pathway. This mechanism provides a new strategy for treating liver IR injury.

## COMMENTS

### Background

Ischemia and reperfusion (IR) injury is an important clinical factor in the process of hepatic surgery. It can affect the prognosis of the patient. Liver IR injury, in nature, is a sterile immune response. All-trans retinoic acid (ATRA) can mitigate liver IR injury, but the underlying mechanism is not clear.

### Research frontiers

ATRA can activate retinoic acid receptor (RAR)  $\alpha$  to induce autophagy, and Foxo3a is the target of ATRA-induced apoptosis and granulocytic polarization in the treatment of acute promyelocytic leukemia (APL). In addition, the expression of Foxo3a is increased by ATRA treatment. Foxo3a also activates the Akt/Foxo1 pathway to regulate autophagy. However, this report is the first to discuss the relationship between ATRA and autophagy in liver IR injury.

### Innovations and breakthroughs

ATRA pretreatment induces autophagy to decrease hepatocyte apoptosis in liver IR injury. This protective role depends on the Foxo3a/p-Akt/Foxo1 pathway through the activation of RAR $\alpha$ .

### Application

This study confirmed that ATRA pretreatment is an effective therapeutic treatment in liver IR injury. Moreover, ATRA has already been applied to treat APL in the clinic. Hence, ATRA is a potential drug for patients undergoing hepatic operations.

### Peer-review

This study demonstrated the mechanism of ATRA-mediated autophagy in liver IR injury. The results are adequate to support the conclusions, and the design is reasonable.

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## Retrospective Study

# Proposal of an ultrasonographic classification for hepatic alveolar echinococcosis: Echinococcosis multilocularis Ulm classification-ultrasound

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**Informed consent statement:** Because of retrospective and

anonymous character of this study the need for informed consent was waived by the Institutional Review Board.

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**Data sharing statement:** No additional data are available.

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## Abstract

**AIM:** To establish an ultrasonographic classification based on a large sample of patients with confirmed hepatic alveolar echinococcosis (AE).

**METHODS:** Clinical data and ultrasonography (US) findings of 185 patients (100 males; 85 females; mean age at diagnosis:  $51.4 \pm 17.6$  years; mean age at time of US examination:  $58.7 \pm 18.2$  years) were retrospectively reviewed with respect to the US morphology of hepatic AE lesions. The sonomorphological findings were grouped according to a five-part classification scheme.

**RESULTS:** Application of the new classification resulted in the following distribution of sonomorphological patterns among the patients examined: hailstorm (54.1%); pseudocystic (13.5%); ossification (13.0%); hemangioma-like (8.1%); and metastasis-like (6.5%). Only 4.9% of lesions could not be assigned to a sonomorphological pattern.

**CONCLUSION:** The sonomorphological classification proposed in the present study facilitates the diagnosis, interpretation and comparison of hepatic alveolar echinococcosis in routine practice and in the context of scientific studies.

**Key words:** Hepatic echinococcosis; *Echinococcus multilocularis*; Classification; Diagnosis; Ultrasonography; Alveolar echinococcosis

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**Core tip:** Alveolar echinococcosis (AE) is a rare but potentially life-threatening parasitic disease. Despite the importance of ultrasonography as an imaging modality in the work-up of hepatic AE, there is no established sonomorphological classification of hepatic AE lesions analogous to the World Health Organization's ultrasonographic classification for cystic echinococcosis. Objective of the present study was to establish an ultrasonographic classification based on a large sample of patients with confirmed hepatic AE. Assignment of hepatic AE lesions to one of the five sonomorphological patterns was successful in 95% of cases based on the ultrasonographic classification scheme proposed in the present study.

Kratzer W, Gruener B, Kaltenbach TEM, Ansari-Bitzenberger S, Kern P, Fuchs M, Mason RA, Barth TFE, Haenle MM, Hillenbrand A, Oeztuerk S, Graeter T. Proposal of an ultrasonographic classification for hepatic alveolar echinococcosis: Echinococcosis multilocularis Ulm classification-ultrasound. *World J Gastroenterol* 2015; 21(43): 12392-12402 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12392.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12392>

## INTRODUCTION

Alveolar echinococcosis (AE) is a rare but potentially life-threatening parasitic disease caused by infection

with the larval stage of the cestode tapeworm, *Echinococcus alveolaris*<sup>[1-3]</sup>. Worldwide, the distribution of the parasite is limited to the cool and temperate regions of the Northern Hemisphere<sup>[4]</sup>. A characteristic feature of AE is its tumor-like growth in the liver, which may infiltrate neighboring organs<sup>[1]</sup>. In a large majority of cases, the liver is the first organ to be infested by the larvae: in seven out of ten cases, hepatic lesions occur in the right hepatic lobe; in 40%, the liver hilus is also involved; while, in only two of ten cases, both hepatic lobes are affected<sup>[5]</sup>.

In its initial phase, the infection is usually asymptomatic. First symptoms and signs may include upper abdominal pain or cholestatic jaundice. The incubation period ranges between five and fifteen years<sup>[6]</sup>. Complications, such as biliary obstruction, portal hypertension and bleeding esophageal varices, have been reported in advanced disease and are ascribed to the invasively growing mass of *Echinococcus alveolaris* in the liver<sup>[7]</sup>. Metastatic infiltration by *Echinococcus alveolaris* has been described for many organs<sup>[8,9]</sup> and is reflected in the PNM classification introduced by Kern *et al*<sup>[10]</sup>.

Radical resection of echinococcal foci is the sole curative therapy for patients with AE. Curative therapy is followed by administration of benzimidazoles for two years; long-term administration of these agents is indicated for non-resectable lesions<sup>[9]</sup>. Left untreated, the disease is associated with a fatal outcome in more than 95% of cases within a period of ten years following diagnosis<sup>[11]</sup>. Only early diagnosis, based on diagnostic imaging and serological markers, can increase the rate of curative resections<sup>[12,13]</sup>. Early diagnostic imaging therefore takes on decisive importance<sup>[14]</sup>.

Beside US, computed tomography (CT) represents the imaging method of choice among currently available diagnostic imaging modalities<sup>[15,16]</sup>. <sup>18</sup>F-fluorodeoxyglucose positron emission tomography (<sup>18</sup>F-FDG-PET) is a sensitive and specific tool that uses <sup>18</sup>F-fluorodeoxyglucose (<sup>18</sup>F-FDG) metabolism to estimate the metabolic activity of hepatic AE lesions<sup>[17-19]</sup>. The development of the US contrast enhanced SonoVue® (Bracco Medical Imaging Deutschland GmbH, Konstanz, Germany) has over the past few years facilitated US assessment of the vitality of AE lesions at follow-up monitoring. Assessment of the vascularization of hepatic AE lesions with contrast-enhanced ultrasound (CEUS) correlates with their metabolic activity at combined <sup>18</sup>F-FDG-PET-CT<sup>[20,21]</sup> and can better delineate the spatial extent of hepatic alveolar echinococcosis lesions<sup>[22,23]</sup>. Lesions characterized by vesicles and small cysts show a high degree of correlation between <sup>18</sup>F-FDG-PET and CEUS findings<sup>[24]</sup>.

In 2003, Kodama *et al*<sup>[25]</sup> introduced a five-part classification for assessing hepatic AE with magnetic resonance imaging (MRI): Type 1: Multiple small round cysts without a solid component; Type 2: Multiple

small round cysts with a solid component; Type 3: A solid component surrounding a large and/or irregular pseudo-cyst with multiple small round cysts; Type 4: A solid component without cysts; Type 5: A large cyst without a solid component.

No corresponding classification has yet been published for either CT or ultrasonography (US). Current studies suggest that the occurrence of alveolar echinococcosis is increasing worldwide and is spreading to previously unaffected regions. Especially in the Northern Hemisphere, there is a growing number of AE lesions occurring as incidental findings at routine upper abdominal US<sup>[14,26,27]</sup>. Knowledge of the typical presentations of hepatic AE at diagnostic imaging may aid in making an early diagnosis<sup>[28]</sup>. Despite the importance of US as an image modality in the work-up of hepatic AE, there is no sonomorphological classification of hepatic AE lesions analogous to the World Health Organization (WHO)'s ultrasonographic classification for cystic echinococcosis, which has achieved worldwide acceptance for assessing the activity of that disease<sup>[14,15,28]</sup>. Objective of the present study was to establish an ultrasonographic classification based on a large sample of patients with confirmed hepatic AE as a way of facilitating the diagnosis, interpretation, classification and comparison of ultrasonographic findings of the rare disease entity.

## MATERIALS AND METHODS

### Study collective

Clinical data and US findings of 185 patients ( $n = 100$  males; 85 females; mean age at diagnosis:  $51.4 \pm 17.6$  years; mean age at time of US examination:  $58.7 \pm 18.2$  years) followed at the Echinococcosis outpatient clinic of Ulm University Hospital ( $n = 385$  patients) were reviewed with respect to the ultrasonographic morphology of hepatic AE lesions. Patients were originally examined between 1999 and 2014. A total of 200 patients were excluded from this analysis due to limitations in image quality impacting interpretation or incomplete data sets. The US findings of all patients ( $n = 185$ ) with confirmed hepatic AE stored in the ViewPoint US documentation system (GE Healthcare Technologies, ViewPoint Bildverarbeitung GmbH, Weßling, Germany) were re-interpreted by a single reviewer (WK) with broad experience in the US of AE and grouped according to a novel five-part sonomorphological classification scheme (Figure 1, Figure 2, Figure 3, Figure 4 and Figure 5).

The study design complies with the requirements of the Helsinki Declaration and was approved by the Ethics Commission of Ulm University.

### Diagnosis and classification

The diagnosis of AE was made in cases with unequivocal seropositivity, positive histological findings

following diagnostic puncture or partial resection of the liver, as well as findings typical for AE in either US, CT, MRI or PET-CT<sup>[15]</sup>. According to the modified WHO criteria of Brunetti *et al.*<sup>[15]</sup> 79 cases were confirmed by positive histopathology and proven specific enzyme linked immunosorbent assay from tissue samples. An additional 85 patients were considered probable cases with positive serology in two different procedures and positive imaging for AE in two imaging techniques, while 21 patients were considered possible cases with a positive medical history and a positive result for imaging and serology in one test each. Based on the reviewer's many years' experience together with reports of sonomorphological findings in patients with hepatic AE in the literature, individual US findings were grouped into one of the following patterns: hailstorm, pseudocystic, hemangioma-like, ossification, and metastasis-like<sup>[14,16,17]</sup> (Figures 1-5). As an acronym, we propose EMUC-US (Echinococcosis Multilocularis Ulm Classification - Ultrasound). In addition to patient-specific parameters, the number, maximum diameter and localization of the largest echinococcus-specific lesion were documented and interpreted. US examinations were performed exclusively using convex transducer heads (1-6 MHz) with different US units (Philips HDI 3000, HDI 5000, IU 22, Toshiba Aplio 500, Siemens S3000, Hitachi Ascendus).

### Statistical analysis

Statistical analyses were performed using the SAS statistical software package (version 9.2; SAS Institute Inc., Cary, NC, United States). Data were analyzed descriptively with regard to absolute and relative frequencies, means and standard deviation. The AE lesions were divided into five morphological patterns. One-way analysis of variance was applied to analyze differences between the patterns.

## RESULTS

The most frequently encountered sonomorphological pattern among the 185 patients was the hailstorm pattern (54.1%,  $n = 100$ ), followed, in 13.5% ( $n = 25$ ) by the pseudocystic appearance and in 13% ( $n = 24$ ) by the ossification appearance. Much less frequently observed were the hemangioma-like appearance (8.1%,  $n = 15$ ) and the metastasis-like appearance (6.5%,  $n = 12$ ). In terms of their mean diameters, the hailstorm lesions measured  $59.6 \pm 27.9$  mm; the pseudocystic lesions,  $120.0 \pm 47.3$  mm; the hemangioma-like lesions,  $68.1 \pm 37.3$  mm; the ossification lesions,  $28.0 \pm 19.4$  mm; and metastasis-like lesions,  $35.3 \pm 33.1$  mm (Figure 6). The diameters of lesions exhibiting pseudocystic sonomorphology were significantly larger than any of the other four lesion types ( $P < 0.05$ ). In terms of their mean diameters, lesions of both the hailstorm and hemangioma-like types differed significantly from



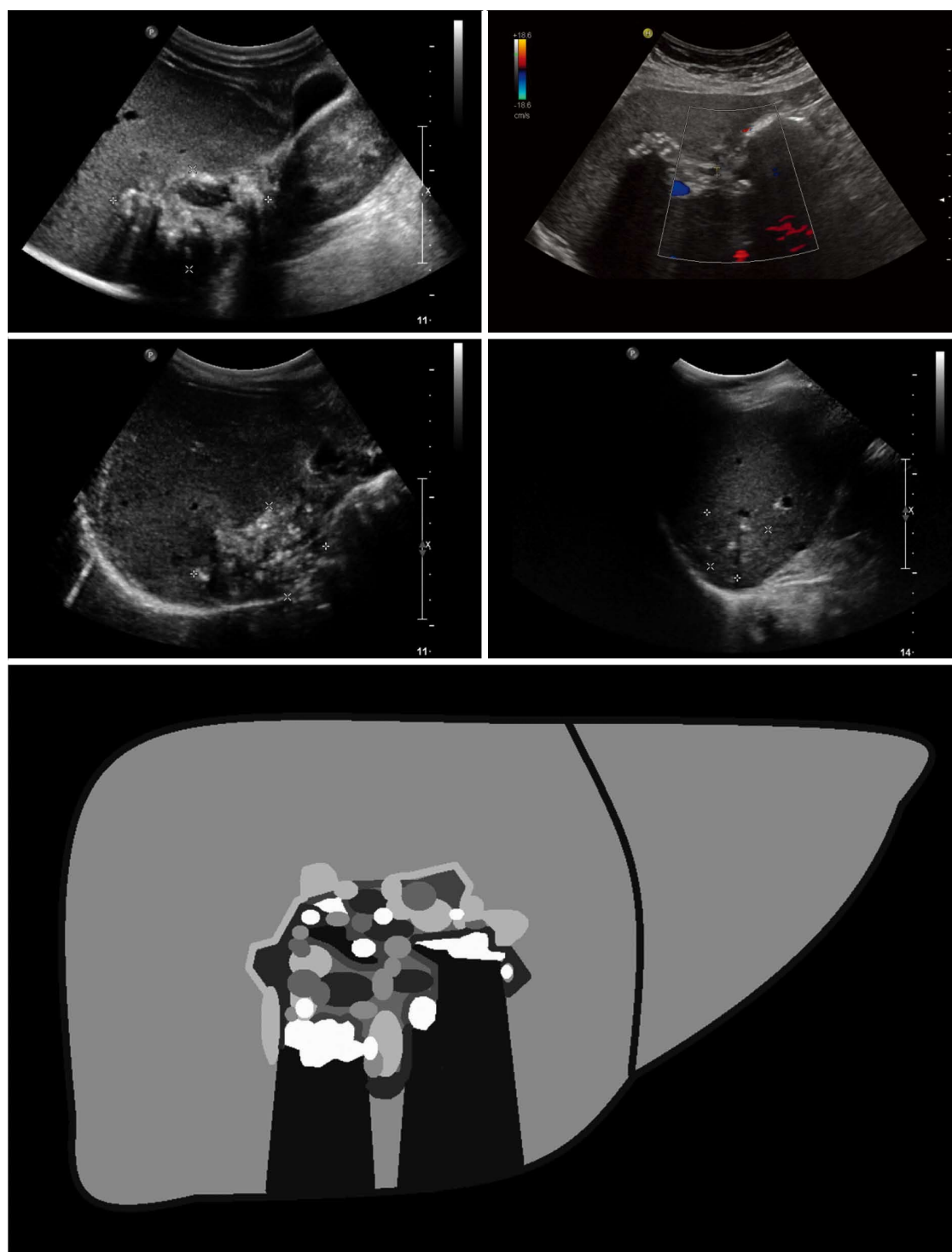
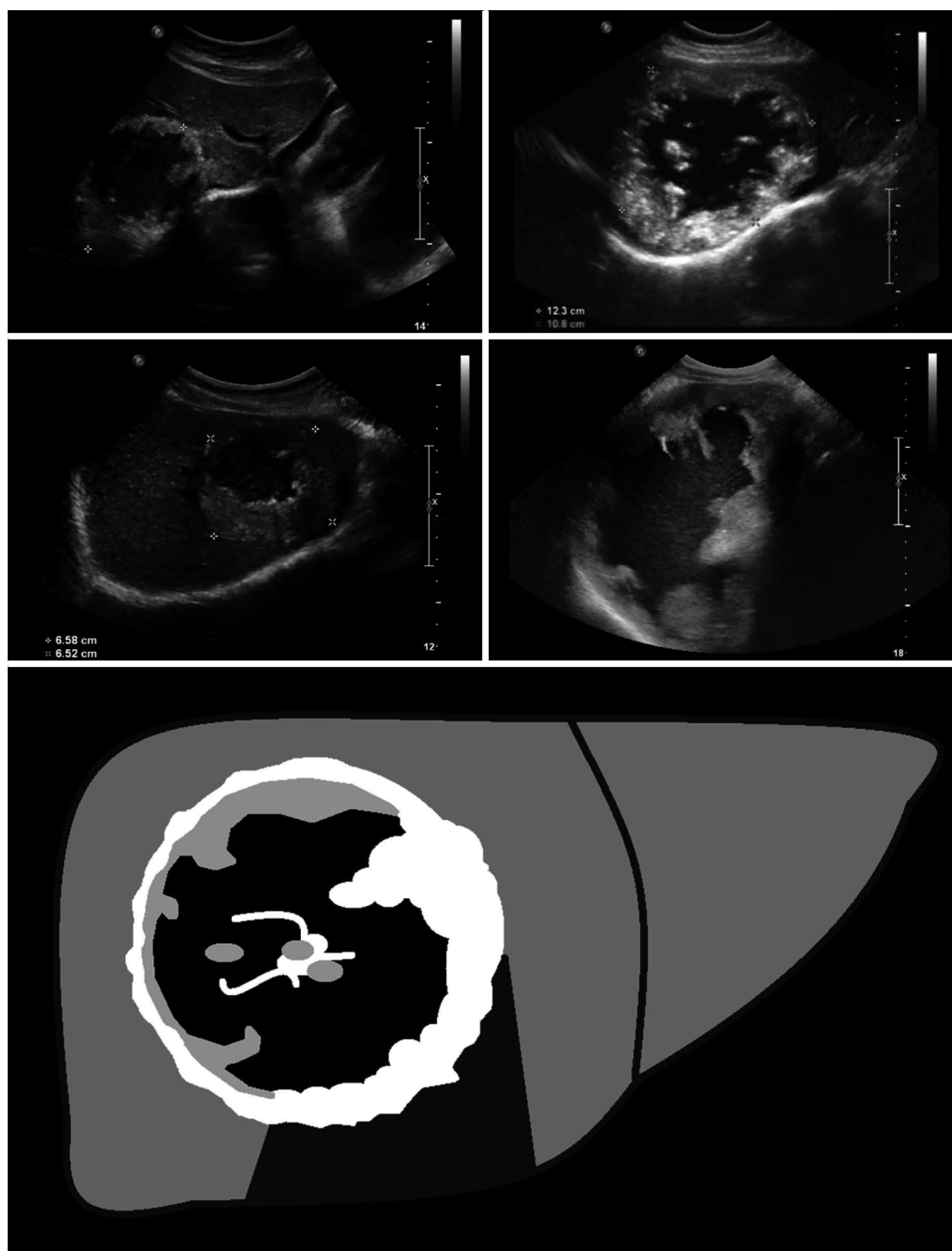


Figure 1 Hailstorm: The typical hailstorm appearance is characterized by indistinct, irregular boundaries, non-homogeneous pattern and hyperechoic formations, with or without dorsal acoustic shadow.

those of the ossification type ( $P < 0.05$ ).

In nine cases (4.9%), the complexity of the sonomorphological appearance or the simultaneous occurrence of characteristics typical for more than one sonomorphological pattern precluded assignment of sonomorphological findings to any one of the defined sonomorphological types in the new classification (Table 1).

Solitary echinococcus foci were by far the most frequent, being observed in 62.7% of cases. Only 13 patients (7%) exhibited more than ten identifiable foci (Table 1). Typical calcifications with dorsal acoustic shadow were visualized ultrasonographically in nearly three-fourths of cases (74.6%). A majority of lesions (61.1%) were localized in the right hepatic lobe compared with only 31.4% in the left hepatic lobe.



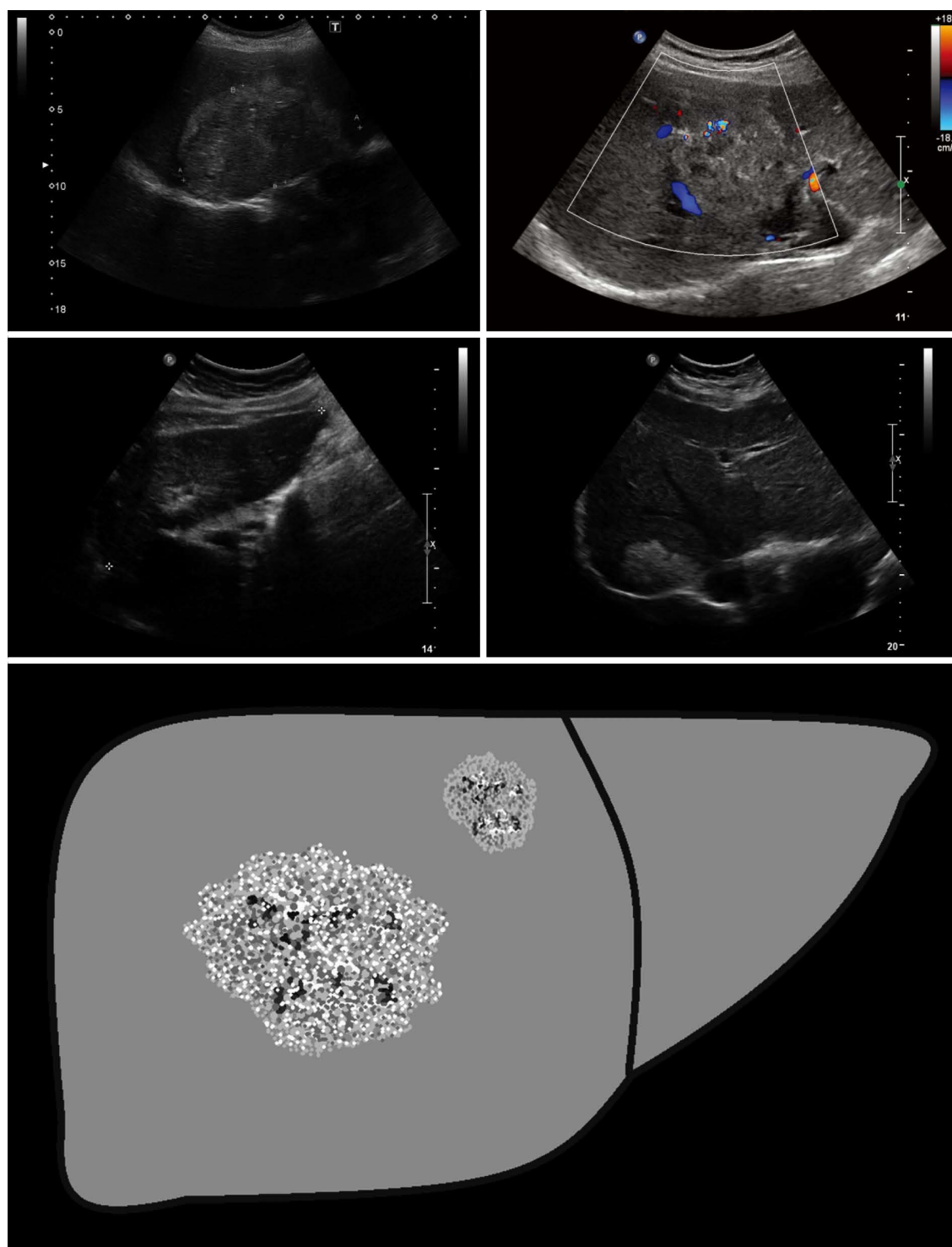
**Figure 2 Pseudocystic:** Pseudocystic alveolar echinococcosis lesions are primarily characterized by an hyperechoic, irregular and non-homogeneous rim that is non-vascularized at power Doppler and color-coded duplex ultrasonography. It may appear to be > 10 mm in thickness. There is a hypo- or anechoic, often non-homogeneous central zone that may contain hyperechoic material. Pseudocystic lesions may be already present at first diagnosis and involve an entire hepatic lobe, or may develop from primary hailstorm lesions following therapy with benzimidazoles.

Echinococcal lesions affecting both hepatic lobes were identified in only 7.6%. Further characteristics and findings are summarized in Table 1.

## DISCUSSION

Alveolar echinococcosis is a rare disease<sup>[14,15,29]</sup>. AE

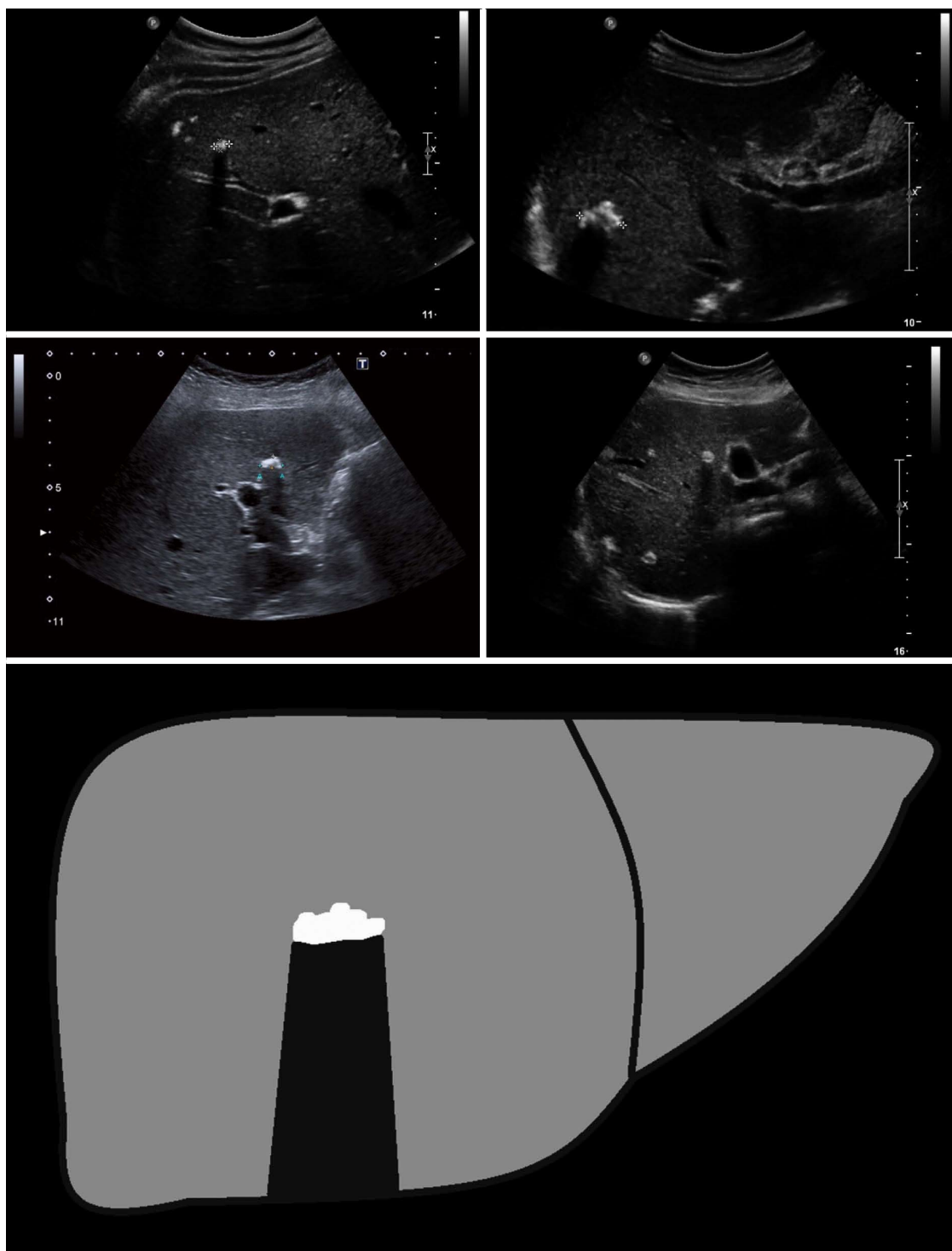
is characterized by destructive growth and exhibits all the characteristics of a malignant disease with infiltration of adjacent organs and formation of distance metastases<sup>[10,15]</sup>. Hence, rapid and definitive diagnosis is essential. Due to the rarity of the disease, however, especially in non-endemic areas, AE presents a significant diagnostic challenge in routine clinical



**Figure 3 Hemangioma-like:** These lesions are difficult to distinguish from atypical (e.g., partially thrombosed) hemangiomas, and often represent a significant diagnostic challenge. Sonomorphologically, the lesions present as a relatively clearly demarcated non-homogeneous tumor that appears hyperechoic in comparison with the surrounding hepatic parenchyma. Echogenicity ranges from slightly and non-homogeneously hyperechoic to strongly and homogeneous hyperechoic.

practice<sup>[30]</sup>. US is the imaging method of choice in the work-up of symptomatic patients and especially as a screening tool<sup>[14,31]</sup>. The widespread use of imaging modalities, such as US, CT and MRI, has led to an increase in the detection of previously unsuspected liver masses in asymptomatic patients<sup>[32]</sup>. These

hepatic incidentalomas in asymptomatic patients are mostly benign and, in most cases, US (including CEUS) will suffice to definitively distinguish them from malignant lesions<sup>[32,33]</sup>. Certain hepatic incidentalomas, such as regenerative nodules, angiomyolipomas of the liver or hepatic AE, however, remain a diagnostic



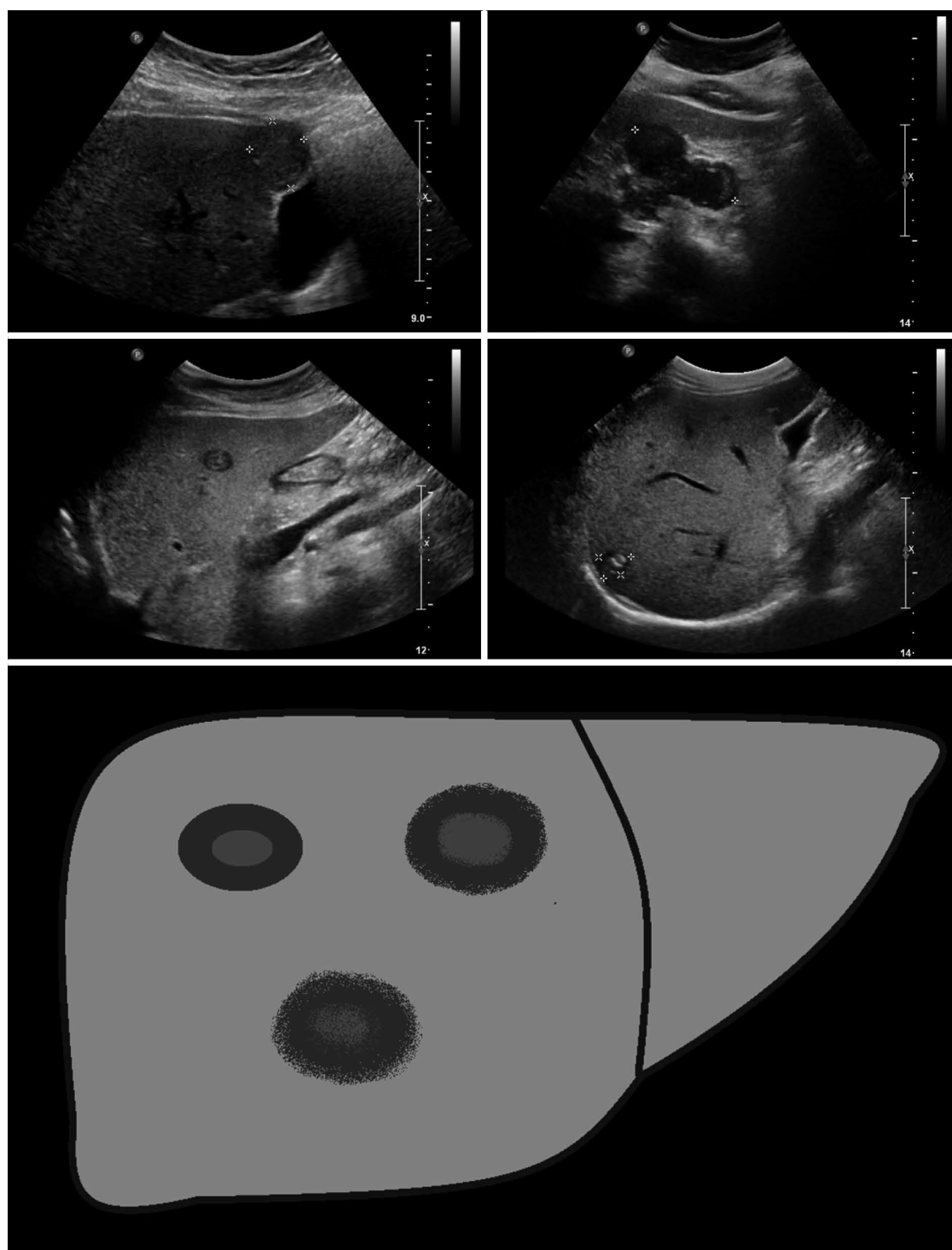
**Figure 4 Ossification:** The ossification pattern presents with solitary or grouped, mostly sharply delineated lesions with dorsal acoustic shadow. In terms of their differential diagnosis, these alveolar echinococcosis (AE) lesions are often difficult to distinguish from inflammatory or hyperechoic metastases of various carcinomas. Very large ossification-type AE lesions represent a rarity. Both uni- and multifocal involvement is possible.

challenge for all imaging modalities<sup>[14,30,34]</sup>. Not infrequently, a final diagnosis is made only upon histopathological examination of material obtained at puncture or resection<sup>[35,36]</sup>.

In the present study population, over 80% of cases corresponded sonomorphologically to the hailstorm, pseudocystic or ossification patterns. These morphologically very characteristic appearances have

already been described by many authors, though not in the context of an ultrasonographic classification<sup>[16,37]</sup>. The so-called "hailstorm" and "pseudocystic" patterns were described as early as 1984 by Didier *et al.*<sup>[37]</sup>. In fact, in their small series of 24 patients, the distribution of the hailstorm and pseudocystic pattern in 62.5% and 12%, respectively, was quite similar to that observed in the present study with 54.1% and 13.5% for the





**Figure 5 Metastasis-like:** Beside the hemangioma-like lesions, the metastasis-like lesions of alveolar echinococcosis represent the greatest diagnostic challenge. Mostly hypoechoic, these lesions exhibit as a typical characteristic-compared to typical hepatic metastases (e.g., of colorectal cancer)-the absence of the halo phenomenon. Instead, there is a central, hyperechoic, non-homogeneous scar.

hailstorm and pseudocystic patterns, respectively<sup>[37]</sup>. Bresson-Hadni *et al.*<sup>[16]</sup> also describe patterns that correspond to our hailstorm and pseudocystic patterns. Taken together, these two forms comprise about 70% of “typical” AE lesions among the lesions studied. The French research group also reported an hemangioma-like pattern as well as a usually small, calcified form of AE lesion (ossification pattern)<sup>[16]</sup>. AE lesions exhibiting

an ossification appearance may present a diagnostic challenge. The differential diagnosis encompasses other hyperechoic, calcified lesions occurring in a wide range of benign, infectious or vascular disorders; with hepatic metastases of colorectal or breast cancer; or metastases of malignant melanomas<sup>[38]</sup>. A metastasis-like appearance for hepatic AE has not previously been described. Unlike typical liver metastases, which

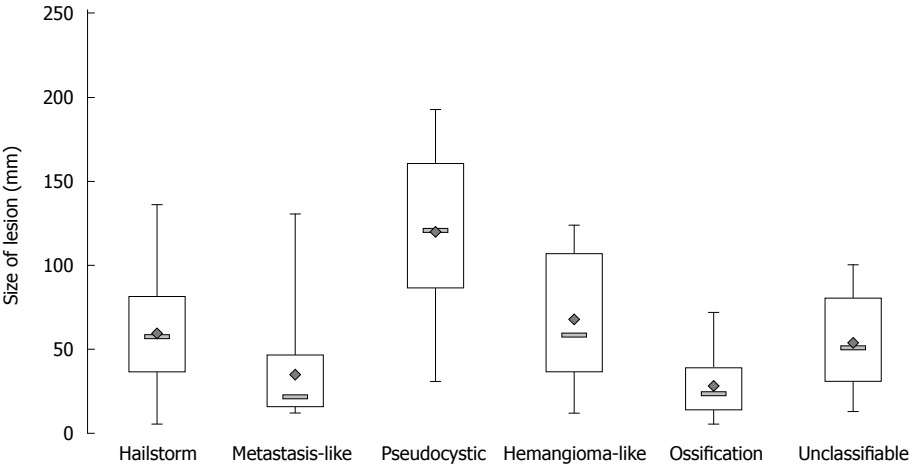


Figure 6 Lesion size depending on the sonomorphological pattern.

Table 1 Patient characteristics n (%)	
Characteristics	mean ± SD
Number of patients	185
Gender	
Female	100 (54.1)
Male	85 (45.9)
Age at diagnosis	51.4 ± 17.6
Age at ultrasonographic examination	58.7 ± 18.2
Sonomorphological classification	
Hailstorm	100 (54.1)
Pseudocystic	25 (13.5)
Ossification	24 (13.0)
Hemangioma-like	15 (8.1)
Metastasis-like	12 (6.5)
Unclassifiable	9 (4.9)
Number of lesions	
1	116
2	24
3	16
4	5
5	7
6-10	4
> 10	13
Mean diameter of the largest lesion	62.5 ± 40.4
Mean lesion diameter according to sonomorphological classification	
Hailstorm	59.6 ± 27.9
Pseudocystic	120.0 ± 47.3
Ossification	28.0 ± 19.4
Hemangioma-like	68.1 ± 37.3
Metastasis-like	35.3 ± 33.1
Unclassifiable	53.9 ± 30.6
Localization of the largest lesion (hepatic lobe)	
Right	113 (61.1)
Left	58 (31.4)
Both	14 (7.6)
Calcification	
No	47 (25.4)
Yes	138 (74.6)
Affected liver segments (multiple segments possible)	
I	6 (3.2)
II	19 (10.3)
III	22 (11.9)
IVa	30 (16.2)
IVb	28 (15.1)
V	44 (23.8)
VI	41 (22.2)

VII	44 (23.8)
VIII	41 (22.2)
Steatosis hepatis	
No	147 (79.5)
Yes	38 (20.5)
Liver size in mid-clavicular line (mm)	146.1 ± 25.2

exhibit an hypoechoic halo, lesions characterized by a metastasis-like appearance may be visualized as a hypoechoic growth without the halo sign or often with a central, hyperechoic scar<sup>[39]</sup>.

In cases with pseudocystic manifestation, especially when the lesion is very large, the differential diagnosis includes liver abscess, cystadenoma or cystic echinococcosis<sup>[14]</sup>. In our series, the pseudocystic lesions were significantly larger than lesions of other sonomorphological types (68.1 ± 37.3 mm, *P* < 0.05).

Since AE is a very rare disease conducting an inter-rater reliability is difficult. The lack of inter-rater reliability remains a limitation of the proposed classification. In the present series, very few hepatic lesions (4.9%) could not be assigned to one of the five sonomorphological patterns. In routine clinical practice, only histopathological confirmation can clarify these unclear hepatic findings<sup>[36]</sup>. Depending on the experience of the pathologist, even the histopathological diagnosis of AE may be difficult. Immunohistochemical examination using Em-specific monoclonal antibodies facilitates a definitive diagnosis even in archived formalin-fixed or paraffin-embedded tissue<sup>[40]</sup>.

In conclusion, ninety-five per cent of cases of hepatic alveolar echinococcosis could be successfully assigned to one of the sonomorphological patterns based on the ultrasonographic classification scheme proposed in the present study. The hailstorm pattern represented the most frequent form, being observed in over 50%. The sonomorphological classification proposed in the present study can facilitate the diagnosis, interpretation, classification and comparison of ultrasonographic findings in patients with alveolar

echinococcosis of the liver, both in routine clinical practice and in the context of scientific studies. The evaluation of different clinical courses (PNM classification) with inclusion of biological markers and other imaging modalities should be investigated in further studies.

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## COMMENTS

### Background

Human alveolar echinococcosis (AE) is the most lethal human helminthic infection and is one of the 17 neglected tropical diseases prioritized by the World Health Organization (WHO). Its incidence is low in endemic regions of Central and Western Europe (0.03-0.05/100000) and high in central Asia. Current studies suggest that the occurrence of alveolar echinococcosis is increasing worldwide and is spreading to previously unaffected regions. Morbidity and treatment costs of the disease are high.

### Research frontiers

Despite the importance of ultrasonography as an image modality in the work-up of hepatic AE, there is no sonomorphological classification of hepatic AE lesions analogous to the WHO's ultrasonographic classification for cystic echinococcosis, which has achieved worldwide acceptance for assessing the activity of that disease.

### Innovations and breakthroughs

Objective of the present study was to establish an ultrasonographic classification based on a large sample of patients with confirmed hepatic AE as a way of facilitating the diagnosis of the disease entity.

### Applications

The sonomorphological classification proposed in the present study can facilitate the diagnosis, interpretation, classification and comparison of ultrasonographic findings in patients with alveolar echinococcosis of the liver, both in routine clinical practice and in the context of scientific studies.

### Peer-review

This is a good study in which the authors introduce a new ultrasound classification for alveolar echinococcosis. The results are interesting and 95% of cases of hepatic alveolar echinococcosis could be successfully assigned to one of the five sonomorphological patterns based on the ultrasonographic classification scheme proposed in the present study. The results of the present study will be important for further research in this field.

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## Retrospective Study

# Surgery in (pre)malignant celiac disease

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## Abstract

**AIM:** To report the outcome of surgery in patients with (pre)malignant conditions of celiac disease (CD) and the impact on survival.

**METHODS:** A total of 40 patients with (pre)malignant conditions of CD, ulcerative jejunitis ( $n = 5$ ) and enteropathy associated T-cell lymphoma (EATL) ( $n = 35$ ), who underwent surgery between 2002 and 2013 were retrospectively evaluated. Data on indications, operative procedure, post-operative morbidity and mortality, adjuvant therapy and overall survival (OS) were collected. Eleven patients with EATL who underwent chemotherapy without resection were included as a control group for survival analysis. Patients were followed-up every three months during the first year and at 6-mo intervals thereafter.

**RESULTS:** Mean age at resection was 62 years. The majority of patients (63%) underwent elective laparotomy. Functional stenosis ( $n = 13$ ) and perforation ( $n = 12$ ) were the major indications for surgery. In 70% of patients radical resection was

performed. Early postoperative complications, mainly due to leakage or sepsis, occurred in 14/40 (35%) of patients. Eight patients required reoperation. More patients who underwent resection in the acute setting ( $n = 3$ , 20%) died compared to patients treated in the elective setting. With a median follow-up of 20 mo, seven patients (18%) required reoperation due to long-term complications. Significantly more patients who underwent acute surgery could not be treated with adjuvant chemotherapy. Patients who first underwent surgical resection showed significantly better OS than patients who received chemotherapy without resection.

**CONCLUSION:** Although the complication rate is high, the preferred first step of treatment in (pre)malignant CD consists of local resection as early as possible to improve survival.

**Key words:** Enteropathy associated T-cell lymphoma; Ulcerative jejunitis; Refractory celiac disease; Celiac disease

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**Core tip:** A small percentage of patients with celiac disease develop (pre)malignant conditions including enteropathy associated T-cell lymphoma. No standardized treatment has been established. Surgery is indicated to relieve clinical symptoms or prevent perforation during chemotherapy. Although the frequency of early- and late post-operative complications is high, local resection is the preferred first step of treatment. Resection is preferred as early as possible after diagnosis since treatment-related mortality seems to rise in the acute setting. Early diagnosis is of utmost importance as elective surgical resection might lower the risk of post-operative mortality and improve overall survival.

van de Water JMW, Nijeboer P, de Baaij LR, Zegers J, Bouma G, Visser OJ, van der Peet DL, Mulder CJJ, Meijerink WJHJ. Surgery in (pre)malignant celiac disease. *World J Gastroenterol* 2015; 21(43): 12403-12409 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12403.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12403>

## INTRODUCTION

Celiac disease (CD) is a common immune-mediated enteropathy affecting approximately 0.6% of individuals in the Western population<sup>[1]</sup>. Enteropathy-associated T-cell lymphoma (EATL) is a very rare peripheral T-cell lymphoma that develops in an estimated 0.04% of adult-onset CD patients<sup>[2]</sup>. In approximately 30%-50%<sup>[3,4]</sup> this is preceded by a state of refractoriness to a gluten-free diet, referred to as refractory celiac disease type II (RCD II). This

disease state is characterized by the occurrence of duodenal T cells with an aberrant phenotype and RCD II is now considered a low-grade lymphoma. Such low-grade lymphomas may also sporadically manifest as ulcerative jejunitis (UJ) with deep ulcerations and stenosis<sup>[5]</sup>.

Due to the rarity of UJ and EATL, no standardized therapeutic regimens have been established for these entities. In general, chemotherapy is the most important factor for improved survival in lymphoma patients<sup>[6]</sup>. Since 2004, this treatment step is usually preceded by surgical resection due to the high risk of bowel perforation or hemorrhage during chemotherapy and to treat symptomatic stenosis or perforation<sup>[6-10]</sup>. In recent years, consolidation therapy with the addition of stem-cell transplantation (SCT) has been successfully applied in some of these patients<sup>[11,12]</sup>. For UJ, no standard treatment is available. The majority of UJ patients are also treated with systemic chemotherapy (cladribine, 2CDA<sup>[13]</sup>) and, depending on stenosis-related symptoms, with resection of the ulcerated segment.

Although surgery is an important first-step in the treatment of both UJ and EATL, the results of this intervention, in terms of outcome, morbidity and mortality, have not been analyzed. Here, we evaluate the indications, resectability, morbidity and mortality in our (pre)malignant CD patients, diagnosed at or referred to our Celiac Center Amsterdam, who underwent surgical intervention. Furthermore, to confirm the additive value of surgery, we compared the outcome of this group with patients who underwent chemotherapy without resection.

## MATERIALS AND METHODS

A total of 40 patients with an established diagnosis of UJ or EATL who underwent surgical resection between January 2002 and November 2013 were included in this analysis. Clinical characteristics, indication for surgery, surgery-related morbidity and mortality and overall survival (OS) were evaluated. Eleven patients with established EATL who underwent chemotherapy without surgical resection were included as a control group for the survival analysis.

### Diagnosis of UJ and EATL

A diagnosis of EATL was histologically established according to the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues<sup>[14]</sup> and was confirmed histologically by a dedicated pathologist.

The diagnosis of UJ was based on the macroscopic appearance of ulcerative lesions using gastro-duodenoscopy, double balloon endoscopy<sup>[15]</sup> and/or video-capsule endoscopy<sup>[16]</sup>.

### Indications and surgical procedure

Both the indication and the need for patients to be either treated in the acute- or in the elective setting

were based on clinical assessment performed by a gastroenterologist and a surgeon. Perforation was defined as the existence of an acute abdomen and/or extraluminal air and mesenteric fatty infiltration seen on computed tomography (CT). During surgery the presence of a perforation was confirmed. When a patient suffered nausea and/or vomiting accompanied by abdominal pain and bowel distension, which was in some cases accompanied by a zone of collapsed bowel seen on CT, the patient was defined as having intestinal stenosis. Surgery in the acute setting was defined as surgical intervention within 24 h after the first presentation. All surgical interventions which occurred after this time-period were defined as elective surgery. A laparoscopic or open-bowel resection was performed depending on localization, the setting (elective/acute) and the preferences of the surgeon performing the surgical intervention. Mobilization and transection of the bowel was performed and the involved segment was resected if possible. Resectability was assessed peri-operatively and defined as radical, partial or unresectable. Radical resection was defined as complete resection of the tumor mass. When some, but not all of tumor mass could be removed it was defined as partial resection. Unresectability was defined as the inability to resect any part of the tumor.

#### **Postoperative morbidity and mortality**

Postoperative patients were either admitted to the intensive care unit (ICU) or the surgical-gastroenterology ward. Data on postoperative hospital stay, morbidity and the need for reoperation were recorded. Morbidity was assessed using early- and late postoperative complications. Early postoperative complications were defined as direct surgery-related complications occurring within 30 d after the initial surgery. When complications occurred after this time-period and showed a direct relation with the initial surgery, these were defined as late postoperative complications. Overall in-hospital and postoperative mortality were documented, the former being defined as mortality due to any cause during the hospital stay. Postoperative mortality was defined as mortality within 30 d after surgery with a direct relation to the operative procedure.

#### **Adjuvant therapy**

Chemotherapy was started within 2-5 wk after resection in both EATL and UJ patients depending on the postoperative condition of the patient. In some cases this was followed by autologous or allogeneic SCT. Detailed information on chemotherapy and SCT will be described elsewhere<sup>[3]</sup>.

#### **Survival outcome**

Follow-up was carried out at 3-mo intervals during the first year and thereafter at 6-mo intervals. Overall

survival (OS) was defined as the time from diagnosis to death. Surviving patients were censored at the time of last follow-up. For the survival analysis, the patients who received chemotherapy without surgical resection were included as a control group.

#### **Statistical analysis**

Continuous variables were compared using the non-parametric Mann-Whitney test. Categorical variables were compared using Fisher's exact test or  $\chi^2$  test. OS was analysed using Kaplan-Meier curves and significance compared using the Log-rank test. *P* values less than 0.05 were considered statistically significant. Statistical analysis was performed using the SPSS software package (SPSS, Chicago, Illinois, United States).

## **RESULTS**

Between January 2002 and November 2013, 40 consecutive patients with UJ or EATL underwent laparoscopic or open resection. Thirty-five patients had EATL (88%) and the remainder had UJ. Mean age at the time of resection was  $62 \pm 7$  years (range: 48-77). Males were overrepresented ( $n = 23$ , 57%). Patient characteristics are described in Table 1.

#### **Indication and surgical procedure**

In the majority of patients the surgical procedure was performed in the elective setting ( $n = 25$ , 63%). Besides local resection in the case of proven EATL to prevent perforation due to chemotherapy ( $n = 11$ ), main indications included stenosis ( $n = 13$ ), perforation ( $n = 12$ ) and others such as sepsis ( $n = 1$ ) or exploratory surgery for diagnostic purposes ( $n = 3$ ). The majority of UJ patients presented with stenosis ( $n = 3$ , 60%), whereas none of the UJ patients presented with perforation. The majority of patients (85%) underwent an open procedure; only six patients (15%) underwent laparoscopic surgery, of which half of the procedures were converted to an open procedure. Radical resection of the tumor mass or the ulcerated segment was performed in 70% of patients. In nine patients (23%) the involved segment was only partially resected due to extensive disease such as vascular or mesenteric root involvement. Three patients were found to be unresectable (9%) during the procedure. The latter two groups consisted of EATL patients. When comparing surgery in the acute setting to the elective setting, patients in the acute setting all suffered from EATL with perforation being a significantly more frequent indication for surgery. Indications and operative procedures are listed in Table 1.

#### **Postoperative morbidity and mortality**

In 10 patients (25%) postoperative ICU-stay was required with a median stay of 9 d (range: 1-37).

**Table 1** Baseline characteristics, indication and operative procedure *n* (%)

Characteristics	Total <i>n</i> = 40	Acute setting <i>n</i> = 15	Elective setting <i>n</i> = 25	<i>P</i> value
Gender M/F	23/17 (57/43)	7/8 (47/53)	16/9 (64/36)	0.28
Mean age (yr) at resection (mean $\pm$ SD, range)	(62 $\pm$ 7, 48-77)	(61 $\pm$ 6, 48-70)	(63 $\pm$ 7, 49-77)	0.25
Diagnosis				0.08
EATL	35 (88)	15 (100)	20 (80)	
UJ	5 (12)		5 (20)	
Indication				< 0.001
Perforation	12 (30)	10 (67)	2 (8)	
Stenosis	13 (32)	4 (27)	9 (36)	
Local resection	11 (28)		11 (44)	
Other	4 (10)	1 (6)	3 (12)	
Procedure				0.25
Open	34 (85)	14 (93)	20 (80)	
Laparoscopic	6 (15)	1 (7)	5 (20)	
Conversion ( <i>n</i> )	<i>n</i> = 3	<i>n</i> = 1	<i>n</i> = 2	0.69
Resected				0.59
Jejunum	27 (67)	9 (60)	18 (72)	
Jejunum and Ileum	7 (18)	3 (20)	4 (16)	
Ileum	4 (10)	2 (13)	2 (8)	
Other	2 (5)	1 (7)	1 (4)	
Median length of resected segment (cm) (range)	35 (9-155)	20 (10-50)	40 (9-155)	0.07
Resectability				0.55
Radical resection	28 (70)	10 (67)	18 (72)	
Partial resection	9 (23)	3 (20)	6 (24)	
Unresectable	3 (7)	2 (13)	1 (4)	

EATL: Enteropathy associated T-cell lymphoma; UJ: Ulcerative jejunitis.

Median hospital stay was ten days (range: 2-63). Early postoperative complications occurred in 14 patients (35%), of which the most important were anastomotic leakage (*n* = 5) and fever/sepsis (*n* = 4). Furthermore, postoperative bleeding (*n* = 2), ileus (*n* = 1), cardiac failure (*n* = 1) and wound dehiscence (*n* = 1) occurred. In eight patients early reoperation was necessary, due to anastomotic leakage (*n* = 5), postoperative bleeding (*n* = 2) or wound dehiscence (*n* = 1). There were no significant differences in early postoperative complications in patients who underwent surgery in the acute setting compared with those who underwent surgery in the elective setting. Postoperative mortality occurred in 3 patients (8%) at a median of 20 d after initial surgery, due to sepsis after anastomotic leakage. This occurred only in patients who underwent surgery in the acute setting (20%; *P* = 0.02). In-hospital mortality occurred in five patients (13%) at a median of 28 d (range: 14-87) after surgery. Besides the postoperative mortality described above, the other two cases were due to progressive EATL (*n* = 2).

With a median follow-up of 14 mo (range: 0.5-125) five patients (13%) experienced long-term complications which required reoperation with a median of 58 mo after initial surgery. Stenosis at the side of the initial anastomosis was the most frequently reported long-term complication (*n* = 4). One patient had anastomotic leakage after stoma reconstruction (*n* = 1). These patients all underwent surgery in the elective setting (20%; *P* = 0.08). There were no

postoperative complications after secondary-surgery and no postoperative mortality. Data on early and late postoperative morbidity and mortality are provided in Table 2.

### Adjuvant therapy

During the median follow-up period of 17 mo (range: 2-146) 28 patients (70%) who underwent surgical resection received adjuvant chemotherapy. Only the patients suitable for an aggressive treatment regimen based on clinical condition and age (< 70 years) received SCT after chemotherapy (*n* = 9, 23%). Long-term follow-up after resection, chemotherapy and SCT will be described elsewhere<sup>[3]</sup>. During adjuvant chemotherapy, one patient died due to a chemotherapy-related complication (pancytopenia-related pneumonia). None of the resected patients experienced perforation as a side effect of the chemotherapy. Twelve patients (30%) were unable to receive adjuvant chemotherapy after initial resection due to poor clinical condition. Comparing patients who underwent surgery in the acute setting with those who underwent surgery in the elective setting, patients who underwent surgery in the elective setting were significantly more able to receive adjuvant chemotherapy (*P* = 0.04) (Table 3).

### Outcome

In total, 26 patients died with an OS of 15 mo. One- and five-year OS in this heterogeneous resected group was 57% and 29%, respectively. Patients who



**Table 2** Early and late postoperative morbidity and mortality *n* (%)

	Total <i>n</i> = 40	Acute setting <i>n</i> = 15	Elective setting <i>n</i> = 25	<i>P</i> value
ICU-stay ( <i>n</i> )	10	5	5	1.0
Median days (range)	9 (1-37)	15 (1-37)	6 (1-28)	0.31
Median hospital stay (d) (range)	10 (2-63)	10 (2-48)	11 (4-63)	1.0
Median follow-up after surgery (mo) (range)	14 (0.5-125)	6 (0.5-125)	16 (1-100)	0.09
Early complications	14 (35)	7 (40)	7 (28)	0.65
Anastomotic leakage	<i>n</i> = 5	<i>n</i> = 3	<i>n</i> = 2	
Fever/sepsis	<i>n</i> = 4	<i>n</i> = 3		
Bleeding	<i>n</i> = 2		<i>n</i> = 2	
Ileus	<i>n</i> = 1		<i>n</i> = 1	
Cardiac failure	<i>n</i> = 1	<i>n</i> = 1		
Wound dehiscence	<i>n</i> = 1		<i>n</i> = 1	
Reoperation necessary	8 (20)	3 (20)	5 (21)	0.44
Late complications,	5 (13)	0 (0)	5 (20)	0.08
Stenosis/ileus	<i>n</i> = 4		<i>n</i> = 4	
Anastomotic leakage after stoma reconstruction	<i>n</i> = 1		<i>n</i> = 1	
Median time after initial surgery (mo)	N.E.		58	
Post-operative mortality	3 (8)	3 (20)	0 (0)	0.02
In-hospital mortality	5 (13)	3 (20)	2 (8)	0.23

N.E.: Could not be estimated.

**Table 3** Postoperative therapy and overall survival *n* (%)

	Total <i>n</i> = 40	Acute setting <i>n</i> = 15	Elective setting <i>n</i> = 25	<i>P</i> value
Treatment				0.04
Resection alone	12 (30)	8 (53)	4 (16)	
Resection and chemotherapy	19 (47)	4 (27)	15 (60)	
Resection, chemotherapy and SCT	9 (13)	3 (20)	6 (24)	
Death	26 (65)	12 (80)	14 (56)	0.12
Overall survival (mo)	15	8	19	0.05
1-yr survival	57%	40%	67%	
5-yr survival	29%	18%	34%	

SCT: Stem-cell transplantation.

underwent surgery in the elective setting showed a significantly better OS of 19 mo compared to 8 mo in patients who underwent surgery in the acute setting. When the outcome of patients who received chemotherapy without surgical resection was compared with patients who underwent surgical resection, the latter group showed a significantly better OS of 14 mo compared to 5 mo in the chemotherapy without resection group ( $P = 0.005$ ). One year survival in these groups was 57% and 18%, respectively. This survival advantage remained after excluding patients who were able to receive SCT (Figure 1).

Two patients (18%) in the chemotherapy without resection group developed bowel perforation during chemotherapy.

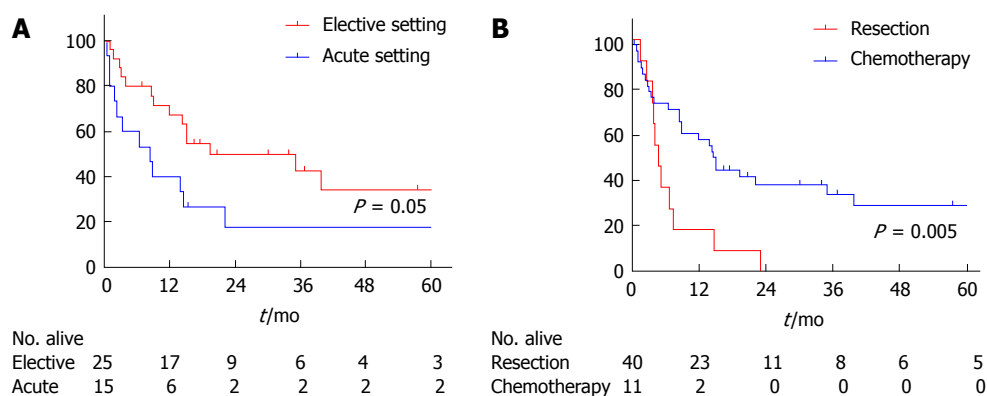
## DISCUSSION

According to our data, surgical resection as the first

step in the treatment of (pre)malignant CD is highly recommended in order to treat symptomatic stenosis or perforation, to prevent perforation during chemotherapy and to improve survival. The present study showed a high incidence of postoperative morbidity which was comparable between patients who underwent resection in the acute and in the elective setting. Postoperative mortality was significantly higher in patients who underwent surgery in the acute setting. In these patients, OS was lower than in those treated in the elective setting. Furthermore, significantly more patients who underwent acute surgery could not be treated with adjuvant chemotherapy.

Small intestinal tumours are rare and generally difficult to detect due to the non-specific nature of symptoms, rare incidence and the need for advanced imaging techniques to detect small-bowel lesions. This may lead to diagnosis at a more advanced stage which may lead to a deteriorated clinical condition in (pre)malignant CD patients. We therefore postulated that acute surgery in these patients is a high risk intervention and may lead to serious complications. However, our data concerning both early and late complications showed no differences when acute and elective surgery was compared. The frequency of both early and late complications was relatively high in both groups which may have been due to a delay in diagnosis in both groups. Hence, patients who underwent resection in the acute setting showed more therapy-related mortality and a less favourable OS compared to patients who underwent elective resection. This stresses the importance of early diagnosis of both UJ and EATL.

In patients who are diagnosed with (pre)malignant CD in a progressive stage of disease, poor clinical



**Figure 1** Kaplan-Meier curve for overall survival of enteropathy associated T-cell lymphoma patients stratified for patients treated in the acute setting and in the elective setting (A) and stratified for patients treated with resection with or without chemotherapy and chemotherapy alone (B). After excluding the patients who underwent stem-cell transplantation.

condition of the patient is partially caused by their poor nutritional status<sup>[17]</sup>. Nutritional status in most EATL patients is very poor as described in one of our recent papers<sup>[18]</sup>. This results in a high risk of surgery-related complications, which could also explain the relatively high rate of early-complications after surgery in our cohort. Moreover, nutritional status is an important factor in the ability to receive adjuvant chemotherapy after resection. Therefore, nutritional status should be meticulously evaluated in all patients at presentation and nutrients should be adequately supplied. Moreover, albumin substitution (> 25 g/L) may play an important role in the preoperative conditioning of EATL and UJ, although no scientific evaluation on albumin infusion in these patients is available<sup>[19]</sup>.

In conclusion, although the frequency of early and late post-operative complications is high, the preferred first step of treatment in (pre)malignant CD patients consists of local resection, preferably as early as possible after diagnosis as treatment-related mortality seems to rise in the acute setting and these patients are less likely to be able to receive chemotherapy. Early diagnosis of both UJ and EATL is of utmost importance as elective surgical resection may lower the risk of post-operative mortality and improve overall survival.

## COMMENTS

### Background

A small percentage of celiac disease (CD) patients develop (pre)malignant conditions including ulcerative jejunitis and enteropathy associated T-cell lymphoma. No standardized treatment has been established. Early diagnosis is of utmost importance as elective surgical resection may lower the risk of post-operative mortality and improve overall survival. Indications for surgery include clinical symptoms or prevention of perforation during chemotherapy.

### Research frontiers

A prospective analysis is important to further evaluate morbidity and mortality in patients treated for (pre)malignant CD.

### Innovations and breakthroughs

In this study we show that resection is important in (pre)malignant CD patients

to improve survival. Optimal treatment, including chemotherapy, is only possible when preceded by surgical resection. Moreover, early diagnosis is important as treatment in the elective setting results in less morbidity and mortality than in the acute setting.

### Applications

The authors advise surgical resection before chemotherapy in patients with (pre)malignant CD to optimize treatment and improve survival.

### Terminology

CD is a common immune-mediated enteropathy triggered by ingestion of gluten in genetically susceptible individuals. Enteropathy associated T-cell lymphoma is a rare peripheral T-cell lymphoma that develops in adult-onset CD patients, mostly preceded by refractoriness to a gluten-free diet.

### Peer-review

This is an interesting paper dealing with a rare complication in a common disease. The paper is well written. The paper focuses on treatment of pre malignant CD and the authors report that both elective and non-elective surgery has a high rate of complications and local de-bulking is the preferred approach.

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## Retrospective Study

# Comparison of selected inflammation-based prognostic markers in relapsed or refractory metastatic colorectal cancer patients

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**Author contributions:** Song A analyzed the data and wrote the draft of manuscript; Eo W suggested the research hypothesis and advised on statistical analyses; Lee S performed the research setup and critically revised the manuscript.

**Institutional review board statement:** This study was reviewed and approved by the Institutional Review Board of Kyung Hee University Hospital at Gangdong (KHNMC-OH-IRB 2013-010).

**Informed consent statement:** Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment before initiation of treatment.

**Conflict-of-interest statement:** We have no conflict of interest to declare.

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## Abstract

**AIM:** To investigate the impact of systemic inflammation-based prognostic markers on overall survival in relapsed/refractory metastatic colorectal cancer (mCRC) patients.

**METHODS:** To investigate prognostic markers in mCRC patients, this study was performed with patients who have experienced relapsed/refractory mCRC with standard chemotherapy or were inapplicable to conventional treatment modality because of poor performance status, age, or comorbidity. We reviewed the medical records of 177 mCRC patients managed with Korean Medicine (KM) treatment modality using an anticancer agent of *Rhus verniciflua* Stokes extract from June 2006 to April 2013. The clinicopathologic characteristics, laboratory test, the systemic inflammation markers including the modified Glasgow prognostic score (mGPS), neutrophil lymphocyte ratio (NLR), platelet lymphocyte ratio (PLR), lymphocyte monocyte ratio (LMR), and prognostic nutritional index (PNI) were analyzed. The overall survival of patients was calculated with the Kaplan-Meier method and the statistical significance was compared using with the log-rank test. To compare the impact of systemic inflammation based markers, the hazard ratio (HR) of mGPS, NLR, PLR, LMR, and PNI for overall survival were evaluated with the Cox proportional hazards regression.



**RESULTS:** The majority of mCRC patients had relapsed/refractory to standard chemotherapy; 128 patients (72.3%) had undergone more than second line chemotherapy, and the median time from diagnosis of mCRC to initiation of KM was 9.4 mo. The median overall survival of enrolled patients was 8.3 mo. On univariate analyses, the inflammation markers of higher mGPS ( $P < 0.001$ ),  $\text{NLR} \geq 5$  ( $P < 0.001$ ),  $\text{PLR} > 300$  ( $P = 0.004$ ),  $\text{LMR} \leq 3.4$  ( $P < 0.001$ ), and  $\text{PNI} \leq 45.3$  ( $P = 0.001$ ) were significantly associated with decreased survival time. On stepwise multivariate proportional hazards model, mGPS at 2 vs 0 (HR = 3.212, 95%CI: 1.437-7.716,  $P = 0.004$ ), and  $\text{LMR} \leq 3.4$  (HR = 1.658, 95%CI: 1.092-2.518,  $P = 0.018$ ) as independent predictors associated with poor overall survival along with carbohydrate antigen 19-9 (HR = 1.482, 95%CI: 1.007-2.182,  $P = 0.046$ ),  $\text{AST} \geq 40$  (HR = 2.377, 95%CI: 1.359-4.155,  $P = 0.002$ ), and the treatment duration for KM less than 2.9 mo (HR = 1.718, 95%CI: 1.160-2.543,  $P = 0.007$ ).

**CONCLUSION:** These results indicate that the inflammatory markers, mGPS and LMR are independent prognostic factors for predicting overall survival in relapsed/refractory mCRC patients.

**Key words:** Colorectal neoplasm; Inflammation; Modified Glasgow prognostic score; Lymphocyte monocyte ratio; Prognosis

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**Core tip:** This is a retrospective study to compare the systemic inflammation based prognostic markers in relapsed or refractory metastatic colorectal cancer (mCRC) patients. We reviewed the medical records of 177 mCRC patients and analyzed the impact of systemic inflammation markers including modified Glasgow prognostic score (mGPS), neutrophil lymphocyte ratio, platelet lymphocyte ratio, lymphocyte monocyte ratio (LMR), and prognostic nutritional index on survival time. The mGPS and LMR were the best inflammatory markers for predicting overall survival time in relapsed or refractory mCRC patients.

Song A, Eo W, Lee S. Comparison of selected inflammation-based prognostic markers in relapsed or refractory metastatic colorectal cancer patients. *World J Gastroenterol* 2015; 21(43): 12410-12420 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12410.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12410>

## INTRODUCTION

Colorectal cancer is the third most common cancer in South Korea, and it is usually diagnosed at an advanced stage<sup>[1]</sup>. The precise prediction of survival

in relapsed/refractory metastatic colorectal cancer (mCRC) patients is important to determine proper management modalities in clinical practice. Over the last couple of decades, there has been a focus on identifying host-related factors, which associated with cancer outcomes. And they have proven to be potential determinants to predict prognosis, guide decisions related to treatment modality, and evaluate treatment efficacy<sup>[2]</sup>. Studies on inflammation markers and host inflammatory responses in cancer have been actively carried out since 2000<sup>[3,4]</sup>.

Systemic inflammation is known to elevate the C-reactive protein (CRP) and change the relative proportion of white blood cells, raising the neutrophil count and lowering the lymphocyte count<sup>[5,6]</sup>. Reflecting the distinct feature of inflammation and cancer, several indices such as the Glasgow prognostic score (GPS), neutrophil lymphocyte ratio (NLR), platelet lymphocyte ratio (PLR), lymphocyte monocyte ratio (LMR), and Onodera's prognostic nutritional index (PNI) have been consistently studied for potential application in cancer prognosis<sup>[7-9]</sup>.

Based on several studies of GPS, hypoalbuminemia without CRP elevation was found to show no significance as a prognostic factor. Thus, recent research has used modified GPS (mGPS) as a potential prognostic factor<sup>[10]</sup>. The mGPS and NLR were studied as independent prognostic factors in advanced colorectal cancer and PLR in pancreatic cancer<sup>[11-14]</sup>. There have also been studies showing a correlation between LMR and prognoses of Hodgkin's lymphoma, and PNI and prognoses of gastrointestinal malignancy<sup>[8,15,16]</sup>.

So far, most studies have focused on the efficacy of inflammatory markers for newly diagnosed advanced cancer patients who were naïve to cancer treatment, and inflammation markers have been used as prognostic markers for the initial treatment, which could be surgery or chemotherapy. However, systemic inflammation is rare in newly diagnosed patients with early conditions of advanced cancers. To enhance the clinical meaning of inflammatory markers as prognostic markers of survival, it is necessary to evaluate far-advanced patients, who relapse after undergoing standard treatments and who show cancer-related symptoms.

Therefore, the aim of this study was to identify the clinical efficacy of systemic inflammation as a prognostic factor to predict overall survival (OS) in mCRC patients who relapsed from standard therapy and to identify which markers from among mGPS, NLR, PLR, LMR, and PNI are useful for identifying patients who should receive palliative care.

## MATERIALS AND METHODS

This study was performed with review of medical records and it was approved by the Institutional Review Board of Kyung Hee University Hospital at

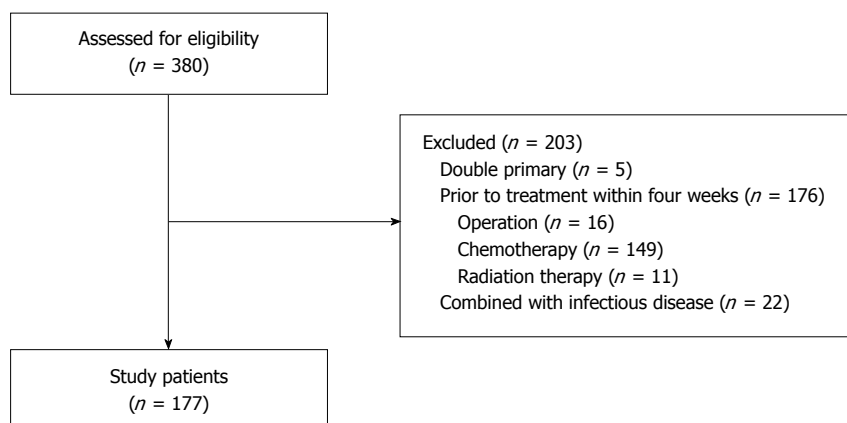


Figure 1 Flow chart of patients' dispositions.

Gangdong (KHNMC-OH-IRB 2013-010).

### Patients

We reviewed the medical records of mCRC patients aged over 20 years who were examined by laboratory tests from June 2006 to April 2013. From 380 cases, we excluded patients with double primary cancers ( $n = 5$ ), and patients who underwent surgery ( $n = 16$ ), chemotherapy ( $n = 149$ ), or radiotherapy ( $n = 11$ ) within four weeks of the initial laboratory examination to avoid the possible influence of treatment modality on inflammation markers. Also, we excluded cases ( $n = 22$ ) that showed comorbidity with specific signs and symptoms of inflammation at the initial examination. Finally, 177 patients with mCRC were included in the study (Figure 1).

### Overall survival

OS was defined as the time from the first day of treatment to the time of death from any cause. When the patient was lost to follow-up or the death was not recorded, the patient was censored. The survival time of censored patients was defined by the period from the initiation of treatment to the last day of follow-up or to September 29, 2013, the date on which the survival was investigated.

### Systemic inflammation markers

Before initiation of treatment, patients were examined for complete blood count with differential and blood chemistry tests including CRP, albumin, tumor markers, and other laboratory tests.

The mGPS was evaluated with CRP and albumin, and it was based on the previous study demonstrating that hypoalbuminemia without elevated CRP has no significant relationship with OS<sup>[10]</sup>. Patients with CRP greater than 10 mg/L and albumin lower than 3.5 g/dL were assigned a score of 2. Patients with only CRP elevation were assigned a score of 1. Patients with a normal value for both CRP and albumin or only low albumin levels were assigned a score of 0. NLR was defined as the absolute neutrophil to absolute

lymphocyte ratio, and PLR was defined as the platelet to absolute lymphocyte ratio. NLR was categorized into two groups based on the cut-off points ( $\geq 5$  or  $< 5$ ), and PLR was classified into three groups ( $< 150$ ,  $150-300$ ,  $> 300$ )<sup>[9]</sup>. LMR was defined as the absolute lymphocyte to absolute monocyte ratio, and PNI was comprised of albumin and lymphocytes. There was no validated cut-off point for the LMR and PNI, so cut-off points of LMR and PNI in this study were presented by the receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC).

### Treatment

The applied treatment modality for relapsed/refractory mCRC patients who had no choice of conventional standard treatment was Korean Medicine (KM). Herbal medication and acupuncture were the major modality for patients underwent KM treatment. The *Rhus verniciflua* stokes (RVS) extract was used as a main anticancer agent. RVS extract was originated from the lacquer tree, which grows in East Asia. RVS has been shown to have an anti-proliferative effect, apoptotic activity, an anti-angiogenic effect, and an anti-tumor migration effect<sup>[17-19]</sup>. RVS was extracted by the standardized method with water at 95 °C, then concentrated and lyophilized in powdered form. After being depleted of toxic allergens, the extract was examined for quality with concentrations of the main compounds. Each capsule contained 500 mg of RVS extract, and patients were typically prescribed 1500 mg of RVS extract daily.

### Statistical analysis

The clinicopathologic features, laboratory tests, and systemic inflammation markers were recorded for the potential prognostic factors. OS was calculated with the Kaplan-Meier method and the statistical significance was compared using the log-rank test. The prognostic factors for survival were identified with the proportional hazards regression. Univariate analysis was performed with each potential prognostic factor and stepwise multivariate proportional hazards

Table 1 Baseline characteristics of patients

Variable		<i>n</i>	%
Clinicopathological factors			
Age (yr)	< 65/≥ 65	123/54	69.5%/30.5%
Sex	Male/female	83/94	46.9%/53.1%
ECOG-PS	0-1/2-4	105/72	59.3%/40.7%
Tumor site	Colon/rectum	125/52	70.6%/29.4%
Liver metastasis	No/yes	69/108	39.0%/61.0%
Prior surgery	No/yes	33/144	18.6%/81.4%
Prior chemotherapy	None/1 <sup>st</sup> line/2 <sup>nd</sup> line/≥ 3 <sup>rd</sup> line	21/28/54/74	11.9%/15.8%/30.5%/41.8%
Prior radiotherapy	No/yes	126/51	71.2%/28.8%
BMI (kg/m <sup>2</sup> )	< 18.5/18.5-22.9/≥ 23	22/88/64	12.6%/50.6%/36.8%
KM treatment duration (mo)	< 2.9/≥ 2.9	89/88	50.3%/49.7%
Laboratory factors			
CEA (ng/mL)	≤ 5/> 5	31/140	18.1%/81.9%
CA19-9 (U/mL)	≤ 27/> 27	59/78	43.1%/56.9%
Hb (g/dL)	> 12.1/≤ 12.1	95/82	53.7%/46.3%
AST (IU/L)	< 40/≥ 40	147/30	83.1%/16.9%
ALT (IU/L)	< 40/≥ 40	157/20	88.7%/11.3%
eGFR (mL/min)	≥ 60/< 60	139/36	79.4%/20.6%
albumin (g/dL)	< 3.5/≥ 3.5	13/164	7.3%/92.7%
CRP (mg/L)	< 10.0/≥ 10.0	114/63	64.4%/35.6%
PLT (× 10 <sup>3</sup> /μL)	< 400/≥ 400	161/16	62.7%/37.3%
WBC (× 10 <sup>3</sup> /μL)	< 10.0/≥ 10.0	161/16	91.0%/9.0%
Neutrophil (%)	≤ 68.2/> 68.2	92/85	52.0%/48.0%
Lymphocyte (%)	≤ 24.3/> 24.3	111/66	62.7%/37.3%
Monocyte (%)	≤ 6.6/> 6.6	59/118	33.3%/66.7%
ANC (cells/μL)	≤ 4505.7/> 4505.7	104/73	58.8%/41.2%
ALC (cells/μL)	≤ 1651.3/> 1651.3	123/54	69.5%/30.5%
AMC (cells/μL)	≤ 460.8/> 460.8	83/94	46.9%/53.1%
mGPS	0/1/2	114/52/11	64.4%/29.4%/6.2%
NLR	< 5/≥ 5	144/33	81.4%/18.6%
PLR	< 150/150-300/> 300	73/78/26	41.2%/44.1%/14.7%
LMR	≤ 3.4/> 3.4	113/64	63.8%/36.1%
PNI	≤ 45.3/> 45.3	52/125	29.4%/70.6%

ECOG-PS: Eastern cooperative oncology group performance status; BMI: Body mass index; KM: Korean Medicine; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; Hb: Hemoglobin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; eGFR: Estimated glomerular filtration rate; CRP: C-reactive protein; PLT: Platelet; WBC: White blood cell; ANC: Absolute neutrophil count; ALC: Absolute lymphocyte count; AMC: Absolute monocyte count; mGPS: Modified Glasgow prognostic score; NLR: Neutrophil to lymphocyte ratio; PLR: Platelet to lymphocyte ratio; LMR: Lymphocyte to monocyte ratio; PNI: Prognostic nutritional index.

models were analyzed with statistically significant factors with *P* value less than 0.05 from univariate analysis for predicting survival. For the precision of model prediction without the complication caused by overlapped factors from univariate analysis, the factors with high priority were entered for multivariate analysis removing the factors with low priority.

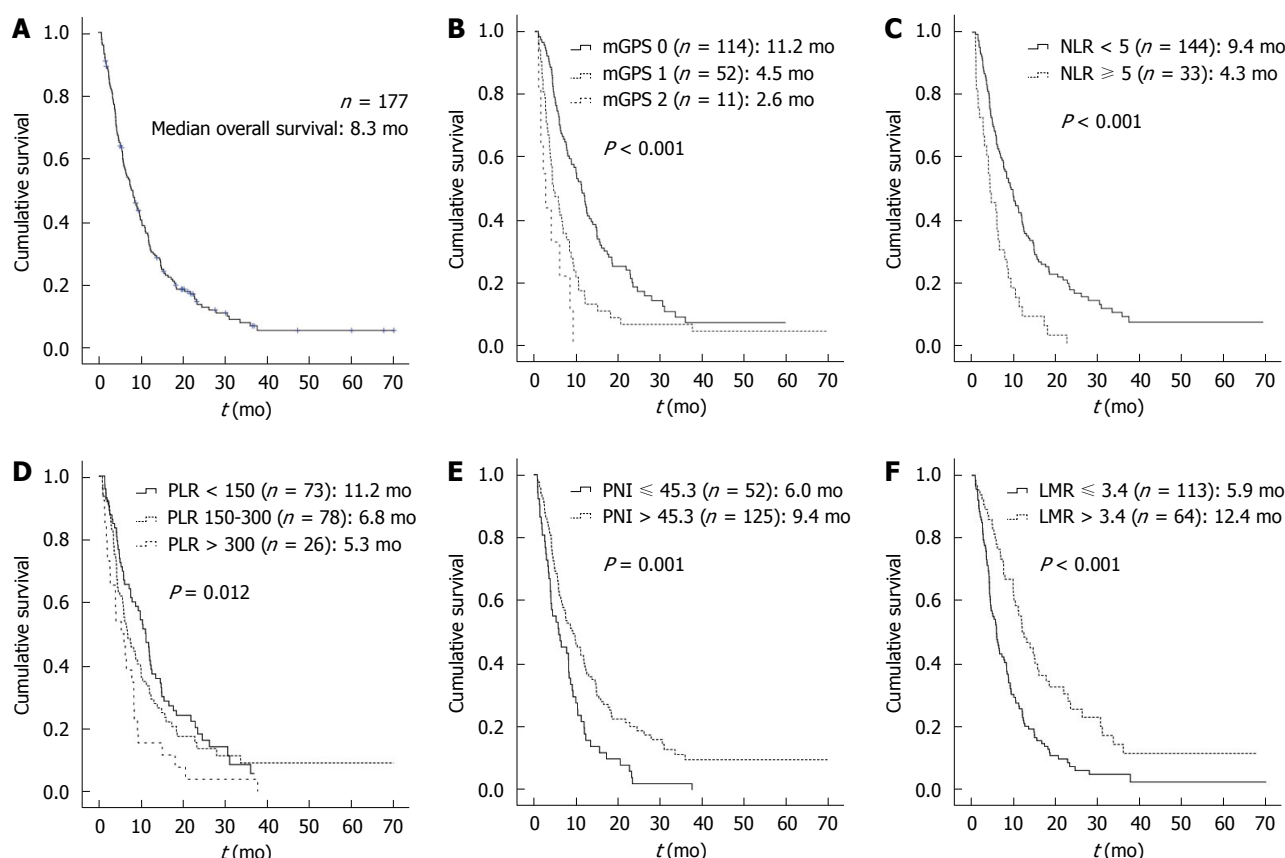
The ROC curve analysis was used to determine the optimal cut-off values of the hemoglobin (Hb), neutrophil%, lymphocyte%, monocyte%, absolute neutrophil count (ANC), absolute lymphocyte count (ALC), absolute monocyte count (AMC), LMR, and PNI. The optimal cut-off values of each factor indicated the discrimination on survival by the maximum joint of sensitivity and specificity. All statistical analyses were conducted using MedCalc Statistical Software (version 12.7.5; MedCalc Software, Ostend, Belgium) and SPSS (version 18.0; SPSS Inc., Chicago, IL, United States). A *P* value less than 0.05 was considered statistically significant.

## RESULTS

The clinicopathological and biochemical characteristics of enrolled patients are summarized in Table 1. Of the 177 colorectal cancer patients, 94 were female (53.1%), the median age was 52 (range, 25-81), and the majority of patients (59.3%) had an ECOG performance status of 0 or 1. There were 125 (70.6%) colon cancer cases and 52 (29.4%) rectal cancer cases, and most patients had liver metastases (61.0%). In total, 171 patients (96.6%) had at least one prior treatment, and 128 patients (72.3%) had experienced more than second line chemotherapy. The median time of KM treatment initiation was 9.4 mo after the diagnosis of mCRC (range, 0.1-81.0 mo).

The median OS was 8.3 mo (range, 0.8-70.0 mo) for all patients (Figure 2). The median follow-up period of patients was 3.1 mo (range, 0.1-33.3 mo), and the median treatment duration of KM was 2.9 mo (range, 0.1-33.3 mo).

The optimal cut-off values, AUCs, and significances



**Figure 2** Overall survival of enrolled patients according to the systemic inflammation markers. Overall survival of total enrolled patients (A), overall survival according to modified Glasgow prognostic score (B), neutrophil lymphocyte ratio (C), platelet lymphocyte ratio (D), prognostic nutritional index (E), and lymphocyte monocyte ratio (F).

of Hb, neutrophil%, lymphocyte%, monocyte%, ANC, ALC, AMC, LMR, and PNI analyzed by ROC curve are shown in Figure 3. The ROC curves demonstrated that LMR of 3.4 was the optimal cut-off for predicting OS (sensitivity = 68, specificity = 62.5, AUC = 0.647,  $P = 0.005$ ) and PNI of 45.3 was the optimal cut-off for predicting OS (sensitivity = 34.0, specificity = 95.8, AUC = 0.559,  $P = 0.256$ ).

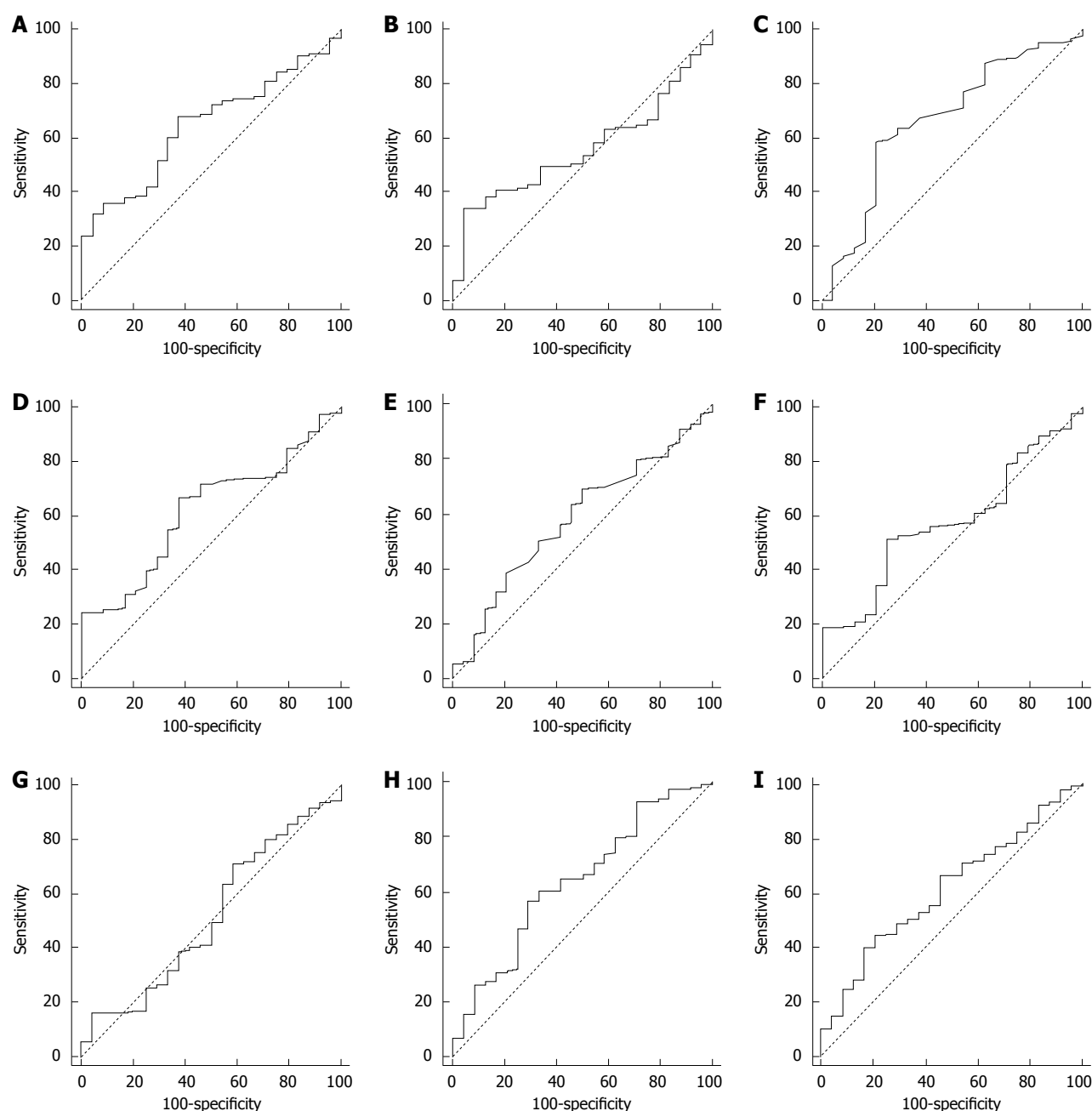
The univariate analysis of the proportional hazards regression was performed to determine predictable factors for multivariable analyses, adjusting for other factors to influence survival. Among the clinicopathological factors, poor ECOG performance status and presence of liver metastasis showed a relationship with poor survival. Regarding biochemical factors, several lab tests showed survival impact, such as tumor markers of carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9), liver enzymes of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), albumin, C-reactive protein (CRP), Hb, neutrophil %, lymphocyte %, ANC, and AMC and treatment duration for KM. The survival of patients was significantly affected by the level of systemic inflammation markers of mGPS, NLR, PLR, and LMR, and the nutrition marker of PNI (Table 2). On mGPS, survival time and hazard were incrementally influenced in accordance with the level of mGPS.

The median OS of mGPS 0, mGPS 1, and mGPS 2 was 11.2 mo, 4.5 mo, and 2.6 mo, respectively ( $P < 0.001$ ). The hazard of mGPS 1 (HR = 1.998, 95%CI: 1.403-2.844) and mGPS 2 (HR = 4.741, 95%CI: 2.418-9.297) increased level-dependently ( $P < 0.001$ ).

The median OS of patients with low NLR (NLR  $< 5$ ) was 9.4 mo, and the high NLR (NLR  $\geq 5$ ) was 4.3 mo ( $P < 0.001$ ), and high NLR (NLR  $\geq 5$ ) reduced the survival period (HR = 2.388, 95%CI: 1.609-3.545,  $P < 0.001$ ). The median OS of patients with low PLR (PLR  $< 150$ ), medium PLR (PLR between 150 and 300), and high PLR (PLR  $> 300$ ) was 11.2 mo, 6.8 mo, and 5.3 mo respectively ( $P = 0.012$ ), and high PLR significantly resulted in poor survival (HR = 1.989, 95%CI: 1.253-3.157,  $P = 0.004$ ), whereas the medium PLR showed no significant influence on survival. The median OS of patients with low LMR (LMR  $\leq 3.4$ ) and high LMR (LMR  $> 3.4$ ) was 5.9 mo and 12.4 mo respectively ( $P < 0.001$ ), and the low LMR group was associated with shortened survival (HR = 2.045, 95%CI: 1.450-2.884,  $P < 0.001$ ). The median OS of patients with low PNI (PNI  $\leq 45.3$ ), and high PNI (PNI  $> 45.3$ ) was 6.0 mo and 9.4 mo respectively ( $P = 0.001$ ), and low PNI was highly associated with a short survival time (HR = 1.785, 95%CI: 1.271-2.507,  $P = 0.001$ ) (Figure 2).

Multivariate analysis using the Cox proportional





**Figure 3** Receiver operating characteristic curves for lymphocyte monocyte ratio (A), prognostic nutritional index (B), hemoglobin (C), neutrophil% (D), lymphocyte% (E), monocyte% (F), absolute neutrophil count (G), absolute lymphocyte count (H), and absolute monocyte count (I) of total enrolled patients. Lymphocyte to monocyte ratio: sensitivity 68.0%, specificity 62.5%, area under the ROC curve (AUC) 0.647, *P* value 0.0048, cut-off 3.4; Prognostic nutritional index: sensitivity 34.0%, specificity 95.8%, AUC 0.559, *P* value 0.2557, cut-off 45.3; Hemoglobin: sensitivity 58.8%, specificity 79.2%, AUC 0.668, *P* value 0.0077, cut-off 12.1g/dL; Neutrophil%: sensitivity 51.6%, specificity 75.0%, AUC 0.578, *P* value 0.1748, cut-off 68.2%. Lymphocyte%: sensitivity 66.7%, specificity 62.5%, AUC 0.620, *P* value 0.0325, cut-off 24.3%. Monocyte%: sensitivity 69.3%, specificity 50.0%, AUC 0.583, *P* value 0.1707, cut-off 6.6%; Absolute neutrophil count: sensitivity 44.4%, specificity 79.2%, AUC 0.614, *P* value 0.0506, cut-off 4505.7; Absolute lymphocyte count: sensitivity 71.2%, specificity 41.7%, AUC 0.516, *P* value 0.8047, cut-off 1651.3; Absolute monocyte count: sensitivity 56.9%, specificity 70.8%, AUC 0.641, *P* value 0.0239, cut-off 460.8.

hazards models with stepwise selection process was performed to identify the independent predictors of survival. The models were analyzed with all significant factors removing overlapped factors. The key potential predictors of hypothesis of this study, mGPS, NLR, PLR, LMR, and PNI of the systemic inflammation markers were calculated with absolute counts of white blood cells and platelet, CRP, and albumin, which was described with detailed formulas in the method section. After

adjusting for confounders, the predictors of survival in the stepwise multivariate proportional hazards model were LMR, mGPS, CA19-9, AST, and treatment duration (Table 3). Among the systemic inflammatory markers, mGPS and LMR were independently associated with survival time in relapsed/refractory mCRC patients. The risk of death was remarkably increased according to the level of mGPS score (*P* = 0.017), the hazard ratio of mGPS 2 (HR = 3.212, 95%CI: 1.437-7.176,

**Table 2 Univariate analyses of factors related to overall survival**

Variable		Hazard ratio (95%CI)	P value
Clinicopathological factors			
Age (yr)	< 65 <i>vs</i> ≥ 65	1.295 (0.922-1.821)	0.136
Sex	Female <i>vs</i> male	0.897 (0.653-1.233)	0.504
ECOG-PS	0-1 <i>vs</i> 2-4	1.528 (1.107-2.109)	0.010
Tumor site	Colon <i>vs</i> rectum	0.823 (0.575-1.177)	0.286
Liver metastasis	No <i>vs</i> yes	1.469 (1.056-2.044)	0.023
Prior surgery	No <i>vs</i> yes	0.797 (0.525-1.211)	0.288
Prior chemotherapy	None <i>vs</i> 1 <sup>st</sup> or 2 <sup>nd</sup> line	0.670 (0.392-1.145)	0.143
	None <i>vs</i> ≥ 3 <sup>rd</sup> line	0.897 (0.526-1.529)	0.689
Prior radiotherapy	No <i>vs</i> yes	0.991 (0.698-1.409)	0.962
BMI (kg/m <sup>2</sup> )	< 18.5 <i>vs</i> 18.5-22.9	0.697 (0.425-1.144)	0.154
	< 18.5 <i>vs</i> ≥ 23	0.700 (0.420-1.169)	0.173
KM treatment duration (mo)	≥ 2.9 <i>vs</i> < 2.9	1.966 (1.425-2.711)	< 0.001
Laboratory factors			
CEA (ng/mL)	≤ 5 <i>vs</i> > 5	1.576 (1.016-2.444)	0.042
CA19-9 (U/mL)	≤ 27 <i>vs</i> > 27	1.654 (1.139-2.402)	0.008
Hb (g/dL)	> 12.1 <i>vs</i> ≤ 12.1	1.721 (1.246-2.378)	0.001
AST (IU/L)	< 40 <i>vs</i> ≥ 40	2.547 (1.678-3.867)	< 0.001
ALT (IU/L)	< 40 <i>vs</i> ≥ 40	1.845 (1.139-2.991)	0.013
eGFR (mL/min)	≥ 60 <i>vs</i> < 60	0.942 (0.634-1.399)	0.766
albumin (g/dL)	≥ 3.5 <i>vs</i> < 3.5	2.202 (1.214-3.993)	0.009
CRP (mg/L)	< 10.0 <i>vs</i> ≥ 10.0	2.202 (1.577-3.076)	< 0.001
PLT (× 10 <sup>3</sup> /μL)	< 400 <i>vs</i> ≥ 400	0.618 (0.367-1.042)	0.071
WBC (× 10 <sup>3</sup> /μL)	< 10.0 <i>vs</i> ≥ 10.0	1.595 (0.945-2.691)	0.080
Neutrophil (%)	≤ 68.2 <i>vs</i> > 68.2	1.576 (1.146-2.168)	0.005
Lymphocyte (%)	> 24.3 <i>vs</i> ≤ 24.3	1.923 (1.369-2.700)	< 0.001
Monocyte (%)	≤ 6.6 <i>vs</i> > 6.6	1.316 (0.931-1.860)	0.120
ANC (cells/μL)	≤ 4505.7 <i>vs</i> > 4505.7	1.832 (1.328-2.527)	< 0.001
ALC (cells/μL)	≤ 1651.3 <i>vs</i> > 1651.3	0.779 (0.549-1.107)	0.164
AMC (cells/μL)	≤ 460.8 <i>vs</i> > 460.8	1.830 (1.325-2.527)	< 0.001
Systemic inflammation markers			
mGPS	0 <i>vs</i> 1	1.998 (1.403-2.844)	< 0.001
	0 <i>vs</i> 2	4.741 (2.418-9.297)	< 0.001
NLR	< 5 <i>vs</i> ≥ 5	2.388 (1.609-3.545)	< 0.001
PLR	< 150 <i>vs</i> 150-300	1.204 (0.849-1.708)	0.297
	< 150 <i>vs</i> > 300	1.989 (1.253-3.157)	0.004
LMR	> 3.4 <i>vs</i> ≤ 3.4	2.045 (1.450-2.884)	< 0.001
PNI	> 45.3 <i>vs</i> ≤ 45.3	1.785 (1.271-2.507)	0.001

ECOG-PS: Eastern cooperative oncology group performance status; BMI: Body mass index; KM: Korean Medicine; CEA: Carcinoembryonic antigen; CA19-9: Carbohydrate antigen 19-9; Hb: Hemoglobin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; eGFR: Estimated glomerular filtration rate; CRP: C-reactive protein; PLT: Platelet; WBC: White blood cell; ANC: Absolute neutrophil count; ALC: Absolute lymphocyte count; AMC: Absolute monocyte count; mGPS: Modified Glasgow prognostic score; NLR: Neutrophil to lymphocyte ratio; PLR: Platelet to lymphocyte ratio; LMR: Lymphocyte to monocyte ratio; PNI: Prognostic nutritional index.

**Table 3 Stepwise multivariate proportional hazards model for predictors of overall survival**

Variable		Hazard ratio (95%CI)	P value
mGPS	0 <i>vs</i> 1	1.135 (0.717-1.797)	0.017
	0 <i>vs</i> 2	3.212 (1.437-7.716)	0.588
LMR	> 3.4 <i>vs</i> ≤ 3.4	1.658 (1.092-2.518)	0.004
CA19-9 (U/mL)	≤ 27 <i>vs</i> > 27	1.482 (1.007-2.182)	0.018
AST (IU/L)	< 40 <i>vs</i> ≥ 40	2.377 (1.359-4.155)	0.002
KM treatment duration (mo)	≥ 2.9 <i>vs</i> < 2.9	1.718 (1.160-2.543)	0.007

mGPS: Modified Glasgow prognostic score; LMR: Lymphocyte to monocyte ratio; CA19-9: Carbohydrate antigen 19-9; AST: Aspartate aminotransferase; KM: Korean Medicine.

$P = 0.004$ ) were increased compared with mGPS 0. The patients with low LMR level showed significantly increased risk of death than patients with high LMR level (HR = 1.658, 95%CI: 1.092-2.518,  $P = 0.018$ ). Of the biochemical markers, the level of CA19-9 and AST were

independent predictors for survival. The high level of CA19-9 (CA19-9 > 27) was associated with increased risk of death (HR = 1.482, 95%CI: 1.007-2.182,  $P = 0.046$ ), and the high level of AST (AST ≥ 40) was significantly associated with poor survival time (HR

= 2.377, 95%CI: 1.359-4.155,  $P = 0.002$ ). Besides, treatment duration less than 2.9 mo was significantly associated with shortened survival time (HR = 1.718, 95%CI: 1.160-2.534,  $P = 0.007$ ).

## DISCUSSION

The systemic inflammation response of the host is one of the representative biomarkers of host-related factors that predict the prognosis of cancer. We investigated the relationship between systemic inflammation markers of GPS, NLR, PLR, LMR, and OS in relapsed/refractory mCRC patients. Besides systemic inflammation markers, the clinicopathologic indexes of the performance status, tumor markers, presence of liver metastasis, and nutritional status were also evaluated to determine their impact on the survival time of mCRC patients.

The mGPS is an inflammation marker comprised of CRP and albumin. A previous study reported that mGPS increases with weight loss and low performance status in colon and lung cancer patients, which seemed to be associated with survival<sup>[20]</sup>. Several studies compared mGPS 0 with mGPS 1-2 or mGPS 0-1 with mGPS 2 for statistical significance<sup>[21,22]</sup>, but mGPS was compared without further grouping in our study. The levels of mGPS were identified as independent prognostic factors for survival. Specifically, patients with a score of mGPS 2 were significantly associated with poor survival, and this result was due to the fact that albumin was one of the highly associated nutritional markers. A high mGPS score before initiation of treatment can predict a poor survival outcome, which may influence the choice of an appropriate treatment modality.

In the tumor microenvironment, it is known that neutrophils affect proliferation, angiogenesis, and metastases by interacting with tumor cells producing cytokines and chemokines<sup>[23,24]</sup>. Also, there has been a study reporting that elevated neutrophil and monocyte counts are associated with poor survival in metastatic melanoma<sup>[25]</sup>. Lymphocytes play a key role in human immunity. Lymphopenia is frequently observed in advanced cancer patients, and the decrease in lymphocyte count is strongly associated with a poor prognosis of progression-free survival and OS in advanced cancer patients<sup>[26]</sup>. Our study demonstrated that an increased percentage of neutrophils and monocytes and a decreased percentage of lymphocytes were related to a short OS. LMR has been studied in Hodgkin's lymphoma patients, and this study indicated that it is a possible prognostic factor along with International Prognostic Score<sup>[7]</sup>. In this study, LMR was found to be an independent prognostic factor for mCRC. Chua *et al.*<sup>[11]</sup> reported that NLR before chemotherapy is an independent prognostic factor of survival in mCRC, but in this study NLR showed an impact on survival only with univariate analysis. PLR was also assessed for its relationship with survival, and

it did not show any impact on survival as shown in the previous study<sup>[27]</sup>.

We also evaluated whether PNI could be an effective variable for survival in mCRC patients. Until recently, PNI has been studied for gastric cancer patients who underwent a gastrectomy, and the OS of those patients was mostly dependent on the nutritional status and the immune system<sup>[8,15]</sup>. Few studies have researched the significance of PNI as a prognostic factor for survival in mCRC.

The efficacy of CA19-9 as a prognostic factor of survival in the previous studies has been reported with diverse outcomes according to the cut-off value of the study<sup>[22,28]</sup>. The optimal cut-off value of CA19-9 of this study was 27 U/mL by the reference value, and it was an independent prognostic marker for survival.

To evaluate the relationship between survival and hepatic or renal function, AST, ALT, and eGFR were also analyzed with the proportional hazards regression. Fahmueller *et al.*<sup>[29]</sup> reported that elevated AST and CRP before selective internal radiation therapy in CRC patients with liver metastases were prognostic factors for survival, and concluded that liver tissue damage before treatment and treatment-induced ischemia resulted in poor survival of CRC patients. In this study liver metastasis was shown in 108 patients and the elevations of AST and ALT were observed in 30 and 20 patients, respectively. The liver metastasis and elevated levels of AST and ALT from univariate analysis showed strongly associated with the short survival time (Table 2).

Based on the results of univariate analyses, the factors that showed a significant effect on OS were ECOG performance status of patients, the presence of liver metastasis, tumor markers CEA and CA19-9, the Hb, AST, and ALT in serum, and the nutrition index of PNI, as well as measured systemic inflammation markers of mGPS, NLR, PLR, and LMR. Interestingly all the systemic inflammation markers were revealed to have significant associations on survival time in univariate analysis. On the basis of this result, we could verify that the survival time of mCRC patients was strongly associated with systemic inflammation.

On multivariate analysis using Cox proportional hazards model in forward and backward stepwise manners, the same predictors from analysis results were obtained that mGPS and LMR of systemic inflammation markers, CA19-9, AST, and treatment duration were independent prognostic predictors for survival time. The prognostic factors to predict survival time in relapsed/refractory mCRC patients were mGPS and LMR to reflect the systemic inflammatory condition of host, CA19-9 of colorectal cancer marker, AST of liver enzyme to indicate the hepatic function. Regarding the treatment duration of KM, the prolongation of survival was seemed to be associated with the length of treated time.

Most of studies on mCRC patients have focused on the prognosis of initial treatment after diagnosis.

The standard regimen of chemotherapy on mCRC is FOLFOX, FOLFIRI, CapeOX, and FOLFOXIRI with the addition of biologic agents such as bevacizumab, cetuximab, and panitumumab in the case of patients who were appropriate for intensive care<sup>[30]</sup>. Cancer patients often consider another approach for management after experiencing a relapse in spite of conventional treatment. Patients who can't undergo radical treatment because of age, performance status, and/or comorbidities also consider cancer management based on traditional medicine in East Asia. In this study, we aimed to focus on relapsed/refractory mCRC patients. The majority, 72.3% of enrolled patients, had received our management after undergoing second- or third-line chemotherapy. It took 9.4 mo to initiate our management after diagnosis of mCRC, demonstrating the status of enrolled patients. Therefore, this study result could be a guide for mCRC patients who relapsed with standard therapy.

Cancer management in East Asian Medicine is focused on host-based treatment to strengthen energy through Qi and Blood, which is the same viewpoint of palliative care, an approach to improve the quality of life in physical, psychosocial, and spiritual ways through the relief of symptoms, pain, and other problems<sup>[31]</sup>.

The anticancer agent of this study, RVS, has been traditionally used since the 15<sup>th</sup> century in Korea for the treatment of abdominal masses because of its reported ability to break up hardness. There has been evidence for an anticancer effect of RVS based on reported anti-proliferative and apoptotic activity in various cancer cells of lymphoma, osteosarcoma, breast cancer, and hepatoma<sup>[17,32-36]</sup>. Furthermore, RVS inhibited the cancer cell migration mediated by matrix metalloproteinases, especially MMP-2 and MMP-9, in human fibrosarcoma cells<sup>[19]</sup>. RVS has also been shown to have antitumor activities involving inhibition of proliferation and migration of human umbilical vein endothelial cells induced by VEGF<sup>[17]</sup>. RVS has been widely used for advanced cancer patients who do not have any further choice in the conventional standard therapies. The previous study on RVS in mCRC patients reported a median OS of 10.9 mo in 2009<sup>[37]</sup>.

This study is limited by the retrospective review of medical records. Another limitation was the relatively short duration of median treatment and follow-up periods. However, considering that enrolled patients had relapsed mCRC after undergoing second line chemotherapy in 30.5% of cases and third line chemotherapy in 41.8% of cases, the treatment duration of 3 mo based on clinical practice was hardly regarded as short term. We identified useful prognostic factors at the initial treatment point. With regard to far-advanced mCRC patients, evaluating repeated points of inflammation markers during management could lead to more confident results<sup>[11,38]</sup>.

In the management of cancer patients, determination

of prognosis could be continuously required to make decisions when undergoing adjuvant therapy, to consider the adverse events of chemotherapy, to apply optimal palliative care, and to determine the best time to initiate supportive care. Especially host-based studies of cancer are becoming a more important issue in various points of cancer management, and research on host-based factors at various cancer will develop to prolong survival and to improve quality of life in cancer treatment. The results of this study could be applied to clinical judgments regarding survival prognosis for relapsed/refractory mCRC patients.

## COMMENTS

### Background

The systemic inflammatory response has been recognized to associate with the progression in cancer patients. In clinical practice C-reactive protein and white blood cell counts has been measured for the assessment of systemic inflammation.

### Research frontiers

Recently, the identification of prognostic biomarkers to predict overall survival and benefit of therapeutic modality is one of hot issues in cancer practice because the prediction on prognosis for survival enables to decide the treatment goal and to determine the treatment modality.

### Innovations and breakthroughs

The value of this study was to compare all the available systemic inflammation markers, modified glasgow prognostic score (mGPS), neutrophil lymphocyte ratio, platelet lymphocyte ratio, lymphocyte monocyte ratio, and prognostic nutritional index in metastatic colorectal cancer (mCRC) patients. Among available markers, the mGPS and lymphocyte monocyte ratio were the strongest prognostic factors to predict overall survival in mCRC patients. Especially, the prediction on survival in relapsed or refractory mCRC patients is important issue to determine the cancer treatment modality. There was none study to compare the systemic inflammation markers for relapsed or refractory mCRC patients.

### Applications

Based on the result of this study, lymphocyte monocyte ratio (LMR) was the powerful prognostic factors than well-known other systemic inflammation markers. LMR showed consistent significance on the prediction for survival after adjusting other markers. Calculating just with the counts of lymphocyte and monocyte, LMR could be easily applied for the survival predictor in clinical practice. The level of mGPS is also a significant predictor of overall survival, especially mGPS 2 indicates the poor survival outcome.

### Peer-review

Song *et al* reported, in 177 mCRC patients, the correlation between systemic inflammation-based prognostic markers and overall survival. The authors demonstrated the prognosis value of modified Glasgow Prognostic Score, Lymphocyte Monocyte ratio, carbohydrate antigen 19-9, and aspartate aminotransferase in stepwise modeling of multivariate proportional hazards regression. Patients were mostly refractory mCRC. The strength of this study is to have excluded patients with chemotherapy within 4 wk what could skew the biological results. The results can help decision making in refractory mCRC and selected patients for other chemotherapy regimen or best supportive care. The results are well analyzed and described. All these prognostic markers can be easily incorporated in everyday practice work.

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## Retrospective Study

# Relationship between virological response and FIB-4 index in chronic hepatitis B patients with entecavir therapy

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## Abstract

**AIM:** To investigate whether long-term low-level hepatitis B virus (HBV) DNA influences dynamic changes of the FIB-4 index in chronic hepatitis B (CHB) patients receiving entecavir (ETV) therapy with partial virological responses.

**METHODS:** We retrospectively analyzed 231 nucleos(t)ide (NA) naïve CHB patients from our previous study (NCT01926288) who received continuous ETV or ETV maleate therapy for three years. The patients were divided into partial virological response (PVR) and complete virological response (CVR) groups according to serum HBV DNA levels at week 48. Seventy-six patients underwent biopsies at baseline and at 48 wk. The performance of the FIB-4 index and area under the receiver operating characteristic (AUROC) curve for predicting fibrosis were determined for the patients undergoing biopsy. The primary objective of the study was to compare the cumulative probabilities of virological responses between the two groups during the treatment period. The secondary outcome was to observe dynamic changes of the FIB-4 index between CVR patients and PVR patients.

**RESULTS:** For hepatitis B e antigen (HBeAg)-positive patients ( $n = 178$ ), the cumulative probability of achieving undetectable levels at week 144 was 95%

and 69% for CVR and PVR patients, respectively ( $P < 0.001$ ). In the Cox proportional hazards model, a lower pretreatment serum HBV DNA level was an independent factor predicting maintained viral suppression. The cumulative probability of achieving undetectable levels of HBV DNA for HBeAg-negative patients ( $n = 53$ ) did not differ between the two groups. The FIB-4 index efficiently identified fibrosis, with an AUROC of 0.80 (95%CI: 0.69-0.89). For HBeAg-positive patients, the FIB-4 index was higher in CVR patients than in PVR patients at baseline ( $1.89 \pm 1.43$  vs  $1.18 \pm 0.69$ ,  $P < 0.001$ ). There was no significant difference in the reduction of the FIB-4 index between the CVR and PVR groups from weeks 48 to 144 ( $-0.11 \pm 0.47$  vs  $-0.13 \pm 0.49$ ,  $P = 0.71$ ). At week 144, the FIB-4 index levels were similar between the two groups ( $1.24 \pm 0.87$  vs  $1.02 \pm 0.73$ ,  $P = 0.06$ ). After multivariate logistic regression analysis, a lower baseline serum HBV DNA level was associated with improvement of liver fibrosis. In HBeAg-negative patients, the FIB-4 index did not differ between the two groups.

**CONCLUSION:** The cumulative probabilities of HBV DNA responses showed significant differences between CVR and PVR HBeAg-positive CHB patients undergoing entecavir treatment for 144 wk. However, long-term low-level HBV DNA did not deteriorate the FIB-4 index, which was used to evaluate liver fibrosis, at the end of three years.

**Key words:** Chronic hepatitis B; Hepatitis B virus DNA; Entecavir; Partial virological response; Liver fibrosis; FIB-4 index

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**Core tip:** Long-term entecavir therapy for patients with chronic hepatitis B (CHB) can result in histological improvement and regression of fibrosis substantially. However, the relationship between the serum low level hepatitis B virus (HBV) DNA and fibrosis is unclear for CHB patients with partial virological response to entecavir. Our study found that although the cumulative probabilities of HBV DNA response showed a significant difference between hepatitis B e antigen-positive CHB patients with complete virological response and partial virological response to 144 wk of entecavir treatment, long-term low level HBV DNA did not deteriorate the FIB-4, which was used to evaluate liver fibrosis, by the end of three years.

Li N, Xu JH, Yu M, Wang S, Si CW, Yu YY. Relationship between virological response and FIB-4 index in chronic hepatitis B patients with entecavir therapy. *World J Gastroenterol* 2015; 21(43): 12421-12429 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12421.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12421>

## INTRODUCTION

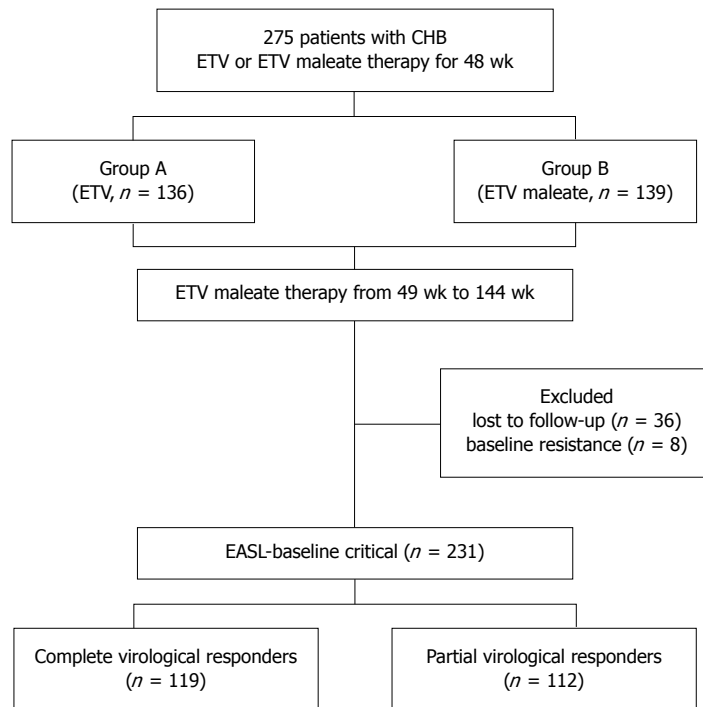
In the Asia-Pacific region, approximately 75 million people die annually from end stage liver diseases caused by hepatitis B virus (HBV)<sup>[1]</sup>. Liver fibrosis and cirrhosis are generally the inevitable and irreversible stages of end stage liver diseases. A large prospective cohort study (REVEAL-HBV study) in Taiwan and several case-control studies have revealed that progression to cirrhosis in chronic hepatitis B (CHB) patients is closely related to the level of circulating virus<sup>[2,3]</sup>. In addition, improvement in histological grade is strongly associated with a decrease in serum HBV DNA levels<sup>[4,5]</sup>. Therefore, HBV replication can be suppressed in a sustained manner, defined as a reduction of serum HBV DNA to undetectable levels, which is the ultimate goal for CHB treatment.

Entecavir (ETV) and tenofovir disoproxil fumarate (TDF) have been suggested as the first-line nucleos(t)ide (NA) therapy for CHB. TDF has not been widely used in China because it only became available for CHB treatment in 2014. Thus, ETV is the preferred first-line agent in patients with NA-naïve CHB<sup>[6]</sup>. Given that early HBV DNA responses to therapy can strongly predict the results of long-term virological responses, modification of the treatment schedule is widely recommended for patients with partial virological response (PVR)<sup>[7]</sup>. However, in contrast to the traditional guidelines, it has been suggested that it is unnecessary to modify antiviral therapy (ETV) in PVR patients, especially if the viral load at week 48 is low<sup>[8]</sup>. Long-term entecavir therapy for patients with CHB can cause histological improvement and regression of fibrosis<sup>[9,10]</sup>. However, the relationship between low serum levels of HBV DNA and fibrosis is unclear for CHB patients with PVR to ETV.

Liver biopsy is the gold standard in assessing liver fibrosis in CHB patients<sup>[11,12]</sup>. However, liver biopsies have limitations of invasiveness, high expense and high sampling error. Thus, observing dynamic changes in liver fibrosis is restricted. Non-invasive tests for the evaluation of liver fibrosis have the advantages of simplicity and repeatability. Although non-invasive tests as an alternative to liver biopsies are restricted either by the cost of the device or by the need for a specific laboratory examination, some of the non-invasive tests have not been proven for the evaluation of fibrosis in CHB<sup>[13]</sup>. The FIB-4 index, a simple non-invasive test, combines age, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and platelet (PLT) to identify HBV-related fibrosis with a moderate sensitivity<sup>[13]</sup>. Therefore, both liver biopsies and the FIB-4 index were used to assess liver fibrosis in this study.

The goal of this study was to assess whether persistence of low-level viral replication influences the regression of liver fibrosis in CHB patients with PVR as determined by the FIB-4 index.





**Figure 1** Flow chart illustrating patient selection and categorization based on EASL. CHB: Chronic hepatitis B; ETV: Entecavir; CVR: Complete virological response; EASL-baseline critical: HBV DNA levels at week 48 in patients treated by entecavir.

## MATERIALS AND METHODS

### Study population

We retrospectively analyzed the clinical data for 275 CHB patients from our previous study from 2008 to 2014 (NCT01926288), which was a randomized, double-blind, double-dummy, controlled, multi-center study<sup>[14,15]</sup>. All patients were hepatitis B surface antigen (HBsAg)-positive for at least 6 mo and NA-naïve prior to ETV treatment. Exclusion criteria were as follows: co-infection with human immunodeficiency virus or hepatitis C virus, liver cirrhosis, and hepatocellular carcinoma. Patients were randomly assigned to receive 48 wk of treatment with 0.5 mg/d ETV (group A;  $n = 136$ ) or 0.5 mg/d ETV maleate (group B;  $n = 139$ ). After 48 wk of treatment, ETV maleate showed similar efficacy and safety profiles as entecavir. Therefore, all patients were given ETV maleate treatment after 48 wk. Forty-four patients were excluded from the study due to loss to follow-up ( $n = 36$ ) and baseline resistance ( $n = 8$ ). A total of 231 patients were eligible, and the ETV maleate treatment was continued for three years (Figure 1). According to the European guideline, the 231 patients were divided into PVR and complete virological response (CVR) groups. The definition of CVR to entecavir was HBV DNA being undetectable at week 48, and PVR to entecavir was defined as a  $> 1$  log decline in HBV DNA from baseline but a detectable viral load at week 48<sup>[6]</sup>.

The study was conducted in compliance with the Declaration of Helsinki and in accordance with Good Clinical Practice guidelines. The study was approved by

the Institutional Review Board of Peking University First Hospital. Informed consent was obtained from each patient enrolled in the study. All patients undergoing liver biopsy signed an informed medical consent form.

### Method for response monitoring

All patients were prospectively monitored every 3 mo. Serum ALT, AST and PLT levels were tested locally with standard laboratory procedures. Serologic marker analyses, including HBsAg, anti-HBsAg, hepatitis B e antigen (HBeAg) and anti-HBe, were measured by commercially available enzyme immunoassays (Abbott Laboratories, Chicago, IL). Serum HBV DNA was determined by a commercially available real-time polymerase chain reaction assay (Cobas Ampliprep/Cobas Taqman) with a lower limit of detection of 20 IU/mL.

### Histologic assessment of liver fibrosis

Percutaneous liver biopsies were performed on 76 of the 231 participants (33%) at baseline and at 48 wk. All liver biopsies were obtained by the local doctor, and liver histology was determined *via* the Ishak score as assessed by two experienced pathologists who were blinded to the patient details. All patients were grouped into two fibrosis stages: mild and moderate (Ishak fibrosis scores 0-3) and advanced (Ishak fibrosis scores 4-6)<sup>[16]</sup>.

### Indirect markers of fibrosis

FIB-4 index was determined according to the following published formula<sup>[17,18]</sup>: FIB-4 index = Age (years)  $\times$

**Table 1** Baseline characteristics and virological responses at week 48 between groups A and B

	All patients ( <i>n</i> = 231)	Group A ( <i>n</i> = 112)	Group B ( <i>n</i> = 119)	<i>P</i> value
Age (yr)	32.5 ± 10.1	32.6 ± 9.78	32.5 ± 10.4	0.96
Male (%)	172 (74.4)	83 (74.1)	89 (74.8)	0.99
ALT (IU/L)	113 ± 115	113 ± 117	113 ± 116	0.75
AST (IU/L)	64 ± 74	65 ± 75	62 ± 67	0.74
PLT (× 10 <sup>9</sup> /L)	167.7 ± 57.3	170.0 ± 53.9	165.7 ± 60.5	0.56
HBeAg-positive (%)	178 (77.0)	89 (79.5)	89 (74.8)	0.43
HBV DNA at baseline (log <sub>10</sub> IU/mL)	7.36 ± 1.20	7.38 ± 1.15	7.35 ± 1.25	0.11
HBV DNA at week 48 (log <sub>10</sub> IU/mL)	1.81 ± 0.85	1.74 ± 0.83	1.88 ± 0.87	0.08
HBV DNA-negative (< 20 IU/mL)	111 (48.1)	54 (48.2)	56 (47.1)	0.86
HBeAg seroconversion in HBeAg-positive patients	19 (8.2)	10 (8.9)	9 (7.6)	0.71
ALT normalization	176 (76.2)	85 (75.9)	91 (76.5)	0.91

Group A patients were treated with 0.5 mg/d ETV. Group B patients were treated with 0.5 mg/d ETV maleate. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelet; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

AST (IU/L)/platelet count (10<sup>9</sup>/L) × ALT (IU/L)<sup>1/2</sup>. The values of the FIB-4 index were compared with the Ishak fibrosis scores. A receiver operating characteristic (ROC) curve for the FIB-4 index to assess liver fibrosis was then developed in 152 person-times under biopsy. Using the previously published cut-off for the FIB-4 index, patients were placed into two classes (FIB-4 index ≤ 1.45 and FIB-4 index > 1.45)<sup>[19]</sup>.

### Study endpoints

The primary objective was to compare the cumulative probabilities of the virological responses in the two groups during the on-treatment follow-up period. The secondary objective was to compare dynamic changes of the FIB-4 index between CVR and PVR patients.

### Statistical analysis

The cumulative probabilities of the virological responses (HBV DNA < 20 IU/mL) were evaluated using Kaplan-Meier analysis. Cox regression analysis was used to determine which of the following baseline factors were associated with complete virological responses to ETV: age, sex, HBeAg status, viral load, ALT/AST level and FIB-4 index. ROC curve analysis was used to evaluate the diagnostic accuracy of the FIB-4 index in predicting the extent of fibrosis (Ishak scoring system). All statistical tests were two-sided, and *P* < 0.05 was considered statistically significant. SPSS version 13.0 (SPSS, Chicago, IL, United States) was used for all statistical analyses.

**Table 2** Baseline characteristics of all populations with respect to hepatitis B virus DNA response at week 48

	Virological response at week 48		<i>P</i> value
	CVR ( <i>n</i> = 119)	PVR ( <i>n</i> = 112)	
Age (yr)	34.9 ± 10.9	30.2 ± 8.8	< 0.001 <sup>1</sup>
Male (%)	78 (70.3)	94 (80.8)	0.16
ALT (IU/L)	120 ± 130	108 ± 88	0.05
AST (IU/L)	78 ± 87	57 ± 41	0.001 <sup>1</sup>
PLT (× 10 <sup>9</sup> /L)	160 ± 55	175 ± 58	0.07
HBeAg-positive (%)	71 (64)	107 (89)	< 0.001 <sup>2</sup>
HBV DNA (log <sub>10</sub> IU/mL)	6.88 ± 1.23	7.78 ± 0.92	< 0.001 <sup>2</sup>
Group A (%)	55 (50)	57 (47.5)	0.75
Genotype			0.77
B	56 (51.4)	53 (48.6)	
C	65 (58.5)	57 (47.5)	
FIB-4 index	2.01 ± 1.43	1.21 ± 0.76	0.003 <sup>1</sup>

<sup>1</sup>This difference was no longer significant after multivariate logistic regression analysis; <sup>2</sup>The two groups still showed a significant difference after multivariate logistic regression analysis. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelet; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

## RESULTS

### Baseline characteristics of the study population

The baseline characteristics and virological responses of the study population treated with ETV or ETV maleate are shown in Table 1. Of the 231 subjects in this study, 74.4% were male, and the mean age was 32.5 years. The mean HBV DNA level was 7.36 log<sub>10</sub> IU/mL, the mean ALT level was 152.0 IU/L, and 77.0% of patients were HBeAg-positive. At week 48, there were no significant differences in HBV DNA level, normalization of ALT and HBeAg loss among HBeAg-positive patients between groups A and B. Due to the efficacy of ETV maleate, all patients were treated with ETV maleate from week 48.

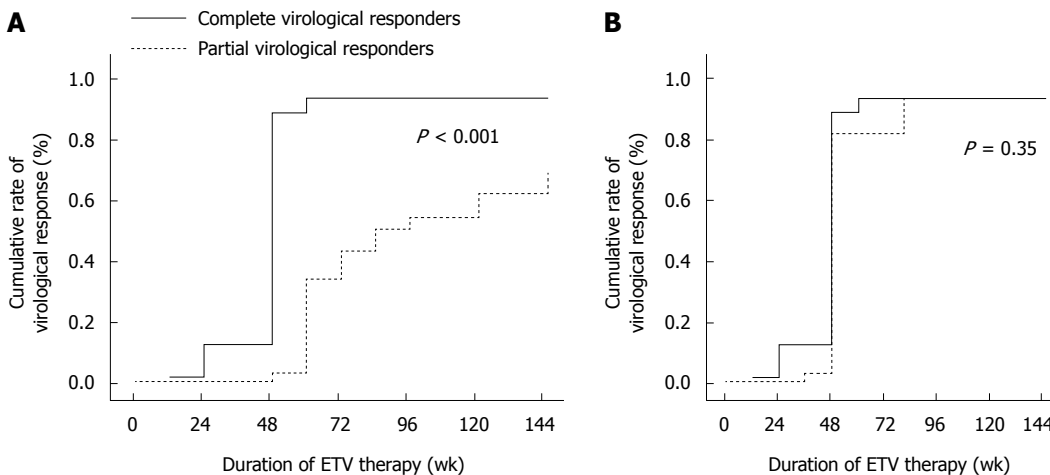
The clinical characteristics of patients with CVR and PVR are shown in Table 2. CVR patients were older compared with PVR patients (*P* < 0.001). Baseline serum HBV DNA levels (*P* < 0.001) and the proportion of HBeAg-positive patients (*P* < 0.001) were higher in PVR than in CVR patients. Pretreatment serum AST levels (*P* = 0.002) and FIB-4 index scores (*P* < 0.001) were higher in CVR patients than in PVR patients. After adjusting for covariates by multivariate logistic regression analysis, pretreatment serum HBV DNA levels and the proportion of HBeAg-positive patients still showed significant differences between the two groups. All other baseline characteristics did not differ between the groups.

The clinical characteristics of the 178 HBeAg-positive and 53 HBeAg-negative patients are shown in Table 3. In HBeAg-positive patients, there were more males in the PVR group than in the CVR group (*P* =

**Table 3** Baseline characteristics of 178 hepatitis B e antigen-positive and 53 hepatitis B e antigen-negative patients with respect to hepatitis B virus DNA response at week 48

	HBeAg-positive ( <i>n</i> = 178)			HBeAg-negative ( <i>n</i> = 53)		
	CVR ( <i>n</i> = 71)	PVR ( <i>n</i> = 107)	<i>P</i> value	CVR ( <i>n</i> = 48)	PVR ( <i>n</i> = 5)	<i>P</i> value
Age (yr)	32.1 ± 9.9	29.7 ± 8.7	0.09	39.2 ± 10.3	35.2 ± 11.7	0.22
Male <i>n</i> (%)	49 (69)	88 (82)	0.04 <sup>1</sup>	29 (72)	6 (46)	0.08
ALT (IU/L)	121 ± 143	107 ± 90	0.108	118 ± 124	120 ± 74	0.89
AST (IU/L)	82 ± 88	56 ± 42	0.002 <sup>1</sup>	73 ± 76	67 ± 92	0.75
PLT (× 10 <sup>9</sup> /L)	158 ± 53	175 ± 58	0.05	163 ± 58	181 ± 75	0.97
HBV DNA (log <sub>10</sub> IU/mL)	7.26 ± 1.05	7.92 ± 0.89	0.001 <sup>1</sup>	6.32 ± 1.29	6.62 ± 0.87	0.15
Group A, <i>n</i> (%)	39 (55)	51 (47)	0.34	19 (39)	4 (80)	0.08
FIB-4 index	1.89 ± 1.43	1.18 ± 0.69	0.001 <sup>1</sup>	2.17 ± 1.44	1.92 ± 1.67	0.84

<sup>1</sup>This difference was no longer significant after multivariate logistic regression analysis; <sup>2</sup>The two groups still showed a significant difference after multivariate logistic regression analysis. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelet; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

**Figure 2** Kaplan-Meier analysis of the cumulative rate of virological response for hepatitis B e antigen-positive (A) or -negative (B) patients between complete virological response and partial virological response groups.

0.04). Baseline serum HBV DNA levels were lower in CVR patients than in PVR patients ( $7.26 \pm 1.05$  vs  $7.92 \pm 0.89$ ,  $P < 0.001$ ). In the CVR group, pretreatment serum AST and the FIB-4 index were higher than those in PVR patients ( $P < 0.01$ ). However, after adjusting for significant covariates in the univariate analysis, these differences were no longer significant. There were no significant differences in the baseline characteristics of HBeAg-negative patients between groups.

#### Virological responses and predictive factors in HBeAg-positive and HBeAg-negative patients

The overall cumulative probabilities of maintained viral suppression at years 1, 2 and 3 were 48.1%, 70.6% and 81.3%, respectively. In HBeAg-positive patients ( $n = 178$ ), the cumulative probabilities of achieving undetectable levels at weeks 48, 96 and 144 were 42%, 67% and 80%, respectively. In the Kaplan-Meier analysis for HBeAg-positive patients, there was

a significant difference in the cumulative probability of achieving undetectable levels at week 144 between the CVR and PVR groups (95% vs 69%,  $P < 0.001$ ; Figure 2A). In the Cox proportional hazards model, lower pretreatment and week 48 serum HBV DNA levels were the independent factors predicting maintained viral suppression (Table 4).

The cumulative probabilities of HBV DNA responses in HBeAg-negative patients ( $n = 53$ ) at weeks 48, 96 and 144 were 87%, 100% and 100%, respectively (Figure 3). Conversely, the cumulative probabilities of achieving undetectable levels of HBV DNA at week 144 for HBeAg-negative patients were similar between the two groups (Figure 2B).

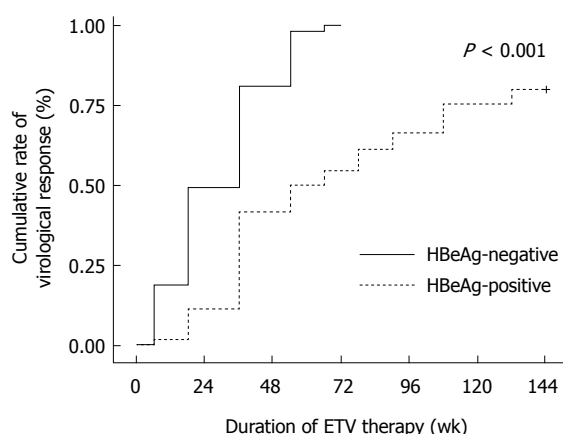
#### Comparison between the FIB-4 index and liver biopsy results

In patients with significant fibrosis (Ishak scores 4-6), the FIB-4 index was significantly higher when compared with patients with no/mild fibrosis (Ishak

**Table 4** Multivariate analysis of factors predicting maintained viral suppression in relation to virological response at week 48 using the Cox proportional hazard model in hepatitis B e antigen-positive patients

	Maintained viral suppression at week 144		
	HR	95%CI	P value
Age (yr)	1.008	0.98-1.04	0.54
Sex	0.927	0.694-1.325	0.68
ALT (IU/L)	1.000	0.997-1.004	0.81
AST (IU/L)	1.001	0.994-1.000	0.77
PLT ( $\times 10^9/L$ )	0.998	0.994-1.000	0.40
HBeAg-positive	1.326	0.918-1.916	0.13
Baseline HBV DNA ( $\log_{10}$ IU/mL)	0.781	0.678-0.900	< 0.001
HBV DNA at week 48 ( $\log_{10}$ IU/mL)	0.768	0.694-0.993	< 0.001
FIB-4 index	0.737	0.452-1.199	0.22

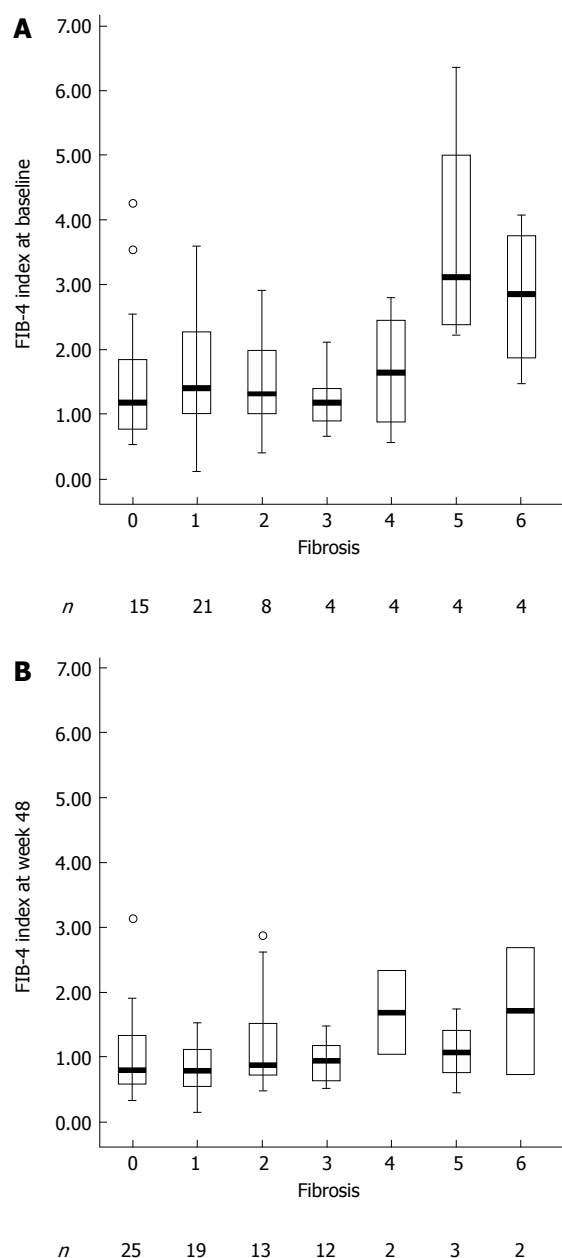
ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PLT: Platelet; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen.

**Figure 3** Kaplan-Meier curve for the probability of achieving virological response according to hepatitis B e antigen status at baseline.

scores 0-3) (Figure 4A). After 48 wk of ETV therapy, the FIB-4 index was reduced significantly (Figure 4B). The FIB-4 index did not show a significant difference among the Ishak scores 0-3 ( $P = 0.76$ ). There was also no significant difference among the Ishak scores 4-6 ( $P = 0.17$ ). The FIB-4 index showed a significant difference between Ishak scores 0-3 and Ishak scores 4-6 ( $P < 0.001$ ). The FIB-4 index efficiently identified fibrosis, with an area under the ROC curve of 0.80 (95%CI: 0.69-0.89; Figure 5). The sensitivity, specificity, PPV and NPV for the FIB-4 index were 0.95, 0.60, 0.25 and 0.64, respectively.

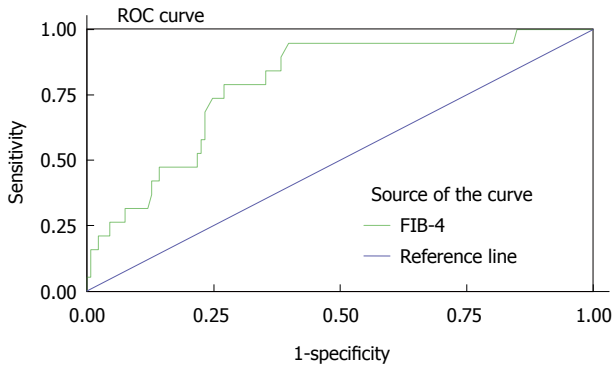
#### Dynamic changes of FIB-4 index and predictive factors

Compared with PVR patients, the FIB-4 index was higher for CVR patients at baseline in HBeAg-positive patients ( $1.18 \pm 0.69$  vs  $1.89 \pm 1.43$ ,  $P < 0.001$ ). The changes in the FIB-4 index were higher in CVR patients than in PVR patients from baseline to week

**Figure 4** Box plots of FIB-4 index according to Ishak score at baseline (A) or at week 48 (B). The FIB-4 index did not show a significant difference among Ishak scores 0-3 ( $P = 0.76$ ). There was also no significant difference among Ishak scores 4-6 ( $P = 0.17$ ). The FIB-4 index show a significant difference between Ishak scores 0-3 and Ishak scores 4-6 ( $P < 0.001$ ). The value of FIB-4 index decreased dramatically from baseline to week 48.

144 ( $0.64 \pm 1.25$  vs  $0.15 \pm 0.72$ ,  $P = 0.004$ ). From week 48 to 144, the FIB-4 index was not significantly reduced between the CVR and PVR groups ( $-0.11 \pm 0.47$  vs  $-0.13 \pm 0.49$ ,  $P = 0.71$ ). At week 144, the levels of the FIB-4 index were similar between the two groups ( $1.24 \pm 0.87$  vs  $1.02 \pm 0.73$ ,  $P = 0.06$ ). After the multivariate logistic regression analysis, lower baseline serum HBV DNA level was associated with improvement of liver fibrosis. In HBeAg-negative patients, dynamic changes of the FIB-4 index did not





**Figure 5** Receiver operating characteristic curve for FIB-4 index in identifying fibrosis (Ishak score) in the total cohort ( $n = 152$ ). FIB-4 had an area under the receiver operating characteristic curve of 0.80. ROC: Receiver operating characteristic.

differ between the two groups (Table 5).

#### Comparison of the dynamic changes of FIB-4 index according to the virological state at 144 wk

According to the level of HBV DNA at week 144, the 178 HBeAg-positive patients were divided into two groups (groups a and b). The patients who achieved undetectable level of HBV DNA was classified as group a ( $n = 120$ ), the other patients was defined as group b ( $n = 58$ ). At baseline, the FIB-4 index did not show a significant difference between groups a and b ( $1.56 \pm 1.14$  vs  $1.27 \pm 0.99$ ,  $P = 0.11$ ). The dynamic changes of FIB-4 index did not differ from baseline to week 144 between groups a and b ( $0.44 \pm 0.99$  vs  $0.17 \pm 0.98$ ,  $P = 0.09$ ). At week 144, the FIB-4 index also did not show a significant difference between groups a and b ( $1.12 \pm 0.81$  vs  $1.10 \pm 0.78$ ,  $P = 0.89$ ) (Table 6).

## DISCUSSION

Even with prolonged ETV monotherapy for 3 years, we found that the cumulative response rates of HBV DNA in HBeAg-positive chronic hepatitis B patients at week 144 were significantly lower for PVR patients to ETV than for CVR patients. Lower pretreatment serum HBV DNA levels were an independent factor predicting maintained viral suppression and contributed to a greater FIB-4 index reduction by entecavir therapy. Although the degree of fibrosis regression was higher in the CVR group than in the PVR group, the mean values of the FIB-4 index were similar and less than 1.45 in the two groups by the end of therapy.

A study in a large cohort of NA-naïve CHB patients treated with ETV monotherapy has shown that long-term ETV therapy leads to a VR for patients with both primary response and nonresponse according to EASL and AASLD<sup>[20]</sup>. The definitions of primary response or nonresponse for the cohort study were based on data from studies of less potent drugs with a higher risk of antiviral resistance<sup>[21,22]</sup>. The study suggested that changing the therapeutic scheme in patients

with a primary nonresponse should depend on drug differences in antiviral potency and resistance risk. Wong *et al.*<sup>[23]</sup> reported that CVRs to ETV at week 48 (HBV DNA < 20 IU/mL) could predict the probability of maintained virological suppression at 3 years. Therefore, patients were stratified on the basis of serum HBV DNA levels at week 48.

Zoutendijk *et al.*<sup>[8]</sup> found that HBV DNA was reduced to undetectable levels (HBV DNA < 80 IU/mL) in the majority of patients with partial VR (84%) after prolonged treatment with ETV. However, our study found that only 58 of the 112 PVR patients to ETV (52%) achieved undetectable HBV DNA levels (HBV DNA < 20 IU/mL) at 144 wk. The differences were likely due to the proportion of HBeAg-positive patients, which was higher in our patients than the above study (95.5% vs 77.7%). For HBeAg-positive patients, the cumulative probability of achieving VR at week 144 was lower than in HBeAg-negative patients (80% vs 100%).

Our study found that the FIB-4 index efficiently identified fibrosis, with an area under the ROC curve of 0.80. In addition, the Ishak score and FIB-4 index were similar in evaluating liver fibrosis at baseline and at week 48. Therefore, the FIB-4 index was identified as a non-invasive method to assess fibrosis in our study. The level of the FIB-4 index at baseline and the changes of the FIB-4 index from baseline to week 144 for HBeAg-positive patients were significantly higher in CVR patients than in PVR patients. Compared with PVR patients, especially for HBeAg-positive patients, CVR patients were characterized by older age, elevated AST, and lower HBV DNA levels. This indicates that patients with higher FIB-4 index values and lower HBV DNA levels at baseline could have better improvement in the FIB-4 index after receiving ETV therapy<sup>[24]</sup>. Xie *et al.*<sup>[25]</sup> also showed that serum ALT, serum HBV DNA levels and age were associated with significant fibrosis in HBeAg-positive patients. Liver fibrosis progression and viral load suppression are caused by T cell-mediated immune responses<sup>[26]</sup>. If the immune response induced by T cells is strong, naïve chronic hepatitis B patients will respond better to ETV therapy. Because of nearly half of PVR patients (45%,  $n = 49$ ) whose HBV DNA levels were undetectable at week 144 in the HBeAg-positive group, it is necessary to evaluate the dynamic changes of FIB-4 index according to the level of HBV DNA at week 144. The dynamic changes of FIB-4 index did not show a significant difference. Because of the low level of HBV DNA at baseline in HBeAg-negative patients, the cumulative probability of maintained viral suppression and dynamic changes of the FIB-4 index were similar between CVR patients and PVR patients.

There are several limitations in our study. The main limitations of this study were retrospective nature, the short duration of observation during entecavir treatment and too small sample of patients who

**Table 5** Comparison of FIB-4 index between complete virological response and partial virological response groups from baseline to 144 wk

	HBeAg-positive ( <i>n</i> = 178)			HBeAg-negative ( <i>n</i> = 53)		
	CVR ( <i>n</i> = 71)	PVR ( <i>n</i> value = 107)	<i>P</i> value	CVR ( <i>n</i> = 48)	PVR ( <i>n</i> = 5)	<i>P</i> value
0 wk	1.89 ± 1.43	1.18 ± 0.69	< 0.001	2.17 ± 1.44	1.92 ± 1.66	0.71
48wk	1.20 ± 0.81	0.94 ± 0.57	0.007	1.50 ± 0.89	1.52 ± 0.82	0.79
144 wk	1.24 ± 0.87	1.02 ± 0.73	0.06	1.49 ± 0.90	1.12 ± 0.44	0.37
48-144 wk	-0.11 ± 0.47	-0.13 ± 0.49	0.71	-0.02 ± 0.34	-0.07 ± 0.38	0.44
0-144 wk	0.64 ± 1.25	0.15 ± 0.72	0.004	0.68 ± 0.95	0.81 ± 1.25	0.79

HBeAg: Hepatitis B e antigen; CVR: Complete virological response; PVR: Partial virological response.

**Table 6** Comparison of FIB-4 index between groups a and b from baseline to 144 wk

	Group a ( <i>n</i> = 120)	Group b ( <i>n</i> = 58)	<i>P</i> value
0 wk	1.56 ± 1.14	1.27 ± 0.99	0.11
144 wk	1.12 ± 0.81	1.10 ± 0.78	0.89
0-144 wk	0.44 ± 0.99	0.17 ± 0.98	0.09

Group a: HBV DNA was undetectable at week 144; Group b: HBV DNA was still detectable at week 144.

underwent liver biopsies. Thus, long-term follow-up assessments are ongoing. Further studies with larger liver biopsies are necessary to remedy these shortcomings and to elucidate the long-term outcomes of ETV treatment. In addition, the study did not observe the dynamic changes of liver inflammation in chronic hepatitis B patients with entecavir therapy and the changes of FIB-4 index have not been reported in the proportion of immunotolerant and immunoactive patients. In the later study, we will observe the changes of FIB-4 index and liver inflammation in the immunotolerant and immunoactive patients infected with HBV.

In conclusion, although the cumulative probabilities of HBV DNA responses showed significant differences between CVR and PVR patients after treatment with entecavir for 144 wk, the FIB-4 index, which was used to evaluate liver fibrosis, did not differ until the end of the observation period. Entecavir therapy resulted in the reversal of fibrosis in CHB patients, especially in HBeAg-positive patients with CVR and HBeAg-negative patients. Long-term low-level HBV DNA in PVR patients did not deteriorate the FIB-4 index at the end of 3 years.

## COMMENTS

### Background

The relationship between low serum levels of hepatitis B virus (HBV) DNA and fibrosis is unclear for chronic hepatitis B (CHB) patients with partial virological response to entecavir (ETV).

### Research frontiers

Non-invasive tests for the evaluation of liver fibrosis have the advantages of simplicity and repeatability. Although non-invasive tests as an alternative to liver biopsies are restricted either by the cost of the device (FibroScan) or by the need for a specific laboratory examination (FibroTest), the FIB-4 index which is

a simple non-invasive test has the ability to identify HBV-related fibrosis with a moderate sensitivity.

### Innovations and breakthroughs

The cumulative probabilities of HBV DNA responses showed significant differences between the CVR and PVR group in hepatitis B e antigen (HBeAg)-positive CHB patients undergoing entecavir treatment for 144 wk. Non-invasive long-term low-level HBV DNA did not deteriorate the FIB-4 index, which was used to evaluate liver fibrosis of CHB patients. Older age and lower HBV DNA levels are associated with FIB-4 index in HBeAg-positive CHB patients.

### Applications

FIB-4 index can be used to evaluate the dynamic changes of liver fibrosis in CHB patients receiving ETV therapy.

### Terminology

FIB-4 index was determined according to the following published formula: FIB-4 index = Age (years) × AST (IU/L)/PLT (10<sup>9</sup>/L) × ALT (IU/L)<sup>1/2</sup>.

### Peer-review

The study reported the easily available FIB-4 index as a marker of hepatic fibrosis in patients on long-term entecavir therapy for chronic HBV hepatitis. The paper confirmed the effectiveness of entecavir therapy for chronic HBV hepatitis and the improvement in liver histology. The improvement in the Ishak score is reflected by a decrease in the FIB-4 score.

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## Observational Study

# Re-re-treatment of hepatitis C virus: Eight patients who relapsed twice after direct-acting-antiviral drugs

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**Conflict-of-interest statement:** Kian Bichoupan is a paid consultant for Gilead Sciences and Janssen Pharmaceuticals, Inc. Dr. Andrea D. Branch is a paid consultant for Gilead Sciences and Janssen Pharmaceuticals, Inc. Dr. Douglas T. Dieterich serves as a paid lecturer, consultant and is a member on scientific advisory boards of companies which either develop or assess medicines used for the treatment of viral hepatitis. These companies include Gilead Sciences, Abbvie, Achillion, Bristol-Myers Squibb, Merck, and Janssen Pharmaceuticals, Inc. Alyson Harty is a paid consultant for Abbvie Pharmaceuticals, Gilead Sciences, and Janssen Pharmaceuticals, Inc. Dr. Sweta Chekuri, Dr. Joshua Hartman, Dr. Neal Patel, and Dr. Ponni V. Perumalswami do not

have any disclosures.

**Data sharing statement:** Technical appendix, statistical code, and dataset available from the corresponding author at [andrea.branch@mssm.edu](mailto:andrea.branch@mssm.edu). Consent was not obtained but the presented data are anonymized and risk of identification is low.

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## Abstract

**AIM:** To determine risk factors associated with hepatitis C virus (HCV) treatment failure after direct acting antivirals in patients with complex treatment histories.

**METHODS:** All HCV mono-infected patients who received treatment at our institution were queried.



Analysis was restricted to patients who previously failed treatment with boceprevir (BOC) or telaprevir (TVR) and started simeprevir (SMV) and sofosbuvir (SOF)  $\pm$  ribavirin (RBV) between December 2013 and June 2014. Patients with human immunodeficiency virus (HIV)/HCV co-infection or patients who received a liver transplant in the past were excluded. Viral loads were recorded while on treatment and after treatment. Data collection continued until December, 31<sup>st</sup> 2014 when data analysis was initiated. Patients missing virologic outcomes data were not included in the analysis. Analysis of 35 patients who had virologic outcome data available resulted in eight patients who were viral load negative at the end of treatment with SMV/SOF but later relapsed. Data related to patient demographics, HCV infection, and treatment history was collected in order to identify risk factors shared among patients who failed treatment with SMV/SOF.

**RESULTS:** Eight patients who were treated with the first generation HCV protease inhibitors BOC or TVR in combination with pegylated-interferon (PEG) and RBV who failed this triple therapy were subsequently re-treated with an off-label all-oral regimen of SMV and SOF for 12 wk, with RBV in seven cases. Treatment was initiated before the Food and Drug Administration approved a 24-wk SMV/SOF regimen for patients with liver cirrhosis. All eight patients had an end of treatment response, but later relapsed. Eight (100%) patients were male. Mean age was 56 (range, 49-64). Eight (100%) patients had previously failed PEG/RBV dual therapy at least once in addition to prior failure with triple therapy. Total number of times treated ranged from 3-6 (mean 3.8). Eight (100%) patients were male had liver cirrhosis as determined by Fibroscan or MRI. Seven (87.5%) patients had genotype 1a HCV. Seven (87.5%) patients had over 1 million IU/mL HCV RNA at the time of re-treatment.

**CONCLUSION:** This study identifies factors associated with SMV/SOF treatment failure and provides evidence that twelve weeks of SMV/SOF/RBV is insufficient in cirrhotics with high-titer genotype 1a HCV.

**Key words:** Hepatitis C; Protease inhibitor; Relapse; Simeprevir; Sofosbuvir; Treatment failure

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**Core tip:** Direct acting antivirals are revolutionizing the treatment of chronic hepatitis C (HCV) infection, but are also increasing the number of patients who have failed multiple rounds of treatment. Information about these patients is needed to plan salvage treatment strategies. We present eight patients who failed treatment with the first generation protease inhibitors and subsequently failed treatment with simeprevir and sofosbuvir. Their shared characteristics include a history of failed treatment with interferon/ribavirin and liver cirrhosis. Seven had genotype 1a HCV and a

high viral load. Our findings suggest that patients with cirrhosis and high viral load remain hard-to-treat.

Hartman J, Bichoupan K, Patel N, Chekuri S, Harty A, Dieterich D, Perumalswami P, Branch AD. Re-re-treatment of hepatitis C virus: Eight patients who relapsed twice after direct-acting-antiviral drugs. *World J Gastroenterol* 2015; 21(43): 12430-12438 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12430.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12430>

## INTRODUCTION

Over 170 million people worldwide have chronic hepatitis C virus (HCV) infection<sup>[1,2]</sup>. HCV is a leading cause of liver-related mortality and is now responsible for more deaths than human immunodeficiency virus (HIV) infection in the United States<sup>[3,4]</sup>. The goal of treatment is to achieve a sustained virologic response (SVR), defined as undetectable HCV viral load 12 or 24 wk after the end of treatment<sup>[5]</sup>. Patients who achieve an SVR have decreased morbidity and mortality compared to null responders<sup>[6]</sup>. For many years, the standard of care (SOC) for chronic HCV infection was dual therapy with pegylated-interferon (PEG) and ribavirin (RBV)<sup>[7]</sup>, but much less toxic and more effective regimens are available now<sup>[8]</sup>. Information about the performance of new agents in various subgroups of patients is needed to optimize care.

Direct acting antiviral (DAA) drugs for HCV target specific viral proteins. The first DAAs to receive Food and Drug Administration (FDA) approval, boceprevir (BOC) and telaprevir (TVR), inhibit the HCV serine protease, NS3/4A<sup>[7]</sup>. BOC and TVR have several shortcomings, however, including their need to be combined with PEG and RBV, a relatively low barrier to resistance, toxicity, and poor activity against non-genotype 1 HCV<sup>[9]</sup>. Newer DAAs include sofosbuvir (SOF), a NS5B polymerase inhibitor, and simeprevir (SMV), a second phase NS3/4A protease inhibitor (PI)<sup>[10-12]</sup>. These medications were approved in the United States in late 2013 and were recommended as first-line agents in the January 2014 AASLD/IDSA practice guidelines<sup>[13]</sup>. Early studies of therapeutic regimens containing these agents reported SVR12 rates ranging from 93%-100%<sup>[12,14-17]</sup>. The success of these antivirals was very encouraging<sup>[18]</sup>; however, the efficacy of the newer DAAs has not been extensively studied in patients with advanced liver disease and complex treatment histories, including those previously exposed to BOC and/or TVR.

## MATERIALS AND METHODS

We examined a cohort of 47 HCV mono-infected patients who previously failed TVR or BOC and started

SMV/SOF  $\pm$  RBV between December 2013 and June 2014. All patients had genotype 1 HCV. All patients received at least one dose of any HCV medication. Patients with HIV/HCV co-infection or patients who received a liver transplant in the past were excluded. Viral loads were recorded for each patient while on-treatment and after treatment. Data collection continued until December, 31<sup>st</sup> 2014 when data analysis was initiated. The analysis was restricted to 35 patients who had virologic outcomes data available. Patients missing virologic outcomes data were not included in the analysis.

Eight patients (described in detail below) who were viral load negative at the end of treatment (EOT) with SMV/SOF later relapsed. The present study was conducted with approval of the Mount Sinai IRB in compliance with the Helsinki accord. Following the case presentations, the use of DAAs in patients with complex histories is reviewed, and suggestions for optimizing HCV treatment in the future are presented.

## RESULTS

### Case 1

Case 1 is a 60-year-old white male with no significant medical history prior to being diagnosed with HCV genotype 1a in 1978. His risk factor for acquisition of HCV was intravenous drug use in the 1970s. He was previously a non-responder to PEG/RBV during two rounds of treatment. He was treated with PEG/RBV for a third time in 2009 after undergoing chemotherapy with cyclophosphamide, doxorubicin, vincristine, prednisone, and rituximab for treatment of non-Hodgkin's B-cell lymphoma. Prior to starting treatment, his viral load was 3300000 IU/mL. At week 4, he had a 1-log drop in viral load (to 567910 IU/mL); however, by week 9, his viral load had increased and remained elevated so treatment was stopped. In 2011, a FibroScan has a score of 22 kPa, consistent with cirrhosis and he was treated with TVR/PEG/RBV. He had a rapid virological response (RVR) with undetectable viral load at week 4 (Figure 1A) that remained undetectable for the remainder of eight weeks of TVR-based triple therapy, however, the viral load was 169000 IU/mL at week 18 of PEG/RBV (five weeks after he completed TVR). Treatment was again stopped. A repeat FibroScan in 2013 suggested worsening cirrhosis (26 kPa) but he remained well compensated. In December, 2013, he began treatment with an off-label all-oral regimen of SMV/SOF/RBV. After 4 wk of treatment, his viral load went from 1449160 IU/mL to undetectable and remained undetected throughout the 12 wk of treatment. Four weeks post-EOT the viral load was 166684 IU/mL, indicating relapse.

### Case 2

Case 2 is a 49-year-old white male with no significant medical history prior to being diagnosed with HCV

genotype 1b in 1993 at the age of 28. His risk factor for acquisition of HCV was a blood transfusion during surgery in his late teens for a sports-related injury. He was first treated in 2011 with TVR/PEG/RBV; however, he stopped TVR after two weeks due to a severe, diffuse rash. He continued PEG/RBV for a total of 12 wk but never had an undetectable HCV viral load. In 2012, his FibroScan was consistent with cirrhosis (33.3 kPa) but he had no evidence of decompensated liver disease. He was started on BOC after a 4-wk lead in with PEG/RBV. The viral load prior to initiation of treatment was 410332 IU/mL (Figure 1B). At week 8 (BOC week 4), he reported a small rash over the legs and arms, dysgeusia, and flu-like symptoms. His continued treatment and his viral load became undetectable at week 16 and remained so through week 48. Four weeks post-EOT, he relapsed with a viral load of 766 IU/mL. In January 2014, he was started on SOF/SMV/RBV, with a viral load of 180020 IU/mL. During the first two weeks of therapy, his viral load was below 43 IU/mL and after 6 wk the viral load was undetectable. Despite his viral load remaining undetectable for the remainder of therapy, he had a viral load of 370430 IU/mL at 4 wk post-EOT, indicating relapse.

### Case 3

Case 3 is a 54-year-old white male with no significant medical history prior to being diagnosed with HCV genotype 1a in 2006. His risk factor for acquisition of HCV was a tattoo that he received over 30 years prior to diagnosis. A liver biopsy in 2006 demonstrated cirrhosis. He was treated with PEG/RBV in 2006 at an outside institution but had difficulty tolerating this regimen due to side effects so treatment was stopped. He was re-treated with PEG/RBV in 2008 for 20 wk and was a partial responder. In 2011, he began treatment with TVR/PEG/RBV and remained on this regimen for 12 wk, although his viral load remained detectable (Figure 1C). In 2013, routine surveillance imaging revealed a 2.0 cm mass consistent with hepatocellular carcinoma. He received transcatheter arterial chemoembolization and then CT-guided thermal ablation of the lesion. Follow-up imaging showed complete ablation of the tumor and no recurrence. A FibroScan later that year was consistent with cirrhosis (32.8 kPa). He required diuretics for minimal ascites but was otherwise well compensated. He was started on treatment with SOF/SMV/RBV in 2014, with a viral load of 10349963 IU/mL. Two weeks after starting therapy, his viral load was 1363 IU/mL. The viral load dropped to less than 43 IU at week 6 and was undetectable for the remainder of therapy; however 6 wk post-EOT, his viral load was 1588696 IU/mL, indicating relapse.

### Case 4

Case 4 is a 50-year-old African American male with a history of diabetes mellitus, hypertension, major

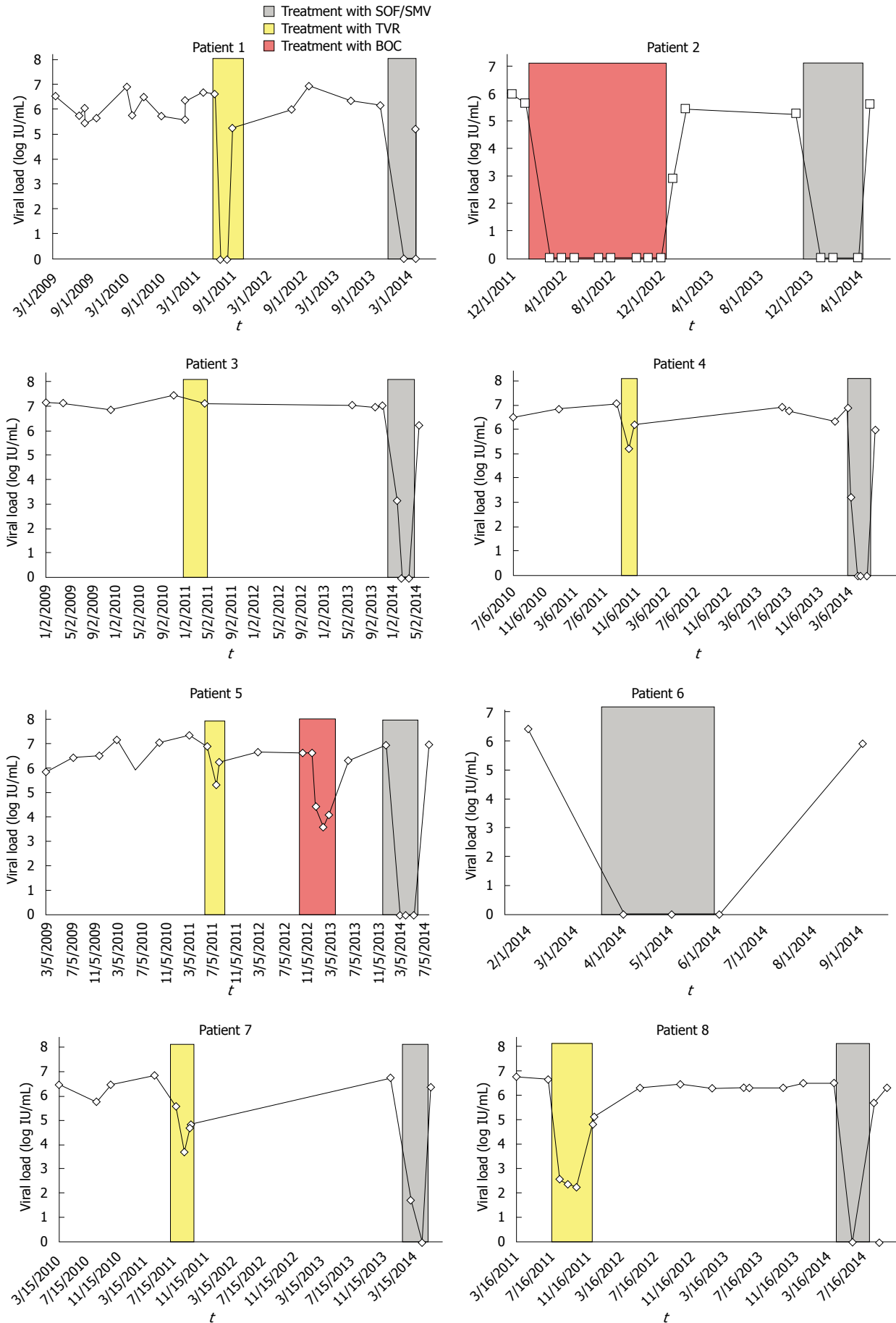


Figure 1 Viral load over time. SOF: Sofosbuvir; SMV: Simeprevir; TVR: Telaprevir; BOC: Boceprevir.

depression, substance abuse requiring inpatient rehabilitation, and HCV genotype 1a. His risk factor for acquisition was being incarcerated multiple times in the 1980s. He was treated once with PEG/RBV at an outside center without achieving an SVR. In 2009, he was re-treated with PEG/RBV. The course was complicated by severe, symptomatic anemia for which he was treated with Epoetin alpha; however, HCV treatment was stopped at 46 wk as he was again a non-responder. Later, an abdominal MRI revealed cirrhosis and portal hypertension. In 2011, he began PEG/RBV/TVR, with a viral load of 11361480 IU/mL. Treatment was complicated by symptomatic anemia, thrombocytopenia, and hemorrhoidal bleeding. He was again prescribed Epoetin. At 4 wk of treatment, the viral load dropped to 152172 IU/mL, a 2-log decrease. Given this response, treatment was continued; however, at week 8, the viral load was 1557865 IU/mL so treatment was stopped early (Figure 1D). In 2014, he was started on SOF/SMV/RBV with a viral load of 7753010 IU/mL. After 4 wk, the viral load dropped to 1629 IU/mL and then became undetectable after 8 wk and remained so throughout the 12 wk of treatment. Four weeks post-EOT, his viral load was 808797 IU/mL, indicating relapse.

#### Case 5

Case 5 is a 63-year-old white male with congenital absence of one kidney, hypertension, and HCV genotype 1a diagnosed in 2007. His risk factor for acquisition of HCV was intravenous drug use decades prior to diagnosis. He was first treated with PEG/RBV at another institution soon after diagnosis but was a non-responder. In 2011, after a FibroScan was consistent with cirrhosis (17 kPa), he was treated with PEG/RBV/TVR. Eight weeks after starting, the medication regimen was discontinued due to acute kidney injury attributed to dehydration requiring hospitalization for intravenous fluid hydration (Figure 1E). Months later, a FibroScan was consistent with worsening cirrhosis (34.3 kPa), although he remained well compensated. He was treated in November 2012 with PEG/RBV/BOC but was a non-responder. He began treatment with SOF/SMV/RBV in 2014, with a viral load of 8450674 IU/mL. After 4 wk, the viral load was undetectable and remained so throughout treatment. At 12 wk post-EOT, his viral load was 9485523 IU/mL, indicating late relapse.

#### Case 6

Case 6 is a 53-year-old white male with no significant past medical history prior to being diagnosed with HCV genotype 1a in 2005. His risk factor for acquisition of HCV was a blood transfusion after a motor vehicle accident in Puerto Rico during his childhood. He had previously been treated with PEG/RBV at an outside center, which was discontinued early due to a severe episode of depression. He underwent treatment with PEG/RBV/TVR in 2012 but treatment was stopped

after eight months due to virologic breakthrough. While awaiting new therapies, he underwent an MRI of the abdomen in 2013 that demonstrated changes consistent with cirrhosis and mild portal hypertension but no ascites or hepatocellular carcinoma. In February 2014, his viral load was 2429839 IU/mL (Figure 1F). He subsequently began treatment with a 12 wk course of SOF/SMV. Labs at week 4 and week 8 of treatment revealed an undetectable viral load. He remained viral load negative after completing treatment, however he had a viral load of 744508 IU/mL 12 wk post-EOT with SOF/SMV, indicating relapse.

#### Case 7

Case 7 is a 64-year-old white male with no significant medical history prior to being diagnosed with HCV genotype 1a (unknown risk factor). He was first treated in 2010 with PEG/RBV but treatment was stopped after six-months as he was a non-responder. After an abdominal MRI in 2011 revealed cirrhosis and portal hypertension, he underwent treatment with TVR/PEG/RBV. Prior to initiation of therapy, his viral load was 3732211 IU/mL. At treatment week 2, his viral load was 4614 IU/mL, which increased to 45205 IU/mL at week 4 (Figure 1G). Triple therapy was discontinued after six weeks of treatment. In 2014, he was started on a 12 wk course of SOF/SMV/RBV. His viral load decreased from 5448450 IU/mL to 49 IU/mL at week 4 of treatment. His viral load subsequently became undetectable and remained undetectable through 12 wk of treatment. At four weeks post-EOT, his viral load was 2166059 IU/mL, indicating relapse.

#### Case 8

Case 8 is a 54-year-old African-American male with a history of hypertension and HCV genotype 1a (risk factor unknown). He had previously failed four courses of PEG/RBV at an outside institution. In 2011, a FibroScan indicated cirrhosis (23.1 kPa). After FDA approval of PIs, he was started on treatment with TVR/PEG/RBV. Viral load prior to starting was 4512245 IU/mL. He initially had a decrease in viral load to 359 IU/mL at week 4 and 224 IU/mL at week 8 (Figure 1H). Treatment course was complicated by anemia treated with Epoetin alpha. He completed 12 wk of TVR but had a viral load of 63858 IU/mL at week 20, so treatment was discontinued. He was monitored throughout 2013 with a FibroScan showing worsening cirrhosis (26.3 kPa). In 2014, he was started on a 12 wk course of SOF/SMV/RBV with a viral load of 3108263 IU/mL. His viral load became undetectable at week 5 and remained so throughout the remainder of treatment. At week 4 post-EOT, he had a viral load of 491287 IU/mL, indicating relapse (Table 1).

## DISCUSSION

As DAA drug treatments for HCV gain widespread



**Table 1** Demographics and treatment history for eight male cases

Case	Race	Age	Genotype	Test to diagnose cirrhosis	IL-28b	Total number of times treated (including DAAs)	Number of times treated with PEG/RBV dual therapy	Prior PI treatment	Time interval between PI and SMV/SOF (mo)	Viral load prior to SMV/SOF treatment (IU/mL)	RBV used with SM/SOF	Duration of treatment (wk)
1	White	60	1a	Fibroscan (24 kPa)	CT	5	3	TVR	30	1449160	Yes	12
2	White	49	1b	Fibroscan (33.3 kPa)	Unknown	3	0	TVR, BOC	19	180020	Yes	12
3	White	54	1a	Fibroscan (32.8 kPa)	Unknown	4	2	TVR	26	10349963	Yes	12
4	African American	50	1a	MRI	TT	4	2	TVR	25	7753010	Yes	12
5	White	63	1a	Fibroscan (34.3 kPa)	Unknown	4	1	TVR, BOC	12	8450674	Yes	12
6	White	53	1a	MRI	Unknown	3	1	TVR	–	2429839	No	12
7	White	64	1a	MRI	Unknown	3	1	TVR	28	5448450	Yes	12
8	African American	54	1a	Fibroscan (26.3 kPa)	Unknown	6	4	TVR	31	3108263	Yes	12

DAA: Direct acting antiviral; PEG: Pegylated-interferon; RBV: Ribavirin; PI: Protease inhibitor; SOF: Sofosbuvir; SMV: Simeprevir; TVR: Telaprevir; BOC: Boceprevir; CT: Computed tomography; MRI: Magnetic resonance imaging.

use, the number of patients who have failed multiple rounds of treatment will inevitably increase. This study provides data about eight patients who relapsed after 12 wk of treatment with SOF/SMV (RBV). Information about their shared characteristics helps to identify patients at high risk of failure, raises questions about what pre-treatment testing might be done in the future to optimize outcomes for patients with complex treatment histories, and highlights the need for future research to determine optimal salvage strategies. The cases have several features in common. All were male and all had cirrhosis. All had previously failed both dual therapy (PEG/RBV) and PI-based (TVR or BOC) triple therapy. Seven had genotype 1a HCV and seven had an HCV viral load over 1 million IU/mL at the time of re-re-treatment. Several of these shared features may have contributed to the most recent treatment failure.

Liver cirrhosis has been associated with treatment failure for many years. With PEG/RBV dual therapy, SVR rates were lower in patients with compensated cirrhosis than in those without cirrhosis, particularly among patients with genotype 1 HCV in whom SVR rates were about 20% lower in cirrhotics<sup>[18–20]</sup>. Patients with advanced fibrosis/cirrhosis accounted for 9%–48% of patients enrolled in the larger trials of PIs<sup>[14,21–24]</sup>. The REALIZE trial, which had the highest proportion of patients with advanced fibrosis and cirrhosis (48%), found SVR rates were inversely related to the stage of fibrosis, with 75% in mild fibrosis, 67% in advanced fibrosis, and 47% in cirrhosis after treatment with PEG/RBV. Additionally, relapse rates were higher in previous partial or null responders with cirrhosis than without (10% vs 4%)<sup>[15,23]</sup>.

The mechanism for reduced SVR rates with more advanced liver disease has not been well elucidated. It is possible that cirrhosis prevents even perfusion of the liver with antiviral drugs, creating pockets that

have low drug concentrations where HCV can persist. Alternatively, patients with cirrhosis have impaired immunity, as indicated by their enhanced susceptibility to infection<sup>[24]</sup>. Studies suggest that prostaglandin E2 (PGE2) may have an immunosuppressive effect by inhibiting the production of proinflammatory cytokines by macrophages. PGE2 has been found in higher concentrations in cirrhotics and additionally has higher bioavailability in cirrhotics due to decreased levels of albumin, which normally binds to PGE2 and therefore decreases its bioavailability<sup>[25]</sup>. Whatever its cause, the immunodeficiency of patients with liver cirrhosis may contribute to treatment failure by slowing the kinetics of the second phase of viral decline—either by reducing the killing of infected cells or by reducing the process that allows infected cells to clear the virus.

Recent studies of all-oral regimens have reported favorable results even in patients with liver cirrhosis. In COSMOS, of 41 treatment-naïve and null responders to PEG/RBV with METAVIR fibrosis stage F3–F4 treated with SMV/SOF ± RBV for 12 wk, only three patients failed<sup>[12]</sup>. The LONESTAR trial contained a cohort of 40 patients who failed PI-based triple therapy with BOC or TVR, over half of the patients had compensated cirrhosis. On SOF and ledipasvir, an NS5A inhibitor, the SVR12 rate was 95% without RBV and 100% with RBV<sup>[16]</sup>. The ELECTRON trial used the same regimen of SOF/ledipasvir and in the cohort with cirrhotics and prior null responders, the SVR12 rate was 70% without RBV and 100% with RBV<sup>[17]</sup>. Many of the case patients in our study had advanced cirrhosis. When considering how the promising published results relate to our investigation of patients who failed treatment, it is important to keep in mind that not all patients with cirrhosis have the same degree of liver damage. Rather, there is a spectrum of disease among cirrhotics. Many of the case patients had

advanced cirrhosis, and this may have increased their susceptibility to treatment failure. FibroScan scores, because they report liver stiffness as a continuous variable, may help stratify the extent of liver scarring and delineate high-risk patients.

The treatment regimen chosen for our patients was based on results of the COSMOS study at a time when the FDA had not yet approved SMV/SOF combination therapy. COSMOS reported SVR rates in patients with METAVIR F3-F4 fibrosis who were treated with SMV/SOF for 12 wk of 93% compared to 93% in patients treated with SMV/SOF/RBV and 100% in patients treated with SMV/SOF for 24 wk<sup>[12]</sup>. In November 2014, the FDA approved a 24 wk regimen of SMV/SOF for patients with cirrhosis. All of the case patients in our case series were treated before this approval with 12 wk of treatment. This longer regimen reflects the growing awareness of the persistent challenge of treating patients with liver cirrhosis despite the availability of DAAs. The treatment failure of our patients highlights a potential limitation with early adoption of HCV treatment regimens that are not yet approved by the FDA.

High viral load is a second factor predisposing to treatment failure. An early study on PEG/RBV by Fried *et al.*<sup>[26]</sup> showed that SVR rates were significantly lower in patients with viral load over 800000 IU/mL than in patients with lower viral load: 41% vs 56%, respectively. Multivariate logistic regression analysis has shown that low baseline viral load is an independent predictor of SVR to treatment with PEG/RBV<sup>[27]</sup>. Showing a similar trend, early studies of SMV had SVR rates of 91% in patients with low viral load vs 77% in patients with high viral load<sup>[28]</sup>. COSMOS did not look at the difference in SVR based on pre-treatment viral load in patients treated with SMV/SOF. Seven of the cases had a viral load over 1 million IU/mL prior to treatment, which likely further increased susceptibility to treatment failure.

In addition to these factors, baseline polymorphisms and mutations that develop during exposure to antiviral drugs may contribute to the failure of DAA-based treatments. Numerous viral sequence variants confer partial or complete resistance to SMV. The barrier to resistance is especially low for genotype 1a HCV. Q80K is one of the most common resistance mutations in the HCV NS3/4A protease. It is often present at baseline. The importance of the Q80K mutation was exhibited in the QUEST-1 study where treatment naïve patients were treated with SMV/PEG/RBV. The SVR rate was 71% in patients with genotype 1a and 90% in patients with genotype 1b, but it was 85% in genotype 1a patients without the baseline Q80K mutation and 52% in genotype 1a patients with the mutation<sup>[28]</sup>. Tests for the Q80K mutation are available, but were not used prior to treating the patients in our case series as there is no formal recommendation to use this test in clinical practice and insurance companies were not providing consistent coverage for this test. There are additional

baseline polymorphisms that exist in genotype 1a HCV that make it challenging to treat<sup>[29]</sup>. In addition, prior exposure to TVR/BOC can promote the development of cross-resistant mutations, such as R155<sup>[30]</sup>. Therefore, patients such as those presented here who previously were treated with PIs could have baseline and/or acquired cross-resistant mutations to SMV. Baseline polymorphisms that reduce effectiveness of SOF have also been described. Using deep-sequencing methods, Donaldson *et al.*<sup>[31]</sup> identified numerous low-frequency substitutions in the target of SOF, the NS5B (nonstructural protein 5B) polymerase. Two, L159F and V321A, were located in the catalytic pocket of the viral enzyme and likely altered drug binding. Research is needed to determine the utility of HCV RNA sequence analysis in selecting optimal first-line and salvage strategies.

The strengths of this report include its timeliness, real-world setting, and case series with eight patients. The real-world setting of this study allows us to report experiences of these new medications in clinical practice. Given the number of cases reported here, we are able to highlight common factors for relapse to better identify and understand patients who may be at higher risk for failure. Data reported in registration trials is not always complete and generalizable to clinical practice. In our cohort, physicians selected the HCV treatment regimen based on their best clinical judgment, which can include early adoption of promising regimens based on data available. Limitations of this report include a lack of resistance data on our case series. Resistance analysis is not yet commonly used in clinical practice but consideration should be given to incorporate into recommendations for patients who fail DAA regimens in order to identify optimal salvage regimens. Barriers to coverage of resistance analysis including Q80K mutation analysis may have a better chance to be overcome once adopted into guidelines.

Early studies examining the efficacy of IFN-free regimens had very high SVR rates in patients with and without liver cirrhosis. The treatment failure in our eight patients was disappointing for the patients and their providers and carries a significant economic burden, as well. The pharmaceutical cost of a 12 wk regimen of SMV/SOF is \$150360. Research is needed to identify the underlying causes of DAA-based treatment failure and to identify the best salvage regimens for patients who have failed on specific drug combinations.

## COMMENTS

### Background

The goal of treatment in patients with chronic hepatitis C virus (HCV) infection is to achieve a sustained virologic response. Historically, the standard of care for chronic HCV infection was dual therapy with pegylated-interferon and ribavirin but less toxic and more effective regimens are available now.

### Research frontiers

Direct acting antiviral (DAA) drugs for HCV target specific viral proteins. The first

DAA to receive Food and Drug Administration (FDA) approval were boceprevir and telaprevir, inhibitors of the HCV serine protease NS3/4A. Newer DAAs include sofosbuvir, a NS5B polymerase inhibitor, and simeprevir, a second phase NS3/4A protease inhibitor. These drugs do not require the addition of interferon and have less toxicity and therefore are now recommended as first-line agents by the FDA for HCV genotype 1. Early studies of therapeutic regimens containing these agents reported sustained virologic response (SVR) 12 rates ranging from 93%-100%.

### Innovations and breakthroughs

DAA drugs are increasing the number of patients who achieve SVR, but are also increasing the number of patients who have failed multiple rounds of treatment. The efficacy of the newer DAAs has not been extensively studied in patients with advanced liver disease and complex treatment histories, including those previously exposed to BOC and/or TVR. Information about patients who repeatedly fail DAA-based therapy may help guide the development of salvage strategies.

### Applications

This study identifies a number of factors associated with SMV/SOF treatment failure, which included prior treatment with earlier DAAs, HCV 1a genotype, liver cirrhosis, and high pre-treatment viral load.

### Terminology

Hepatitis C is an infectious disease caused by the HCV, a small infectious agent, which primarily infects cells of the liver. Cirrhosis is advanced scarring of the liver, often induced by chronic viral infection, including Hepatitis C, or chronic alcohol abuse, which render the liver unable to conduct a number of necessary functions. Sustained virologic response describes when there are no viral particles detected in the blood 12 or 24 wk after the end of treatment. Direct-acting antiviral drugs are medications that target specific parts of the HCV in order to prevent the virus from duplicating.

### Peer-review

This is a comprehensive observational study in which the authors characterized and analyzed patients who failed treatment with new DAAs to uncover risk factors associated with SMV/SOF for treatment of chronic HCV infection. Identifying factors associated with treatment failure prior to initiating treatment is important in order to optimize treatment strategies and reduce health care costs. The results suggest that patients with complex histories may benefit from individualized risk analysis prior to treatment.

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## Observational Study

# Combined transjugular intrahepatic portosystemic shunt and other interventions for hepatocellular carcinoma with portal hypertension

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**Author contributions:** Qiu B and Zhao MF contributed equally to this work; Qiu B, Zhao MF and Liu FQ designed the research; Liu FQ supervised the study; Qiu B, Zhao MF, Yue ZD, Zhao HW, Wang L and Fan ZH performed the research; He FL, Dai S and Yao JN analyzed the data; and Qiu B wrote the paper.

**Institutional review board statement:** The study was reviewed and approved by Institutional Review Board at Beijing Shijitan Hospital. All procedures were conducted according to the guidelines of the Ethics Committee at Beijing Shijitan Hospital.

**Informed consent statement:** Informed consent was acquired from each participant or their legal guardian before the operation.

**Conflict-of-interest statement:** The authors declared that there are no conflicts of interest in this study.

**Data sharing statement:** No additional data are available.

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## Abstract

**AIM:** To evaluate combination transjugular intrahepatic portosystemic shunt (TIPS) and other interventions for hepatocellular carcinoma (HCC) and portal hypertension.

**METHODS:** Two hundred and sixty-one patients with HCC and portal hypertension underwent TIPS combined with other interventional treatments (transarterial chemoembolization/transarterial embolization, radio-frequency ablation, hepatic arterio-portal fistulas embolization, and splenic artery embolization) from January 1997 to January 2010 at Beijing Shijitan Hospital. Two hundred and nine patients (121 male and 88 female, aged 25-69 years, mean  $48.3 \pm 12.5$  years) with complete clinical data were recruited. We evaluated the safety of the procedure (procedure-related death and serious complications), change of portal vein pressure before and after TIPS, symptom relief [e.g., ascites, hydrothorax, esophageal gastric-fundus variceal bleeding (EGVB)], cumulative rates of survival, and tributary channel restenosis. The characteristics of the patients surviving  $\geq 5$  and  $< 5$  years were also analyzed.

**RESULTS:** The portosystemic pressure was decreased from  $29.0 \pm 4.1$  mmHg before TIPS to  $18.1 \pm 2.9$  mmHg after TIPS ( $t = 69.32$ ,  $P < 0.05$ ). Portosystemic pressure was decreased and portal hypertension symptoms were ameliorated. During the 5 year follow-up, the total recurrence rate of resistant ascites or hydrothorax was 7.2% (15/209); 36.8% (77/209) for EGVB; and 39.2% (82/209) for hepatic encephalopathy. The cumulative rates of distributary channel restenosis at 1, 2, 3, 4, and 5 years were 17.2% (36/209), 29.7% (62/209), 36.8% (77/209), 45.5% (95/209) and 58.4% (122/209), respectively. No procedure-related deaths and serious complications (*e.g.*, abdominal bleeding, hepatic failure, and distant metastasis) occurred. Moreover, Child-Pugh score, portal vein tumor thrombosis, lesion diameter, hepatic arterio-portal fistulas, HCC diagnosed before or after TIPS, stent type, hepatic encephalopathy, and type of other interventional treatments were related to 5 year survival after comparing patient characteristics.

**CONCLUSION:** TIPS combined with other interventional treatments seems to be safe and efficacious in patients with HCC and portal hypertension.

**Key words:** Transjugular intrahepatic portosystemic shunt; Interventional treatment; Hepatocellular carcinoma; Portal hypertension

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**Core tip:** There are conflicting results about the safety and efficacy of transjugular intrahepatic portosystemic shunt (TIPS) combined with other interventional treatments for patients with hepatocellular carcinoma (HCC) and portal hypertension. We reviewed 209 patients with HCC and portal hypertension who underwent TIPS and other interventional treatments. Portosystemic pressure was decreased and portal hypertension symptoms were ameliorated, and no procedure-related deaths and serious complications occurred. The survival rates for TIPS in combination seem better than those reported for transarterial chemoembolization or radiofrequency ablation alone.

Qiu B, Zhao MF, Yue ZD, Zhao HW, Wang L, Fan ZH, He FL, Dai S, Yao JN, Liu FQ. Combined transjugular intrahepatic portosystemic shunt and other interventions for hepatocellular carcinoma with portal hypertension. *World J Gastroenterol* 2015; 21(43): 12439-12447 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12439.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12439>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer death worldwide<sup>[1]</sup>, and

the second most common malignancy in China<sup>[2]</sup>, with an estimated 391250 new cases and 372750 deaths in 2012<sup>[3]</sup>. The mortality rate of HCC in China was 20.4 per 100000 according to the 2015 annual report from the American Society of Clinical Oncology (ASCO), and patient survival has increased significantly in recent years<sup>[4]</sup>. There is a variety of treatments for HCC depending on the nature of the tumor (size, stage, degree, and complications), and interventional treatments, including transarterial embolization/chemoembolization (TAE/TACE)<sup>[5,6]</sup> and radiofrequency ablation (RFA)<sup>[7]</sup>, have become important for HCC recently. HCC often stems from hepatitis B cirrhosis and combines with portal hypertension<sup>[2]</sup>, leading to esophageal gastric-fundus variceal bleeding (EGVB) and/or refractory ascites (or hydrothorax)<sup>[8,9]</sup>. Patients with HCC and portal hypertension often have no opportunity to receive radical surgery, liver transplantation, or even some interventional treatments. It is important to manage portal hypertension urgently in patients with HCC<sup>[10]</sup>. Transjugular intrahepatic portosystemic shunt (TIPS) is an expandable metal stent inserted *via* the jugular vein that creates a shunt from the portal vein to the systemic circulation *via* an artificial communication through the liver. TIPS is widely used as a treatment of portal hypertension and its complications<sup>[11-14]</sup> (such as EGVB, refractory ascites, hepatic hydrothorax, hepatorenal syndrome, and hepatopulmonary syndrome) and as a bridge to liver transplantation. Patients with portal hypertension have improvements in symptoms after TIPS, especially timely termination of acute EGVB and refractory ascites, which create opportunities for further treatment without affecting overall survival<sup>[15-17]</sup>. However, HCC and portal hypertension have been considered as relative contraindications for TIPS combined with other interventional treatments. There are conflicting results about the safety and efficacy of TIPS combined with other interventional treatments for patients with HCC and portal hypertension<sup>[18-26]</sup>. In this study, we reviewed and analyzed the data from 209 patients with HCC and portal hypertension who underwent TIPS and other interventional treatments from January 1997 to January 2010 at Beijing Shijitan Hospital.

## MATERIALS AND METHODS

### Clinical materials

Two hundred and sixty-one patients with HCC and portal hypertension underwent TIPS combined with other interventional treatments (TACE/TAE, RFA, hepatic arterio-portal fistulas embolization, and splenic artery embolization) from January 1997 to January 2010 at Beijing Shijitan Hospital. We recruited 209 patients (121 male and 88 female, aged 25-69 years, mean  $48.3 \pm 12.5$  years) who had complete clinical data; the remaining the patients who lacked such

data were excluded. Thirty-seven cases of HCC were diagnosed by pathological biopsy and 172 cases by ultrasound, computed tomography (CT), magnetic resonance imaging (MRI),  $\alpha$ -fetoprotein (AFP), and hepatic artery angiography. Hepatitis B cirrhosis was detected in 180 cases, hepatitis C cirrhosis in 12, overlapping hepatitis B and C cirrhosis in two, primary cholestasis cirrhosis in one, alcoholic liver cirrhosis in 13, and autoimmune liver cirrhosis in one. EGVB was seen in 182 cases, refractory ascites (and/or pleural effusion) in 39, and refractory ascites (and/or pleural effusion) combined with EGVB in 12. Preoperative splenectomy cutoff was undertaken in eight cases, hardening or ligation treatments in 41, and preoperative surgical excision of tumor in 35.

### Methods

We evaluated the safety (procedure-related death and serious complications, such as abdominal bleeding, hepatic failure, and distant metastasis), efficacy (change of portal vein pressure before and after TIPS, symptom relief, including ascites, hydrothorax, EGVB, and distributary channel restenosis) of the procedure, and the cumulative rates of survival. We also retrospectively analyzed and compared the clinical characteristics of patients living  $\geq 5$  and  $< 5$  years, including sex, age, Child-Pugh score before TIPS, portal vein tumor thrombosis (PVTT), tumor lesion, lesion diameter, hepatic arterio-portal fistulas, cancer diagnosed before and after TIPS, stents used, treatments received (RFA, TACE/TAE, and RFA+TACE/TAE), and complications (recurrence of ascites/bleeding, hepatic encephalopathy, and distributary channel function) that occurred during follow-up.

### TIPS

**Indications:** Acute or repeated variceal bleeding that failed conservative and endoscopic treatment; rebleeding after surgical shunting or laparosplenectomy; bleeding after preventive endoscopic/drug treatment; gastric or ectopic variceal bleeding; or refractory hepatic ascites/hydrothorax.

**Relative contraindications:** Serious dysfunction of blood coagulation and bleeding tendency; hepatic encephalopathy; serious infections; portal vein thrombosis; cavernous transformation of portal vein; or tumor too large to avoid during TIPS. The predicted survival of these patients was  $\leq 3$  mo.

**Contraindications:** Liver failure, severe cardiopulmonary dysfunction, multiple hepatic cysts, and refractory biliopancreatic obstruction.

The TIPS procedure was performed in the Interventional Radiology Suite under local anesthesia. The right jugular vein was punctured by RUPS-100 (Cook, Bloomington, United States) with a 10-F sheath. A 5-F multipurpose catheter was used to engage

the hepatic vein (right usually) and the portal vein, perform portal vein angiography, and measure portal vein pressure before the shunting. A balloon catheter (6 or 8 mm in diameter) was used to expand the shunt along a guidewire, and the stents (7, 8, or 10 mm in diameter) were placed. Portal vein angiography and measurement of portal vein pressure were then conducted again.

Bare stents were used for 112 cases (Protégé stent, EV3 Company, Nathan Lane North, Plymouth, MN, United States; Smart stent, Cordis Company, Miami Lakes, Florida, United States). Covered stents were used for 97 cases (Fluency stent, Bard Company, Karlsruhe, Germany). One stent was used in 150 cases, and more than two stents were used in 59 cases. Stents with diameter 7, 8, and 10 mm were used for 3, 154, and 52 cases, respectively. The shunts were entered *via* the hepatic vein in 185 cases and high inferior vena cava in 24 cases. TIPS was combined with percutaneous liver biopsy and portal vein venography in 28 cases and indirect portal venography in 34 cases, providing that it was hard to puncture the portal vein, the risk of direct TIPS was high, or direct TIPS was failed. Varicose vein embolism was conducted in 199 cases.

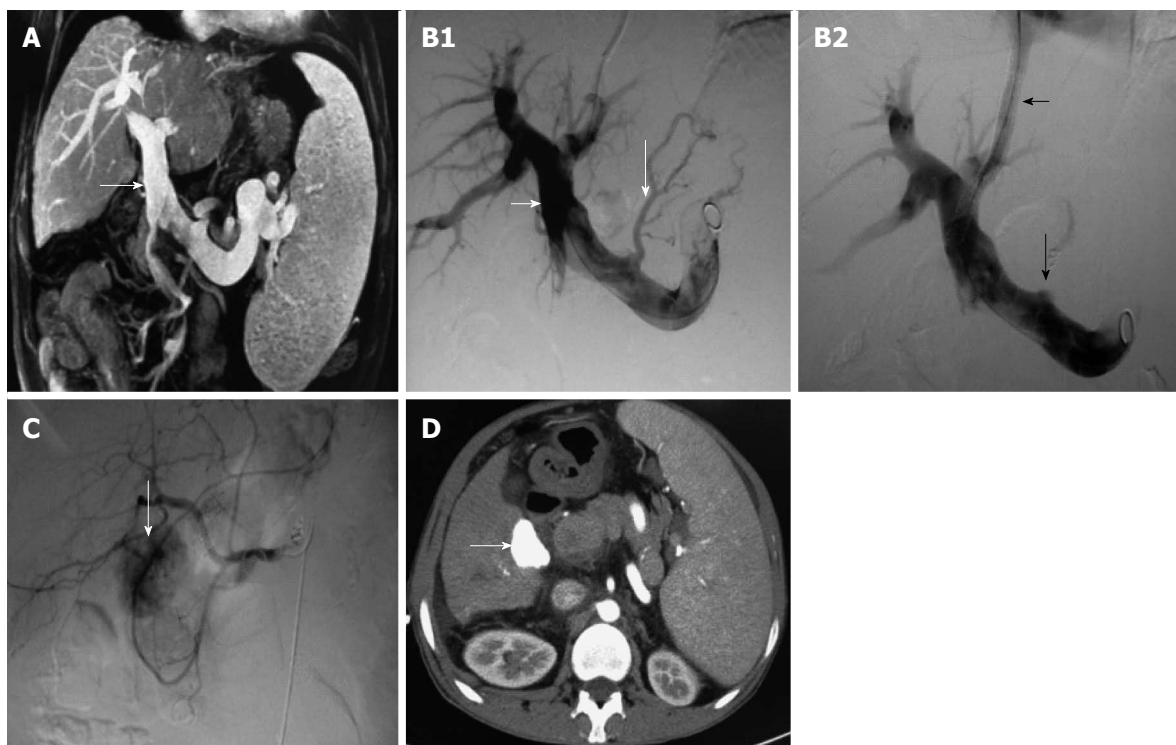
### Other interventional treatments

TACE or TAE was conducted before or after TIPS in 185 cases, from one to 11 times per patient. It was usually conducted once monthly for the first 3 mo, and the treatment intensity and interval were determined by the blood supply to the tumor lesion. RFA was conducted in 113 cases (mainly for lesions  $< 3$  cm in diameter and lack of blood supply), which was conducted alone in 24 cases and combined with TACE/TAE in 89 cases several days before RFA. TACE or TAE was conducted alone in 96 cases, hepatic arterio-portal fistulas embolization was used in 22 cases, and transcatheter splenic arterial embolization was used in 14 cases (Figure 1 and Figure 2).

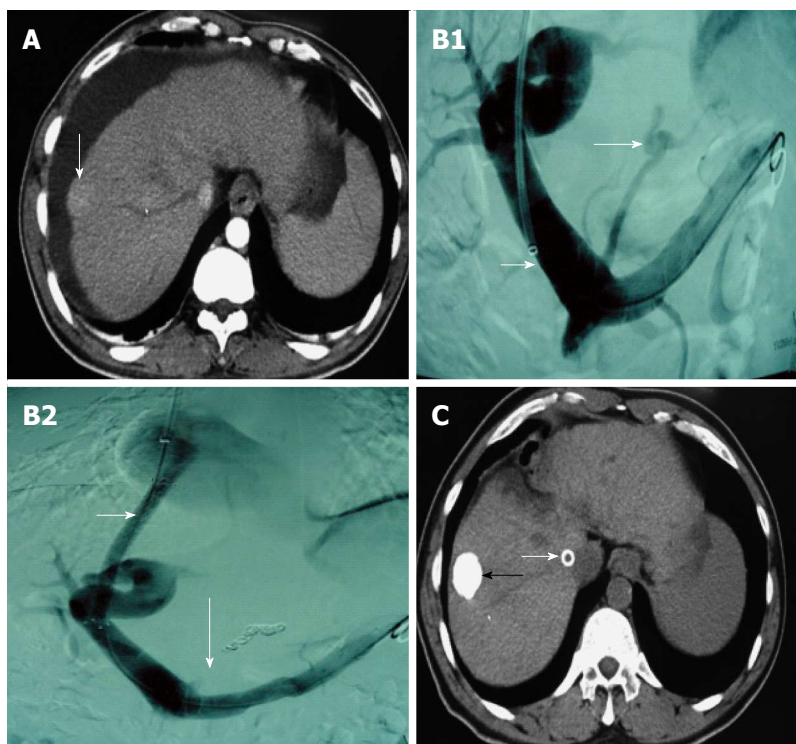
### Follow-up

All cases were followed up until death or 5 years. We observed clinical symptoms, medical events, routine blood measurements, AFP, blood coagulation, liver and kidney functions, and blood ammonia and performed color ultrasound, barium meal, or gastroscopy and CT/MRI. If the distributary channel was narrow, as shown by color ultrasound, or EGVB, resistant ascites, or hydrothorax occurred, we reviewed the distributary channel by radiography and measured the portal vein pressure at the same time. If the blood flow was normal, while portal pressure increased or the distributary channel was narrow or occluded under the radiography, we conducted balloon expansion and/or stent placement again. Then, if the original distributary channel was hard to reopen or the blood flow was insufficient, we conducted TIPS again to





**Figure 1** Patient with hepatitis C cirrhosis associated with esophageal gastric-fundus variceal bleeding was diagnosed with hepatocellular carcinoma 37 mo after transjugular intrahepatic portosystemic shunt. We treated HCC with TACE. A: Obvious portal vein dilation (white arrow) revealed by magnetic resonance cholangiopancreatography; B1: Obvious portal vein dilation (short white arrow) and gastric coronary vein varicosis (long white arrow); B2: Gastric coronary vein embolism (long white arrow) and distributary channel (short white arrow); C: Tumor lesion with rich blood supply in the right hepatic lobe 37 mo after TIPS by hepatic angiography (white arrow); D: Iodine oil deposited in the lesion after TACE, shown by contrast-enhanced CT (white arrow). HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization; TIPS: Transjugular intrahepatic portosystemic shunt; CT: Computed tomography.



**Figure 2** Patient with hepatitis B cirrhosis was diagnosed with hepatocellular carcinoma, refractory ascites, and esophageal gastric-fundus variceal bleeding. Patient underwent TACE after TIPS. A: Tumor lesion revealed by contrast-enhanced CT arterial phase (white arrow). B1: Obvious portal vein dilation (short white arrow) and gastric coronary vein varicosis (long white arrow). B2: Gastric coronary vein embolism (long black arrow) and distributary channel (short black arrow). C: Iodine oil deposited in the lesion after TACE, shown by CT (black arrow); ascites disappeared and distributary channel stent (white arrow). TACE: Transarterial chemoembolization; TIPS: Transjugular intrahepatic portosystemic shunt; CT: Computed tomography.



**Table 1 Results of 5-years follow-up *n* (%)**

Items	Patients
Refractory ascites or hydrothorax	15 (7.2)
EGVB	77 (36.8)
Hepatic encephalopathy	82 (39.2)
Distributary channel restenosis (yr)	
1	36 (17.2)
2	62 (29.7)
3	77 (36.8)
4	95 (45.5)
5	122 (58.4)
Interventional treatments	
RFA	113 (54.1)
Once	34 (30.1)
Twice	42 (37.2)
Thrice or more	37 (32.7)
TACE/TAE	185 (88.5)
Once	31 (16.8)
Twice	42 (22.7)
Thrice or more	112 (60.5)
Fistula embolization	22 (10.5)
Once	6 (27.3)
Twice	14 (63.6)
Thrice or more	2 (9.1)
Splenic artery embolization	14 (6.7)
Once	7 (50.0)
Twice	6 (42.9)
Thrice or more	1 (7.1)
Interventional re-treatments	102 (48.8)
Twice	62 (60.8)
Thrice or more	40 (39.2)
Balloon angioplasty	9 (8.8)
Stent angioplasty	93 (91.2)

EGVB: Esophageal gastric-fundus variceal bleeding; TACE: Transarterial chemoembolization; TAE: Transarterial embolization; RFA: Radiofrequency ablation.

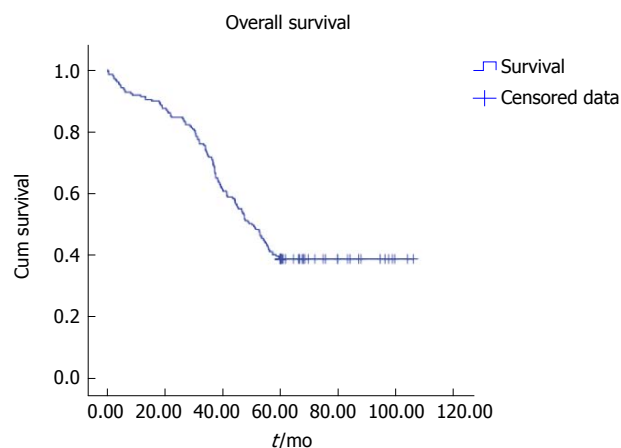
create a second channel. For the patients that needed sequential treatments for HCC after TIPS, we followed up or treated them once monthly in the first 3 mo, and then we followed up or treated them according to circumstances, usually once every 1-3 mo. For the patients who did not need sequential treatments for HCC, we followed up once every 3 mo. Follow-up results are shown in Table 1. Overall survival is shown in Table 2 and Figure 3.

### Statistical analysis

Statistical analysis was conducted using SPSS version 17.0 (Chicago, IL, United States). A *t* test was used for comparison of continuous measurement data, and  $\chi^2$  test for comparison of data between patients living  $\geq 5$  and  $< 5$  years.  $P < 0.05$  was considered as statistically significant. The overall survival time was estimated using the Kaplan-Meier method. The median survival time was calculated using censored observations only.

## RESULTS

The pre-TIPS portosystemic pressure was  $29.0 \pm 4.1$  mmHg, which decreased to  $18.1 \pm 2.9$  mmHg ( $t =$



**Figure 3** Survival functions of all patients by Kaplan-Meier survival curves.

69.32,  $P < 0.05$ ) after TIPS. The portal hypertension symptoms were relieved and improved; the rates of resistant ascites, hydrothorax, EGVB, hepatic encephalopathy, and distributary channel restenosis during follow-up were relatively impressive. Details including the interventional re-treatments for distributary channels and interventional treatments for tumor lesions are all presented in Table 1.

Clinical characteristics of patients living  $\geq 5$  and  $< 5$  years were analyzed and compared: patients' sex, mean age, lesion number, recurrence (ascites/bleeding), and distributary channel function did not differ significantly between the two groups ( $P > 0.05$ ). Moreover, Child-Pugh score, with or without PVTT, lesion diameter, hepatic arterio-portal fistulas, cancer diagnosed before or after TIPS, stent type, hepatic encephalopathy, and interventional treatment differed significantly between the two groups ( $P < 0.05$ ) (Table 3).

No procedure-related deaths or serious complications (e.g., abdominal bleeding, hepatic failure, and distant metastasis) occurred. The main cause of death during follow-up was gastrointestinal rebleeding (36 cases), which caused hemorrhagic shock, acute liver failure, and hepatic encephalopathy. Thirty-one cases died of liver failure or multiple organ failure; 29 of abdominal or lung infection; and 19 of tumor progression, which led to respiratory and circulatory failure. Other causes of death were hepatorenal syndrome and cardiovascular and cerebrovascular diseases.

Thus, the portal hypertension symptoms were ameliorated after TIPS and other interventional treatments with no procedure-related deaths and serious complications. Moreover, Child-Pugh score, PVTT, lesion diameter, hepatic arterio-portal fistulas, HCC diagnosed before or after TIPS, stent type, hepatic encephalopathy, and type of other interventional treatments were related to 5 year survival after comparing the characteristics of patients living  $\geq 5$  and

**Table 2 Means and medians for survival time**

Mean <sup>1</sup> estimate	SE	95%CI		Median estimate	SE	95%CI	
		Lower	Upper			Lower	Upper
62.067	2.582	57.007	67.128	50.300	2.995	44.431	56.169

<sup>1</sup>Estimation limited to the largest survival time if it is censored.**Table 3 Clinical characteristics of patients living  $\geq 5$  and  $< 5$  years *n* (%)**

Items	Total ( <i>n</i> )	Patients living		$t/\chi^2$	<i>P</i> value
		$\geq 5$ yr	$< 5$ yr		
Sex	209	81 (38.8)	128 (61.2)	0.367	0.545
Male	121	49 (60.5)	72 (56.2)		
Female	88	32 (39.5)	56 (43.8)		
Age (mean $\pm$ SD, yr)		49.2 $\pm$ 4	47.8 $\pm$ 8	2.868	0.488
Child-Pugh score				6.888	0.032 ( <i>P</i> < 0.05)
A	83	38 (46.9)	45 (35.2)		
B	72	30 (37.0)	42 (32.8)		
C	54	13 (16.1)	41 (32.0)		
PVTT				10.249	0.001 ( <i>P</i> < 0.05)
With	31	4 (4.9)	27 (21.1)		
Without	178	77 (95.1)	101 (78.9)		
Lesion number				0.263	0.608
Single	148	59 (72.8)	89 (69.5)		
Multiple	61	22 (27.2)	39 (30.5)		
Lesion diameter				18.351	0.0001 ( <i>P</i> < 0.05)
$\leq 3$ cm	52	27 (33.3)	25 (19.5)		
$> 3, \leq 5$ cm	103	46 (56.8)	57 (44.6)		
$> 5$ cm	54	8 (9.9)	46 (35.9)		
Hepatic arterio-portal fistulas				6.537	0.011 ( <i>P</i> < 0.05)
With	22	3 (3.7)	19 (14.8)		
Without	187	78 (96.3)	109 (85.2)		
Cancer diagnosed				5.973	0.015 ( <i>P</i> < 0.05)
Pre-TIPS	142	47 (58.0)	95 (74.2)		
Post-TIPS	67	34 (42.0)	33 (25.8)		
Stents				4.446	0.035 ( <i>P</i> < 0.05)
Bare stent	112	36 (44.4)	76 (59.4)		
Covered stent	97	45 (55.6)	52 (40.6)		
Recurrence (ascites/bleeding)				3.624	0.057
Yes	92	29 (25.8)	63 (49.2)		
No	117	52 (64.2)	65 (50.8)		
Hepatic encephalopathy				3.887	0.049 ( <i>P</i> < 0.05)
Yes	82	25 (30.9)	57 (44.5)		
No	127	56 (69.1)	71 (55.5)		
Interventional therapy				7.556	0.023 ( <i>P</i> < 0.05)
RFA	24	13 (16.0)	11 (8.6)		
TACE/TAE	96	28 (34.6)	68 (53.1)		
RFA + TACE/TAE	89	40 (49.4)	49 (38.3)		
Distributary channel function				3.274	0.070
Restenosis	122	41 (50.6)	81 (63.3)		
Unobstructed	87	40 (49.4)	47 (36.7)		

PVTT: Portal vein tumor thrombosis; TIPS: Transjugular intrahepatic portosystemic shunt; TACE: Transarterial chemoembolization; TAE: Transarterial embolization; RFA: Radiofrequency ablation.

&lt; 5 years.

## DISCUSSION

HCC tends to be associated with liver cirrhosis and portal hypertension, even gastrointestinal hemorrhage and/or refractory ascites<sup>[27]</sup>. In such cases, the priority is to manage the symptoms and complications of

portal hypertension, rather than HCC itself. TIPS is an effective method for resolving the symptoms of portal hypertension<sup>[10]</sup>. After TIPS, the portal vein blood flow to the liver is reduced, and the hepatic artery blood flow is increased to compensate for the reduction<sup>[28,29]</sup>, based on the interdependence of the portal vein and hepatic artery<sup>[30]</sup>. In theory, the extra blocking of the blood supply from the hepatic artery (such as

TAE/TACE) aggravates liver damage after TIPS, due to ischemia<sup>[18]</sup>. However, the impact on the clinical curative effect is still not clear. Kuo *et al.*<sup>[18]</sup> reported that the rate of complete response was significantly greater in non-TIPS patients compared with TIPS patients (74% vs 30%,  $P = 0.03$ ) after TACE. Objective response rate (complete and partial response) tended to be greater in the non-TIPS group (83% vs 50%,  $P = 0.09$ ). TACE was less effective in achieving a complete or partial response in TIPS patients compared with those without TIPS. Kohi *et al.*<sup>[21]</sup> reported that the incidence of severe hepatobiliary adverse events after TACE was nearly two times higher in patients with TIPS (70%) than in those without TIPS (36%,  $P = 0.046$ ). Thus, patients with HCC and patent TIPS were more likely to develop significant hepatotoxicity after TACE than comparable patients without TIPS. Conversely, Kang *et al.*<sup>[26]</sup> reported that after TACE, 14 of 20 (70%) patients showed a tumor response, with only one (5%) experiencing a TACE-related major complication. Selective TACE may be safe and effective for the palliative treatment of HCC in patients with TIPS. In patients with HCC and TIPS receiving locoregional tumor therapy, Padia *et al.*<sup>[23]</sup> showed that ablation procedures resulted in low rates of hepatotoxicity. However, the above studies lacked long-term clinical observation and had a small numbers of cases. In this study, we reviewed 209 patients with HCC and portal hypertension who underwent successful TIPS. Among them, 96 cases were treated in combination with TACE/TAE; 89 in combination with TAE/TACE and RFA; and 24 in combination with RFA. Hepatic arterio-portal fistulas embolization was also used in 22 cases, and splenic artery embolization in 14. There were no procedure-related deaths and serious complications (e.g., abdominal bleeding, hepatic failure, and distant metastasis). Thus, TIPS combined with other interventional treatments, such as RFA and TACE, are technically feasible and safe for patients with HCC and portal hypertension.

Regarding efficacy, some studies have shown that TIPS combined with TACE/TAE is effective<sup>[22-24,26]</sup>. However, reports of TIPS combined with RFA and other interventional treatments are rare. Padia *et al.*<sup>[23]</sup> reported the outcomes of locoregional tumor therapy in 48 cases of HCC treated with TIPS. Twenty-nine of 48 (60%) patients were assigned to the local treatment group, which had received RFA 39 times, chemoembolization 17 times, and yttrium-90 radioembolization 10 times. Nineteen of 48 (40%) patients received best supportive care (*i.e.*, symptomatic management only). Follow-up imaging response showed an objective response for all ablation procedures, 67% of radioembolization procedures, and 50% of chemoembolization procedures ( $P = 0.001$ ). When censored for orthotopic liver transplantation, patients undergoing treatment survived longer than those receiving supportive care (2273 d vs 439 d,  $P = 0.001$ ). It was concluded that ablation appears to be

safe and efficacious for patients with HCC treated with TIPS. Moreover, the survival rates at 1, 2, 3, 4, and 5 years in our study were 91.4% (191/209), 84.7% (177/209), 71.8% (150/209), 51.2% (107/209), and 38.8% (81/209), respectively. The 5 year survival rate seems better than that for treating by TACE alone, which was reported as 26%<sup>[31]</sup>. It also seems better than the survival rates at 1, 2, and 3 years for RFA of 60%, 39%, and 35%, respectively<sup>[32]</sup>. We compared the clinical characteristics of patients living  $\geq 5$  and  $< 5$  years, and Child-Pugh score, PVTT, lesion diameter, hepatic arterio-portal fistulas, HCC diagnosed before or after TIPS, stent type, hepatic encephalopathy, and type of other interventional treatments had significant differences between the two groups. A prospective randomized controlled trial may be needed to confirm that the above factors affect 5 year survival. In addition, with TIPS using bare stents, it is easy to develop shunt stenosis or occlusion<sup>[16]</sup>. An increase in symptom recurrence rate and the incidence of hepatic encephalopathy after TIPS, which may adversely affect quality of life and accelerate liver function deterioration, is the main cause of death. In the study, the cumulative rates of tributary channel restenosis tended to be similar to that of expanded polytetrafluoroethylene covered stent<sup>[33]</sup>. TACE was used alone mainly for patients that were not suitable for combination with RFA or in whom the lesions had a large diameter, which made it difficult to achieve necrotic collapse of the lesions. Therefore, the curative effects were inferior to those in patients treated by TACE combined with RFA. In normal follow-up, early detection of recurrent liver lesions and timely interventional treatment are crucial. Thus, the curative effect for patients with HCC diagnosed after TIPS was better than that for patients with HCC diagnosed before TIPS.

In conclusion, TIPS combined with other interventional treatments seems a safe and efficacious choice for patients with HCC and portal hypertension.

## COMMENTS

### Background

Patients with hepatocellular carcinoma (HCC) accompanied with portal hypertension often have no opportunity to receive radical surgery or liver transplantation. For some interventional treatments, it is important to manage portal hypertension urgently in patients HCC. Transjugular intrahepatic portosystemic shunt (TIPS) is widely used as a treatment for portal hypertension. However, the safety and efficacy of TIPS combined with other interventional treatments for patients with HCC and portal hypertension are still not clear.

### Research frontiers

There are few English language studies concerning combination of TIPS with other interventional treatments for patients with HCC and portal hypertension. The small number of studies has yielded conflicting results for safety and efficacy, and they were conducted in small patient populations. Kuo *et al.* reported that transarterial chemoembolization (TACE) was less effective in achieving complete or partial response, in TIPS patients compared with those without TIPS. Kohi *et al.* reported that patients with HCC and patent TIPS were

more likely to develop significant hepatotoxicity after TACE than comparable patients without TIPS. However, Kang *et al* reported that TACE may be safe and effective for the palliative treatment of HCC in patients with TIPS. Outcomes of locoregional tumor therapy for patients with HCC and TIPS by Padia *et al* showed that ablation procedures resulted in low rates of hepatotoxicity.

### Innovations and breakthroughs

Most of the studies reporting the safety and efficacy of TIPS combined with other interventional treatments for patients with HCC and portal hypertension had no more than 20 patients, which could have affected the clinical observations and conclusions. However, the authors reviewed and analyzed complete 5 year clinical data of 209 HCC patients with portal hypertension who were treated with TIPS and other interventional treatments. The authors also analyzed the characteristics of patients living  $\geq 5$  and  $< 5$  years. Moreover, the results were encouraging.

### Applications

This results suggested that TIPS combined with other interventional treatments seemed a safe and efficacious choice for patients with HCC and portal hypertension. There were no procedure-related deaths and serious complications (e.g., abdominal bleeding, hepatic failure, and distant metastasis). The survival rates seem better than those reported for TACE or radiofrequency ablation alone.

### Terminology

TIPS is an expandable metal stent inserted via the jugular vein that creates a shunt from the portal vein to the systemic circulation via an artificial communication through the liver.

### Peer-review

In this study, the authors evaluated the long-term clinical safety and efficacy of TIPS combined with other interventional treatments for patients with HCC and portal hypertension. The main concept is interesting because there is limited data in this patient population.

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## Randomized Clinical Trial

# Epidural anesthesia improves pancreatic perfusion and decreases the severity of acute pancreatitis

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## Abstract

**AIM:** To study the safety of epidural anesthesia (EA), its effect on pancreatic perfusion and the outcome of patients with acute pancreatitis (AP).

**METHODS:** From 2005 to August 2010, patients with predicted severe AP [Ranson score  $\geq 2$ , C-reactive protein  $> 100$  or necrosis on computed tomography (CT)] were prospectively randomized to either a group receiving EA or a control group treated by patient

controlled intravenous analgesia. Pain management was evaluated in the two groups every eight hours using the visual analog pain scale (VAS). Parameters for clinical severity such as length of hospital stay, use of antibiotics, admission to the intensive care unit, radiological/clinical complications and the need for surgical necrosectomy including biochemical data were recorded. A CT scan using a perfusion protocol was performed on admission and at 72 h to evaluate pancreatic blood flow. A significant variation in blood flow was defined as a 20% difference in pancreatic perfusion between admission and 72 h and was measured in the head, body and tail of the pancreas.

**RESULTS:** We enrolled 35 patients. Thirteen were randomized to the EA group and 22 to the control group. There were no differences in demographic characteristics between the two groups. The Balthazar radiological severity score on admission was higher in the EA group than in the control group (mean score  $4.15 \pm 2.54$  vs  $3.38 \pm 1.75$ , respectively,  $P = 0.347$ ) and the median Ranson scores were 3.4 and 2.7 respectively ( $P = \text{NS}$ ). The median duration of EA was 5.7 d, and no complications of the epidural procedure were reported. An improvement in perfusion of the pancreas was observed in 13/30 (43%) of measurements in the EA group vs 2/27 (7%) in the control group ( $P = 0.0025$ ). Necrosectomy was performed in 1/13 patients in the EA group vs 4/22 patients in the control group ( $P = 0.63$ ). The VAS improved during the first ten days in the EA group compared to the control group ( $0.2$  vs  $2.33$ ,  $P = 0.034$  at 10 d). Length of stay and mortality were not statistically different between the 2 groups (26 d vs 30 d,  $P = 0.65$ , and 0% for both respectively).

**CONCLUSION:** Our study demonstrates that EA increases arterial perfusion of the pancreas and improves the clinical outcome of patients with AP.

**Key words:** Severe acute pancreatitis; Epidural anesthesia; Pancreatic necrosectomy; Pancreatic perfusion; Computed tomography

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**Core tip:** We conducted this prospective randomized study to explore the safety of epidural anesthesia (EA), its effect on pancreatic perfusion and the outcome of patients with acute pancreatitis, as high mortality is linked with necrosis of the gland. We found an improvement in perfusion of the pancreas in the EA group. Necrosectomy was performed in 1/13 patients in the EA group vs 4/22 patients in the control group.

Sadowski SM, Andres A, Morel P, Schiffer E, Frossard JL, Platon A, Poletti PA, Bühler L. Epidural anesthesia improves pancreatic perfusion and decreases the severity of acute pancreatitis. *World J Gastroenterol* 2015; 21(43): 12448-12456 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12448.htm> DOI:

## INTRODUCTION

Acute pancreatitis (AP) is a common disease whose incidence in the US reaches 35 per 100000 population annually<sup>[1]</sup>. The main causes of AP in adults are gallstone migration into the common bile duct and alcohol abuse. Approximately 80% of patients with AP will develop a mild disease for which the management is mainly conservative<sup>[2]</sup>. However 20% will develop a severe form that is associated with the development of local complications such as pancreatic and peripancreatic necrosis, pseudocysts, as well as systemic complications such as adult respiratory distress syndrome or renal failure. In the severe form of AP, the mortality rate can reach 17% and is primarily due to multiple organ failure and pancreatic necrosis. In particular, pancreatic necrosis is associated with a death rate of up to 40%<sup>[3]</sup>.

The pathophysiology of necrotizing AP is not yet fully understood, though animal studies<sup>[4-6]</sup> suggest that an alteration in pancreatic microcirculatory blood flow as well as arterial vasoconstriction and ischemia-reperfusion injury are contributing factors. Microcirculatory dysfunction results in part from hypercoagulability and an increase in microvascular permeability that is mediated by the local and systemic inflammatory response (leukocyte activation as well as release of free radicals and cytokines). Kusterer *et al*<sup>[7]</sup> showed that, in animals, pancreatitis is associated with early arteriolar vasoconstriction and hypoperfusion of the pancreatic microcirculation. Thus, early in the course of AP, a decrease in pancreatic blood flow occurs that potentially plays a role in the development of necrotizing AP<sup>[4]</sup>.

Epidural anesthesia (EA) is widely used to induce analgesia in the perioperative period and has also been used to decrease pain in patients with AP<sup>[8]</sup>. In addition, experimental studies have shown a specific beneficial effect of EA in AP attributed to a sympathetic nerve blockade that redistributes splanchnic blood flow to non-perfused pancreatic regions<sup>[9,10]</sup>.

We previously performed an animal study showing that EA improves pancreatic hypoperfusion induced by AP and decreases the severity of metabolic acidosis and tissue injury, thus preventing progression of an edematous disease to necrotizing AP<sup>[4]</sup>. Clinically this can be measured with radiological perfusion studies of the pancreas<sup>[11]</sup>.

We conducted a clinical randomized pilot study aimed at evaluating: (1) the safety of EA in predicted severe AP patients; (2) whether EA increases the perfusion of the pancreatic gland, which explains a decrease in severity of the disease; and (3) whether EA improves the clinical outcome of patients with predicted severe AP.

## MATERIALS AND METHODS

### Study design

We conducted a prospective randomized controlled study and included data from all adult patients admitted to the surgical unit of the University Hospital of Geneva for predicted severe AP.

The study began in July 2005 and ended in August 2010. The Ethics Board of Geneva's University Hospital approved the study in 2004 (HUG 02-0555). The trial began as a pilot study when registration was not mandatory. However, registration for randomized clinical trials was subsequently performed: NCT01607996.

Upon admission, the severity of pancreatitis was established according to the Ranson classification<sup>[12,13]</sup>. We included all patients presenting an AP with a Ranson score  $\geq 2$  and/or a C-reactive protein  $> 100$  (mg/L) and/or necrosis on computed tomography (CT). After obtaining written consent from the patients who met the inclusion criteria, they were randomized into two study groups: (1) study group treated with EA; and (2) control group treated by patient controlled intravenous analgesia (PCA). Randomization was established with a closed envelope.

Exclusion criteria were absence of severe pancreatitis as defined previously, patients with contraindications to epidural anesthesia (skin infection of the vertebral region, coagulation disorders, iodine allergy), inability to obtain consent and concurrent participation in another clinical trial. According to our standardized protocol, all information on admission was gathered, including demographic characteristics such as age, sex and race. All medical information on past history, diagnostic, radiological and biochemical data and past and current medication was included.

For patients allocated to group 1 (treated with EA), EA was inserted immediately after the admission CT scan was obtained and used for up to 5 d following randomization. The standardized protocol for EA contained a mix of Carbostesine (Bupivacaine) at a concentration of 0.1% and Fentanyl at 2  $\mu$ g/mL. The doses were established according to a pain score at rest and at mobilization and according to the sensitivo-motor bloc (ether test and Bromage scale). It was administered with a continuous infusion of the mix at a minimum of 6 mL to a maximum of 15 mL/h. A bolus between 3 to 5 mL every 30 to 60 min could be added upon request.

Patients allocated to group 2 received standardized intravenous analgesia using a PCA, which was started as soon as the patient was randomized. The medication contained Fentanyl at a concentration of 10  $\mu$ g/mL. The continuous debit was used at a rate of 10 to 20  $\mu$ g/h in association with pushes on demand of 1 to 2 mL every 5 to 10 min. The maximum dose was 400  $\mu$ g every four hours. The duration of this therapy was between 3 to 5 d and was conducted using the same criteria as the EA.

We evaluated pain management in the two groups every eight hours using the visual analog pain scale (VAS)<sup>[14,15]</sup>, scaled from zero to ten (zero meant no pain, and ten meant the worst pain tolerable). All modifications in pain management, blood pressure surveillance, cardiac and respiratory frequency and venous oxygen saturation were reported.

The same management was applied to the two groups, including a nothing by mouth regimen, a urinary catheter, prophylactic anticoagulation (Lique-mine 2-3  $\times$  5000 U/d sc according to weight), an antisecretory medication (Omeprazol 40 mg/d iv) and parenteral nutrition according to patient weight and local protocol.

### CT scan protocol

Both groups were evaluated according the same protocol. A CT scan was obtained on admission and 72 h after admission along with a radiological perfusion study of the pancreas to evaluate pancreatic blood flow. A significant variation in blood flow was defined as a 20% difference in perfusion between admission and 72 h measured in the head, body and tail of the pancreas, which is higher than the maximal standard deviation associated with the perfusion measures (19.4%), as shown in a previous study at our center<sup>[11]</sup>. Perfusion series were anonymized and downloaded separately from the rest of the CT examination, onto a dedicated workstation. These perfusion series were analyzed in a delayed fashion, once all patients had been included in the series. For this reading the radiologists were blinded to the treatment allocation and especially to the presence of an epidural catheter because perfusion images were limited to the upper abdomen.

Perfusion studies were obtained on a 16 row CT-scanner (MX 8000, Philips Medical Systems, Best, the Netherlands). Dynamic series (90 kV, 100 mAs) were performed using four slices with a beam collimation of 6 mm, targeted on the pancreas. Acquisition started simultaneously with an IV injection of a 40-mL bolus of iodinated contrast [Ultravist 300 (iopromide), Schering] at a flow-rate of 5 mL/s, performed during a single breath-hold. A total of 160 images (40 images for each slice level) were obtained during the dynamic examination.

Perfusion images were analyzed on a dedicated workstation (Advantage Windows, GE healthcare) using the positive enhancement integral (PEI) method, compatible with Contrast Enhanced Dynamic Acquisitions, based on rates of contrast uptake by the parenchyma.

Whenever possible, two different perfusion measurements by PEI were obtained on the head, body, and tail of the pancreas for both the admission and control CT. Perfusion measures were never performed in necrotic tissue. For each of these areas, the examination was considered relevant when the region of interest remained within the pancreas parenchyma during



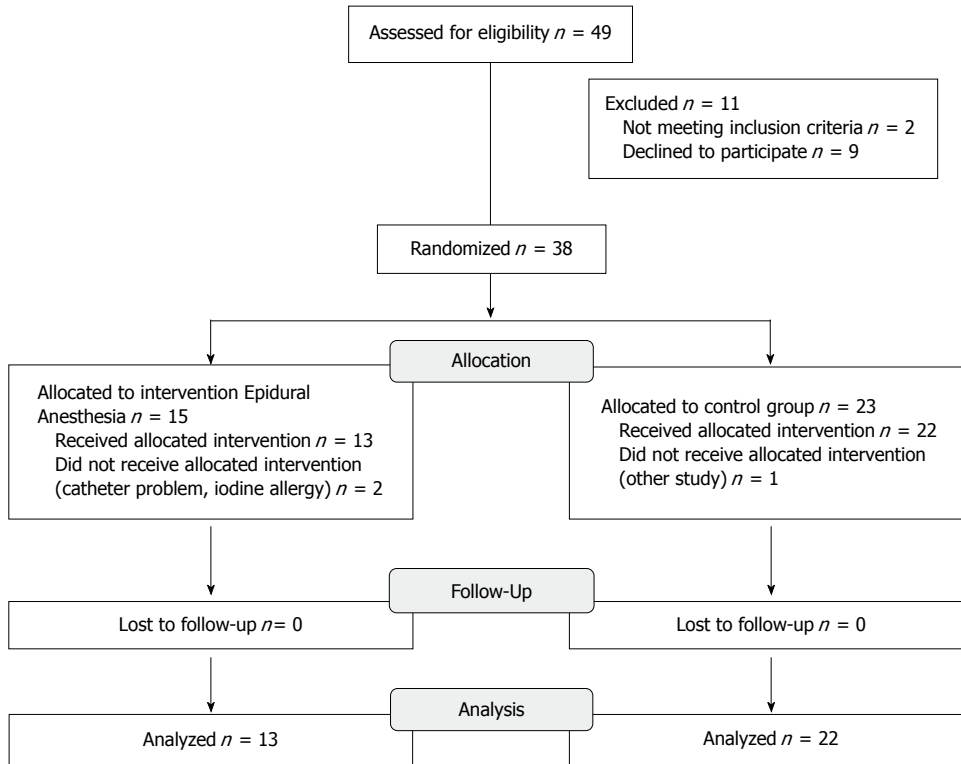


Figure 1 CONSORT diagram showing randomization and allocation of the study cohort.

the entire acquisition. The perfusion of a given area was considered to be improved (respectively, to be impaired) between the first and the second CT when the PEI value measured on the second CT was at least 20% higher (respectively, lower) when compared to the admission CT. When the difference in perfusion was less than 20% between the two CT, the perfusion was considered unchanged. When one of the two measures was not feasible, no value was reported.

#### Definition of the “primary endpoint”

Our primary endpoint was determined by “Safety of EA in patients with AP”.

#### Definition of covariates

Including: (1) pancreatic blood perfusion determined by the CT analysis according to our perfusion protocol; (2) parameters for clinical severity: length of stay in the hospital, use of antibiotics, admission to the intensive care unit, the clinical systemic and loco-regional complication score established by Clavien<sup>[16]</sup>, and requirements for surgical necrosectomy; (3) pain was evaluated using the VAS to measure the effect of EA on the severity of the pain due to AP; and (4) complications detected by imaging studies: cysts or fluid collections, necrotic collections and other infectious adverse events.

#### Statistical analysis

Fisher’s exact test and the Wilcoxon Mann-Whitney test

were used to compare dichotomous and continuous variables between the two groups and results were expressed as means with standard deviation and proportions. Crude odds ratios (OR) and 95% CIs were calculated. All statistical analyses were performed using SPSS version 18.0.

We calculated that 50 patients were needed in each group to detect an OR of > 2.5 with a significance level of 0.05 and a power of 80% using power and sample size calculation software version 2.1.31.

## RESULTS

Between July 2005 and August 2010, a total of 49 patients met the inclusion criteria, as shown in Figure 1. After obtaining a written consent, 15 patients were randomized to the EA group and 23 patients to the control group. Of these, we had to exclude 2 patients in the EA group because of contraindications to the insertion of an epidural catheter and 1 patient in the control group because of involvement in another study. In summary, 13 patients were randomized to the EA group and 22 patients to the control group. Follow-up was the duration of the hospital stay.

#### Demographics of study population

Characteristics of each group and the results of univariate analyses are summarized in Table 1. Univariate analyses revealed that both groups were similar with respect to age, sex, BMI, and comorbid

**Table 1 Clinical characteristics of patients with severe acute pancreatitis**

Factor	Epidural anesthesia ( <i>n</i> = 13)	Control group ( <i>n</i> = 22)	<i>P</i> value <sup>1</sup>
Demographics			
Age (yr)	66.08 (11.67)	57.36 (17.97)	0.092
Male sex, <i>n</i> (%)	7 (55.8)	12 (54.5)	0.968
BMI (kg/m <sup>2</sup> )	27.82 (6.13)	29.15 (7.08)	0.623
Pain duration prior hospitalization (h)	87.46 (198.85)	90.86 (208.36)	0.962
Comorbid conditions, <i>n</i> (%)			0.561
Diabetes	3 (23.1)	3 (13.6)	
Hypertension	2 (15.4)	7 (31.8)	
High cholesterol	2 (15.4)	3 (13.6)	
Obesity	1 (7.7)	5 (22.7)	
Chronic alcoholism	3 (23.1)	3 (13.6)	
Cardiovascular condition	2 (15.4)	1 (4.5)	
Aetiology of pancreatitis, <i>n</i> (%)			0.901
Alcoholic	3 (23.1)	6 (27.27)	
Biliary	7 (53.8)	13 (59.1)	
Hyperlipidaemia	1 (7.7)	1 (4.5)	
Medication	0	0	
Unknown	2 (15.4)	2 (9.1)	
Ranson score at admission	3.38 (1.12)	2.68 (0.945)	0.056

<sup>1</sup>Groups were compared with a *t*-test for continuous variables (or Wilcoxon Mann-Whitney test) and a Pearson  $\chi^2$  test (or Fisher exact test) for categorical variables. Values are mean (standard deviation) for continuous variables and *n* (%) for categorical variables.

conditions.

The etiology of pancreatitis was not different between the two groups (alcoholic AP in 23% of the EA group vs in 27% of the control group, biliary AP 54% vs 59%, hyperlipidemic AP 7.7% vs 4.5%, unknown causes 15% vs 9.1% respectively, *P* = NS).

In the EA group, the mean Ranson score was higher than in the control group (Ranson score  $3.38 \pm 1.12$  vs  $2.68 \pm 0.9$ , *P* = 0.056), although this did not reach statistical significance.

### **Safety of epidural anesthesia in patients with acute pancreatitis**

Our study showed no complications of the epidural procedure in patients with predicted severe AP. There were no catheter-related infections and no cases of hemodynamic complications during procedure. The median time of EA was 5.7 d.

### **Epidural anesthesia improves pancreatic perfusion**

Altogether, 57 comparative perfusion measurements were obtained in the same pancreatic area in both groups on the first and on the second CT (114 measures in total). When comparing perfusion studies on admission to those obtained at 72 h (Figures 2 and 3), a significant improvement in arterial perfusion of the pancreas was observed in 13 (43%) of 30 measurements in the EA group, and in 2 (7%) of 27 measurements in the control group. No change in perfusion was observed in 10 (33%) and 9 (33%)

perfusion measurements in the EA group and in the control group respectively. The difference between the perfusion improvement (*n* = 15) and all other cases (no change or decrease, *n* = 42) was statistically significant using a two-tailed Fisher's exact test (*P* = 0.0025) (Figure 4).

### **Comparison of pancreatic necrosis on CT scan**

On the CT scans, we compared the radiological severity score "Balthazar severity index"<sup>[17]</sup> between the two groups ranging from 1 to 10 and based on pancreatic morphology, pancreatic necrosis, and retroperitoneal complications. As shown in Table 2, the Balthazar score on admission was higher in the EA group than in the control group (mean score  $4.15 \pm 2.54$  vs  $3.38 \pm 1.75$ , respectively, *P* = 0.347) and was higher as well at 48 h (mean score  $4.69 \pm 2.59$  in the EA group and  $4.17 \pm 2.01$  in the control group, *P* = 0.548).

Analysis of radiological interventions showed that five patients in the EA group and ten patients in the control group had a CT guided puncture of peri-pancreatic fluid or of necrotic collections (38.5% vs 45.5%, *P* = 0.68). Of these punctures, one in the EA group and six in the control group were infected (20% vs 54.5%, *P* = 0.3). This was not a statistically significant difference.

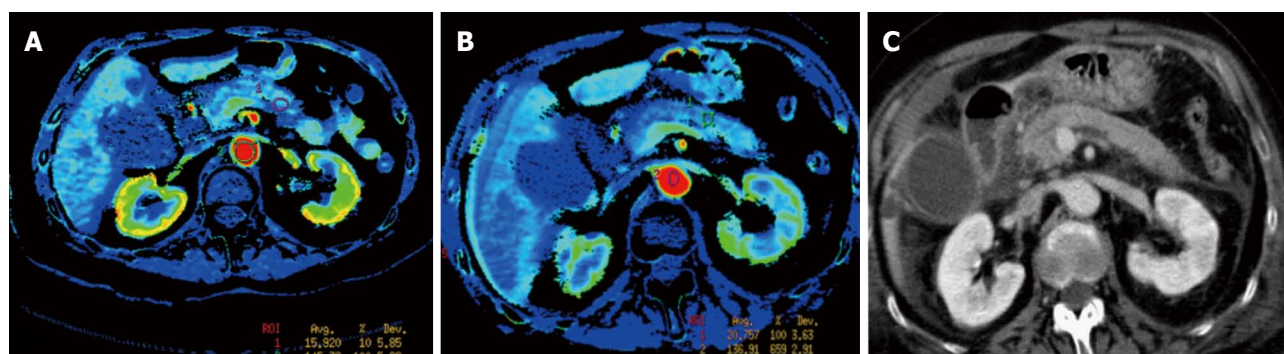
### **Epidural anesthesia and requirements for necrosectomy**

Surgical pancreatic necrosectomy was performed in one patient in the EA group compared to four patients in the control group (7.7% vs 18.2%, *P* = 0.63) (Table 2). There was a trend towards EA reduction in the risk of necrosectomy that did not reach statistical significance.

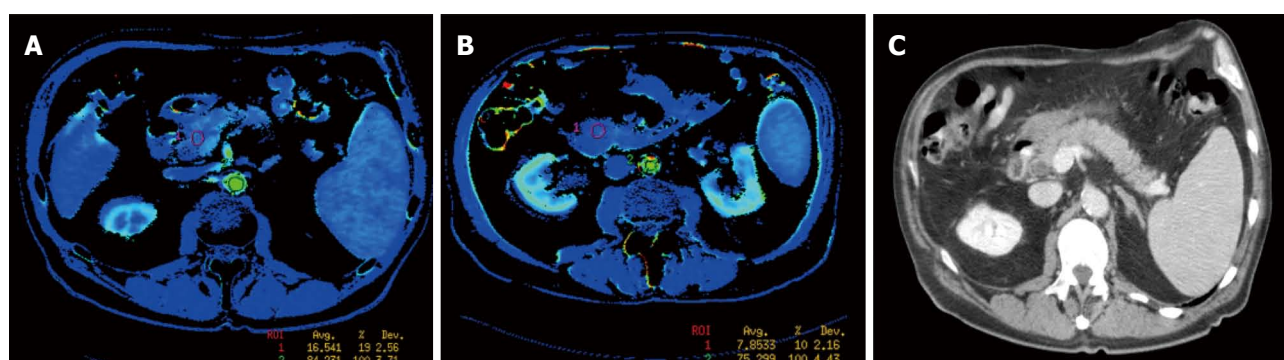
### **Clinical outcome and mortality**

To evaluate the clinical severity of AP, we looked at sepsis, organ failure and the need for an ICU stay. As shown in Table 2, the biochemical data on admission and on day 2 (48 h) did not differ between the two groups. Further, four patients in the EA group and ten in the control group had to be admitted to the intensive care unit (33% vs 45.5%, *P* = 0.493). In the EA group, none of the patients developed clinical sepsis and only one needed intubation, whereas in the control group, six patients needed intubation for acute respiratory distress (7.7% vs 27.3% had an intubation, respectively, *P* = 0.22). Furthermore, the use of antibiotics was not different between the two groups (61.5% of patients of the EA group and 68.2% of the control group, *P* = 0.689), nor was the duration of therapy.

During hospitalization, the EA group developed 9 cases of loco-regional complications and 10 cases of systemic complications compared to the control with 12 cases of loco-regional complications and 13 systemic complications. According to Claviens' grading system from one to four, there was no significant



**Figure 2** Perfusion computed tomography color map and standard computed tomography of the pancreas obtained in a 75-year-old woman admitted for acute severe pancreatitis, randomized to group 1 (epidural anesthesia). A: Axial image obtained at admission shows a positive enhancement integral (PEI) value of 15.9 in the pancreatic body; B: Axial image obtained with control computed tomography (CT), 72 h after admission, shows a 29% improvement of the perfusion in the pancreatic body (PEI of 20.5) when compared to admission values; C: Standard CT axial oblique image at the level of the pancreas obtained on admission, during portal phase.



**Figure 3** Perfusion computed tomography color map and standard computed tomography of the pancreas obtained in a 73-year-old man admitted for acute severe pancreatitis, randomized to group 2 (control group). A: Axial image obtained on admission shows a positive enhancement integral (PEI) value of 16.5 in the pancreatic head; B: Axial image obtained with control computed tomography (CT), 72 h after admission, shows a 53% impairment of the perfusion in the pancreatic head (PEI of 7.8) when compared to admission values; C: Standard CT axial oblique image, at the level of the pancreas obtained on admission, during portal phase.

difference between the two groups in the mean score for loco-regional and for systemic complications (loco-regional:  $1.54 \pm 1.45$  vs  $1.55 \pm 1.65$ ,  $P = 0.99$  and systemic:  $1.77 \pm 1.64$  vs  $1.73 \pm 1.75$ ,  $P = 0.95$ ) (Table 2).

Also, length of stay and mortality were not statistically different between the two groups (26 d in the EA group vs 30 d in the control group,  $P = 0.65$ , and 0 mortality in both groups).

### **Epidural anesthesia improves pain management**

The VAS showed an improvement in subjective pain during the first twelve days in the EA group compared to the control group, with a significant difference on the day of EA implementation and at ten days. The results for the mean pain score on a scale from one to ten were before randomization  $6.55$  vs  $7.31$ ,  $P = 0.57$ ; after EA implementation  $1.6$  vs  $3.5$ ,  $P = 0.02$ ; at day one  $0.57$  vs  $2$ ,  $P = 0.06$ ; at day five  $1.86$  vs  $1.38$ ,  $P = 0.69$ ; at day ten  $0.2$  vs  $2.33$ ,  $P = 0.034$ ; at day twelve  $0$  vs  $2.8$ ,  $P = 0.071$  (Table 2).

## **DISCUSSION**

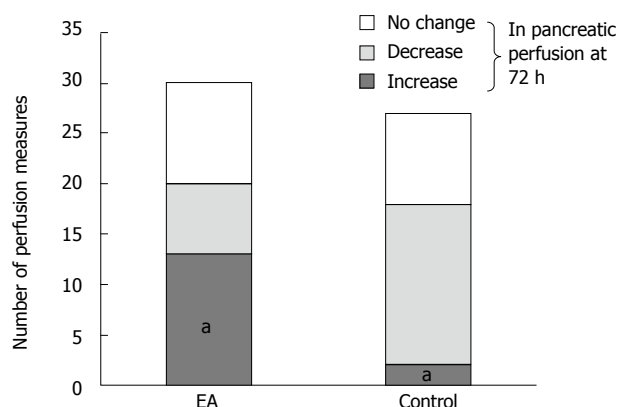
This study is the first to randomize the use of EA for

the treatment of AP. Previous studies have shown a beneficial effect of EA on pain management in patients with predicted severe AP<sup>[8,18]</sup>. Furthermore, the safety of EA has been widely documented in the literature<sup>[19,20]</sup> and its benefit on postoperative morbidity and mortality are well known<sup>[21-23]</sup>.

We chose to present the results as a pilot study because one primary and one secondary endpoint showed statistical significance and several others showed a trend towards better outcome after EA. Our data indicated a better outcome for the EA group, though this should be confirmed by a multi-center phase II clinical trial using a larger sample size.

More than one factor seems to activate the pathological process leading to AP. The activation of pancreatic enzymes leading to edema and necrosis<sup>[24]</sup>, vasoconstriction and pancreatic ischemia may convert mild disease to predicted severe AP with parenchymal necrosis<sup>[13,25,26]</sup>. This has been demonstrated by Klar *et al*<sup>[6]</sup> who have shown experimentally that pancreatic blood flow decreases with severe pancreatitis.

Previous studies have shown that EA increases blood flow and delays metabolic acidosis, though this has been largely investigated in the gut<sup>[9,27]</sup>. These



**Figure 4** Changes in pancreatic perfusion measurements at 72 h compared to the measurements. On admission in the epidural anesthesia (EA) and control group (significant increase, decrease or no change in perfusion). <sup>a</sup>*P* = 0.0025 vs control.

effects have been attributed to a sympathetic nerve blockade that redistributes blood flow to non-perfused regions<sup>[9,10]</sup>.

At our center, using an animal model reproducing AP, Demirag *et al.*<sup>[4]</sup> showed that EA has a beneficial effect on the severity of AP, suggesting that EA leads to an improvement in pancreatic blood flow with a concomitant decrease in the severity of metabolic acidosis and diminished tissue injury.

Development of pancreatic necrosis is a critical event in AP that determines patient prognosis because it is often accompanied by infection and multiple organ dysfunction syndromes and, thus, is associated with a high mortality<sup>[3,28-30]</sup>. Therefore, early detection of necrosis is important for the appropriate treatment of predicted severe AP. The literature supports the use of CT scan perfusion studies to measure blood flow and diagnose necrosis in the pancreas<sup>[11,31,32]</sup>. Our measures of pancreatic perfusion showed significant improvement of the parenchymal blood flow within the pancreatic gland in the group treated with EA when compared to the control group on admission and at 72 h. This observation substantiates the theory that the severity of AP may be related to a vasoconstriction phenomenon, which can be attenuated by EA. It also suggests that the use of EA decreases progression from edematous to severe necrotizing pancreatitis caused by early ischemia of the gland and thus could reduce the severity of the disease.

In our study, there was a significant drop in subjective pain feeling at ten days as well as fewer organ failures and admissions to the ICU in the group treated with EA. Furthermore, the number of patients with infected necrosis and subsequent sepsis requiring necrosectomy was lower in the EA group, although this was not statistically significant. In general, infection occurs in about 40%-70% of patients with necrotizing pancreatitis requiring surgical debridement with necrosectomy<sup>[33]</sup>. However, these procedures have a high complication and mortality rate, ranging from

**Table 2** Clinical outcome of patients with severe acute pancreatitis

Factor	Epidural anesthesia (n = 13)	Control group (n = 22)	P value <sup>1</sup>
Biochemical data			
Blood pH at admission	7.4 (0.05)	7.38 (0.07)	0.270
Blood pH day 2	7.43 (0.04)	7.4 (0.04)	0.180
CRP at admission (mg/L)	58 (102)	87 (118)	0.470
CRP day 2	274 (108)	245 (145)	0.550
Glucose at admission (mmol/L)	10.66 (4.29)	9.5 (4.76)	0.480
Glucose day 2	8.36 (2.1)	7.9 (3.07)	0.630
Amylase at admission (U/L)	1479 (1183)	1700 (1374)	0.640
Amylase day 2	239 (188)	306 (262)	0.440
Lipase at admission (U/L)	1844 (1568)	1804 (1675)	0.940
Lipase day 2	108 (68)	211 (261)	0.190
CT at admission Balthazar score	4.15 (2.54)	3.38 (1.75)	0.347
CT day 2 Balthazar score	4.69 (2.59)	4.17 (2.01)	0.548
CT guided puncture	5 (38.5%)	10 (45.5%)	0.686
Infected	1 (20%)	6 (54.5%)	0.308
Surgical treatments			
Cholecystectomy	5 (38.5%)	13 (59.1%)	0.240
Necrosectomy	1 (7.7%)	4 (18.2%)	0.630
Caudal pancreatectomy		1	
Clinical severity			
ICU	4 (33.3%)	10 (45.5%)	0.493
Sepsis	0 (0%)	2 (10%)	0.508
Intubation	1 (7.7%)	6 (27.3%)	0.220
Medical treatments			
Antibiotics	8 (61.5%)	15 (68.2%)	0.689
Nb of days	19.7 (13.2)	16.3 (12.75)	0.580
Systemic complications	10 (76.9%)	13 (59.1%)	0.283
Grading 1-4	1.77 (1.64)	1.73 (1.75)	0.945
Loco regional complications	9 (69.2%)	12 (54.5%)	0.392
Grading 1-4	1.54 (1.45)	1.55 (1.65)	0.990
Length of stay (d)	26.15 (21.94)	30.05 (25.06)	0.646
Death	0	0	1
Pain score VAS			
Before randomization	6.55 (3.39)	7.31 (3.44)	0.572
VAS day0, EA implementation	1.6 (1.838)	3.5 (2.2)	0.020
VAS day 1	0.57 (1.51)	2.0 (2.89)	0.066
VAS day 2	1.63 (3.46)	1.67 (2.693)	0.637
VAS day 5	1.86 (3.485)	1.38 (1.768)	0.694
VAS day 7	3 (2.38)	2 (2.39)	0.346
VAS day 10	0.2 (0.447)	2.33 (2.309)	0.034
VAS day 12	2.8 (2.28)	0 (0)	0.071

<sup>1</sup>Groups were compared with a *t*-test for continuous variables (or Wilcoxon Mann-Whitney test) and a Pearson  $\chi^2$  test (or Fisher exact test) for categorical variables. Values are mean  $\pm$  SD for continuous variables and *n* (%) for categorical variables.

14%-26%<sup>[34,35]</sup>.

There is one main limitation to our study. The study was interrupted and enrollment was closed after 49 patients because of the extreme difficulty encountered in recruiting patients from the emergency setting with severe acute pancreatitis. In addition, patients randomized for EA had a higher dropout rate compared to the standard therapy group. This resulted in uneven study groups, which could bias our results. Further trials are needed to increase the study population.

We propose that EA is beneficial for preventing early tissue damage during AP by enhancing pancreatic blood flow, as shown by our previous animal study<sup>[4]</sup>



and by the CT perfusion images in this study. In conclusion, our study showed that EA significantly increased arterial perfusion of the pancreatic gland and suggested a trend towards improvement of the clinical outcome for patients with predicted severe AP. To confirm this statement, we plan to initiate a multi-center phase II study.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Severe acute pancreatitis is linked to necrosis of the pancreatic gland, which is worsened by local vasoconstriction. This can lead to high mortality for patients. Animal studies have shown that epidural anesthesia restores pancreatic microcirculation and decreases the severity of acute pancreatitis by inducing a blockade of sympathetic nerves.

### Research frontiers

Epidural anesthesia is widely used to induce analgesia in the perioperative period and has also been used to decrease pain in patients with acute pancreatitis. In addition, experimental studies have shown a specific beneficial effect of epidural anesthesia in acute pancreatitis, attributed to a sympathetic nerve blockade that redistributes splanchnic blood flow to non-perfused pancreatic regions. The hotspot in current research is focusing on how to prevent or reverse pancreatic necrosis (and the risk of infection and sepsis), which in its severe form has a high mortality rate primarily due to multiple organ failure.

### Innovations and breakthroughs

The innovation in this study is the use of epidural anesthesia because of its effect on splanchnic blood-flow rather than its widely approved use for analgesia. Furthermore, we present the clinical application of a computed tomography (CT) scan perfusion measuring protocol for better assessment of the perfusion status of the pancreatic gland. Different perfusion measurements using the Positive Enhancement Integral method were performed in the head, body, and tail of the pancreas, for both admission and follow-up CT.

### Applications

The study results suggest that epidural anesthesia increases arterial perfusion of the pancreatic gland and shows a trend towards an improved clinical outcome for patients with predicted severe acute pancreatitis. Therefore, the authors propose that epidural anesthesia could be beneficial for preventing early tissue damage during acute pancreatitis by enhancing its blood flow.

### Terminology

Acute pancreatitis is a process caused by inflammation of the pancreatic gland that is mainly due to migration of gallstones into the common bile duct or to alcohol abuse. It can lead to the development of local complications, such as pancreatic and peri-pancreatic necrosis and pseudocysts, as well as systemic complications such as adult respiratory distress syndrome or renal failure, with a risk of death in its most severe form. Epidural anesthesia is a technique that involves injection of drugs through a catheter placed into the epidural space. When injecting anesthetic drugs, it leads to loss of sensation (like pain), by blocking the transmission of signals through nerve fibers in or near the spinal cord.

### Peer-review

This is a randomized prospective study evaluating the use of epidural anesthesia in patients with acute pancreatitis. Although the study did not reach its targeted accrual, the results are new and show that epidural anesthesia

is safe in patients with acute pancreatitis. Further, the study demonstrates that there could be the benefit of preventing early tissue damage that could potentially improve the patient's clinical outcome.

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## Randomized Clinical Trial

# Sulforaphane-rich broccoli sprout extract improves hepatic abnormalities in male subjects

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**Institutional review board statement:** The clinical trial has been registered with UMIN-CTR (#UMIN000012855); the protocol was approved by the Institutional Review Board for Clinical Research of the Tokai University School of Medicine (#13R-169) and the Ethics Committee of Kagome Co., Ltd (#2013-R05); the study was conducted in accordance with the International Ethical Guidelines and Declaration of Helsinki.

**Institutional animal care and use committee statement:** The animal experiment was approved by the Animal Care and Use Committee of Kagome Co., Ltd. in accordance with the guidelines established by the Japanese Society of Nutrition and Food Science (Law and Notification 6 of the Japanese Government). The animal experiment was designed to minimize pain or discomfort to the animals.

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## Abstract

**AIM:** To evaluate effects of dietary supplementation of sulforaphane (SF)-rich broccoli sprout (BS) extract on hepatic abnormalities in Japanese male participants.

**METHODS:** In a randomized, placebo-controlled, double blind trial, male participants with fatty liver received either BS capsules containing glucoraphanin [GR; a precursor of SF ( $n = 24$ )] or placebo ( $n = 28$ ) for 2 mo. Liver function markers, serum levels of aspartate and alanine aminotransferases (AST and ALT, respectively) and  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GTP) and an oxidative stress marker, urinary levels of 8-hydroxydeoxyguanosine (8-OHdG), were measured and compared in participants before and after the trial period. In an animal model, chronic liver failure was induced in Sprague-Dawley rats by successive

intraperitoneal injection with *N*-nitrosodimethylamine (NDMA) for 4 wk. Concomitantly, rats received AIN-76 diets supplemented with or without BS extract. Thereafter, rats were sacrificed, and their sera and livers were collected to measure serum liver function markers and hepatic levels of thiobarbituric acid reactive substances (TBARS) levels and hepatic glutathione *S*-transferase (GST) activity, a prototypical phase 2 antioxidant enzyme.

**RESULTS:** Dietary supplementation with BS extract containing SF precursor GR for 2 mo significantly decreased serum levels of liver function markers, ALT [median (interquartile range), before: 54.0 (34.5-79.0) *vs* after supplementation: 48.5 (33.3-65.3) IU/L,  $P < 0.05$ ] and  $\gamma$ -GTP [before: 51.5 (40.8-91.3) *vs* after: 50.0 (37.8-85.3) IU/L,  $P < 0.05$ ], as well as the alkali phosphatase activity. Placebo showed no significant effects on the markers. The urinary level of 8-OHdG, an established oxidative stress marker, was significantly reduced in participants who had received BS capsules but not the placebo [before: 6.66 (5.51-9.03) *vs* after: 5.49 (4.89-6.66) ng/mg-creatinine,  $P < 0.05$ ]. The reduction of urinary 8-OHdG was significantly correlated with decreased levels of both ALT and  $\gamma$ -GTP [ $\Delta$ 8-OHdG and  $\Delta$ ALT: Spearman  $r$  ( $r$ ) 0.514 and  $P = 0.012$ ,  $\Delta$  8-OHdG and  $\Delta\gamma$ -GTP:  $r = 0.496$  and  $P = 0.016$ ]. Intake of BS extract prevented NDMA-induced chronic liver failure in rats, which was attributable to the suppression of the increase in TBARS through induction of hepatic phase 2 antioxidant enzymes including hepatic GST ( $86.6 \pm 95.2$  *vs*  $107.8 \pm 7.7$  IU/g,  $P < 0.01$ ).

**CONCLUSION:** Dietary supplementation with BS extract containing the SF precursor GR is likely to be highly effective in improving liver function through reduction of oxidative stress.

**Key words:** Sulforaphane; Glucoraphanin; Broccoli sprout; Nrf2; Hepatic abnormality; Oxidative stress; Phase 2 enzymes

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**Core tip:** A randomized, placebo-controlled, double blind trial was conducted to assess the efficacy of dietary supplementation with broccoli sprout extract containing glucoraphanin (GR), a sulforaphane (SF) precursor, on hepatic abnormalities in Japanese men without changing their lifestyle or habits. Supplementation for 2 mo significantly decreased serum levels of liver function markers such as alanine aminotransferase and  $\gamma$ -glutamyl transpeptidase. The effect was associated with a reduction of urinary 8-hydroxydeoxyguanosine, an oxidative stress marker. Dietary supplementation with SF precursor GR is effective in improving liver function, and represents a potent method for maintaining good liver condition.

Kikuchi M, Ushida Y, Shiozawa H, Umeda R, Tsuruya K, Aoki Y, Suganuma H, Nishizaki Y. Sulforaphane-rich broccoli sprout extract improves hepatic abnormalities in male subjects. *World J Gastroenterol* 2015; 21(43): 12457-12467 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12457.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12457>

## INTRODUCTION

The prevalence of viral hepatitis has rapidly decreased alongside the notable improvements in the development therapeutic agents against viral hepatitis over the past 25 years. Meanwhile, the incidence of lifestyle-related "obesity and hepatic abnormalities" has increased and thus, has focused the attention of many researchers and clinicians involved in the care and management of liver diseases<sup>[1]</sup>. Non-alcoholic fatty liver disease (NAFLD) is a lifestyle-related liver disease that occurs in patients without or with almost no history of alcohol intake and has a high risk of accompanying necrosis/inflammation and fibrosis of the liver. This so-called non-alcoholic steatohepatitis (NASH) is one of the leading causes of cirrhosis and liver cancer<sup>[2]</sup>. In 2010, the Japanese Society of Hepatology published "Guidelines for the management of NASH/NAFLD 2010", but there are only a few established consensus for the treatment of NASH and NAFLD. A diet and exercise therapy is preferentially chosen for the purpose of weight loss; however, it is difficult to encourage patients to change their lifestyles and habits. Prolonged medical treatment has some risk of adverse effects. Alcoholic liver disease (ALD), which comprises a spectrum of diseases including alcoholic fatty liver, hepatitis, cirrhosis, and hepatocellular carcinoma, remains a major health problem worldwide<sup>[3]</sup>. Stopping alcohol consumption is the most effective strategy for the improvement of ALD, but it is not always easy to preach temperance to patients who thrive on alcohol consumption. We therefore have been keen on developing dietary methods for improving liver health and function of patients without adverse effects and drastic modification of lifestyle patterns including excessive diet restriction and ergotherapy.

Sulforaphane (SF), identified in broccoli<sup>[4]</sup>, is one of the most fascinating phytochemicals in the world because it protects aerobic cells against carcinogens and toxic DNA-damaging electrophiles and oxidants by induction of a network of phase 2 detoxification and antioxidant enzymes, and by suppressing inflammatory responses<sup>[5,6]</sup>. Major cytoprotective effects of SF are mediated by the transcriptional upregulation of the Kelch-like ECH-associated protein 1 (Keap1)-NF-E2-related factor 2 (Nrf2) pathway and other anti-inflammatory mechanisms including inhibition



of the NF- $\kappa$ B pathway<sup>[7,8]</sup>. Nrf2 has recently been suggested to play a critical role in protecting liver health not only from hepatotoxic chemicals but also from lifestyle-related factors such as high-energy food consumption<sup>[9-11]</sup>. Based on these mechanisms, in animal experimental models, dietary SF has been demonstrated to protect against a wide variety of liver diseases caused by hepatotoxic chemicals<sup>[12-15]</sup>, alcohol<sup>[16]</sup>, and high energy diets<sup>[17,18]</sup>. To our knowledge, however, the hepatoprotective effects of SF in human subjects have never been reported so far.

Several clinical trials<sup>[19-21]</sup> have been conducted with the focus on prevention or therapeutic effects of SF against various types of cancer, because SF was originally expected to be used as a cancer chemopreventive agent. In previous trials, doses of glucoraphanin (GR), a glucosinolate (GSL) precursor of SF, were set at more than 400  $\mu$ mol/d, which appears to be much higher compared to the estimated daily intake of GSLs (less than 100  $\mu$ mol/d) according to previous surveys<sup>[22,23]</sup>. From the aspect of safety based on eating experience, a lower dose is suitable for dietary supplementation with GR.

We herein describe the effects of dietary supplementation with a lower dose of SF precursor GR (approximately 69  $\mu$ mol/d) on hepatic abnormalities in Japanese men who did not undergo any fundamental changes in their lifestyle and habits during the 2 mo of the randomized clinical trial. This report will contribute to develop potent dietary methods for improving liver health and function.

## MATERIALS AND METHODS

### *Preparation of broccoli sprout extract and test capsules for the clinical trial*

BS extract powder, which is industrially produced by Kagome Co., Ltd., was used in the present study. BS was grown from specially selected seeds (Caudill Seed Co. Inc., Louisville, KY) for 1 d after the germination. Then the 1-d-old BS was added into boiling water and maintained at 95 °C for 30 min, and the sprout residues were removed by filtration. The boiling water extract was mixed with a dextrin and then spray dried to yield the BS extract powder containing 135 mg (approximately 310  $\mu$ mol) of GR per gram, which was confirmed by a high performance liquid chromatograph analysis as previously described<sup>[24-26]</sup>. For the clinical trial, the BS extract powder was blended with waxy corn starch, crystalline cellulose, and calcium stearate, and then encapsulated in hydroxypropyl methylcellulose capsules to yield 10 mg (approximately 23  $\mu$ mol) of GR per a capsule (BS capsule). Placebo capsules were prepared similarly but without the BS extract powder. These were prepared by a Good manufacturing practices facility (Sansho Pharmaceutical Co., Ltd., Shizuoka, Japan).

### *Design, protocol, participants, and outcome of the clinical study*

We conducted a randomized, placebo-controlled, double blind trial from January through May 2014 at a single institute, Tokai University Tokyo Hospital (Tokyo, Japan) in accordance with the International Ethical Guidelines and Declaration of Helsinki. The trial has been registered with UMIN-CTR (#UMIN000012855). The protocol was approved by the Institutional Review Board for Clinical Research of Tokai University School of Medicine (#13R-169) and the Ethics Committee of Kagome Co. Ltd (#2013-R05).

Participants were recruited from among male outpatients, aged between 30 and 69, who had higher activity of at least one of three liver function markers, alanine aminotransferase (ALT)  $\geq$  40 IU/L, aspartate aminotransferase (AST)  $\geq$  35 IU/L, or  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GTP)  $\geq$  80 IU/L for at least the last 2 consecutive months, and who were diagnosed with fatty liver using ultrasonography. Patients were excluded if they had serious liver diseases, were suspected of acute liver failure, viral hepatitis, or other serious diseases including cardiac disease, renal dysfunction (serum creatinine > 2.0 mg/dL), and bile duct cancer, or if they habitually consumed higher amounts of alcohol (more than 60 g of alcohol per day). Written informed consent was obtained from all participants.

Randomization was performed in a 1:1 ratio using a random number table. Study treatment was randomly assigned and labeled with the participant numbers before the study. Participants were numbered consecutively in the order in which they entered the study. They received either 3 BS capsules containing 30 mg of GR, the precursor of SF, or the placebo for 2 mo. The participants as well as investigators were blinded to the treatment until the study completion.

Primary outcome measures were decreased levels of serum ALT, AST, and  $\gamma$ -GTP. Secondary outcome measures were improvement of the following physical parameters: body weight, body mass index (BMI), and waist circumference; blood biochemical markers: albumin, total bilirubin, alkali phosphatase (ALP), choline esterase (ChE), ferritin, urinary acid (UA), triglyceride (TG), total-cholesterol, high-density lipoprotein (HDL)-cholesterol, and low-density lipoprotein (LDL)-cholesterol, fasting blood sugar (FBS), hemoglobin A1c (HbA1c), high-sensitivity C-reactive protein (hs-CRP), serum adipokines (leptin and adiponectin), urinary oxidative marker 8-hydroxy-deoxyguanosine (8-OHdG); and diagnosis of fatty liver on ultrasonography.

### *Experimental protocols for animal models*

Animal experiments were approved by the Animal Care and Use Committee of Kagome Co., Ltd. in accordance with the guidelines established by the

**Table 1** Composition of animal diets

Contents, g/100 g	Normal diet	BS low	BS middle	BS high
Casein	25.00	25.00	25.00	25.00
Corn starch	40.25	39.83	39.41	38.56
Sucrose	20.00	20.00	20.00	20.00
Corn oil	5.00	5.00	5.00	5.00
Mineral mix <sup>1</sup>	3.50	3.50	3.50	3.50
Vitamin mix <sup>1</sup>	1.00	1.00	1.00	1.00
Choline bitartrate	0.25	0.25	0.25	0.25
Cellulose	5.00	5.00	5.00	5.00
BS extract	0.00	0.42	0.84	1.69
Glucoraphanin	0.00	0.063	0.13	0.25

<sup>1</sup>Based on AIN-76 diet. BS: Broccoli sprout.

Japanese Society of Nutrition and Food Science (Law and Notification 6 of the Japanese Government). The animal experiment was designed to minimize pain or discomfort to the animals.

Male Sprague Dawley rats (7-wk-old) were acclimatized on an AIN-76 diet (CLEA Japan, Tokyo, Japan) in individual stainless steel cages in a room maintained at  $20 \pm 2^\circ\text{C}$ , and a relative humidity of  $65\% \pm 6\%$ , with a 12/12-h light cycle. Rats were divided into five groups: sham, control, BS-low, BS-middle, and BS-high groups (sham:  $n = 6$ , others:  $n = 8$  each). Sham and control rats received the AIN-76 diet for 4 wk. BS-low, BS-middle, and BS-high rats received 62.5, 125, and 250 mg of GR per 100 g of the AIN-76 diet, respectively (Table 1). Except for sham, all rats were intraperitoneally injected with *N*-nitrosodimethylamine (NDMA) at a dose of 5 mg/kg body weight on 3 consecutive days (every Tuesday to Thursday) of the week for 4 wk. Sham rats were injected with the vehicle (saline) in the same manner. During this period, their body weights and food intakes were monitored. After 4 wk, rats were sacrificed with no pain, and their blood and livers were harvested, frozen in liquid  $\text{N}_2$ , and stored at  $-80^\circ\text{C}$  until analyzed.

### Biological assays

Parameters of blood biochemistry of participants were automatically measured using an Auto Blood Biochemistry Analyzer. Serum levels of adiponectin and leptin were determined with an ELISA kit (Human adiponectin ELISA kit, Otsuka Pharmaceutical Co., Ltd, Tokushima, Japan) and an enzyme immune assay kit (Human leptin highly sensitive assay kit, Immuno-Biological Laboratories Co., Ltd., Gunma, Japan), respectively. Urinary levels of 8-OHdG were measured with a commercial ELISA kit (New 8-OHdG check, Japan Institute for the Control of Aging, Shizuoka, Japan), and standardized to creatinine concentrations that were measured using a creatinine urinary assay kit (Cayman Chemical Company, MI).

Activities of AST and ALT in rat sera were determined using a transaminase CII-test kit (Wako Pure Chemical Institute, Osaka, Japan). Levels of albumin and total bilirubin in rat sera were measured by

using a modified bromocresol purple method and a chemical oxidation method, respectively (SRL Inc., Tokyo, Japan). Hepatic levels of thiobarbituric acid reactive substances (TBARS), byproducts of lipid peroxidation, were measured according to the method by Kikugawa *et al.*<sup>[24]</sup>. In brief, a portion of rat liver (0.5 g) was homogenized in 4.5 mL of ice-cold 10 mmol/L Tris-HCl (pH 7.4) with a polytron homogenizer. The homogenate was centrifuged at  $12000 \times g$ , for 20 min at  $4^\circ\text{C}$ , and the supernatant (0.2 mL) was added to a glass tube containing 0.65 mL of a reaction mixture, 0.04 mL of 5.0% sodium dodecyl sulfate, and 0.01 mL of 0.8% butylhydroxytoluene, 0.28 mL of 0.8% thiobarbituric acid, and 0.32 mL of distilled water. After the addition of 0.15 mL of 20% acetate buffer (pH 3.5), the test tube was carefully sealed with a plastic paraffin film and then incubated at  $100^\circ\text{C}$  for 1 h. TBARS was extracted with 1 mL of butanol/pyridine (15/1) and then measured by spectrophotometric assay (OD 532 nm). Levels of TBARS in the liver were calculated using a standard curve of 1,1,3,3-tetramethoxypropane dissolved in ethanol. The enzyme activity of glutathione *S*-transferase (GST) was determined with 1-chloro-2,4-dinitrobenzene as the substrate as described previously<sup>[25]</sup>.

### Statistical analysis

Parametric data are represented as the mean  $\pm$  SD and non-parametric data as the median with percentile distribution. In the clinical trial, differences between the two groups were analyzed using the non-parametric Mann-Whitney *U* test, and compared before and after intervention by using the Wilcoxon single rank tests. Correlations between variables were examined by using the Spearman rank correlation test. In the animal experiment, differences among five groups were analyzed with the Kruskal-Wallis test followed by the Steel-Dwass test for non-parametric data or with the ANOVA followed by the Tukey Kramer test or the Dunnett test for parametric data. *P* values less than 0.05 were considered significant.

The sample size in the clinical study was calculated using an estimated 10% loss in the intervention period, a confidence level of 95%, and a power of 80% (at least 20 in each group). Statistical significance was inferred at  $P < 0.05$ .

Statistical analyses were carried out using JMP (SAS institute, Cary, NC).

## RESULTS

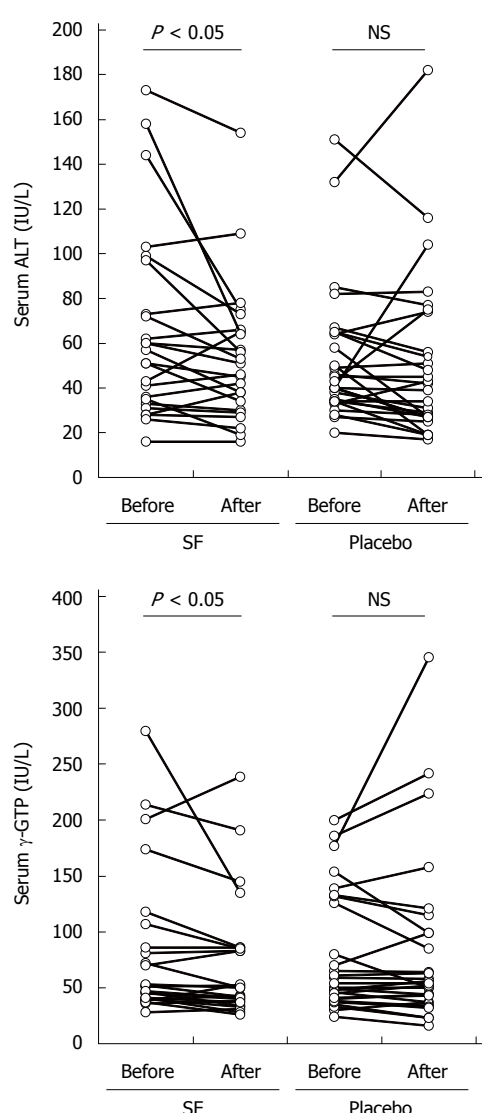
### Profile of participants

A total of 55 male outpatients were enrolled in this clinical study, and assigned to one of the following two groups: SF group ( $n = 27$ ) and placebo group ( $n = 28$ ). Of those, 24 and 28 participants, respectively, completed the study protocol and were available for efficacy analyses. Three participants in the SF group were excluded for following reasons: 2 had poor

**Table 2** Initial clinical profiles of participants in the sulforaphane and placebo groups

<i>n</i>	Median (IQR)	
	SF group	Placebo group
Age, yr	51.5 (42.0-57.5)	56.0 (49.8-63.3)
BMI, kg/cm <sup>2</sup>	25.7 (24.2-27.6)	25.9 (24.5-27.2)
Waist circumference, cm	86.5 (81.0-95.0)	92.3 (88.3-95.3)
AST, IU/L	37.5 (24.8-48.3)	30.0 (27.5-39.5)
ALT, IU/L	54.0 (34.5-79.0)	41.5 (34.0-64.3)
γ-GTP, IU/L	51.5 (40.8-91.3)	52.0 (39.5-91.5)

<sup>†</sup>Calculated by the Mann-Whitney *U* test. IQR: Interquartile range; AST: Aspartate-aminotransferases; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; SF: Sulforaphane.



**Figure 1** Effect of supplementation with broccoli sprout extract containing the sulforaphane precursor glucoraphanin on liver function markers in male participants with hepatic abnormalities. A, B: Serum levels of alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (γ-GTP) in sulforaphane (SF) and placebo group participants (*n* = 24 and 28, respectively) before and after 2 mo of intervention. Each line between the circle symbols represents individual change in serum levels of ALT and γ-GTP. *P* values were analyzed by using the Wilcoxon single rank test.

compliance and 1 had gallstone pancreatitis during the intervention period. There were no adverse events due to the treatment or to the study protocol. Table 2 shows the characteristics and baseline parameters of primary outcome measures of participants in both groups. No significant differences were observed in the parameters including liver function markers, ALT, AST, and γ-GTP.

### Effect of BS treatment on primary outcome measures

Primary outcome measures of the present study were significant decreases in serum levels of ALT, AST, and γ-GTP by dietary supplementation with 30 mg of SF precursor GR per day. Serum levels of those markers before and after 2 mo of intervention are displayed in Figure 1 and Table 3. A non-parametric Wilcoxon single rank test revealed that serum levels of ALT and γ-GTP, but not AST, were significantly decreased comparing levels of ALT and γ-GTP before and after intervention in the SF group, whereas, there were no significant differences in the placebo group. The mean percent changes in ALT and γ-GTP levels from before intervention were -10.7% and -8.9% in the SF group, which seemed to be larger than those (-4.3% and -1.1%, respectively) in the placebo group (no significant differences).

As shown in Table 3, serum levels of ALP and albumin, relevant markers of liver function, were significantly changed following treatment with capsules containing GR. The median change in ALP levels (IU/L) before and after the trial was -6.0 with an interquartile range (IQR) of 17.8 in the SF group, and 3.5 with an IQR of 25.5 in the placebo group, respectively (*P* < 0.05). Serum albumin levels were significantly lowered in the SF group, but the change was very slightly, and was within the reference level range.

### Effects on other markers

Dietary supplementation with 30 mg of GR in BS capsules did not show any remarkable impact on physical parameters (BMI and waist circumference) and markers of sugar metabolism (FBS and HbA1c). Only BMI significantly improved in the placebo group, but the reason was unknown. However, relevant markers of lipid metabolism were partly improved in the SF group. At baseline, the median level of serum total cholesterol was more than 200 mg/dL, which is considered a borderline-to-high range according to the American Heart Association. The level was lowered to less than 200 mg/dL after supplementation with BS capsules containing GR. Conversely, the level was elevated in the placebo group. Neither HDL- nor LDL-cholesterol levels were significantly changed in the study. In association with the improvement of total cholesterol level, serum activity of ChE, which is known to be higher in those developing obesity and fatty liver, was significantly decreased in the SF group. Furthermore, a representative marker of oxidative

**Table 3** Clinical parameters in sulforaphane and placebo group participants before and after trial, Median (interquartile range)

	SF group (n = 24)		Placebo group (n = 28)	
	Before	After	Before	After
BMI, kg/m <sup>2</sup>	25.7 (24.2-27.6)	25.8 (24.2-27.3)	25.9 (24.6-26.8)	25.6 (24.5-26.8) <sup>a</sup>
Waist Circumference, cm	86.5 (81.0-95.0)	88.8 (82.5-95.0)	92.3 (88.4-95.3)	91.4 (88.5-97.1)
AST, IU/L	37.5 (24.8-48.3)	37.5 (27.0-39.8)	30.0 (27.5-39.5)	26.0 (22.5-42.8)
ALT, IU/L	54.0 (34.5-79.0)	48.5 (33.0-65.3) <sup>a</sup>	41.5 (34.0-64.3)	40.5 (27.0-60.5)
γ-GTP, IU/L	51.5 (40.8-91.3)	50.0 (37.8-85.3) <sup>a</sup>	52.0 (39.5-127.5)	53.0 (37-99)
Alb, g/dL	4.6 (4.4-4.9)	4.5 (4.3-4.7) <sup>a</sup>	4.6 (4.5-4.7)	4.6 (4.5-4.8)
TB, mg/dL	0.9 (0.6-1.1)	0.9 (0.7-1.2)	0.9 (0.6-1.1)	0.8 (0.7-1.0)
ALP, IU/L	210 (168-248)	182 (166-140) <sup>a</sup>	230 (206-274)	231 (214-281)
ChE, IU/L	390 (359-436)	388 (358-421) <sup>a</sup>	398 (369-458)	406 (370-424)
Ferritin, ng/mL	281 (131-381)	216 (136-365)	231 (162-322)	215 (168-321)
UA, mg/dL	6.1 (5.5-7.0)	6.0 (5.4-6.8)	6.4 (5.4-6.8)	6.1 (5.6-6.7)
TG, mg/dL	146 (118-193)	135 (95.5-208)	133 (99.3-166)	113 (83.0-160)
TC, mg/dL	201 (182-219)	194 (184-211) <sup>a</sup>	196 (183-224)	207 (184-225)
HDL-C, mg/dL	50.0 (44.0-59.0)	48.5 (44.8-54.0)	47.5 (44.8-58.0)	49.5 (45.0-57.0)
LDL-C, mg/dL	123 (107-138)	123 (111-141)	125 (116-142)	137 (118-146)
HbA1c, %	5.7 (5.4-6.1)	5.7 (5.5-6.0)	5.8 (5.5-6.1)	5.8 (5.5-6.1)
FBS, mg/dL	105 (99.8-113)	106 (101-115)	105 (99-115)	106 (97.5-115)
hs-CRP, mg/dL	0.067 (0.036-0.13)	0.059 (0.031-0.13)	0.056 (0.037-0.14)	0.068 (0.028-0.15)
8-OHdG, ng/mg-CRE	6.8 (5.5-9.0)	5.5 (4.9-6.7) <sup>a</sup>	6.6 (5.3-8.4)	6.1 (4.7-7.6)
Adiponectin, μg/mL	6.1 (4.8-7.4)	4.8 (4.1-7.7) <sup>a</sup>	6.3 (4.6-9.5)	6.1 (4.6-7.9) <sup>a</sup>
Leptin, ng/mL	5.9 (4.2-7.5)	5.8 (3.0-8.7)	7.3 (5.2-11.4)	6.6 (4.1-10.1)

<sup>a</sup>P < 0.05 vs before. AST: Aspartate-aminotransferase; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; Alb: Albumin; TB: Total bilirubin; ALP: Alkali phosphatase; ChE: Choline esterase; UA: Urinary acid; TG: Triglyceride; TC: Total cholesterol; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; HbA1c: Hemoglobin A1c; FBS: Fasting blood sugar; hs-CRP: High-sensitivity C-reactive protein; 8-OHdG: 8-hydroxydeoxyguanosine; CRE: Creatinine; SF: Sulforaphane.

**Table 4** Protective effect of broccoli sprout extract against hepatotoxicity by *N*-nitrosodimethylamine

Group	n	Food intake (g/d)	Body weight gain (g)	Liver weight (g)
Sham	6	20.3 ± 1.3	210.0 ± 23.7	17.1 ± 1.4
Control	8	19.2 ± 1.7	174.4 ± 20.5	12.1 ± 1.5 <sup>b</sup>
BS-low	8	19.3 ± 1.6	181.4 ± 20.3	12.9 ± 1.6
BS-middle	8	19.2 ± 2.1	186.8 ± 21.1	13.9 ± 2.2
BS-high	8	18.9 ± 1.0	188.3 ± 8.7	15.1 ± 1.4 <sup>d</sup>

<sup>b</sup>P < 0.01 vs sham; <sup>d</sup>P < 0.01 vs control. BS: Broccoli sprout.

stress, urinary 8-OHdG level was significantly reduced by approximately 20% by dietary supplementation with GR.

#### Relationship between reduction of oxidative stress and improved liver function

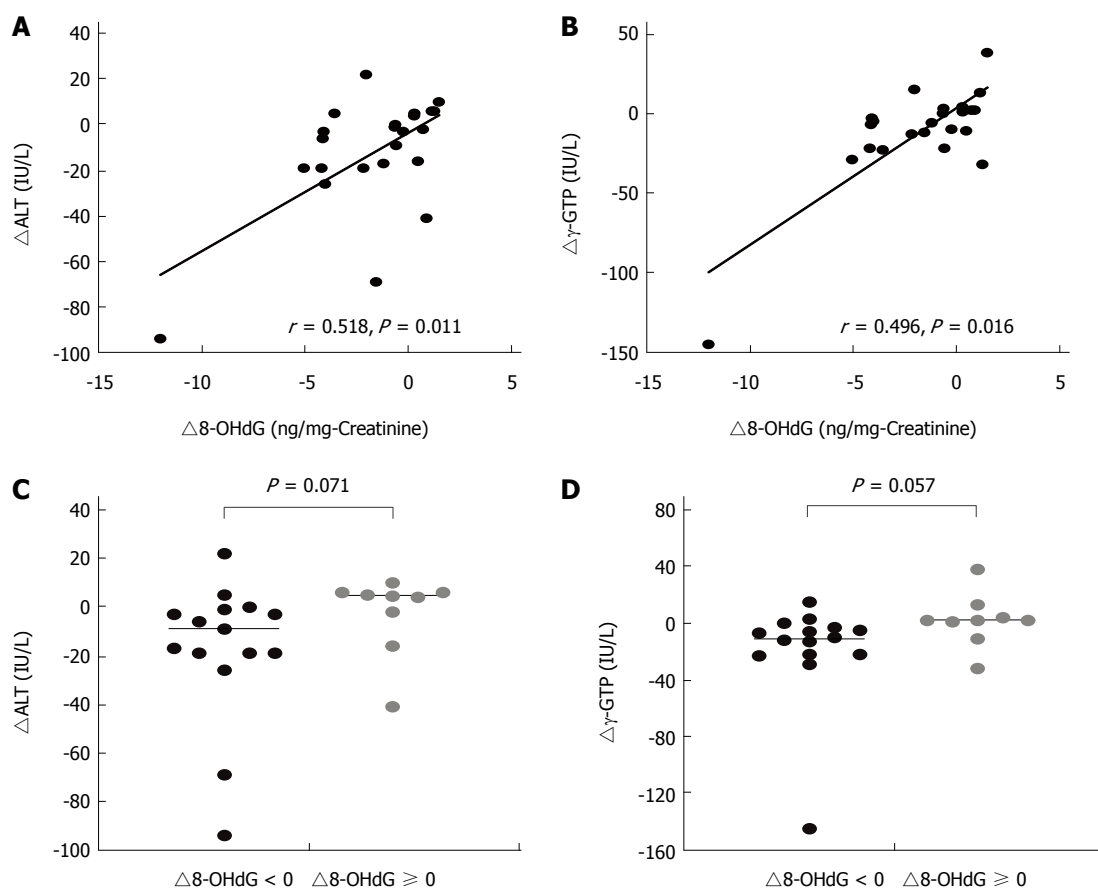
The Spearman rank correlation test identified significant positive relationships between changes in levels of urinary 8-OHdG (Δ8-OHdG) and in liver function markers (ΔALT and Δγ-GTP) in participants in the SF group (Figure 2A and B). No significant relationships between in these levels were observed in the placebo group. To further understand the contribution of reduced oxidative stress in the improvements of liver function markers, ΔALT and Δγ-GTP were compared in participants with or without reduction in 8-OHdG levels (Δ8-OHdG < 0 and ≥ 0, respectively). In the SF group, 15 participants showed a reduction in 8-OHdG levels,

but the other 9 participants did not. In participants with Δ8-OHdG < 0 in the SF group, stratified analysis showed clear trends of lowering both levels of ΔALT and Δγ-GTP, where the median levels were lowered by 17 and 11.5 IU/L, respectively, compared with those participants with Δ8-OHdG ≥ 0.

#### Protective effects of BS extract on NDMA-induced chronic liver failure in rats

Repeated intraperitoneal injection of NDMA resulted in a body weight loss of approximately 17% and a significant reduction of liver weight (29%) in control rats compared with sham rats (Table 4). The NDMA-induced hepatotoxicities were prevented by the intake of BS extract in a dose-dependent manner. A significant protective effect on reducing liver weight was observed in rats belonging to the BS-high group. There was no significant difference in food intake among groups. Serum levels of representative liver function markers, AST and ALT, were dramatically elevated in control rats by more than 2-fold compared to sham rats, which clearly showed that NDMA toxicity induced chronic liver failure in the control rats (Figure 3A and B). On the other hand, elevations of those levels were dose-dependently prevented in rats that had been fed diets containing serial doses of BS. Significant differences from control were observed in BS-middle and BS-high group rats. Furthermore, the intake of BS resulted in the improvement of serum levels of albumin and bilirubin, which are also used to evaluate functions of the liver and bile duct (Figure 3C and D).





**Figure 2** Parallel improvements of an oxidative stress marker and liver function markers in sulforaphane group participants. A, B: Change levels of liver function markers, alanine aminotransferase and  $\gamma$ -glutamyl transpeptidase ( $\Delta$ ALT and  $\Delta$  $\gamma$ -GTP) were respectively plotted against changes in levels of urinary 8-hydroxydeoxyguanosine ( $\Delta$ 8-OHdG), an *in vivo* oxidative stress marker, in SF group participants ( $n = 24$ ). Each circle represents individual data. Spearman  $r$  and  $P$  were determined; C, D:  $\Delta$ ALT and  $\Delta$  $\gamma$ -GTP were compared between participants with  $\Delta$ 8-OHdG  $< 0$  ( $n = 15$ ) and  $\Delta$ 8-OHdG  $\geq 0$  ( $n = 9$ ). Each circle symbol represents an individual data point. Bars in graphs represent median values.  $P$  values were obtained by using the Mann-Whitney  $U$  test.

### Effect of BS on oxidative stress and antioxidant enzymes in rat livers

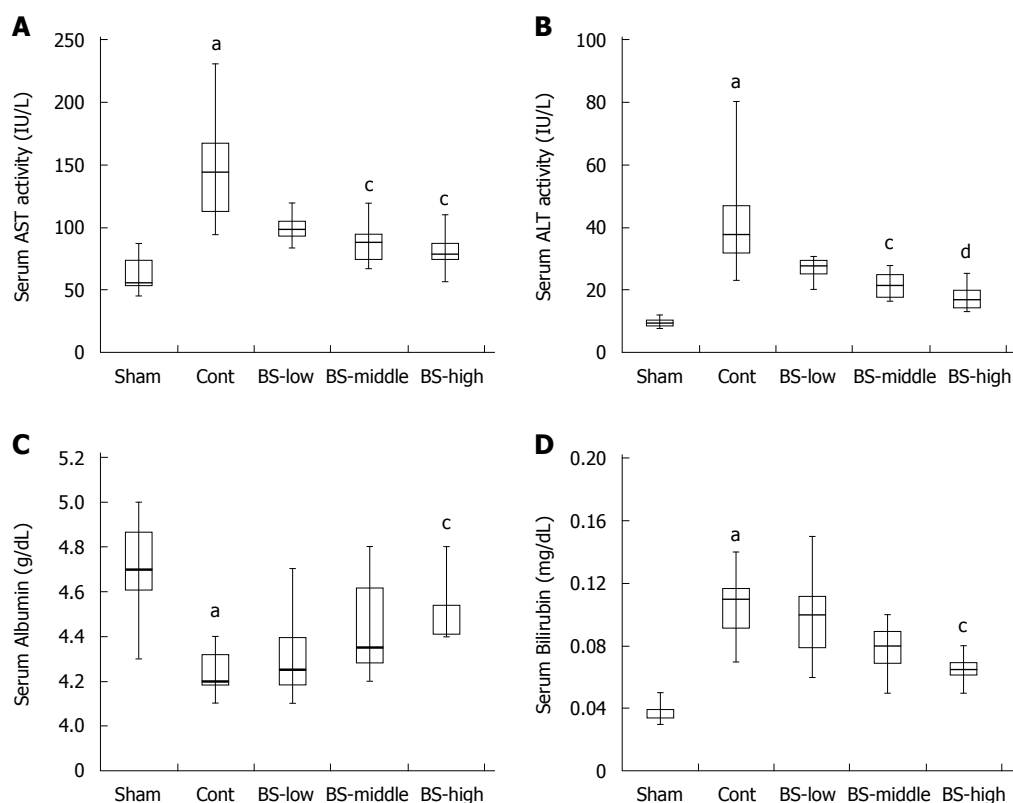
In connection with the elevation of serum AST and ALT activities, hepatic TBARS levels were significantly increased in control rats, which indicated that NDMA toxicity resulted in excess oxidative stress in rat livers (Figure 4A). However, lower levels of hepatic TBARS were observed in BS-fed rats. In particular, these levels in the BS-high group rats improved almost completely. Figure 4B shows the enzyme activities of hepatic GST, a typical cytoprotective phase 2 enzyme that plays an important role in the detoxification of NDMA and in antioxidant activity. GST activity was very slightly increased by repeated injection of NDMA, whereas it increased significantly by continuous intake of BS.

## DISCUSSION

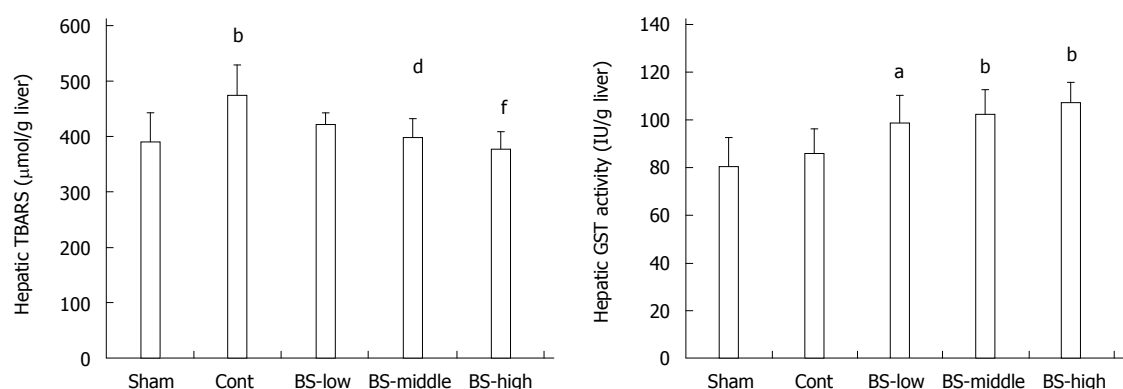
The liver is so called a silent organ; symptoms tend not to appear until a disease is well advanced. Therefore, for the prevention of chronic liver failure, it is quite important to manage liver condition on the basis of serum levels of liver function markers, which can be easily measured during routine health checkups. We

describe herein the first demonstration that dietary supplementation with BS extract containing a low dose of GR (30 mg or approximately 69  $\mu$ mol), a precursor of SF, is much likely to be effective in the improvement of liver function by reducing oxidative stress in male participants with abnormally higher levels of liver function markers.

The improvement on liver function was judged from the results of the present randomized, placebo-controlled, double blind trial, where serum levels of representative liver function markers, ALT and  $\gamma$ -GTP, as well as the relevant marker ALP were significantly lowered in male participants with fatty liver in SF group who had taken BS capsules containing 30 mg of GR per day for 2 mo, but not in placebo group participants. Unfortunately, no significant differences were observed in the decreased levels of the markers between the SF and placebo groups, probably due to the design of the present trial such as a small sample size and a no limit on criteria for subject inclusion: at least one higher activity of three liver function markers, ALT  $\geq 40$  IU/L, AST  $\geq 35$  IU/L, or  $\gamma$ -GTP  $\geq 80$  IU/L. In the present trial, serum levels of liver function markers were decreased in some participants



**Figure 3** Effects of intake of broccoli sprout extract on liver function markers in serum from *N*-nitrosodimethylamine-induced chronic liver failure model rats. A, B: Serum activities of aspartate-aminotransferases (AST) and alanine aminotransferase (ALT) in male Sprague-Dawley rats who had received an AIN-76 diet containing no (sham,  $n = 6$  and control,  $n = 8$ ) or different amounts of BS extract at glucoraphanin (GR) doses of 62.5, 125, and 250 mg per 100 g diet (BS-low, BS-middle, and BS-high, respectively,  $n = 8$ ) for 4 wk. All rats, except for the sham group, were intraperitoneally injected with *N*-nitrosodimethylamine (5 mg per kg body weight) on 3 consecutive days of the week for 4 wk; C, D: Serum levels of albumin and bilirubin in the rats. Data are shown as boxplots representing the minimum (bottom of the bar), 25th percentile (bottom of the box), median (horizontal line), 75th percentile (top of the box), and the maximum observations (top of the bar). <sup>a</sup> $P < 0.05$  vs sham; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs control (Steel-Dwass test). BS: Broccoli sprout.



**Figure 4** Effects of intake of broccoli sprout extract on oxidative stress and enzyme activity of glutathione S-transferase in livers from *N*-nitrosodimethylamine-induced chronic liver failure model rats. A: Thiobarbituric acid reactive substances (TBARS) levels were determined in livers from serum activities of alanine aminotransferase (AST) and alanine aminotransferase (ALT) in male Sprague-Dawley rats who had received an AIN-76 diet containing no (sham,  $n = 6$  and control,  $n = 8$ ) or different amount of BS extract at glucoraphanin (GR) doses of 62.5, 125, and 250 mg per 100 g diet (BS-low, BS-middle, and BS-high, respectively,  $n = 8$ ) for 4 wk. All rats, except for sham group, were intraperitoneally injected with NDMA (5 mg per kg body weight) in 3 consecutive days of the week for 4 wk; B: GST activities in rat livers were determined with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Data are shown as mean  $\pm$  SE. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs sham, <sup>c</sup> $P < 0.01$ , <sup>d</sup> $P < 0.001$  vs control (A: Tukey Kramer; B: Dunnett test).

even in the placebo group possibly because of the so-called placebo effect or subtle lifestyle related bias, although actual reasons are unclear. However, the lowering activities of the markers were not associated with reduction of urinary levels of 8-OHdG as a biological marker of oxidative stress in the placebo group, which was distinctly different from that in the SF group participants. This suggested that continuous intake of GR for 2 mo surely affected physiologies of participants beyond the placebo effect, and the reduction of oxidative stress was much likely to be involved in the improvement of liver function. The urinary level of 8-OHdG has been established to reflect oxidative damage of DNA occurring in various cells and tissues in the body<sup>[26,27]</sup>, and thereby widely used for evaluating *in vivo* oxidative stress<sup>[28]</sup>. As for the liver, a previous clinical study showed significant correlations between hepatic expression levels and urinary levels of 8-OHdG in patients with chronic hepatitis C<sup>[29]</sup>. In the present clinical trial, although a biopsy test was not conducted, hepatic oxidative stress might also be reduced in the SF group participants.

As previously mentioned, there is no report describing the efficacy of SF or BS preparations on the improvement of hepatic abnormalities in humans to date, whereas many animal experiments have been carried out to demonstrate the hepatoprotective effects of SF and clarify the underlying mechanism involved. Traditional experimental models in toxicology have demonstrated that intake of SF or the precursor GR can markedly prevent liver damage induced by a wide variety of hepatotoxic chemicals such as cisplatin<sup>[12]</sup>, mycocystin<sup>[13]</sup>, carbon tetrachloride<sup>[14]</sup>, acetaminophen (unpublished our preliminary data), and D-galactosamine and lipopolysaccharide<sup>[15]</sup>. Additionally, it has been suggested that SF is potent in preventing lifestyle-related liver diseases caused by excessive consumption of alcohol<sup>[16]</sup> and high energy diets<sup>[17,18]</sup>. These hepatoprotective effects have been suggested to be attributable to eminent inducer potency of SF for phase 2 cytoprotective proteins including antioxidant and detoxifying enzymes through activation of the transcriptional factor Nrf2. This speculation is strongly supported by numerous basic studies using Nrf2 knockout mice and cells, which revealed that Nrf2 plays an essential role in the prevention of liver diseases<sup>[30,31]</sup>.

In the present study, we assessed the potency of the BS extract containing the SF precursor GR as an inducer of Nrf2 and its downstream phase 2 cytoprotective enzymes in animal models. Intake of the BS extract for 4 wk significantly increased the hepatic activity of GST, a typical phase 2 enzyme. Consequently, elevation of oxidative stress and resulting liver failure by successive exposure to NDMA was significantly prevented in rats fed the BS extract. This finding ascertained the inducer potency of the BS

extract used in the present clinical trial, and inferred that Nrf2-regulated phase 2 enzymes might also be induced in participants by supplementation with BS capsules in lieu of biopsy data of the human liver.

Of special note is that the dose of SF precursor GR was set at 30 mg (approximately 69  $\mu$ mol) per day in the present clinical trial, which was much lower than previous clinical studies evaluating cancer prevention effects of SF. The therapeutic effect on *Helicobacter pylori* infection was demonstrated by a continuous daily intake of 70 g of fresh BS containing 420  $\mu$ mol GR<sup>[19]</sup>. Furthermore, in a large-scale randomized clinical study in China, a detoxification effect on airborne pollutants was clearly observed in participants given a BS beverage containing 800  $\mu$ mol GR<sup>[20,21]</sup>. It is generally recognized that GR is a safer compound based on a long-term eating habit of broccoli and other cruciferous vegetables, which was also demonstrated by previous clinical trials including phase I studies<sup>[32,33]</sup> and the above-mentioned high-dose tests. However, we set our study to the lower GR dose, which was considered a sufficiently safe dose even for participants with hepatic abnormalities, because of the estimated range of daily intake of GSLs from cruciferous vegetables. A dose-dependent effect should be examined in future trials.

Our study has several limitations. First, in addition to the small sample size as mentioned above, only male participants were recruited for this randomized clinical trial. Thus, the efficacy of the BS extract on female participants is unclear. Second, the trial period was only 2 mo, which was shorter than the previously reported randomized controlled trials describing the efficacy of silymarin from milk thistle<sup>[34]</sup>. Although the longer term effects of BS extract is yet unknown, striking outcomes could be obtained in future intervention studies with a longer period of observation. Third, the BS extract but not the purified SF was used in the present study. Similar to a number of previous studies, our BS extract contains a rich SF precursor of GR; therefore, we consider SF as the predominant active compound in the BS extract responsible for the reduction in oxidative stress and the resulting improvement in liver function. Fourth, we did not perform a histological and pathological examination of fat deposition, inflammation, or fibrosis in the liver. In a future randomized clinical trial that is being planned, we will address the above-mentioned limitations to comprehend the efficacy of the BS extract containing the SF precursor in liver diseases.

## ACKNOWLEDGMENTS

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## COMMENTS

## Background

Instead of viral hepatitis, the growing incidence of lifestyle-related "obesity and hepatic abnormalities" has been of great concern to many researchers and clinicians involved in the care and management of liver diseases. The modification of lifestyle is preferentially chosen for preventing and improving the hepatic abnormalities. However, it is difficult to encourage patients to change their lifestyles and habits such as diet, alcohol consumption, and exercise.

## Research frontiers

A phytochemical sulforaphane (SF) from broccoli shows potent cytoprotective effects through activation of a transcription factor Nrf2 that has recently been suggested to play a critical role in protecting liver health not only from hepatotoxic chemicals but also from lifestyle-related factors. Hepatoprotective effects of SF have been demonstrated in various animal models.

## Innovations and breakthroughs

The present randomized, placebo-controlled, double blind study demonstrates that supplementation with a low dose of glucoraphanin (GR), a precursor of SF for 2 mo significantly improved serum levels of alanine aminotransferase and  $\gamma$ -glutamyl transpeptidase, representative liver function markers in Japanese male subjects with hepatic abnormalities. The improvement effect is associated with a reduction of urinary 8-hydroxydeoxyguanosine (8-OHdG), an oxidative stress marker.

## Applications

The daily dose of SF precursor GR (69  $\mu$ mole) in the present study was within the estimated daily intake amount of glucosinolates (less than 100  $\mu$ mol), and thus is applicable to dietary supplements. The present findings will contribute to develop potent dietary methods for improving liver health and function.

## Terminology

GR, a glucosinolate precursor of SF, is highly contained in cruciferous vegetables in particular broccoli sprout. SF was identified as a potent inducer for cytoprotective genes such as phase 2 detoxification and antioxidant enzymes. The induction is mediated by the transcriptional upregulation of the Kelch-like ECH-associated protein 1-NF-E2-related factor 2 pathway. Urinary level of 8-OHdG is widely used as an *in vivo* oxidative stress marker.

## Peer-review

The purpose of the research was to determine whether SF will reduce/prevent hepatic abnormalities. The authors have selected well known markers to evaluate the state of the liver, and are able to draw a relatively reliable conclusion with regard to the effects of the treatment of the male participants in the study. The animal studies also support the conclusion that SF improves liver function through reduction of oxidative stress. The data obtained are interesting. In a follow-up project it would in particular be of interest to determine the effects of higher doses of SF-precursor and longer trial periods.

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## Self-expandable metal stents for malignant gastric outlet obstruction: A pooled analysis of prospective literature

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**Data sharing statement:** All data in this systematic review were extracted from the original articles and are presented in the literature Table 1. No additional data are available.

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### Abstract

**AIM:** To provide an overview of the clinical outcomes of self-expandable metal stent (SEMS) placement for malignant gastric outlet obstruction (MGOO).

**METHODS:** A systematic literature search was performed in PubMed of the literature published between January 2009 and March 2015. Only prospective studies that reported on the clinical success of stent placement for MGOO were included. The primary endpoint was clinical success, defined according to the definition used in the original article. Data were pooled and analyzed using descriptive statistics. Subgroup analyses were performed for partially covered SEMSs (PCSEMSs) and uncovered SEMSs (UCSEMSs) using Fisher's exact test.

**RESULTS:** A total of 19 studies, including 1281 patients, were included in the final analysis. Gastric (42%) and pancreatic (37%) cancer were the main causes of MGOO. UCSEMSs were used in 76% of patients and PCSEMSs in 24%. The overall pooled technical success rate was 97.3% and the clinical success rate was 85.7%. Stent dysfunction occurred in 19.6% of patients, mainly caused by re-obstruction (12.6%) and stent migration (4.3%), and was comparable between PCSEMSs and UCSEMSs (21.2% vs 19.1%, respectively,  $P = 0.412$ ). Re-obstruction was more common with UCSEMSs (14.9% vs 5.1%,  $P < 0.001$ ) and stent migration was more frequent after PCSEMS placement (10.9% vs 2.2%,  $P < 0.001$ ). The overall perforation rate was 1.2%. Bleeding was reported in 4.1% of patients, including major bleeding in 0.8%. The median stent patency ranged from 68 to 307 d in five studies. The median overall survival ranged from 49 to 183 d in 13 studies.

**CONCLUSION:** The clinical outcomes in this large population showed that enteral stent placement was feasible, effective and safe. Therefore, stent placement

is a valid treatment option for the palliation of MGOO.

**Key words:** Stents; Gastric outlet obstruction; Stomach neoplasms; Pancreatic neoplasms; Intestinal obstruction; palliative care; Systematic review

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**Core tip:** In this pooled analysis of the prospective literature published since January 2009, we provide an extensive overview of the clinical outcomes of stent placement for malignant gastric outlet obstruction. We analyzed the technical and clinical success, stent dysfunction, stent patency, perforation, bleeding and overall survival in 1281 patients treated with enteral stent placement.

van Halsema EE, Rauws EAJ, Fockens P, van Hooft JE. Self-expandable metal stents for malignant gastric outlet obstruction: A pooled analysis of prospective literature. *World J Gastroenterol* 2015; 21(43): 12468-12481 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12468.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12468>

## INTRODUCTION

Gastric outlet obstruction is a syndrome characterized by nausea (90%), vomiting (83%), regurgitation (69%) and abdominal pain (66%)<sup>[1]</sup>. The majority of patients (> 75%) presenting with malignant gastric outlet obstruction (MGOO) cannot tolerate solids, and approximately 40% of patients have no oral intake at all<sup>[1]</sup>. Pancreatic cancer is the most common cause of MGOO in Western countries<sup>[1-3]</sup>, while gastric cancer is the leading cause of MGOO in Eastern Asian studies<sup>[4-6]</sup>. Gastric outlet obstruction is usually a late sign of a locally advanced or metastatic cancer, requiring palliative management. These patients have a poor prognosis with a mean survival of approximately 100 d (3.3 mo)<sup>[7]</sup>, and an impaired quality of life<sup>[8,9]</sup>. The aim of palliative therapy is to relieve obstructive symptoms and to allow oral intake. Treatment options for MGOO are endoscopic stent placement (Figure 1), surgical bypass by means of a gastrojejunostomy, a percutaneous gastrostomy (PEG) serving for gastric decompressing with subsequent jejunal feeding tube placement, and pharmacological therapy aiming for improvement in gastric emptying, relief of symptoms and comfort<sup>[7,10-12]</sup>. Comparison of enteral stenting and gastrojejunostomy revealed sooner return to oral intake and shorter hospital stay after stent placement<sup>[7,13]</sup>. On the long term, however, patients with an enteral stent have more recurrent obstruction and require more re-interventions<sup>[9]</sup>. Therefore, one might argue that patients with a relatively short survival benefit the most from enteral stent placement.

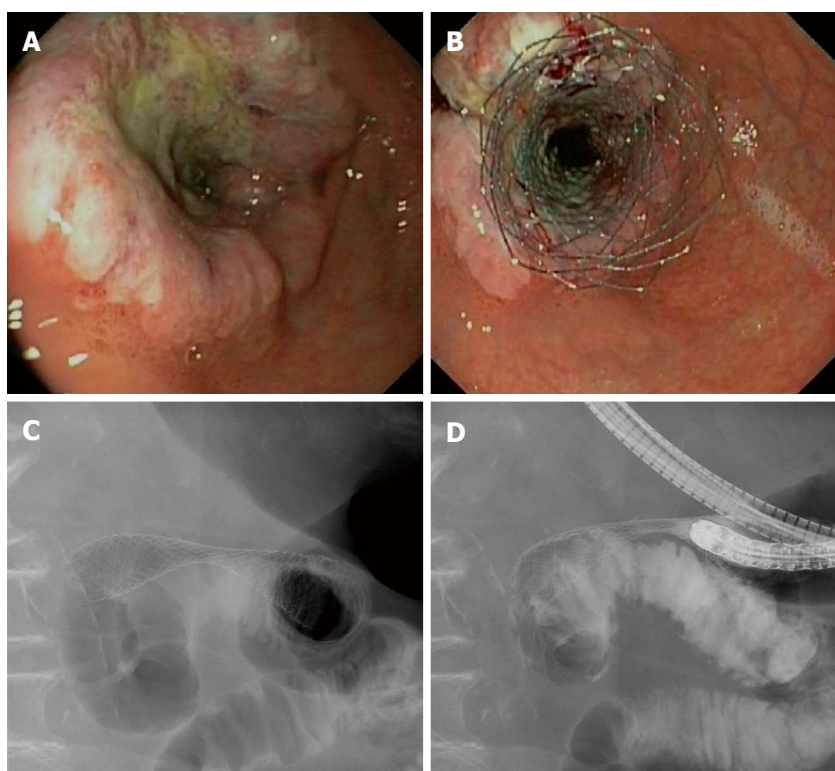
The stents used for the endoscopic treatment of MGOO are self-expandable metal stents (SEMSs) (Figure 2). They consist of a flexible framework of wire mesh made of nitinol, a metal alloy of nickel and titanium, and are either uncovered or covered by a polytetrafluoroethylene, polyurethane or silicone membrane. Over the past years many studies have been published on the clinical outcomes of enteral stent placement for MGOO. With a pooled analysis of the recent literature we aim to provide an overview of the clinical outcomes of SEMS placement for MGOO, including subgroup analyses for covered and uncovered SEMSs.

## MATERIALS AND METHODS

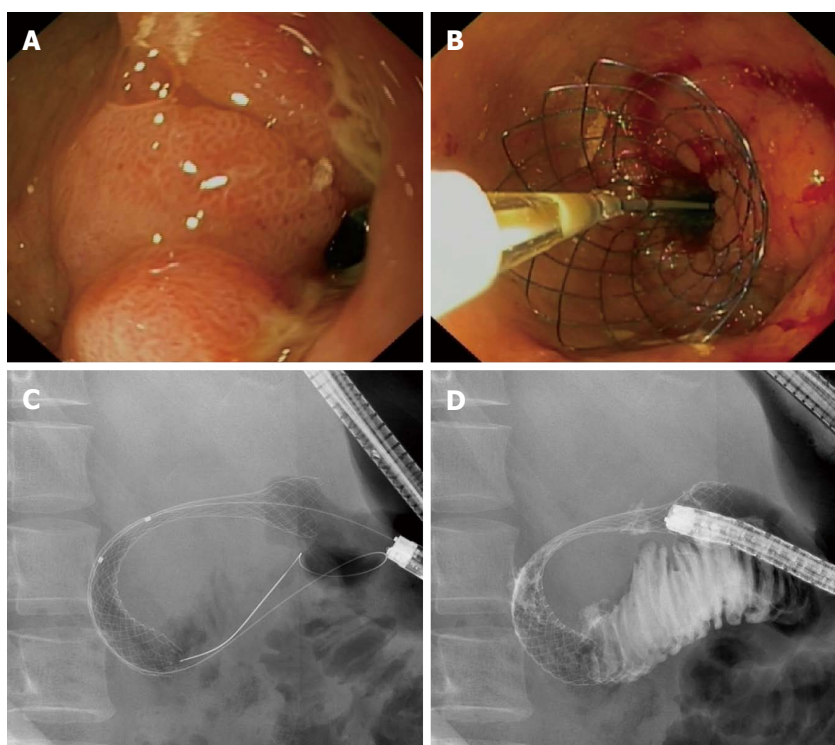
The PubMed database was searched for relevant articles published between January 2009 and March 2015. This period was chosen because during the past years new stent designs have emerged and before 2009 the studies were usually small and retrospective. The search terms used were gastric outlet obstruction, duodenal obstruction, malignant and stents. A single reviewer (van Halsema EE) selected relevant articles by title and abstract. Only prospective studies that reported on the clinical success and safety of stent placement for MGOO were included. Studies with a sample size of less than 10 patients were excluded to avoid pilot studies with experimental stent designs and because the average series in this field usually contains a minimum of at least 30 patients. The search strategy and exclusion criteria are presented in Figure 3. The primary endpoint was clinical success of stent placement. Secondary endpoints were technical success of stent placement, stent dysfunction, stent patency, perforation, bleeding and survival. Clinical success was defined according to the definition used in the original article. These definitions all comprised the ability to tolerate oral intake, improvement in Gastric Outlet Obstruction Severity Score or relief of obstructive symptoms, up to 14 d after enteral stent placement. Stent dysfunction included re-obstruction by tumor in- or overgrowth, stent migration, stent compression by tumor pressure, insufficient expansion after deployment, stent fracture and food occlusion. Technical success was defined as successful stent placement across the obstructing tumor. Perforation and bleeding were analyzed when reported, regardless whether they were thought to be unrelated to enteral stent placement.

### Statistical analysis

Data were pooled and analyzed as an intention-to-treat analysis. Pooled data were presented as frequency and proportion. The median in days was used to report the stent patency and overall survival, because the median was reported most frequently in the original articles. Fisher's Exact Test was used to compare two



**Figure 1** Endoscopic view of a gastric antrum adenocarcinoma involving the pylorus and causing obstructive symptoms (A) for which an uncovered WallFlex stent (Boston Scientific) was placed (B). Fluoroscopic view shows the fully deployed stent across the pylorus (C) with good passage of contrast to the duodenum (D).



**Figure 2** Endoscopic view of an adenocarcinoma of the distal stomach invading the duodenal bulb causing a gastric outlet obstruction (A) for which an uncovered WallFlex stent (Boston Scientific) was placed (B). Fluoroscopic view shows the fully deployed stent in the duodenal bulb (C) with good passage of contrast to the distal duodenum (D).



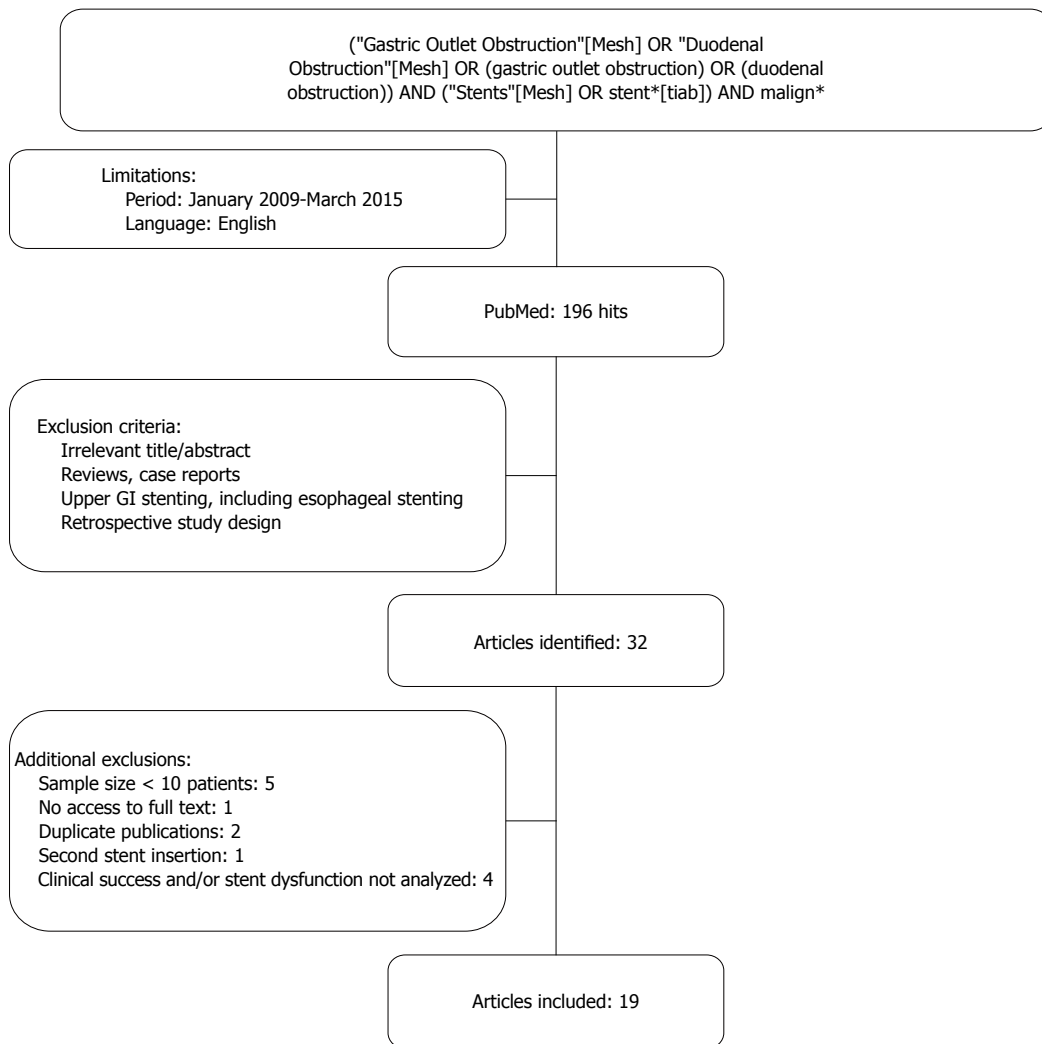


Figure 3 PubMed search. GI: Gastrointestinal.

proportions using WinPepi, Version 11.26, freeware computer programs for epidemiologists<sup>[14]</sup>. Two-sided *P* values < 0.05 were considered statistically significant.

## RESULTS

Thirty-two relevant prospective studies were identified. Figure 3 shows the results of the literature search. Thirteen articles were excluded because of the following reasons: the sample size was insufficient ( $n = 5$ )<sup>[30-34]</sup>, the primary endpoint as defined before was not analyzed ( $n = 4$ )<sup>[8,35-37]</sup>, second stent insertion was analyzed ( $n = 1$ )<sup>[38]</sup>, the full text was not accessible ( $n = 1$ )<sup>[39]</sup> or because of duplicate publication ( $n = 2$ )<sup>[40,41]</sup>. Nineteen prospective studies, including four randomized controlled trials (RCTs), were included in the final analysis (Table 1<sup>[1,2,5,9,15-29]</sup>). A total of 1281 patients underwent enteral stent placement for MGOO. Gastric cancer (42%) was the most common indication for stent placement, followed by pancreatic cancer (37%). Uncovered SEMSs (UCSEMS) were used in 75.7% of patients and partially covered SEMSs (PCSEMS) in 24.3%. The majority of patients (93.5%,

692/740) received a single stent during the initial procedure and 6.5% (48/740) required two stents. The baseline characteristics are summarized in Table 2.

### Technical and clinical success

Technical success was achieved in 97.3% (range 89.1%-100%) of patients and was significantly higher for PCSEMSs in comparison with UCSEMSs: 99.4% vs 96.6% ( $P = 0.008$ ). The main reasons for technical failure were the inability to pass the guidewire across the stenosis (1.0%), stent migration during deployment (0.3%) and insufficient deployment (0.3%). Technical failure due to a procedure-related perforation was reported in one case (0.1%)<sup>[25]</sup>. The overall clinical success rate was 85.7% (range, 57.8%-97.4%). PCSEMSs had a significantly higher clinical success rate than UCSEMSs: 92.3% vs 83.6% ( $P < 0.001$ ). Four studies compared the clinical outcomes of PCSEMSs and UCSEMSs<sup>[5,15,16,24]</sup>. In those comparative studies, the pooled clinical success rates of PCSEMSs and UCSEMSs were 94.3% (164/174) and 93.6% (175/187), respectively ( $P = 0.829$ ). Further details are summarized in Table 3.

Table 1 Literature table

Ref.	Design, patients, indication	No. of stents <sup>1</sup> , biliary obstruction	Stent type	Technical success	Clinical success, definition	Adverse events	Stent dysfunction	Stent patency, median (range)	Survival, follow-up, median (range)
Maefta <i>et al</i> <sup>[15]</sup> 2014	RCT, <i>n</i> = 62 GC: <i>n</i> = 27 PC: <i>n</i> = 26 BDC: <i>n</i> = 7 OT: <i>n</i> = 2	No. of stents: 1: <i>n</i> = 58 2: <i>n</i> = 4 Biliary drainage: Yes: <i>n</i> = 24 No: <i>n</i> = 38	UCSEMS: -Niti-S  PCSEMS: -Niti-S Comvi	100% (62/62)	90.3% (56/62) -UCSEMS: 93.5% (29/31) -PCSEMS: 87.1% (27/31) ≥ 1 grade of improvement in GOOSS at any visit compared to baseline	Perforation: 1.6% (1/62) -PCSEMS: 3.2% (1/31) Major bleeding: 1.6% (1/62) -UCSEMS: 3.2% (1/31)	Overall: 22.6% (14/62) -UCSEMS: 29.0% (9/31) -PCSEMS: 16.1% (5/31) Re-obstruction: 9.7% (6/62); -UCSEMS: 19.4% (6/31) -PCSEMS: 0% (0/31) Migration: 4.8% (3/62) -UCSEMS: 3.2% (1/31) -PCSEMS: 6.5% (2/31) Fracture: 4.8% (3/62) -UCSEMS: 6.5% (2/31) -PCSEMS: 3.2% (1/31) Insufficient expansion: 3.2% (2/62) -UCSEMS: 0% (0/31) -PCSEMS: 6.5% (2/31)	PCSEMS: 68 d UCSEMS: 88 d ( <i>P</i> = 0.70)	PCSEMS: 73 d UCSEMS: 93 d ( <i>P</i> = 0.34)  FU: 83.5 d; until death
Shi <i>et al</i> <sup>[16]</sup> 2014	RCT, <i>n</i> = 65 GC: <i>n</i> = 65	No. of stents: 1: <i>n</i> = 65 Biliary obstruction: NR	UCSEMS: -Micro-Tech  PCSEMS: -Micro-Tech (tailored cup- or funnel-shaped)	96.9% (63/65) -UCSEMS: 96.9% (31/32) -PCSEMS: 97.0% (32/33)	93.7% (59/63) -UCSEMS: 93.5% (29/31) -PCSEMS: 93.8% (30/32) Resolution of symptoms and the ability to restart a low residue diet after stent placement	Mild bleeding: 20% (13/65) -UCSEMS: 6.3% (2/32) -PCSEMS: 33.3% (11/33) Mild abdominal pain: 21.5% (14/65) -UCSEMS: 3.1% (1/32) -PCSEMS: 39.4% (13/33)	Overall: 18.5% (12/65) -UCSEMS: 25% (8/32) -PCSEMS: 12.1% (4/33) Re-obstruction: 12.3% (8/65) -UCSEMS: 21.9% (7/32) -PCSEMS: 3.0% (1/33) Migration: 3.1% (2/65) -UCSEMS: 0% -PCSEMS: 6.1% (2/33) Food impaction: 3.1% (2/65) -UCSEMS: 3.1% (1/32) -PCSEMS: 3.0% (1/33)	NR	Tailored PCSEMS: mean 231 (30-387) d  Standard UCSEMS: mean 212 (43-267) d  FU: until death
Tringali <i>et al</i> <sup>[1]</sup> 2014	Pros, <i>n</i> = 108 PC: <i>n</i> = 58 GC: <i>n</i> = 14 BDC: <i>n</i> = 7 GBC: <i>n</i> = 7 DC: <i>n</i> = 5 APC: <i>n</i> = 3 OT: <i>n</i> = 14	No. of stents: 1: <i>n</i> = 106 2: <i>n</i> = 2 Biliary obstruction: Yes: <i>n</i> = 56 No: <i>n</i> = 52	UCSEMS: -Evolution	99.1% (107/108)	84.5% (82/97) Relief of symptoms and/or improvement of oral intake at 14 d	Overall: 32.4% (35/108), including 19.4% (21/108) stent-related Perforation: 1.9% (2/108) Bleeding: 4.6% (5/108) No intervention required Abdominal pain: 1.9% (2/108) Other GI events: 15.7% (17/108)	Overall: 17.6% (19/108) Re-obstruction: 15.7% (17/108) Migration: 1.9% (2/108)	Estimated patency rates: -At 14 d: 94.6% (88/93) -At 60 d: 86.2% -At 180 d: 63.4%	Patients who completed 6 mo follow-up (11/108): 182 (178-195) d  Patients who died before 6 mo follow-up: 52 (9-180) d
Shi <i>et al</i> <sup>[17]</sup> 2013	Pros, <i>n</i> = 37 GC: <i>n</i> = 37	No. of stents: 1: <i>n</i> = 35 2: <i>n</i> = 2 Biliary obstruction: NR	PCSEMS: -Micro-Tech (cup- or funnel-shaped)	97.3% (36/37)	94.4% (34/36) Relief of obstructive symptoms	Mild bleeding: 40.5% (15/37) Major hemorrhage: 2.7% (1/37) Abdominal pain: 37.8% (14/37) Perforation: 0%	Overall: 5.4% (2/37) Food impaction: 5.4% (2/37) Migration: 0% Re-obstruction: 0%	NR	FU: until 6 mo, death or re-intervention Mean 232 (28-387) d  FU: until death

van den Berg <i>et al</i> <sup>[18]</sup> 2013	Pros, <i>n</i> = 46 PC: <i>n</i> = 25 GC: <i>n</i> = 5 BDC: <i>n</i> = 7 DC: <i>n</i> = 3 GBC: <i>n</i> = 1 OT: <i>n</i> = 5	No. of stents: 1: <i>n</i> = 43 2: <i>n</i> = 3  Biliary drainage: Yes: <i>n</i> = 34 No: <i>n</i> = 12	UCSEMS: -Evolution	89.1% (41/46)	71.7% (33/46)  Improvement of GOOSS of ≥ 1 point and/or relief of symptoms after 1 wk	Overall: 56.5% (26/46) <i>Procedure-related:</i> -Perforation: 2.2% (1/46) -Pancreatitis: 2.2% (1/46) -Pain: 2.2% (1/46) -Cholangitis: 2.2% (1/46) <i>Non procedure-related:</i> -Acute abdomen: 2.2% (1/46) -Cholangitis: 13.0% (6/46) -Jaundice: 4.3% (2/46) -Anemia: 8.7% (4/46) -Pneumonia: 2.2% (1/46) -CVA: 6.5% (3/46) -Ascites: 4.3% (2/46) -Motility disorder: 10.9% (5/46)	Overall: 30.4% (14/46) Re-obstruction: 19.6% (9/46) Stent compression: 4.3% (2/46) Migration: 4.3% (2/46) Food impaction: 2.2% (1/46)	67% for up to 395 d, accounting for death unrelated to stent	87 (IQR 35-237) d  FU: until death
Costamagna <i>et al</i> <sup>[2]</sup> 2012	Pros, <i>n</i> = 202 PC: <i>n</i> = 104 GC: <i>n</i> = 37 DC: <i>n</i> = 18 BDC: <i>n</i> = 12 GBC: <i>n</i> = 12 APC: <i>n</i> = 2 OT: <i>n</i> = 17	No. of stents: 1: <i>n</i> = 192 2: <i>n</i> = 10  Biliary drainage: Yes: <i>n</i> = 127 No: <i>n</i> = 75	UCSEMS: -WallFlex	98.0% (198/202)	91% (177/195)  Relief of obstruction as measured by oral intake	Overall: 20.3% (41/202) Transient periprocedural symptoms: 3.5% (7/202) Bleeding: 3.0% (6/202) -Major: 2.0% (4/202) -Self-limiting: 1.0% (2/202) Perforation: 0.5% (1/202)	Re-obstruction: 12.4% (25/202) Migration: 1.5% (3/202) Food impaction: 0.5% (1/202)	Maintaining GOOS score of 2-3 ( <i>n</i> = 149); 91 d (95%CI: 87-182)	Survival rate at 9 mo: 28.2%  FU: until 9 mo
Italy, Czech Republic, South Africa, Canada, Sweden, Brazil, France, Germany, Finland, Spain Isayama <i>et al</i> <sup>[19]</sup> 2012	Pros, <i>n</i> = 50 PC: <i>n</i> = 26 GC: <i>n</i> = 14 BDC: <i>n</i> = 9 OT: <i>n</i> = 1	No. of stents: NR  Biliary obstruction: Yes: <i>n</i> = 30 No: <i>n</i> = 20	PCSEMS: -ComVi Niti-S (modified)	100% (50/50)	90% (45/50)  Relief of symptoms or improvement in GOOSS after 3 d	Cholangitis: 2% (1/50) Mild pancreatitis: 2% (1/50) Minor perforation: 2% (1/50)	Overall: 18% (9/50) Re-obstruction: 10% (5/50) Stent migration: 6% (3/50) Insufficient expansion: 2% (1/50)	Mean ± SD: 149.8 ± 8.9 d	106 d  FU: NR
Japan  Moura <i>et al</i> <sup>[20]</sup> 2012	Pros, <i>n</i> = 15 PC: <i>n</i> = 9 GC: <i>n</i> = 3 BDC: <i>n</i> = 1 OT: <i>n</i> = 2	No. of stents: NR  Biliary drainage: Yes: 8 No: 7	UCSEMS: -WallFlex	100% (15/15)	80% (12/15)  Improvement of GOOSS at 15 d	Removal of foreign body: 7% (1/15)	Re-obstruction: 13% (2/15) Migration: 13% (2/15)	Mean time to first failure to maintain GOOS 2-3: 2.35 mo	NR  FU: until 180 d
Brazil  Dolz <i>et al</i> <sup>[21]</sup> 2011	Pros, <i>n</i> = 77 GC: <i>n</i> = 29 PC: <i>n</i> = 20 DC: <i>n</i> = 5 GBC: <i>n</i> = 4 BDC: <i>n</i> = 3 APC: <i>n</i> = 3 OT: <i>n</i> = 6 Unk: <i>n</i> = 7	No. of stents: NR  Biliary obstruction: NR	UCSEMS: -WallFlex -Wallstent -Ultraflex	92.2% (71/77)	81.7% (58/71)  GOOSS 2-3 post-stenting	Pneumonia: 1.4% (1/71) Central catheter infection: 1.4% (1/71) Self-limiting bleeding: 7.0% (5/71) Late perforation: 2.8% (2/71) Intense pain: 2.8% (2/71)	Re-obstruction: 14.1% (10/71) Insufficient expansion: 4.2% (3/71)	NR	91 (9-552) d  FU: NR

	Pros, <i>n</i> = 50	No. of stents: NR	PCSEMS: -Niti-S Comvi	100% (50/50)	88% (44/50)	Hyperamylasemia: 2% (1/50) Obstructive jaundice: 10% (5/50)	Re-intervention rate: 28% (14/50) Stent migration: 10% (5/50) Re-obstruction: 8% (4/50) Stent compression: 10% (5/50)	Mean 92 (4-238) d	Mean 110 (30-290) d
Kim <i>et al</i> <sup>[23]</sup> 2011 South Korea	GC: <i>n</i> = 31 PC: <i>n</i> = 11 BDC: <i>n</i> = 6 GBC: <i>n</i> = 2	Biliary drainage: Yes: <i>n</i> = 17 No: <i>n</i> = 33	-Niti-S Comvi	100% (50/50)	Ability to tolerate oral food intake without vomiting				FU: until death
van Hooft <i>et al</i> <sup>[23]</sup> 2011	Pros, <i>n</i> = 52 PC: <i>n</i> = 32 GC: <i>n</i> = 7 BDC: <i>n</i> = 10 APC: <i>n</i> = 1 DC: <i>n</i> = 1 OT: <i>n</i> = 1	No. of stents: 1: <i>n</i> = 45 2: <i>n</i> = 7  Biliary obstruction: NR	UCSEMS: -Niti-S D-Weave	96.2% (50/52)	76.9% (40/52)  Relief of symptoms or improvement of GOOSS after 1 wk	Overall complications: 23.1% (12/52) -Pain: 7.7% (4/52) -Cholangitis: 1.9% (1/52) Non procedure-related: -Anemia: 3.8% (2/52) -Pneumonia: 1.9% (1/52) -Ascites: 1.9% (1/52) -Gastroenteritis: 1.9% (1/52) -Peritonitis carcinomatosis: 1.9% (1/52) -Bacteremia: 1.9% (1/52) Bacterial infection: 4.8% (1/21)	Overall: 25% (13/52) Re-obstruction: 21.2% (11/52) Migration: 3.8% (2/52)	75% for up to 190 d, accounting for death unrelated to stent	82 (IQR 31-135) d  FU: until death
Jeumink <i>et al</i> <sup>[9]</sup> , 2010 Netherlands	RCT, <i>n</i> = 21 PC: <i>n</i> = 15 GC: <i>n</i> = 2 DC: <i>n</i> = 3 OT: <i>n</i> = 1	No. of stents: 1: <i>n</i> = 17 2: <i>n</i> = 4  Biliary drainage: Yes: <i>n</i> = 12 No: <i>n</i> = 9	UCSEMS: -WallFlex	95.2% (20/21)	85.7% (18/21); persistent obstruction within 4 wk in 3/21	Delayed gastric emptying: 14.3% (3/21) Jaundice post stent: 19.0% (4/21) Cholangitis: 4.8% (1/21)	Overall: 19.0% (4/21) Re-obstruction: 9.5% (2/21) Migration: 4.8% (1/21) Food obstruction: 9.5% (2/21)	NR	56 d  FU: until death
Kim <i>et al</i> <sup>[24]</sup> 2010 South Korea	RCT, <i>n</i> = 80 GC: <i>n</i> = 80	No. of stents: NR  Biliary obstruction: NR	PCSEMS: -Niti-S pyloric -Niti-S Comvi  UCSEMS: -Wallstent -WallFlex	100% (80/80)	92.5% (74/80) -PCSEMS: 95% (38/40) -UCSEMS: 90% (36/40)  Relief of symptoms or improvement of GOOSS at 3 d	Perforation by migrated stent: 1.5% (1/67) -PCSEMS: 3.2% (1/31) Intestinal obstruction by migrated stent fragment after fracture: 1.5% (1/67) -PCSEMS: 3.2% (1/31)	Re-obstruction: 25.4% (17/67) -PCSEMS: 3.2% (1/31) -UCSEMS: 44.4% (16/36) Migration: 19.4% (13/67) -PCSEMS: 32.3% (10/31) -UCSEMS: 8.3% (3/36) Fracture: 4.5% (3/67) -PCSEMS: 9.7% (3/31) Stent collapse: 1.5% (1/67) -PCSEMS: 3.2% (1/31) Overall: 18.9% (10/53)	PCSEMS: 14 wk (95%CI: 8.9-19.1) UCSEMS: 13 wk (95%CI: 9.5-16.5)	PCSEMS: 26 wk (95%CI: 11-41) UCSEMS: 19 wk (95%CI: 10-28)  FU: until 8 wk
Maetani <i>et al</i> <sup>[25]</sup> 2010 Japan	Pros, <i>n</i> = 53 GC: <i>n</i> = 29 PC: <i>n</i> = 14 BDC: <i>n</i> = 5 OT: <i>n</i> = 5	No. of stents: 1: <i>n</i> = 44 2: <i>n</i> = 9  Biliary drainage: Yes: <i>n</i> = 17 No: <i>n</i> = 36	UCSEMS: -Niti-S	98.1% (52/53)	94.3% (50/53)  Ability to tolerate oral intake without vomiting	Procedure-related perforation: 1.9% (1/53) Obstructive jaundice: 1.9% (1/53) Major bleeding: 1.9% (1/53)	Insufficient expansion: 3.8% (2/53) Re-obstruction: 13.2% (7/53) Food impaction: 1.9% (1/53) Fracture: 1.9% (1/53)	NR	88 d  FU: until death
							Re-intervention rate: 20.8% (11/53)		



Shaw <i>et al</i> <sup>[26]</sup> , 2010	Pros, <i>n</i> = 70	No. of stents: NR	UCSEMS: -WallFlex	92.9% (65/70)	88.6% (62/70)	Minor bleeding: 2.9% (2/70) Perforation: 0%	Overall: 7.1% (5/70) Re-obstruction: 4.3% (3/70) Insufficient expansion: 1.4% (1/70) Stent migration: 1.4% (1/70)	NR	1.8 (0.1-19) mo FU: 54 (range 1-570) d; until death
South Africa	GC: <i>n</i> = 19 PC: <i>n</i> = 34 GBC: <i>n</i> = 5 DC: <i>n</i> = 2 BDC: <i>n</i> = 3 OT: <i>n</i> = 7	Biliary drainage: Yes: <i>n</i> = 35 No: <i>n</i> = 35			Resumption of intake that enabled the patient to return home independent of nutritional support				
Havemann <i>et al</i> <sup>[27]</sup> , 2009	Pros, <i>n</i> = 45	No. of stents: NR	UCSEMS: -Hanaro	91.1% (41/45)	63.4% (26/41)	Procedure-related perforation: 4.4% (2/45) Biliary obstruction: 17.8% (8/45)	Re-obstruction: 8.9% (4/45) Migration: 6.7% (3/45)	NR	Mean 121 (95%CI: 62-181) d FU: NR
Denmark Lee <i>et al</i> <sup>[6]</sup> , 2009	PC: <i>n</i> = 30 GC: <i>n</i> = 5 OT: <i>n</i> = 10 Pros, <i>n</i> = 154	Biliary drainage: Yes: <i>n</i> = 11 No: <i>n</i> = 34 No. of stents: NR	UCSEMS: -Niti-S PCSEMS: -Niti-S	100% (154/154)	Improvement in GOOSS by ≥ 1 point 97.4% (150/154) -PCSEMS: 98.6% (69/70) -UCSEMS: 96.4% (81/84)	No procedure-related complications	Re-intervention rate: 13.3% (6/45) Migration: 7.8% (12/154) -PCSEMS: 17.1% (12/70) -UCSEMS: 0% Re-obstruction: 13.6% (21/154) -PCSEMS: 7.1% (5/70) -UCSEMS: 19.0% (16/84)	UCSEMS: 73 (95%CI: 44-102) d PCSEMS: 75 (95%CI: 47-134) d	UCSEMS: 108 (95%CI: 60-151) d PCSEMS: 115 (95%CI: 80-156) d FU: until death
South Korea	GC: <i>n</i> = 122 PC: <i>n</i> = 19 GBC: <i>n</i> = 3 BDC: <i>n</i> = 3 APC: <i>n</i> = 4 DC: <i>n</i> = 2 OT: <i>n</i> = 1	Biliary obstruction: NR			Relief of vomiting and resumption of diet		Re-intervention rate: 17.5% (27/154) -PCSEMS: 21.4% (15/70) -UCSEMS: 14.3% (12/84) Overall: 18.6% (8/43) Re-obstruction: 9.3% (4/43) Malposition: 2.3% (1/43) Stent collapse: 2.3% (1/43) Incomplete expansion: 4.7% (2/43) Occlusion by jejunal wall: 2.3% (1/43) Migration: 0%	GOOS score increase of ≥ 1 until death or (19/43) up: 45% (95%CI: 27-74)	49 d; At 24 wk: 44.1% (19/43) FU: until 24 wk
Piesman <i>et al</i> <sup>[28]</sup> , 2009	Pros, <i>n</i> = 43	No. of stents: 1: <i>n</i> = 39 2: <i>n</i> = 4	UCSEMS: -WallFlex	95.3% (41/43)	81.4% (35/43)	Duodenal perforation: 4.7% (2/43) Vomiting: 9.3% (4/43) Cholangitis: 2.3% (1/43) Hemorrhage: 2.3% (1/43) -Endoscopy performed Nausea: 2.3% (1/43) Sepsis: 2.3% (1/43)	Re-obstruction: 11.8% (6/51) Migration: 2.0% (1/51)	307 d; 75% functional at 135 d, 25% functional at 470 d	62 d; 75% alive at 35 d, 25% alive at 156 d FU: until death
United States	PC: <i>n</i> = 21 GC: <i>n</i> = 8 BDC: <i>n</i> = 3 GBC: <i>n</i> = 1 OT: <i>n</i> = 9 Unk: <i>n</i> = 1	Biliary drainage: Yes: <i>n</i> = 23 No: <i>n</i> = 20			GOOSS increase of ≥ 1 point				
Van Hooft <i>et al</i> <sup>[29]</sup> , 2009	Pros, <i>n</i> = 51	No. of stents: 1: <i>n</i> = 48 2: <i>n</i> = 3	UCSEMS: -WallFlex	98.0% (50/51)	84.3% (43/51)	Motility dysfunction: 3.9% (2/51) Intermittent pain: 3.9% (2/51) Cholangitis: 5.9% (3/51) Major bleeding: 3.9% (2/51)	Re-obstruction: 11.8% (6/51) Migration: 2.0% (1/51)	307 d; 75% functional at 135 d, 25% functional at 470 d	62 d; 75% alive at 35 d, 25% alive at 156 d FU: until death
Netherlands	PC: <i>n</i> = 35 GC: <i>n</i> = 2 BDC: <i>n</i> = 3 DC: <i>n</i> = 3 GBC: <i>n</i> = 2 APC: <i>n</i> = 1 OT: <i>n</i> = 5	Biliary drainage: Yes: <i>n</i> = 38 No: <i>n</i> = 13			Relief of symptoms or improvement of GOOSS after 1 wk				

<sup>1</sup>Number of enteral stents inserted at the initial procedure. Pros: Prospective; Retro: Retrospective; RCT: Randomized controlled trial; GC: Gastric cancer; PC: Pancreatic cancer; BDC: Bile duct cancer; GBC: Gallbladder cancer; APC: Ampullary cancer; DC: Duodenal cancer; OT: Other malignancies; UCSEMS: Uncovered self-expandable metal stent; PCSEMS: Partially covered self-expandable metal stent; FU: Follow-up; GOOSS: Gastric outlet obstruction severity score; NR: Not reported; IQR: Interquartile range; GI: Gastrointestinal; CVA: Cerebrovascular accident.

**Table 2** Baseline characteristics *n* (%)

Patients with MGOO	1281 (100)
Cause of MGOO	
Gastric cancer	536 (41.8)
Pancreatic cancer	479 (37.4)
Bile duct cancer	79 (6.2)
Duodenal cancer	42 (3.3)
Gallbladder cancer	37 (2.9)
Ampullary cancer	14 (1.1)
Other malignancies	86 (6.7)
Unknown	8 (0.6)
Biliary obstruction <sup>1</sup>	
Yes	432 (52.9)
No	384 (47.1)
Stent type	
Uncovered SEMS	970 (75.7)
Partially covered SEMS	311 (24.3)
No. of enteral stents inserted at initial procedure <sup>2</sup>	
Single stent	692 (93.5)
Two stents	48 (6.5)

<sup>1</sup>Total group: *n* = 816, no data of 465 patients; <sup>2</sup>Total group: *n* = 740, no data of 541 patients. MGOO: Malignant gastric outlet obstruction; SEMS: Self-expandable metal stent.

### Stent dysfunction

Stent dysfunction occurred in 19.6% (range, 5.4%–42.5%) of patients. There was no difference between the stent dysfunction rate of PCSEMSs and UCSEMSs: 21.2% vs 19.1%, respectively ( $P = 0.412$ ). The main reasons for stent failure were re-obstruction by tumor in- or overgrowth (12.6%) and stent migration (4.3%). Re-obstruction was more common with the use of UCSEMSs compared with PCSEMSs: 14.9% vs 5.1% ( $P < 0.001$ ). The stent migration rate was significantly higher after PCSEMS placement: 10.9% vs 2.2% ( $P < 0.001$ ). Stent compression or collapse by tumor pressure occurred in 0.7% of patients, and was significantly higher for PCSEMSs: 1.9% vs 0.3% ( $P = 0.008$ ). Other reasons for stent dysfunction were insufficient expansion (0.9%), food occlusion (0.7%), stent fracture (0.5%) and other (0.2%) (Table 4).

### Perforation and bleeding

The overall perforation rate was 1.2% and was comparable for PCSEMSs and UCSEMSs (Table 4). Perforation within 30 d was reported in 0.7% and late perforations in 0.5% of patients. Six (0.5%) perforations occurred during or immediately after the initial stent placement procedure. A description of the perforation cases is provided in Table 5.

Bleeding was reported in 4.1% of patients and was more frequent in patients treated with PCSEMSs: 8.7% vs 2.6% ( $P < 0.001$ ) (Table 4). Major bleeding, requiring an intervention, occurred in 10 (0.8%) cases.

### Stent patency and overall survival

The median stent patency was reported in five studies<sup>[2,5,15,24,29]</sup>, including 549 patients, and ranged from 68 d to 98 d, with exception of one study that

reported a median stent patency of 307 d<sup>[29]</sup>.

The median overall survival ranged from 49 d to 183 d in thirteen studies, including 867 patients<sup>[1,5,9,15,18,19,21,23-26,28,29]</sup>. When the majority ( $\geq 50\%$ ) of the study sample included patients with pancreatic cancer, the median overall survival ranged from 49 d to 106 d<sup>[1,9,18,19,23,26,28,29]</sup>. When the majority of the study sample included patients with gastric cancer, the median overall survival ranged from 88 d to 183 d<sup>[5,24,25]</sup>.

## DISCUSSION

This pooled analysis of 1281 patients identified from the prospective literature, showed that palliative SEMS placement for MGOO is feasible, effective and safe. Stent placement can therefore be regarded as a good alternative for surgery in the palliative setting. The clinical success rate was high (85.7%) and although stent dysfunction was frequently encountered (19.6%), it could usually be managed endoscopically by additional stent placement. Large, recently published, retrospective studies, each including more than 125 patients, reported comparable results<sup>[4,42,43]</sup>.

In subgroup analysis, the technical and clinical success rates of PCSEMS placement were significantly higher than those of UCSEMSs. The reasons for technical failure (Table 3) were rather procedure-related than stent-related. The higher technical success rate of PCSEMSs can therefore not be easily explained. The higher clinical success rate of PCSEMSs is a notable finding, suggesting that these stent models have more capacity in relieving MGOO, for instance by a higher radial force than UCSEMSs. However, the validity of this finding may be questioned because of heterogeneity, such as the difference in definitions of clinical success between the included studies. To exclude this heterogeneity, a subgroup analysis was performed of the four studies that compared the outcomes of PCSEMSs and UCSEMSs, showing similar pooled clinical success rates for PCSEMSs and UCSEMSs. In addition, a meta-analysis of comparative studies found no difference in technical and clinical success between covered and uncovered SEMSs<sup>[44]</sup>. The data were insufficient and the samples would be too small to analyze the outcomes of the eleven different stent models, including modified and patient-tailored stents, used in the 19 included studies.

Several factors have been identified as predictors for the outcomes of stent placement for MGOO. One prospective cohort study, including 71 patients, found a significantly lower clinical success rate for stents placed in the gastric antrum (29%) compared with success rates of stent placement in the duodenum (70%) or at the gastrojejunal anastomosis (87%)<sup>[21]</sup>. The authors speculated that antral tumors have to be larger to cause obstruction, resulting in more antral rigidity<sup>[21]</sup>. The two main indications for enteral stent placement in our pooled analysis were obstruction

**Table 3 Technical and clinical success of enteral stent placement *n* (%)**

	Overall ( <i>n</i> = 1281)	UCSEMS ( <i>n</i> = 970)	PCSEMS ( <i>n</i> = 311)	<i>P</i> value <sup>1</sup>
Technical success	1246 (97.3)	937 (96.6)	309 (99.4)	0.008
Reasons for technical failure				
Inability to pass guidewire	13 (1.0)	13 (1.3)	0	
Looping/buckling of delivery system	2 (0.2)	0	2 (0.6)	
Stent malposition	1 (0.1)	1 (0.1)	0	
Stent migration during deployment	4 (0.3)	4 (0.4)	0	
Insufficient deployment	4 (0.3)	4 (0.4)	0	
Colonic stent inserted	1 (0.1)	1 (0.1)	0	
No stenosis at endoscopy	1 (0.1)	1 (0.1)	0	
Procedural perforation	1 (0.1)	1 (0.1)	0	
Not specified	8 (0.6)	8 (0.8)	0	
Clinical success	1098 (85.7)	811 (83.6)	287 (92.3)	< 0.001

<sup>1</sup>Comparison of UCSEMS and PCSEMS using Fisher's exact test. UCSEMS: Uncovered self-expandable metal stents; PCSEMS: Partially covered self-expandable metal stents.

**Table 4 Adverse events *n* (%)**

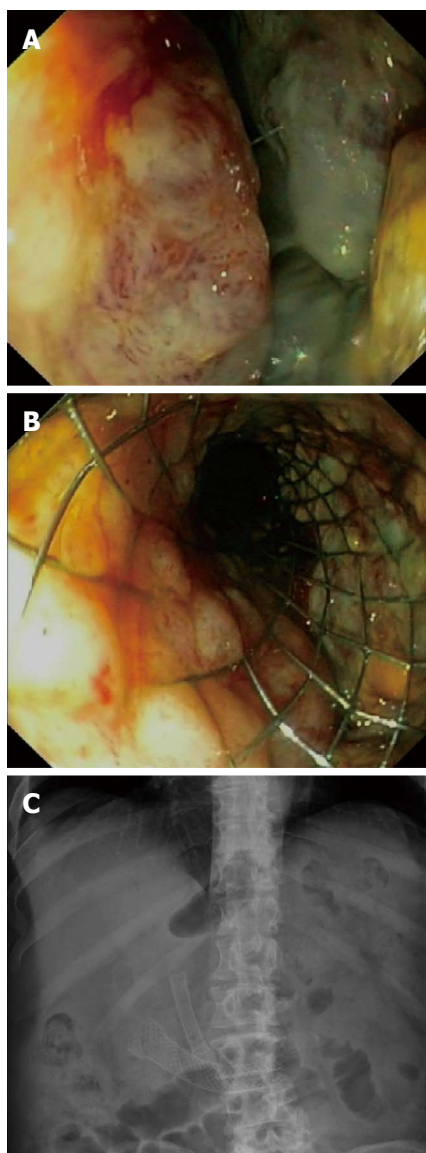
	Overall ( <i>n</i> = 1281)	UCSEMS ( <i>n</i> = 970)	PCSEMS ( <i>n</i> = 311)	<i>P</i> value <sup>1</sup>
Stent dysfunction	251 (19.6)	185 (19.1)	66 (21.2)	0.412
Re-obstruction by tumor growth	161 (12.6)	145 (14.9)	16 (5.1)	< 0.001
Stent migration	55 (4.3)	21 (2.2)	34 (10.9)	< 0.001
Stent compression by tumor pressure	9 (0.7)	3 (0.3)	6 (1.9)	0.008
Stent fracture	7 (0.5)	3 (0.3)	4 (1.3)	0.064
Insufficient expansion	11 (0.9)	8 (0.8)	3 (1.0)	0.734
Food occlusion	9 (0.7)	6 (0.6)	3 (1.0)	0.460
Other	2 (0.2)	2 (0.2)	0	-
Perforation	15 (1.2)	12 (1.2)	3 (1.0)	1.000
Bleeding	52 (4.1)	25 (2.6)	27 (8.7)	< 0.001
Major bleeding requiring intervention	10 (0.8)	9 (0.9)	1 (0.3)	0.466

<sup>1</sup>Comparison of UCSEMS and PCSEMS using Fisher's exact test. UCSEMS: Uncovered self-expandable metal stents; PCSEMS: Partially covered self-expandable metal stents.

**Table 5 Details on the perforation cases**

No.	Description	Day of onset	Treatment
1	Jejunal perforation at distal end of the stent <sup>[15]</sup>	173	Surgical closure
2	Intraprocedural perforation while the stricture was crossed with the catheter and guidewire <sup>[1]</sup>	0	Successfully treated with covered SEMS
3	Duodenal perforation after biliary stent placement <sup>[1]</sup>	82	Laparotomy, abdominal drainage and duodenal covered SEMS
4	Acute abdomen <sup>[18]</sup>	42	Refused treatment
5	Guidewire perforation <sup>[18]</sup>	0	Conservative treatment with antibiotics
6	Perforation likely due to stent-induced ischemia <sup>[2]</sup>	15	Surgical suture and gastrojejunostomy
7	Minor perforation after balloon dilation because of insufficient stent expansion <sup>[19]</sup>	7	Recovered without surgery
8	Late perforation, not related to dilatation <sup>[21]</sup>	NR	NR
9	Late perforation, not related to dilatation <sup>[21]</sup>	NR	NR
10	Late intestinal perforation by migrated stent <sup>[24]</sup>	NR	Surgical intervention
11	Perforation while pushing the delivery system across the initially placed stent <sup>[25]</sup>	0	Surgical closure and gastrojejunostomy
12	Perforation by the guidewire and/or ERCP catheter with subsequent misplacement of the stent <sup>[27]</sup>	0	Surgical suture, bowel patch and gastroenteric bypass
13	Perforation by the guidewire and/or ERCP catheter with subsequent misplacement of the stent <sup>[27]</sup>	0	Surgical suture, bowel patch and gastroenteric bypass
14	Abdominal pain and pneumoperitoneum immediately after stent placement <sup>[28]</sup>	0	Loop gastrojejunostomy and combined gastrostomy-jejunostomy tube placement
15	Abdominal pain, distension, vomiting, and free air on x-ray 6 d after second stent placement <sup>[28]</sup>	12	Nasogastric tube placement and hospitalized; died two days later of sepsis

SEMS: Self-expandable metal stent; NR: Not reported; ERCP: Endoscopic retrograde cholangiopancreatography.



**Figure 4** Endoscopic view of an ulcerative, obstructing gastrointestinal stromal tumor of the peri-ampullary region of the duodenum (A) for which an uncovered WallFlex stent (Boston Scientific) was placed (B). Fluoroscopic view shows the fully deployed duodenal stent overlapping the previously placed biliary SEMS (C).

by gastric (42%) and pancreatic (37%) cancer. Unfortunately, the data were insufficient to analyze the clinical outcomes according to cause and site of obstruction. However, other retrospective and prospective studies never identified type of cancer and site of obstruction as predictors for success of enteral stent placement<sup>[4,42,45-47]</sup>. The main factors associated with a poor stent outcome in the literature are a poor performance status and peritoneal dissemination with ascites<sup>[4,36,37,43,48]</sup>.

One fifth of the patients experienced stent dysfunction, mainly because of re-obstruction by tumor in- or overgrowth and stent migration. PCSEMSs were associated with the occurrence of stent migration, while re-obstruction was more frequently seen with the use of UCSEMSs. The overall stent dysfunction rates

were comparable between both stent types, which is consistent with a recently published meta-analysis<sup>[44]</sup>. The fact that stent covering precludes tumor ingrowth, but provokes stent migration, has already been demonstrated<sup>[44]</sup>. A large retrospective analysis, including 583 patients with MGOO mainly caused by gastric cancer (57%), found that duodenal lesions, a shorter stricture length and longer survival time were associated with the occurrence of re-obstruction by tumor overgrowth<sup>[49]</sup>. Also short time to progression has been identified as a predictor for re-obstruction, while administration of first line chemotherapy was protective against re-stenosis<sup>[50]</sup>. Regarding the occurrence of stent migration, chemotherapy after stent placement was associated with migration in two studies, although only in univariate analysis<sup>[50,51]</sup>. In a prospective pilot study of 25 patients with MGOO, covered SEMSs were anchored into the mucosa by three endoscopic clips at the proximal end of the stent to prevent stent migration<sup>[41]</sup>. No cases of stent migration occurred, suggesting that endoscopic clipping may prevent stent migration<sup>[41]</sup>. Regarding the stent patency, one of the included studies estimated with a Kaplan-Meier analysis that 63% of the stents were patent at six months<sup>[1]</sup>. Another prospective cohort reported that the GOOS score increase persisted until death or end of follow-up in 45% (95%CI: 27%-74%) of patients<sup>[28]</sup>.

Perforation and major bleeds were rare, both occurring in approximately 1% of patients. Seven of the 15 perforations were procedure- or balloon dilatation-related. A recently published, retrospective study reported perforation in 3.4% (10/292) of patients treated with SEMSs for MGOO<sup>[42]</sup>. The perforation rate according to the cause of obstruction was 4.6% (9/196) for pancreatic cancer and 1.0% (1/96) for nonpancreatic cancer<sup>[42]</sup>, suggesting that the cause of obstructing may be associated with the occurrence of perforation after enteral stent placement. However, data are lacking to support this assumption. Minor bleeding was more frequently seen in patients treated with PCSEMSs, mainly contributed by two studies from the same institution that reported 56% (29/52) of bleedings using tailored, funnel- and cup-shaped, PCSEMSs<sup>[16,17]</sup>. Therefore, these tailored PCSEMSs may not be directly comparable with the other PCSEMS designs used in the literature. Nevertheless, the overall major bleeding rate in our pooled analysis was only 0.8%.

This analysis of the prospective literature has several limitations. Heterogeneity between the included studies is the main limitation. As mentioned before, the causes of MGOO, the definitions used for clinical success and the stent designs differed between the included studies. Furthermore, the included prospective studies are prone to selection-by-indication, since only one RCT was included that compared surgical gastrojejunostomy with enteral stent placement<sup>[9]</sup>. The patients included in the



remaining articles therefore represent a selected population, because it was decided upfront that stent placement was indicated. This may overestimate the outcomes of enteral stent placement. Another issue is the clinically relevant question whether duodenal stent placement should be preceded by biliary stenting to maintain biliary drainage (Figure 4). However, that question was beyond our literature search.

In conclusion, this pooled analysis of the recently published, prospective literature provides an extensive overview of the clinical outcomes of stent placement for MGOO. In this large population enteral stent placement was feasible, effective and safe. Therefore, stent placement is a valid option for the palliation of MGOO.

## COMMENTS

### Background

Gastric outlet obstruction is usually a late sign of a locally advanced or metastatic cancer, requiring palliative management. Endoscopic self-expandable metal stent placement to relieve obstructive symptoms and allow oral intake, is a well-established treatment option in patients with malignant gastric outlet obstruction.

### Research frontiers

Comparison of enteral stenting and gastrojejunostomy revealed sooner return to oral intake and shorter hospital stay after stent placement. On the long term, however, patients with an enteral stent have more recurrent obstruction and require more re-interventions.

### Innovations and breakthroughs

To improve the long term patency of self-expandable metal stents, many different stent designs have been developed to reduce the risk of stent migration and re-obstruction by tumor ingrowth. In this systematic review, the authors provide an extensive overview of the prospective literature published since January 2009 on the clinical outcomes of stent placement for malignant gastric outlet obstruction.

### Applications

This pooled analysis may be helpful for the endoscopist in the decision-making on the indication for duodenal stent placement and also to inform the patient on the risks and benefits of stent therapy.

### Terminology

Gastric outlet obstruction is an obstruction at the level of the pylorus (gastric antrum, pylorus, duodenal bulb) causing problems with the passage of food into the small intestine. Self-expandable metal stents consist of a flexible framework of wire mesh made of nitinol, a metal alloy of nickel and titanium, and are either uncovered or covered by a polytetrafluoroethylene polyurethane or silicone membrane.

### Peer-review

Interesting study, well written and deeply described.

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## Cooperative laparoscopic endoscopic and hybrid laparoscopic surgery for upper gastrointestinal tumors: Current status

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### Abstract

**AIM:** To investigate the cooperative laparoscopic and endoscopic techniques used for the resection of upper gastrointestinal tumors.

**METHODS:** A systematic research of the literature was performed in PubMed for English and French language articles about laparoscopic and endoscopic cooperative, combined, hybrid and rendezvous techniques. Only original studies using these techniques for the resection of early gastric cancer, benign tumors and gastrointestinal stromal tumors of the stomach and the duodenum were included. By excluding case series of less than 10 patients, 25 studies were identified. The study design, number of cases, tumor pathology size and location, the operative technique name, the endoscopy team and surgical team role, operative time, type of closure of visceral wall defect, blood loss, complications and length of hospital stay of these studies were evaluated. Additionally all cooperative techniques found were classified and are presented in a systematic approach.

**RESULTS:** The studies identified were case series and retrospective cohort studies. A total of 706 patients were operated on with a cooperative technique. The tumors resected were only gastrointestinal stromal tumors (GIST) in 4 studies, GIST and various benign submucosal tumors in 22 studies, early gastric cancer (pT1a and pT1b) in 6 studies and early duodenal cancer in 1 study. There was important heterogeneity between the studies. The operative techniques identified were:



laparoscopic assisted endoscopic resection, endoscopic assisted wedge resection, endoscopic assisted trans-gastric and intragastric surgery, laparoscopic endoscopic cooperative surgery (LECS), laparoscopic assisted endoscopic full thickness resection (LAEFR), clean non exposure technique and non-exposed endoscopic wall-inversion surgery (NEWS). Each technique is illustrated with the roles of the endoscopic and laparoscopic teams; the indications, characteristics and short term results are described.

**CONCLUSION:** Along with the traditional cooperative techniques, new procedures like LECS, LAEFR and NEWS hold great promise for the future of minimally invasive oncologic procedures.

**Key words:** Cooperative laparoscopic endoscopic; Hybrid laparoscopic; Laparoscopic endoscopic cooperative surgery; Endoscopy; Laparoscopy; Minimally invasive surgery; Gastrointestinal stromal tumors; Gastric cancer; Submucosal tumor

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**Core tip:** Cooperative laparoscopic and endoscopic surgery for the resection of upper gastrointestinal tumors combines the advantages of intraluminal and extraluminal approach: precise lesion localization, safe excision and reconstruction. It has been used for the resection of benign submucosal tumors and Gastrointestinal stromal tumors. Novel techniques like inverted laparoscopic endoscopic cooperative surgery, laparoscopic assisted endoscopic full thickness resection, clean non exposure technique and non-exposed endoscopic wall-inversion surgery have emerged for the minimally invasive treatment of early gastric cancer. Their oncologic principles are sound and the first results encouraging. Soon, the close collaboration of laparoscopic and endoscopic teams will be "*conditio sine qua non*" for the institutions that seek excellence in the treatment of upper gastrointestinal neoplasias.

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## INTRODUCTION

In the last decade there has been an important development in the minimally invasive treatment of benign and borderline benign tumors of the upper gastrointestinal tract. The creation of new operative tools and the invention of new operative techniques

offer more options for the resection of these tumors than ever before.

For the interventional endoscopist, the possibilities for local excision with techniques such as endoscopic submucosal dissection (ESD) and endoscopic full thickness resection (EFTR) are approaching those that only a surgical approach could offer in the past<sup>[1,2]</sup>. In a parallel course, thanks to the evolution of laparoscopic surgery, the gastrointestinal surgeons can now offer precise minimally invasive segmental resections that favor a more functional outcome and a faster patient recovery in comparison with more aggressive resections<sup>[3]</sup>.

In this competitive race each of these two approaches have their strong points and weaknesses. Nevertheless we are noticing the emergence of a third approach: the cooperative laparoscopic and endoscopic approach, also called hybrid laparoscopic approach. This group of techniques aims to accumulate the strong points of intraluminal and intraperitoneal procedures, and at the same time, negate their limitations.

This is not a novel concept since many laparoscopic and endoscopic cooperative techniques have been described with different names: combined laparoscopic and endoscopic, hybrid laparoscopic, cooperative laparoscopic-endoscopic, laparoscopic-endoscopic rendezvous. In spite of the nomenclature used, the idea remains the same: a simultaneous endoluminal and intraperitoneal approach for the localization of the lesion, its precise resection respecting oncologic principles and the closure of the defect.

Interestingly in the last decade, our better knowledge on the biological behavior of gastrointestinal stromal tumors (GIST) and early gastric cancer, as well as the more frequent detection of these two pathologies has increased the interest in precise, segmental and mini invasive resection techniques based on sound oncologic principles<sup>[4,5]</sup>. Teams from Asiatic countries and pioneers like Hiki lead the effort of discovering new cooperative laparoscopic and endoscopic techniques.

The aim of this article is to review the data on cooperative laparoscopic and endoscopic techniques, identify and classify the different techniques described, define their indications and elaborate their characteristics.

## MATERIALS AND METHODS

A systematic review of the literature from the databases of PubMed and MEDLINE from January 1960 to March 2015 was performed. The search algorithm used the following strategy:

("tumour"[All Fields] OR "tumor"[All Fields] OR "submucosal tumor"[All Fields] OR "early gastric cancer"[All Fields] OR "GIST"[All Fields] OR "gastrointestinal stromal tumor"[All Fields] OR "neoplasms"[MeSH Terms] OR "neoplasms"[All Fields]).

And [("laparoscopic"[All Fields] or "laparoscopy"[All Fields] OR "laparoscopy"[MeSH Terms]) and ("endoscopic"[All Fields] or "endoscopy"[All Fields] or "endoscopy"[MeSH Terms]) and ("intra-gastric"[All Fields] or "transgastric"[All Fields] or "cooperative"[All Fields] or "combined"[All Fields] or "rendezvous"[All Fields] or "hybrid"[All Fields])].

And ("stomach"[MeSH Terms] or "stomach"[All Fields] or "gastric"[All Fields] or "upper gastrointestinal"[All Fields] or "Upper Gastrointestinal Tract"[MeSH Terms]).

### **Inclusion criteria**

The search results were screened by the two reviewers (DN, GM) for relevant publications. All original articles describing the combined techniques of laparoscopy and endoscopy for the treatment of tumors of the upper gastrointestinal tract were included. The list of references of each eligible article was manually evaluated for any relevance to the specific topic. Only the articles that were published in English and French were included.

### **Exclusion criteria**

Animal studies and experimental studies were excluded. Duplicate publications, review articles without any original data and studies that did not clearly state in their methods that a combined endoscopic and laparoscopic approach was used were also excluded. When there was an overlap in the data between the two studies, only the most recent study was considered. Technical articles not reporting patient results were also excluded.

### **Data extraction and analysis**

The included studies were assessed; and the relevant data that was extracted by the reviewers was filled onto a spreadsheet data extraction form. The extracted data for each study focused on: first author's name, year of publication, country involved, study design, number of cases, lesion pathology, its' size and location, operative technique name, endoscopy team and surgical team role, operative time, type of closure of visceral wall defect, any blood loss or complications and length of hospital stay. If a technique has a commonly used acronym this is given in a parenthesis after its name. The investigated outcomes for each technique were: type of technique used, number of cases, lesion size, tumor location, tumor pathology, role of endoscopic and of laparoscopic team, technique of visceral wall closure, operative time duration, adverse events and complications. Only descriptive statistics were used. Weighted averages were calculated based only on the number of patients of each study. Results are given as means  $\pm$  SD.

## **RESULTS**

The search returned 414 abstracts published from

June 1971 to March 2015; 339 of these were excluded on the basis of their title or the abstract contents. From the remaining 75 abstracts, 50 were case reports or studies with less than 10 patients. The remaining 25 articles included 8 prospective case series, 4 retrospective cohort studies, and 13 retrospective series (Table 1). The total number of patients operated by a cooperative laparoscopic endoscopic technique in these studies was 706.

Concerning the resected lesion pathology, 4 studies included only GIST, while 22 studies involved a variety of benign submucosal tumors (SMT) and GIST, 6 studies early gastric cancer (pT1a and pT1b) and only 1 study had to do with early duodenal cancer. The mean tumor size varied from 5 to 55 mm. Almost all tumors were located in the stomach, with only one study dealing with duodenal tumors. Heterogeneity existed concerning the name of the procedures, the patients' characteristics and the reported outcomes (Table 2). For consistency reasons, throughout this paper the term Cooperative Laparoscopic Endoscopic Techniques is used to include all the techniques described.

Eleven operative techniques using a cooperative laparoscopic and endoscopic approach were identified from the literature. In all publications the procedure was undertaken in the operating theater under general anesthesia. The operative techniques were classified into three categories according to the roles of the endoscopic and laparoscopic teams: the laparoscopy-assisted endoscopic resection (LAER) group where the resection was performed primarily by the endoscopic team under laparoscopic control; the endoscope-assisted laparoscopic resection (EALR) group where the laparoscopic team performed the resection under endoscopic monitoring; the combined laparoscopic endoscopic resection (CLER) group where the resection was performed jointly by the laparoscopic and the endoscopic team. The characteristics of these techniques, their indications and their reported outcomes are reported herein (Table 3).

Weighted averages based on patient number were calculated: for the tumor size they were 3.1 mm for the LAER, 33.3 mm for the EARL and 28.5 mm for the CLER group; for the operative time they were 88.4 min for the CLER, 72.1 min for the LAER and 103.1 min for the EALR group; for the estimated blood loss were 3.5 mL, 29.8 mL and 50.6 mL for the CLER, LAER and EARL groups respectively; and for the hospital stay time were 9.9 d for the CLER group, 2 d for the LAER group and 8.5 d for the EARL group.

### **Group 1: LAER**

In this category, endoscopic mucosal resections (EMR) and ESD are undertaken under laparoscopic control (Figure 1).

The endoscopic team performs an EMR or ESD while the laparoscopic team has a backup role; it monitors the endoscopic resection and provides an extraluminal treatment of any adverse effect like

**Table 1 Publications of cooperative laparoscopic endoscopic techniques with > 10 patients**

Authors	Country	Year	Study type	No. Cases
Choi <i>et al</i> <sup>[20]</sup>	Korea	2000	Retrospective series	32
Shimizu <i>et al</i> <sup>[26]</sup>	Japan	2002	Retrospective cohort	11
Matthews <i>et al</i> <sup>[30]</sup>	United States	2002	Retrospective cohort	33
Ludwig <i>et al</i> <sup>[27]</sup>	Germany	2002	Prospective case series	18
Bouillot <i>et al</i> <sup>[19]</sup>	France	2003	Multicenter retrospective case series	56 <sup>1</sup>
Walsh <i>et al</i> <sup>[36]</sup>	United States	2003	Retrospective series	13
Hindmarsh <i>et al</i> <sup>[21]</sup>	United Kingdom	2005	Retrospective series	30
Schubert <i>et al</i> <sup>[28]</sup>	Germany	2005	Retrospective series	26
Mochizuki <i>et al</i> <sup>[24]</sup>	Japan	2006	Retrospective series	12
Novitsky <i>et al</i> <sup>[13]</sup>	United States	2006	Prospective case series	50
Huguet <i>et al</i> <sup>[22]</sup>	United States	2008	Retrospective series	33
Privette <i>et al</i> <sup>[15]</sup>	United States	2008	Retrospective series	12
Wilhelm <i>et al</i> <sup>[16]</sup>	Germany	2008	Prospective case series	93
Sasaki <i>et al</i> <sup>[25]</sup>	Japan	2010	Prospective case series	45
Kang <i>et al</i> <sup>[14]</sup>	China	2013	Retrospective series	101
Ohata <i>et al</i> <sup>[29]</sup>	China	2014	Retrospective series	22
Qiu <i>et al</i> <sup>[6]</sup>	China	2013	Retrospective series	69
Dong <i>et al</i> <sup>[68]</sup>	China	2014	Retrospective cohort	18
Tsujimoto <i>et al</i> <sup>[49]</sup>	Japan	2012	Retrospective series	20
Kawahira <i>et al</i> <sup>[46]</sup>	Japan	2012	Retrospective cohort	16
Hoteya <i>et al</i> <sup>[45]</sup>	Japan	2014	Retrospective series	25
Cho <i>et al</i> <sup>[56]</sup>	Korea	2011	Prospective case series	14
Hur <i>et al</i> <sup>[57]</sup>	Korea	2014	Prospective case series	13
Mori <i>et al</i> <sup>[54]</sup>	Japan	2015	Prospective case series	16
Shiwaku <i>et al</i> <sup>[71]</sup>	Japan	2010	Prospective case series	16

<sup>1</sup>Only 20 patients had a cooperative laparoscopic endoscopic procedure.**Table 2 Reported outcome for cooperative laparoscopic endoscopic techniques with > 10 patients**

Authors	Year	Technique	Cases	Lesion	Location	Lesion size (mm)	Operative time (min)	Blood loss (mL)	LOS (d)
Choi <i>et al</i> <sup>[20]</sup>	2000	EAWR	21	SMT,	Stomach	(20-60)	(80-180)	NR	(6-7)
		LIGS	10	leiomyo-					
		Proximal gastrectomy	1	sarcoma					
Shimizu <i>et al</i> <sup>[26]</sup>	2002	EAWR	11	SMT	Stomach	NR	145 ± 43	98 ± 107	13.2 ± 3.7
Matthews <i>et al</i> <sup>[30]</sup>	2002	EAWR	15	GIST	Stomach	45 (17-82)	169 (65-300)	106 (20-200)	3.8 (2.7)
		EATR	3						
		Needlescopic LIGS (enucleations)	3						
Ludwig <i>et al</i> <sup>[27]</sup>	2002	EAWR	18	SMT,	Stomach	NR	44.3 (31-67)	NR	7.5 (3-11)
		LIGS	8	EGC			67.1 (49-102)		10.2 (6-16)
Bouillot <i>et al</i> <sup>[19]</sup>	2003	EAWR	20	SMT	Stomach	38 (15-100)	104 (40-120)	NR	6 (2-12)
Walsh <i>et al</i> <sup>[36]</sup>	2003	LIGS	13	SMT	Stomach	38 (15-70)	186	NR	3.8 (3-8)
Hindmarsh <i>et al</i> <sup>[21]</sup>	2005	EAWR	30	SMT	Stomach	46.6 (12-90)	73.8 (26-160)	196 (0-1000)	5 (1-11)
Schubert <i>et al</i> <sup>[28]</sup>	2005	EAWR	16	SMT,	Stomach	36 (16-47)	53 (35-115)	NR	NR
				EGC					
		LIGS	7	SMT,	Stomach	36 (16-47)	83 (56-130)		
				EGC					
Mochizuki <i>et al</i> <sup>[24]</sup>	2006	EAWR	12	SMT	Stomach	27 (15-48) <sup>1</sup>	100 (65-180) <sup>1</sup>	0 (0-100) <sup>1</sup>	7 (5-12) <sup>1</sup>
Novitsky <i>et al</i> <sup>[13]</sup>	2006	EAWR	30	SMT	Stomach	44 (10-85)	135 (49-295)	NR	NR
		LIGS	17						
		other	3						
Huguet <i>et al</i> <sup>[22]</sup>	2008	EAWR	11	SMT	Stomach	39 (5-10.5) <sup>1</sup>	NR	NR	3 (1-40) <sup>1</sup>
		EATR							
Privette <i>et al</i> <sup>[15]</sup>	2008	EAWR	5	SMT	Stomach	52 (25-60)	180 (122-262)	80 (50-100)	3.4 (2-5)
		Distal gastrectomy	3			55 (35-70)	322 (256-340)	167 (100-200)	8.3 (8-9)
		LIGS	4			46 (25-75)	236 (202-265)	100 (50-200)	3.3 (3-4)
Wilhelm <i>et al</i> <sup>[16]</sup>	2008	LAER	1	SMT	Stomach	5	25	NR	2 (2)
		EAWR	55			25 (3-65)	81.2 (35-202)		7.68 (4-19)
		EATR	34			26 (5-55)	114 (40-275)		7.48 (2-14)
Sasaki <i>et al</i> <sup>[25]</sup>	2010	EAWR	35	SMT	Stomach	32 (16-74)	73 (30-150)	3 (1-80)	7 (5-14)
		LIGS	3				145 (100-240)	10 (3-65)	8 (5-9)

		Single port LIGS	3						
		EATR	4						
Kang <i>et al</i> <sup>[14]</sup>	2013	EAWR	97	SMT	Stomach	(10-82)	113 ± 36	36 ± 18	4.5 ± 2.1
Ohata <i>et al</i> <sup>[29]</sup>	2014	EAWR	22	SMT, EDC	Duodenum	13.3 ± 11.6	133 ± 45	16 ± 21.1	15.1 ± 7.7
Qiu <i>et al</i> <sup>[6]</sup>	2013	LAER	5	GIST	Stomach	28 ± 16	81.6 ± 31.8	29.8 ± 15.4	4.6
		EAWR	64	GIST	Stomach		86.3 ± 28.5	31.4 ± 11.6	
Dong <i>et al</i> <sup>[68]</sup>	2014	MLIGS	8	SMT	Stomach	27.5 ± 10.7	85 ± 25.77	20 ± 10.4	7.5 ± 1.1
		EFR	10			16.5 ± 5.9	120 ± 34.72	48 ± 31.9	10.2 ± 9.1
Tsujimoto <i>et al</i> <sup>[49]</sup>	2012	LECS	20	SMT	Stomach	37.9 (18-66)	157.5 (89-316)	3.5 (0-20)	11.6 (6-13)
Kawahira <i>et al</i> <sup>[46]</sup>	2012	LECS	16	SMT	Stomach	NR	172	NR	10
Hoteya <i>et al</i> <sup>[45]</sup>	2014	LECS	25	SMT	Stomach	NR	156	NR	10.5
Cho <i>et al</i> <sup>[56]</sup>	2011	LAEFR +	14	EGC	Stomach	26 (12-90) <sup>1</sup>	143 (110-253) <sup>1</sup>	16 (5-30) <sup>1</sup>	6 (4-10) <sup>1</sup>
		Lymphadenectomy							
Hur <i>et al</i> <sup>[57]</sup>	2014	LAEFR +	9	EGC	Stomach	12 (4-32)	181 (125-240)	NR	5.9 ± 1.3 (4-8)
		Lymphadenectomy							
		LADG	4						
Mori <i>et al</i> <sup>[54]</sup>	2015	LAEFR	16	GIST	Stomach	28.3 (8-54)	271 (100-480)	NR	12.3 (10-15)
Shiwaku <i>et al</i> <sup>[71]</sup>	2010	Clean-NET	16	EGC	Stomach	NR	182.1	19.4	NR

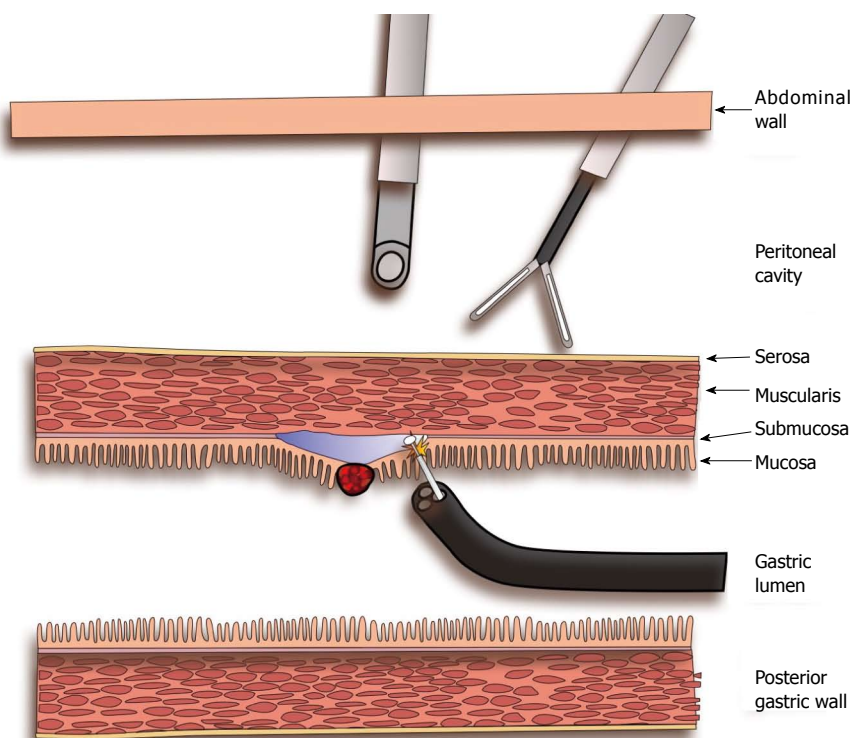
Numbers are given as means ± SD, or means (range). <sup>1</sup>Median (range). EAWR: Endoscope-assisted laparoscopic wedge resection; LIGS: Laparoscopic intragastric surgery; ELIS: Endoscope-assisted laparoscopic intragastric stapling; EATR: Endoscope-assisted laparoscopic transgastric surgery; LAER: Laparoscopy-assisted endoscopic resection; EFR: Endoscopic full thickness resection; LECS: Laparoscopic endoscopic cooperative surgery; LAEFR: Laparoscopy-assisted endoscopic full thickness resection; NEWS: Non-exposed endoscopic wall-inversion surgery; Clean-NET: Clean non exposure technique; SMT: Submucosal tumors; EGC: Early gastric cancer (T1a, T1b); EDC: Early duodenal cancer; LOS: Length of hospital stay in days; NR: Not reported.

**Table 3 Cooperative laparoscopic endoscopic techniques data comparison**

Technique name	Lesion	Location	Endoscopy team role	Surgical team role	Closure type	Specimen retrieval	No. papers <sup>2</sup>	No. cases <sup>2</sup>
LAER <sup>[6,8,9,16]</sup>	SMT	Stomach, Duodenum	Endoscopic resection	Monitoring	No closure	Endoscopic	4	10
EAWR <sup>[6,13-29]</sup>	SMT, EGC	Stomach, Duodenum	Tumor localization, exposure	Full thickness resection	Stapler/sutures	Surgical	17	523
EATR <sup>[16,18,22,25,30,31]</sup>	SMT	Stomach	Tumor localization	Mucosal resection, full thickness resection	Stapler/sutures	Surgical	6	70
LIGS <sup>[13,15,20,25,27, 28,30,32,34-38]</sup>	SMT, EGC	Stomach	Tumor localization, exposure	Mucosal resection, full thickness resection	Stapler/sutures/ endo clips	Endoscopic, surgical	13	101
ELIS <sup>[39-41]</sup>	SMT	Stomach	Tumor localization, exposure, endoscopic guidance	Stapling	Stapler/sutures	Endoscopic, surgical	3	13
single port LIGS <sup>[25,42,43]</sup>	SMT	Stomach	Tumor localization	Mucosal resection, full thickness resection	Open sutures	Surgical	3	13
LECS <sup>[44-51]</sup>	SMT, EDC	Stomach, Duodenum	Submucosal dissection	Seromuscular dissection	Stapler	Surgical	8	72
Inverted LECS <sup>[52]</sup>	EGC	Stomach	Submucosal dissection	Seromuscular dissection	Stapler	Endoscopic	1	1
LAEFR <sup>[53,55-57]</sup>	SMT, EGC <sup>1</sup>	Stomach	Full thickness resection	Full thickness resection	Sutures	Surgical, endoscopic	5	48
Clean-NET <sup>[58,71]</sup>	SMT, EGC	Stomach	Tumor localization, submucosal injection	Seromuscular dissection	Stapler	Surgical	1	16
NEWS <sup>[60,61]</sup>	GIST, EGC <sup>1</sup>	Stomach	Submucosal dissection	Seromuscular dissection	Sutures	Endoscopic	2	7

<sup>1</sup>Associated with sentinel lymph node dissection; <sup>2</sup>Including publications with < 10 patients and case reports. LAER: Laparoscopy assisted endoscopic resection; LECS: Laparoscopic endoscopic cooperative surgery; LAEFR: Laparoscopy-assisted endoscopic full thickness resection; NEWS: Non-exposed endoscopic wall-inversion surgery; EAWR: Endoscope-assisted laparoscopic wedge resection; EATR: Endoscope-assisted laparoscopic transgastric surgery; LIGS: Laparoscopic intragastric surgery; ELIS: Endoscope-assisted laparoscopic intragastric stapling; SMT: Submucosal tumors; EGC: Early gastric cancer (T1a, T1b); EDC: Early duodenal cancer; Clean-NET: Clean non exposure technique.





**Figure 1 Laparoscopy-assisted endoscopic resection.** The endoscopist is performing an endoscopic submucosal dissection while the laparoscopic team facilitates the exposure by external manipulations.

accidental perforation or difficulty in controlling blood loss<sup>[6]</sup>. It may also provide assistance by presenting the tumor to the endoscopic team with manipulations on the serosal side of the gastric wall<sup>[7]</sup>. Laparoscopy has been used for verification of the tumor location and orientation and for intraoperative ultrasound<sup>[8]</sup>.

Benign SMT and GIST in the stomach and the duodenum have been resected with these methods<sup>[6-9]</sup>. Concerning the complications, Qiu *et al.*<sup>[6]</sup> reported in a series of five patients, one late perforation and one episode of hemorrhage were both treated conservatively.

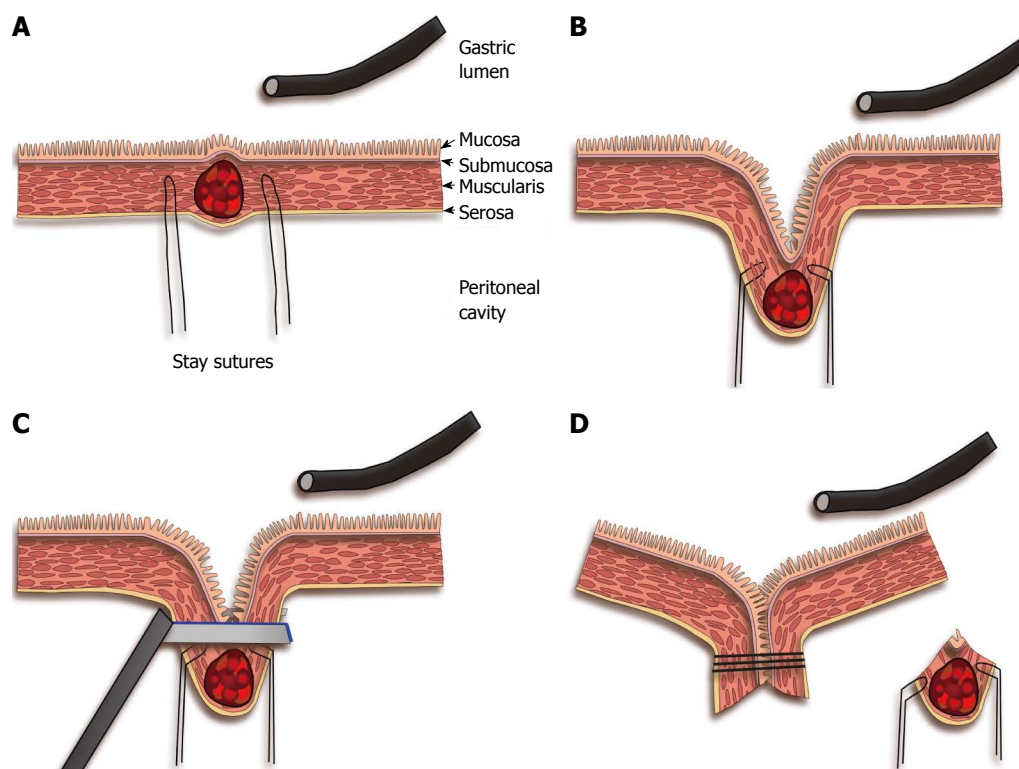
### Group 2: EALR

In this group, the tumor is resected by laparoscopy while endoscopy plays an important role in locating the tumor, assisting with exposure and monitoring the laparoscopic procedure. Minimally invasive robot-assisted resections have also been combined with intraluminal endoscopy<sup>[10-12]</sup>.

**Endoscope-assisted wedge resection:** The patient is placed in the operating theater in the standard position for laparoscopic upper gastrointestinal surgery. A 10-12 mm port for the optic is placed in the supraumbilical midline or, according to some teams, at about one third of the distance between the umbilicus and the xiphoid<sup>[13]</sup>. Two to three 5-mm ports and a 12 mm port for the endoscopic stapler are added according to the surgical team habitude. A laparoscopic bowel clamp can be placed

in the small bowel just distally to the angle of Treitz to avoid small bowel insufflation that hinders the laparoscopic exposure. Using the endoscopic vision and laparoscopic vision and palpation, the location of the SMT is confirmed and marked. Blood vessels in the excision area around the tumor are dissected and controlled in order to avoid hemorrhage. The gastric wall, including the SMT, is lifted by the laparoscopic team with seromuscular sutures placed or by traction from laparoscopic grasping forceps. The tumor, as well as a cuff of normal gastric tissue around it, is removed with a linear endoscopic gastrointestinal stapler in a wedge resection. The staple line can be reinforced with a running suture. Usually two to three stapler cartridges are used for the resection. The tumor is retrieved through an enlarged port hole, protected inside a specimen bag. At the end of the procedure, endoscopy confirms the complete resection and the absence of bleeding or leak (Figure 2).

For lesions located at the posterior gastric wall, the gastrocolic omentum is dissected and the greater curvature of the stomach mobilized laparoscopically. The greater curvature is retracted cephalad, exposing the posterior gastric wall *via* the lesser sac. Then the same technique is applied<sup>[14]</sup>. If the tumor is located near the esophagogastric junction or pyloric ring, the endoscope is placed distally into the stomach or duodenum to protect the normal gastric wall from stenosis or damage. In these cases and in the case of large tumors it is proposed to resect the tumor by using laparoscopic ultrasound shears or a vascular



**Figure 2 Endoscope-assisted laparoscopic wedge resection.** A: The tumor is located by endoscopy and laparoscopy; two laparoscopic stay sutures are placed; B: Traction by the stay sutures creates tenting of the gastric wall; C: An endoscopic stapler is applied at the normal gastric wall distally to the tumor under endoscopic control; D: Full thickness resection with everted stapling of the gastric wall.

sealing system as the application of a stapler may result in deformation of the stomach and stenosis<sup>[15]</sup>. The gastric wall defect may be closed by laparoscopic sutures or a laparoscopic stapler<sup>[16]</sup>.

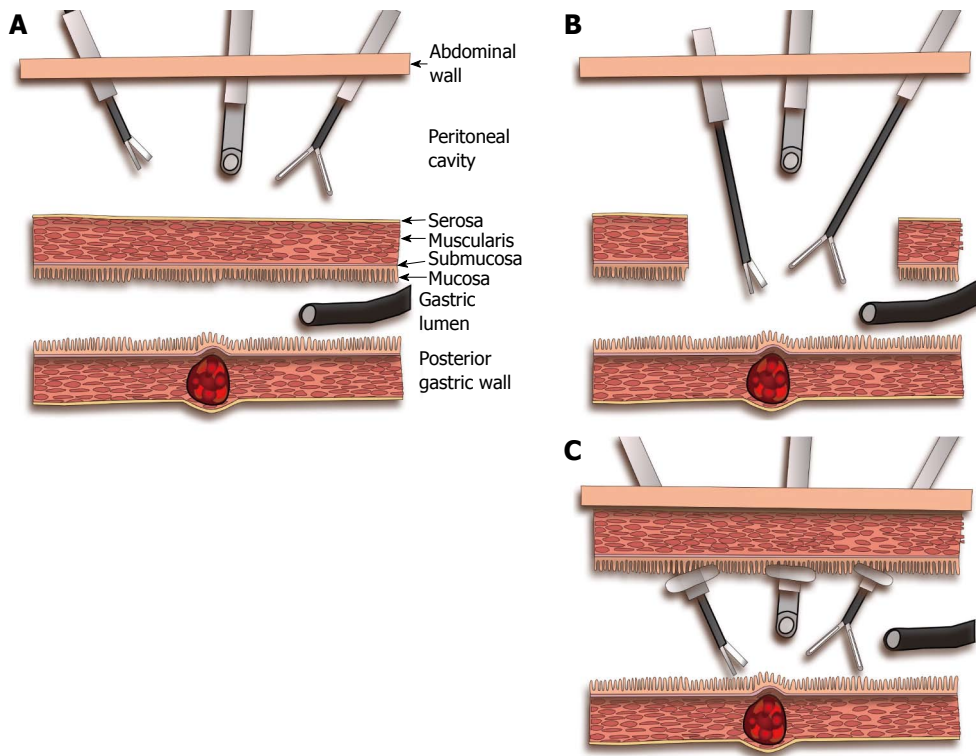
This is by far the most common combined technique with more than 500 cases published. It has been used for the resection of gastric SMT and GIST<sup>[6,13-26]</sup>. A few teams have described the resection of early gastric cancer of stages pT1aNx<sup>[27,28]</sup> as well as the resection of duodenal SMT and duodenal submucosal cancer<sup>[29]</sup>. The mean operative time is 44.3-180 min, the mean estimated blood loss is 3-196 mL and the mean hospitalization time is 3.4-15.1 d. The reported complications include: hemorrhage, hypoperistalsis, bowel injury, staple or suture line insufficiency and incomplete resection<sup>[14,15,19,20,22,24,27,29,30]</sup>; in large series, the complication rate is 0%-3%<sup>[6,14,16,19]</sup>.

**Endoscope-assisted laparoscopic transluminal (transgastric) surgery:** The same principles for the patient and the surgical team installation as the previous technique are applied. Once the lesion is located, the laparoscopic team makes a laparoscopic incision (gastrotomy) in the anterior abdominal wall overlying the lesion. The exact position of the gastrotomy is defined by palpation and transillumination by the gastroscope (Figure 3). By applying traction on the adjacent normal mucosa, the lesion is delivered through the gastrotomy into the peritoneal cavity and is resected with an inverted wedge excision with a

laparoscopic stapler<sup>[20]</sup>. Alternatively, the laparoscopic team can perform a submucosal or full thickness excision of the lesion<sup>[25]</sup>. The gastric wall opening is closed with an endoscopic stapler or with sutures<sup>[18]</sup>.

This technique is used predominantly to resect lesions of the posterior gastric wall<sup>[16,18,22,25,30,31]</sup> and posterior lesions of the duodenum<sup>[10]</sup>. The reported mean operative time is 114-145 min, the mean blood loss 3-8 mL, and the mean hospital stay 3-8 d<sup>[16,22,25]</sup>. There are rare incidents of hemorrhage, leak and surgical wound infection<sup>[22,25]</sup>.

**Endoscope-assisted laparoscopic intraluminal (intra-gastric) surgery:** In this approach, first described by Ohashi in 1995, the laparoscopic ports are passed through the abdominal wall and then through the gastric wall, inside the gastric cavity<sup>[32]</sup>. The procedure starts with a laparoscopic exploration of the peritoneal cavity and, if needed, mobilization of the gastric ligaments. Once the tumor location is pinpointed by endoscopy, the laparoscopic team punctures the gastric wall and inserts an optical port and two working ports inside the stomach (Figure 3). The ports rest in their place by means of an inflatable balloon at their tip; some authors prefer using standard laparoscopic ports and keep them in place by suspending the stomach at the anterior abdominal wall with sutures<sup>[33]</sup>. The lesion can be removed by laparoscopic mucosal resection, full thickness resection or laparoscopic stapling. The endoscopist can facilitate



**Figure 3 Endoscope-assisted laparoscopic intragastric and transgastric resection technique.** A: Localization of the tumor at the posterior gastric wall by endoscopy and the position of the anterior gastric wall overlying the tumor is shown to the laparoscopic team by transillumination; B: Transgastric resection: the anterior gastric wall is sectioned (gastrotomy) to give laparoscopic access to the gastric lumen and the tumor; C: Intragastric resection: the laparoscopic ports are inserted through the abdominal wall and through the gastric wall inside the gastric lumen; the gastric wall is held in contact with the abdominal wall by means of the balloon-tipped laparoscopic ports.

the procedure by applying traction with a snare and in the end retrieve the resected lesion through the mouth. Large lesions can be retrieved by the surgical team through an enlarged gastrotomy. In the end, the gastric wall perforations are sutured and the stomach is inflated to verify the closure.

A modified laparoscopic intraluminal (intragastric) surgery (LIGS) procedure has been described by Dong *et al.*<sup>[33]</sup>. The stomach is suspended from the anterior abdominal wall and under the combined laparoscopic and endoscopic control, two 5 mm laparoscopic ports are inserted into the gastric cavity. Inside the stomach the surgeon performs a partial thickness resection of the tumor down to the muscular layer under vision from the gastroscope. The specimen is retrieved through the mouth by the endoscopist. The gastric wall defect and the gastric port holes are closed with endoscopic titanium clips.

LIGS has been used in more than 100 cases for the removal of benign SMT and GIST<sup>[13,15,20,25,27,28,30,32,34-38]</sup>, as well as early gastric cancer<sup>[28,32]</sup>. The mean operative time is 74.6-236 min, the mean blood loss is 10-100 mL and the mean hospital stay is 3.3-10.2 d; reported complications include hemorrhage, posterior wall perforation and leak<sup>[13,15,20,25,27,28,30,32,35-38]</sup>.

**Endoscope-assisted laparoscopic intragastric stapling:** Only one 12 mm laparoscopic port is

inserted into the stomach. The endoscopist visualizes and exposes the tumor. A laparoscopic stapler is used to resect the lesion with an inverted wedge resection (Figure 3). The gastric port hole is stapled shut. This procedure is used mainly for endophytic benign lesions and has an operative time ranging 85-105 min<sup>[39-41]</sup>.

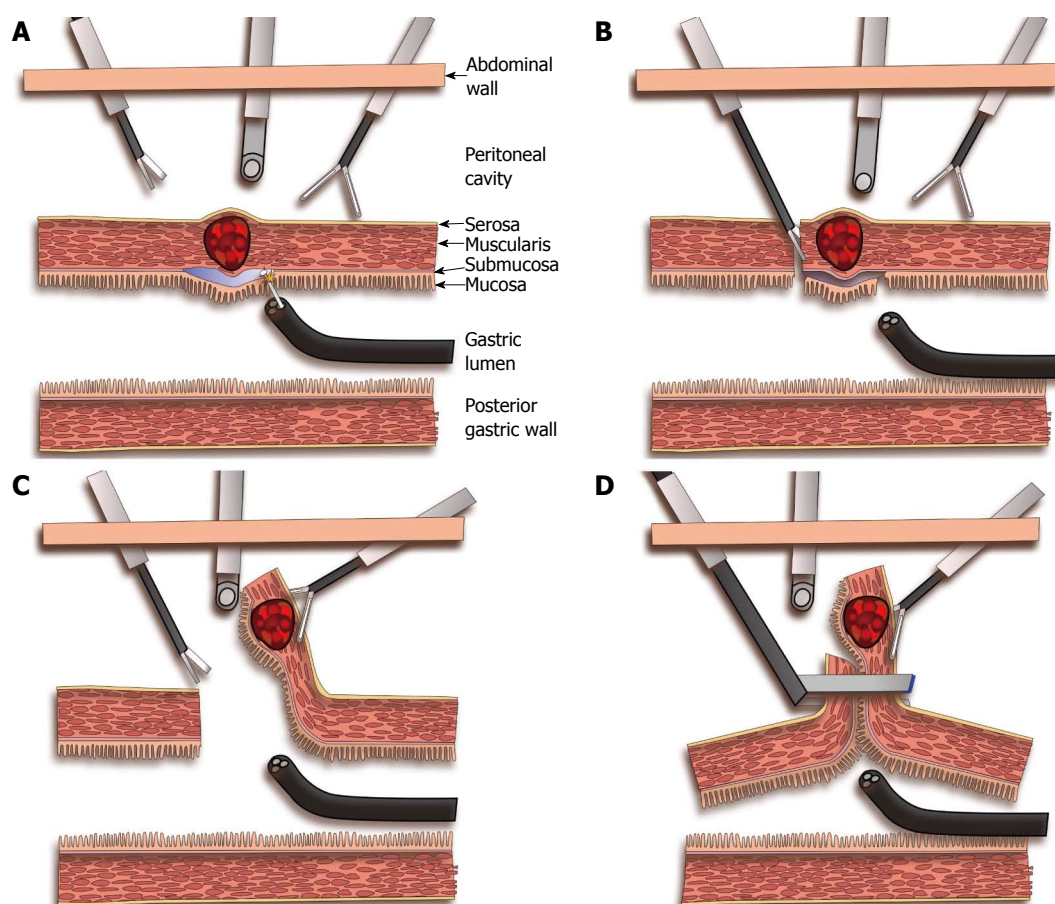
**Single Port LIGS:** A 3 cm umbilical mini laparotomy is made, and through it the stomach is grasped and a 2 cm gastrotomy is performed. It is possible to make the mini laparotomy higher and to the left of the midline in order to get a better access to the stomach. A single port laparoscopic device is inserted through the abdominal wall into the stomach; the device's shape maintains the gastric wall in contact with the abdominal wall. The single port allows the introduction of a laparoscope, laparoscopic instruments, and stapler (Figure 3). In the end of the procedure, the gastrotomy can be closed through the mini laparotomy by open suturing or by stapling<sup>[25,42,43]</sup>.

This technique is used for posterior gastric wall benign lesions. It has a mean operative time of 74.6-145 min, a mean blood loss of 10 mL and a mean hospitalization of 5-8 d. Leak from the gastric wall closure has been reported in a single case<sup>[25,42,43]</sup>.

**Group 3: combined laparoscopic-endoscopic resection**

This group is comprised of operative techniques





**Figure 4 Laparoscopic endoscopic combined surgery.** A: Submucosal injection and section of the mucosa around the tumor by the endoscopic team; B: After perforation of the gastric wall by the endoscopic team, the laparoscopic team makes a full thickness incision following the path of the endoscopic resection; C: Eversion of the tumor towards the peritoneal cavity and laparoscopic resection around the 2/3 of the gastric wall around it; D: Resection of the tumor and closure of the gastric wall by application of a laparoscopic stapler.

where the endoscopic and the laparoscopic team are cooperating for the joint dissection of the tumor.

**Laparoscopic endoscopic cooperative surgery:** In this procedure, described by Hiki *et al*<sup>[44]</sup> in 2008, the lesion is located and partially dissected by ESD. The resection is completed by laparoscopy.

The laparoscopic team is installed and the operative ports are placed in the standard position for upper gastrointestinal procedures. Then the endoscopic team marks by coagulation the periphery of the tumor keeping a safety margin. After submucosal injection, three-fourths of the marked area around the tumor are cut down to the submucosal layer using an insulated tip electrosurgical knife (IT Knife, Olympus, Japan). The presence of an insulated ceramic ball at the tip of this needle-knife is believed to reduce the chance of accidental muscle layer dissection. Following that, the endoscopist makes a perforation of the gastric wall at the dissection line, with the tip of a standard needle knife. Next, the surgical team intervenes laparoscopically. The tip of an ultrasonic shears device or alternatively of a vessel sealing system is inserted into the perforation and three-

fourths of the circumference around the tumor are dissected following the endoscopic dissection path. Subsequently, the tumor is turned over, towards the peritoneal cavity. The serosa of the non-resected part of the tumor is grasped and retracted, exposing the extremity of the incision. Finally, the incision line is closed using a laparoscopic stapler (Figure 4). The specimen is retrieved and protected in a plastic bag, through a laparoscopic port hole. For the tumors located near the esogastric junction or pyloric ring, the gastric wall defect is closed with laparoscopic sutures, since any application of a stapling device in these areas may cause deformity of the stomach or stenosis.

Laparoscopic endoscopic cooperative surgery (LECS) has been used in 70 patients for the resection of benign SMT of the stomach and the duodenum<sup>[44-50]</sup> and in one case for the resection of an early duodenal cancer<sup>[51]</sup>. The mean operative time of this procedure is 120-182 min, the mean blood loss 7.4-11.6 mL and the patient is hospitalized for a mean 5-11.6 d. Interestingly, there are no complications reported from the highly specialized team performing this procedure<sup>[44-51]</sup>.

**Inverted LECS:** In order to prevent spillage of gastric



content and consequently reduce the risk of tumor cell dissemination in the peritoneal cavity, a variation of the classic LECS procedure was described by Nunobe *et al*<sup>[52]</sup> in 2012 in a single case of early gastric cancer resection. The Inverted LECS procedure makes use of suspension sutures passed through the abdominal wall to lift the gastric wall around the tumor like a crown; this step reduces the risk of gastric content spillage. In addition, to prevent contact between the tumor and the peritoneal cavity, the tumor is inverted to face the intragastric cavity. The resection is terminated by application of a laparoscopic stapler that resects the tumor and closes the gastric wall defect and the specimen is removed trans-orally. Thus, the tumor is always kept inside the stomach and is never in direct contact with the perigastric viscera.

**Laparoscopy-assisted endoscopic full-thickness resection:** Laparoscopy-assisted endoscopic full-thickness resection (LAEFR) was described by Abe *et al*<sup>[53]</sup>. It is based on the same principles of LECS but instead of a submucosal dissection, the endoscopic team performs a full thickness resection around the tumor. The laparoscopic team facilitates the exposure by traction from the serosal side of the gastric wall. After the 2/3 of the resection is completed, the exposure for the endoscopic team is hindered by the loss of CO<sub>2</sub> towards the peritoneal cavity. Then the laparoscopic team completes the full thickness resection with ultrasonic scissors or a vessel sealing device. The specimen is retrieved either per-orally or protected in a plastic bag through a port site and the gastric wall defect is hand-sewn by the laparoscopic team.

This technique has been used in 20 patients with GIST<sup>[53,54]</sup> and other benign SMT and in 24 patients with early gastric cancer; in the case of early gastric cancer a sentinel node selective lymph node dissection was performed by laparoscopy<sup>[55-57]</sup>. This difficult procedure has a mean operative time of 181-389 min, minimal blood loss and a mean hospitalization of 5.9-12.3 d. The expert teams performing LAEFR report no complications in their series<sup>[54-57]</sup>.

**Clean non exposure technique:** Clean non exposure technique (Clean-NET) is a combined laparoscopic and endoscopic "non exposure" full-thickness gastric wall resection described by Inoue *et al*<sup>[58]</sup> in 2012. First the boundary of the lesion is marked by coagulation endoscopically with a 10 mm safety circumferential margin. The surgical team places 4 full thickness stay sutures around the lesion in order to fix the mucosa to the other layers. The endoscopic team injects the submucosal layer with a solution in a circle outside of the stay sutures. Then, the surgical team dissects the seromuscular layer around the tumor and outside of the stay sutures, down to the submucosal layer, using a laparoscopic electrocautery knife. Once the dissection is finished, traction is applied to the stay sutures and

the specimen with the surrounding mucosa is pulled out of the stomach, sealed inside a protective mucosal "net". A laparoscopic linear stapler is used to close the wall defect and resect the mucosal "net" containing the specimen. In the end, the specimen is put into a laparoscopic bag and is withdrawn from a port hole<sup>[58]</sup>.

#### **Non-exposed endoscopic wall-inversion surgery:**

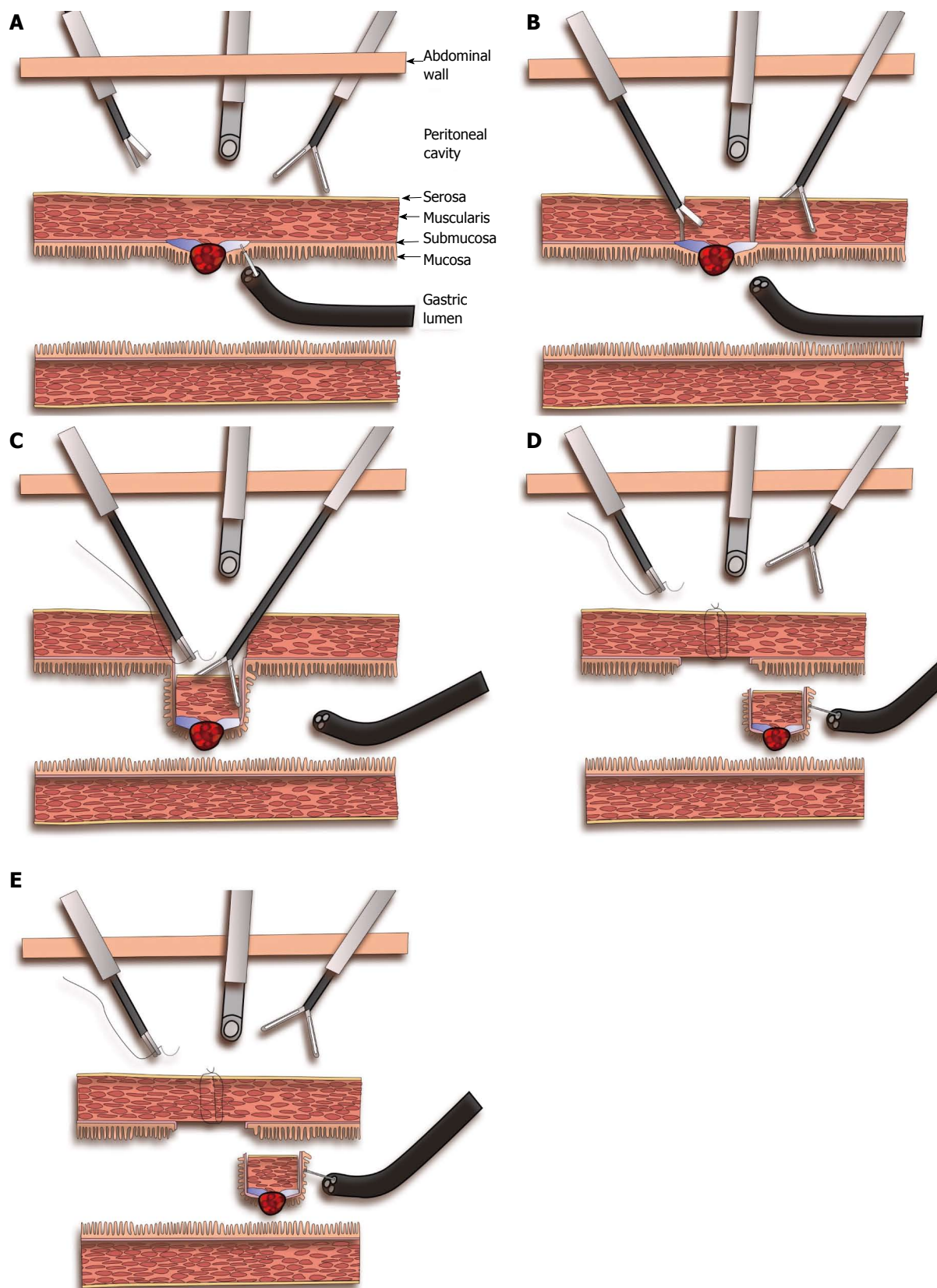
Non-exposed endoscopic wall-inversion surgery (NEWS) was developed as a new full-thickness resection technique without intentional perforation mainly aimed at being a minimally-invasive procedure for early gastric cancer.

The NEWS procedure starts by placing marks with the coagulation around the tumor; on the mucosal surface by the endoscopic team and on the serosal surface by the laparoscopic team. A submucosal injection of sodium hyaluronate with an Indigo Carmine dye solution is performed by the endoscopist; this helps dissect the submucosal plane around the tumor. In the case of early gastric cancer, Indocyanine Green is used in order to stain the regional sentinel lymph nodes. The whole lymph node group containing the sentinel node is dissected and sent for frozen section; the procedure continues once the lymph nodes are confirmed free of cancer. Then the laparoscopic team proceeds with a circumferential seromuscular dissection down to the colored submucosal layer, around the tumor. Once the dissection is terminated, the tumor is inverted and the seromuscular layer is closed with a laparoscopic running suture; a surgical spacer (Securea®; Hogen Medical Co., Ltd., Tokyo, Japan), cut to fit the resection site, can be placed between the tumor and the suture line to facilitate the procedure. Finally the resection is completed by a circumferential muco-submucosal incision under endoscopy and the spacer is dug out. The resected specimen is retrieved through the mouth, and the mucosal defect at the resection site is closed with endoscopic clips (Figure 5).

The technique was initially described in a porcine model by Goto *et al*<sup>[59]</sup> in 2011 and was applied to 6 patients with gastric GIST<sup>[60]</sup>. It has been used in combination with a selective lymph node dissection to resect an early gastric cancer<sup>[61]</sup>. The mean operative time is reported at 270 min and the mean blood loss is 10-113 mL. In two patients, the mucosal barrier was unintentionally breached during the dissection but no other adverse events have been reported<sup>[60,61]</sup>.

## **DISCUSSION**

A large armamentarium of cooperative laparoscopic and endoscopic procedures are available for the multidisciplinary teams that wish to make use of them. What are, then, the indications for these cooperative procedures? The three largest published series totaling 256 patients involved primarily the resection of GISTs (82%) and secondly the resection of various benign SMT<sup>[6,14,16]</sup>. According to the 2010 National



**Figure 5 Non-exposed endoscopic wall-inversion surgery.** A: Endoscopic submucosal injection around the tumor; B: Laparoscopic seromuscular dissection around the tumor down to the stained submucosal plane; C: Retraction of the tumor with its overlying normal gastric wall towards the gastric lumen, and closure of the gastric wall by laparoscopic seromuscular extramucosal suturing; D: The gastric wall opening is completely closed and the tumor is invaginated inside the gastric lumen; endoscopic mucosal resection around the tumor with a needle knife. E: Endoscopic extraction of the tumor, and the gastric mucosa is approximated with clips.

Comprehensive Cancer Network (NCCN) GIST and the 2015 NCCN sarcoma guidelines, GISTs smaller than 5 cm may be treated by a laparoscopic resection by surgeons who have the appropriate experience; provided that a margin free resection is done, the GIST capsule is preserved and tumor spillage is avoided. It is recommended that the specimens should be removed from the abdomen in a plastic bag<sup>[62]</sup>. Consequently, patients with gastric GISTs smaller than 5 cm irrespectively of their localization are good candidates for these cooperative techniques. The LECS procedure is on the national insurance list in Japan for benign SMT and small GIST since February 2014; it is currently widely being carried out and its use is rapidly increasing in Japanese Institutions<sup>[63]</sup>.

In our data there were also reports and small case-series of cooperative resections for early gastric cancer for a total of 46 patients<sup>[27,28,32,52,55-57,61]</sup>. Early gastric cancers (T1a) are amenable to endoscopic resection if they are well-differentiated,  $\leq 2$  cm, confined to the mucosa and not ulcerated; the associated lymph node metastatic risk is virtually zero for this group<sup>[64]</sup>. The Japanese National Cancer Centre has expanded the inclusion criteria for ESD to tumors with intestinal-type histology, no evidence of lymphovascular involvement and to intramucosal cancers without ulceration regardless of tumour size; intra-mucosal cancers  $< 3$  cm with ulceration or cancers with early invasion into the submucosa (sm1) measuring  $< 3$  cm<sup>[65]</sup>. A large retrospective study of 1485 patients comparing patients who had received a curative resection by means of ESD following the expanded criteria to those following the classic inclusion criteria did not find any difference in long term survival and outcomes<sup>[66]</sup>. Even though the ESD achieves excellent en bloc resection rates in early gastric cancer it also presents a high possibility of bleeding, perforation, and long procedure times<sup>[67]</sup>. Various "non exposure" combined laparoscopic and endoscopic techniques have been developed for the treatment of early gastric cancer with the purpose of diminishing the risk of tumor cell seeding in the peritoneal cavity: inverted LECS, LAEFR, Clean-NET and NEWS. They may be considered for early gastric cancer lesions difficult to treat with ESD, including large intramucosal lesions located at the greater curvature of the gastric body and fornix, or for lesions with a strong ulcer scar<sup>[52]</sup>. Clinical experience remains limited to case reports and case series from expert Asiatic centers, nevertheless, preliminary results are promising.

Cooperative laparoscopic endoscopic techniques present many theoretical advantages. Is there any clinical benefit to be gained by their application? In the series of endoscopy assisted laparoscopic resections from Wilhelm *et al.*<sup>[16]</sup>, tumor identification and localization solely by visual exploration and palpation of the abdominal cavity during initial laparoscopy was successful in 21 out of 93 patients (22.6%). In addition, the tumor localization was exact enough to

permit a secure resection in only three patients. This was due to the inability to define whether a lesion was located in the anterior or posterior gastric wall. With the endoscopic endoluminal view, 92 tumors (98.9%) were exactly located<sup>[16]</sup>. This fivefold increase in the tumor detection is clinically and statistically significant ( $P < 0.001$ ,  $\chi^2 = 93.8$ ) and achieves a detection rate similar to that of open surgery. Laparoscopic SMT wedge resection is comparable to the open surgical resection in respect to the duration of surgery and the complication rate, but has a significantly less intraoperative blood loss, faster first flatus passage and earlier resumption of oral intake<sup>[24]</sup> as well as an earlier mobilization<sup>[26]</sup> and a shorter hospital stay<sup>[30]</sup>.

A small case-control study by Dong *et al.*<sup>[68]</sup> compared eight patients operated with a modified laparoscopic intragastric surgery (MLIGS) technique for GISTs to ten patients operated with an endoscopic full-thickness resection (EFR). The MLIGS technique was found to have a significantly shorter operative time (mean time, 85 vs 120 min,  $t = 2.371$ ,  $P < 0.05$ ) and blood loss (mean blood loss, 20 mL vs 48 mL,  $t = 2.372$ ,  $P < 0.05$ ). Also the patients operated by MLIGS had a tendency for bigger tumor size, shorter hospital stay and shorter abdominal pain duration in comparison to those treated by EFR<sup>[68]</sup>. Therefore there is some evidence that a laparoscopic endoscopic cooperative approach has certain benefits over open surgery and other endoscopic techniques as well.

Some studies have tried to propose recommendations for the choice of the ideal cooperative technique according to the tumor characteristics. Tagaya *et al.*<sup>[69]</sup> suggest that the endoscopy-assisted laparoscopic wedge resection (EAWR) is better suited for tumors located on the anterior wall and for exophytic tumors, while the Endoscopy-assisted laparoscopic transgastric resection (EATR) should be used for tumors  $< 4$  cm located on the posterior wall. EAWR can resect easily lesions of the gastric fundus and of the greater curvature, while LIGS is more adapted for tumors of the lesser curvature and the gastroesophageal junction<sup>[15]</sup>. A ROC curve analysis and multivariate logistic regression of 57 patients with SMT treated by EAWR and EATR, proposed a decision making algorithm based on the tumor size, location and growth pattern. An EATR seems to be the ideal choice for endophytic SMT  $\leq 2$  cm, situated high or low on the posterior gastric wall or lesser curvature, whereas an EAWR can be proposed for all other cases<sup>[70]</sup>. The size of the SMT plays an important role in the choice of operative technique. For a wedge resection with a linear stapler, theoretically the radius of the resected gastric wall must be more than  $2 \pi \times$  the radius of the tumor. This translates to a gastric wall defect of more than three times the size of the tumor and for large tumors this may be the cause for deformation of the stomach and stenosis. Tsujimoto *et al.*<sup>[49]</sup> showed that a LECS requires resection of gastric wall less than 1.5 times the tumor size, reducing this

**Table 4 Advantages and disadvantages of the various cooperative laparoscopic endoscopic techniques**

Technique name	Pros	Cons
LAER	Minimally invasive approach Monitoring and backup from the laparoscopic team in case of accidental perforation	Suitable for small lesions with intraluminal expansion Requires advanced endoscopy skills
EAWR	No requirement of advanced laparoscopic or endoscopic skills Good entry level for teams starting cooperative techniques	Leaves larger wall defects compared to other methods Risk of gastric deformation or stenosis from stapling Requires gastrotomy closure
EATR	Favorable access to lesions $\leq 2$ cm, situated high on the posterior wall or lesser curvature without mobilizing the stomach	May lead to spillage with peritoneal contamination and dissemination
LIGS	Similar to EATR	Risk of gastric deformation or stenosis from stapling
ELIS	Same as EATR	Same as LIGS
Single port LIGS	Less invasive than the classic LIGS The gastrotomy can be closed through the single port incision	Difficulty in orienting the stapler under endoscopic view Requires previous experience in single port laparoscopy More difficult than EATR and LIGS
LECS	Combines the advantages of both endoscopy and laparoscopy. No restriction in the size or location of the tumor	Requires advanced endoscopy and laparoscopy skills. More adapted in high volume centers Risk of spillage and contamination Not adapted for early gastric cancer
Inverted LECS	Diminishes the risk of peritoneal cancer dissemination	Not adapted for early gastric cancer
LAEFR	Minimal invasive endoscopic resection	Requires advanced endoscopy skills in dissection techniques and closure of wide wall defects with macro-clips or suturing devices
Clean-NET	The procedure is facilitated by the laparoscopic view and exposure Diminishes the risk of peritoneal dissemination of gastric cancer	Limited literature Requires special training Risk of mucosal tear with cancer cell dissemination
NEWS	Diminishes the risk of peritoneal dissemination of early gastric cancer	Limited literature. Requires special training

LAER: Laparoscopy assisted endoscopic resection; LECS: Laparoscopic endoscopic cooperative surgery; LAEFR: Laparoscopy-assisted endoscopic full thickness resection; NEWS: Non-exposed endoscopic wall-inversion surgery; EAWR: Endoscope-assisted laparoscopic wedge resection; EATR: Endoscope-assisted laparoscopic transgastric surgery; LIGS: Laparoscopic intragastric surgery; ELIS: Endoscope-assisted laparoscopic intragastric stapling; Clean-NET: Clean non exposure technique.

risk. In addition, the endoscopic incision that traces the margin of resection for LECS is believed to provide a more accurate incision line in comparison to the extraluminal approaches<sup>[63]</sup>.

The choice of which cooperative technique to use extensively depends on the team's expertise (Table 4). For example Endoscope assisted laparoscopic resections do not require endoscopic dissection skills since the role of the endoscopist is to locate, inspect and guide the laparoscopic resection; these procedures can be reproduced by any team with expertise in laparoscopic surgery. This approach is attractive for starting endoscopic/laparoscopic teams and could be the first step towards more advanced combined procedures. Interestingly the combined procedures like LECS offer a significant advantage: the technical difficulty is weighed-down and shared between the endoscopic and the laparoscopic team. Additionally, any adverse events encountered by the endoscopic team may be easily controlled by the laparoscopic team and vice versa. The future may prove that the cooperative techniques will be used by all endoscopic and laparoscopic teams aiming for excellence.

surgical resection or lymph node dissection is required. These lesions are excellent candidates for mini-invasive procedures, either surgical or endoscopic. Each technique has its advantages and shortcomings and several groups have combined the two approaches into what is called cooperative laparoscopic endoscopic surgery, or hybrid laparoscopic surgery. The primary aim of this systematic review is to present in a comprehensive way the different combined laparoscopic and endoscopic techniques that have been described. In addition, the authors aim to delineate the indications for each technique with a special interest in describing its advantages and disadvantages.

### Research frontiers

Endoscopy was found to be an invaluable tool for the localization of small gastric or duodenal lesions that were hard to identify during laparoscopic resection. With the evolution of interventional endoscopy, the role of the endoscopist during these cooperative procedures became progressively more active.

### Innovations and breakthroughs

Hiki *et al* described first in 2008 an innovative procedure called laparoscopic endoscopic cooperative surgery (LECS) that combined the strongest points of interventional endoscopy and laparoscopic surgery for the removal of gastric wall lesions. Following that, a series of other innovative techniques such as inverted LECS, laparoscopic endoscopic full thickness resection, clean non exposure technique and non-exposed wall-inversion surgery have emerged. When performed by expert teams they show a lot of promise and achieve solid oncologic results.

### Applications

The combined laparoscopic endoscopic techniques are mainly used to resect benign gastric wall lesions that are not resectable by endoscopic submucosal dissection. They have been extensively used for the resection of small GIST. Interestingly, highly expert teams from Asiatic countries are reporting good results in the application of these techniques for the treatment of early gastric cancer.

## COMMENTS

### Background

Benign upper gastrointestinal tumors can be treated with local excision. Small gastrointestinal stromal tumors (GIST) can also be treated likewise as long as the resection margins are free in the pathological examination; no extensive



## Terminology

The numerous procedures that have been described can be classified into three groups. The endoscopic assisted laparoscopic resection group is making use of endoscopic guidance while the laparoscopic team performs a wedge resection. In the laparoscopic assisted endoscopic resection group, the laparoscopic team has a backup role while the endoscopic team performs the resection. Finally the combined laparoscopic endoscopic group assembles the procedures that make use of the operative skills of both teams in order to achieve an optimal result.

## Peer-review

This review investigated the cooperative laparoscopic and endoscopic techniques used for the resection of upper gastrointestinal tumors. This review was clearly written, and was reported all techniques used for this type of tumour.

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## Intraductal papillary neoplasm of the bile ducts: A case report and literature review

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### Abstract

Intraductal papillary neoplasm of the bile duct (IPNB) is a rare bile duct neoplasm mostly found in far eastern nations where hepatolithiasis and clonorchiasis infections are endemic. In western countries, it is very rare and the etiology is unknown. In this article, we report the first IPNB patient we encountered in our clinic and a literature review. The patient is a 38-year-old female with a history of choledocholithiasis who presented with obstructive jaundice. She was found to have a papillary mass at the junction of the right hepatic duct and common hepatic duct with six masses in the liver parenchyma. The immunophenotypic and histologic features of the tumor are consistent with IPNB, gastric subtype. The patient had a partial hepatectomy and has been receiving palliative chemotherapy. In a search of PubMed database, we collected 354 IPNB patients reported in 22 articles. In these patients, 52.8% were from Japan and 27.7% were from western countries including the United States (11.0%). The age of the patients ranged from 35 to 80 years old with an average of 64.6. Male/female ratio was 1.5. Macroscopically, 57.5% of the tumors were in the left lobe and 29.5% were in the right lobe. The average size of the tumor were 4.2 cm at the time of diagnosis. Histologically, pancreato-biliary subtype accounted for 41.8%, intestinal 28.0%, gastric 13.5% and oncocytic 16%. An invasive component is most often present in the pancreato-biliary and gastric subtypes. Despite recent advanced technologies, diagnosis of IPNB is still challenging, especially in western countries due to its rarity. Defined clinico-pathologic features are in demand for the accurate diagnosis and proper treatment.

**Key words:** Intraductal papillary neoplasm; Mucinous cystic neoplasm; Bile duct carcinoma; Bile duct tumor

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**Core tip:** Intraductal papillary neoplasm of the bile duct (IPNB) is very rare in the United States. In this article, we reported the first IPNB patient we encountered. The patient is a 38-year-old female who is one of the youngest patients that have been reported. She was found to have intraductal papillary masses in the common hepatic duct and liver parenchyma. The diagnosis was IPNB, gastric subtype. In a search of PubMed database, we collected 354 IPNB patients from 22 articles and summarized the clinico-pathologic features including geographic distribution, age, gender, symptoms, location, microscopic subtypes, differential diagnosis, pathogenesis and therapeutic options.

Tan Y, Milikowski C, Toribio Y, Singer A, Rojas CP, Garcia-Buitrago MT. Intraductal papillary neoplasm of the bile ducts: A case report and literature review. *World J Gastroenterol* 2015; 21(43): 12498-12504 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12498.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12498>

## INTRODUCTION

Intraductal papillary neoplasm of the bile duct (IPNB) is a bile duct neoplasm characterized by a predominantly papillary growth pattern in dilated bile ducts. An invasive component is present in approximately 40%-80% of reported cases. IPNB is a rare tumor, mostly found in far eastern nations like Taiwan, Japan, and North and South Korea where hepatolithiasis and clonorchiasis are endemic<sup>[1-5]</sup>. In western countries, it is sporadic and the etiology is unknown. As a precursor lesion of cholangiocarcinoma, IPNB was recently included in the World Health Organization (WHO) classification of the bile duct tumors to include the previous categories of biliary papilloma and papillomatosis. IPNBs are considered the biliary counterpart of pancreatic intraductal papillary mucinous neoplasms (IPMNs). These two types of neoplasms show a mucin-secreting columnar epithelium with varying degrees of atypia and are similarly classified into four subtypes based on the histomorphology and immunophenotypic profile: pancreato-biliary, intestinal, gastric, and oncocytic subtypes (Table 1)<sup>[3,6]</sup>. However, pancreato-biliary is the most common one for IPNBs, while gastric subtype is the most common one for IPMNs. IPMNs have better prognosis with a lower frequency of an invasive component.

As a new entity, the characteristics of IPNB, including the clinico-pathologic features, the prognostic factors and oncogenic pathways are still ill-defined, especially of those of the sporadic neoplasms seen in western countries. Its identification can represent a diagnostic challenge. In this case report, we describe the cytologic and histopathologic features of IPNB

**Table 1** Histologic subtype of intraductal papillary neoplasm of the bile ducts by mucin core protein

	MUC1	MUC2	MUC5AC
Pancreato-biliary	+	-	+
Intestinal	-	+	+
Gastric	-	-	+
Oncocytic	+(focal)	+(focal)	+

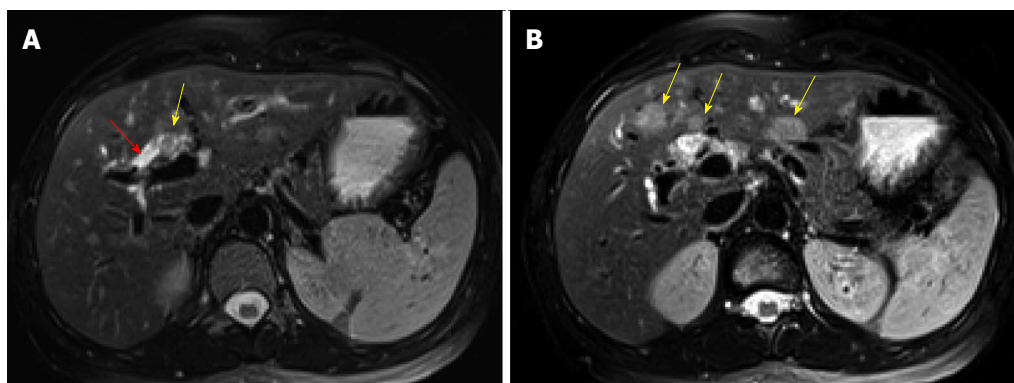
MUC: Mucin.

with invasive adenocarcinoma in a very young female patient.

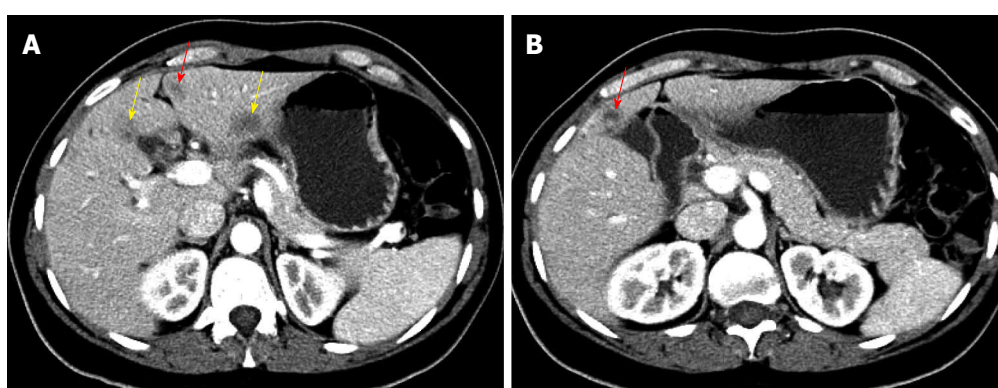
## CASE REPORT

A 38-year-old female presented to an outside hospital in December 2012 due to one-week of acholic stools, jaundice, pruritus and dark colored urine. Her past medical history was significant for choledocholithiasis. Blood workup was consistent with obstructive jaundice. She underwent endoscopic retrograde cholangiopancreatogram (ERCP), percutaneous biliary drainage and stone extraction. Subsequently, the patient's symptoms resolved. Three months later, the patient developed recurrent symptoms and presented to the same hospital. Another ERCP was performed and a biliary stent was placed. While placing the stent, a mucoid mass in the common hepatic duct was identified and biopsied. It was diagnosed as adenocarcinoma with papillary architecture. The patient was referred to our institution for further management.

In April 2013, the patient presented to our institution. Her laboratory tests demonstrated a total bilirubin 2.0 mg/dL (reference range: 0.2-1.3 mg/dL), direct bilirubin 1.5 mg/dL (reference range: 0-0.4 mg/dL), alkaline phosphatase 195 UI/L (reference range: 38-126 UI/L), AST 83 UI/L (reference range: 15-46 UI/L), ALT 187 UI/L (reference range: 9-52 UI/L), and CA19-9 37.2 UI/mL (reference range: 0-35 UI/mL). Serology was negative for hepatitis A, B and C infection. Magnetic resonance imaging revealed an ill-defined soft tissue fullness at the bifurcation of the common hepatic duct causing biliary occlusion. The left and right hepatic ducts were also involved. Additionally, there were three ill-defined but suspicious intrahepatic masses in segments IVB and III that measured up to 1.8 cm. They were considered as metastases (Figure 1). Based on the radiologic findings, the patient was considered as a poor candidate for resection; therefore, chemotherapy was initiated using Cisplatin 25 mg/m<sup>2</sup> IV at day #1 with Gemcitabine 1000 mg/m<sup>2</sup> IV at day #1 and day #8 for total of 4 to 6 cycles. After initiation of the treatment, the patient showed signs of improvement including decreased CA19-9 level and stable radiologic findings. During the sixth to seventh cycle of the chemotherapy in October 2013, the patient complained of mild right upper quadrant discomfort. At this time, laboratory



**Figure 1 Non-enhanced magnetic resonance imaging.** A: Axial T2 with fat saturation (96/3000) demonstrates dilatation of the proximal right hepatic duct (red arrow) with soft tissue noted within the lumen (yellow arrow); B: Axial T2 with fat saturation (96/3000) demonstrates three ill defined, rounded hyperintense lesions in segments III and IVB (arrows).



**Figure 2 Computed tomography of abdomen.** A: Axial contrast enhanced (portal phase, liver window, 200/50) demonstrates three hypodense lesions. The previously seen segment III and IVB lesions have increased in size (yellow arrows) and the one medial to the falciform ligament is now apparent (red arrow); B: Axial contrast enhanced (portal phase, liver window, 200/50) demonstrates a new liver lesion (arrow).

tests showed a total bilirubin 0.6 mg/dL, alkaline phosphatase 86 UI/L, AST 40 UI/L, ALT 39 UI/L, and CA19-9 161.3 UI/mL. Contrast enhanced computed tomography (CT) demonstrated interval increased size of the previously identified intrahepatic lesions and an additional new liver lesion (Figure 2). A decision of salvage surgery was made after positron emission tomography (PET)/CT confirmed that the tumor was confined to the liver and bile ducts. In December 2013, the patient underwent a left hepatic lobectomy with cholecystectomy and hepaticojejunostomy.

### Pathology findings

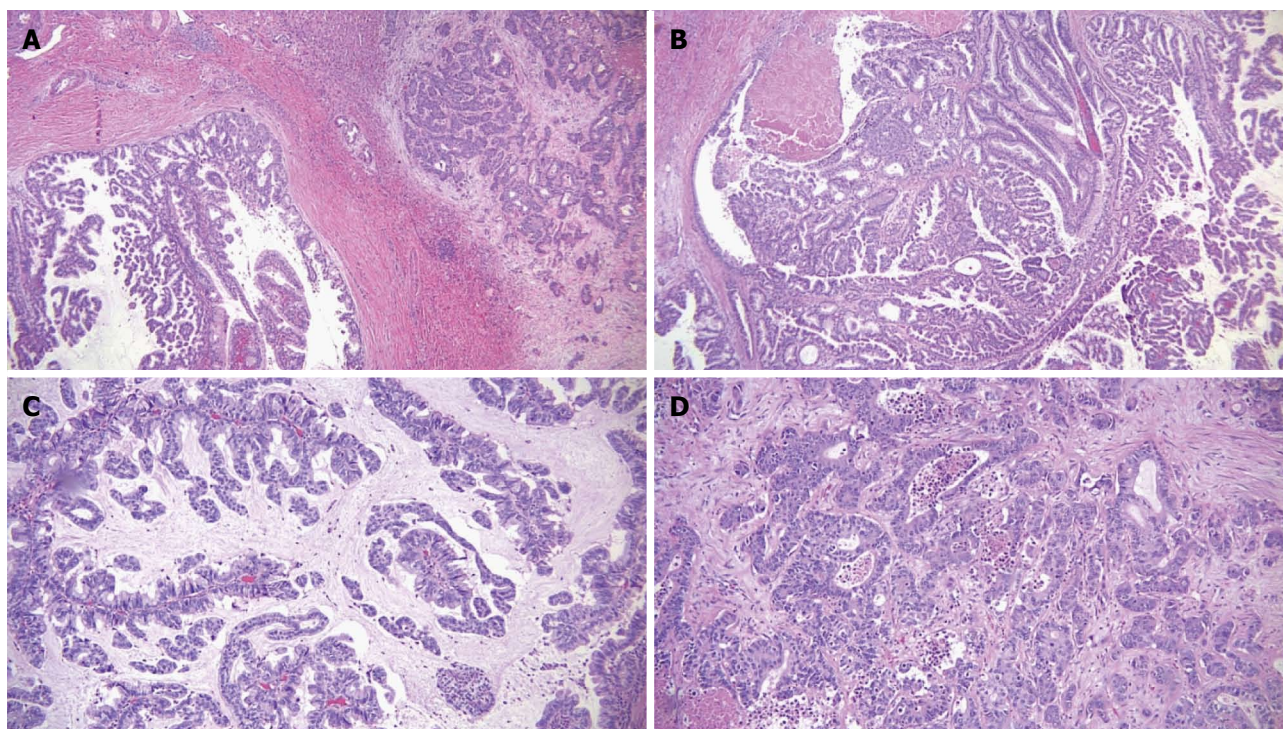
Gross examination of the 438-g partial hepatectomy specimen revealed a 1.5 cm × 1.5 cm × 1.0 cm papillary friable tan mass at the junction of the right hepatic duct and common hepatic duct. The mass was confined to the dilated bile duct. Additionally, there were six well-circumscribed homogenous tan masses ranging from 1.0 to 6.0 cm in the liver parenchyma. One of these masses was immediately adjacent to the papillary mass in the bile duct.

Microscopic examination of the biliary mass showed a papillary mass dilating the bile duct lumen

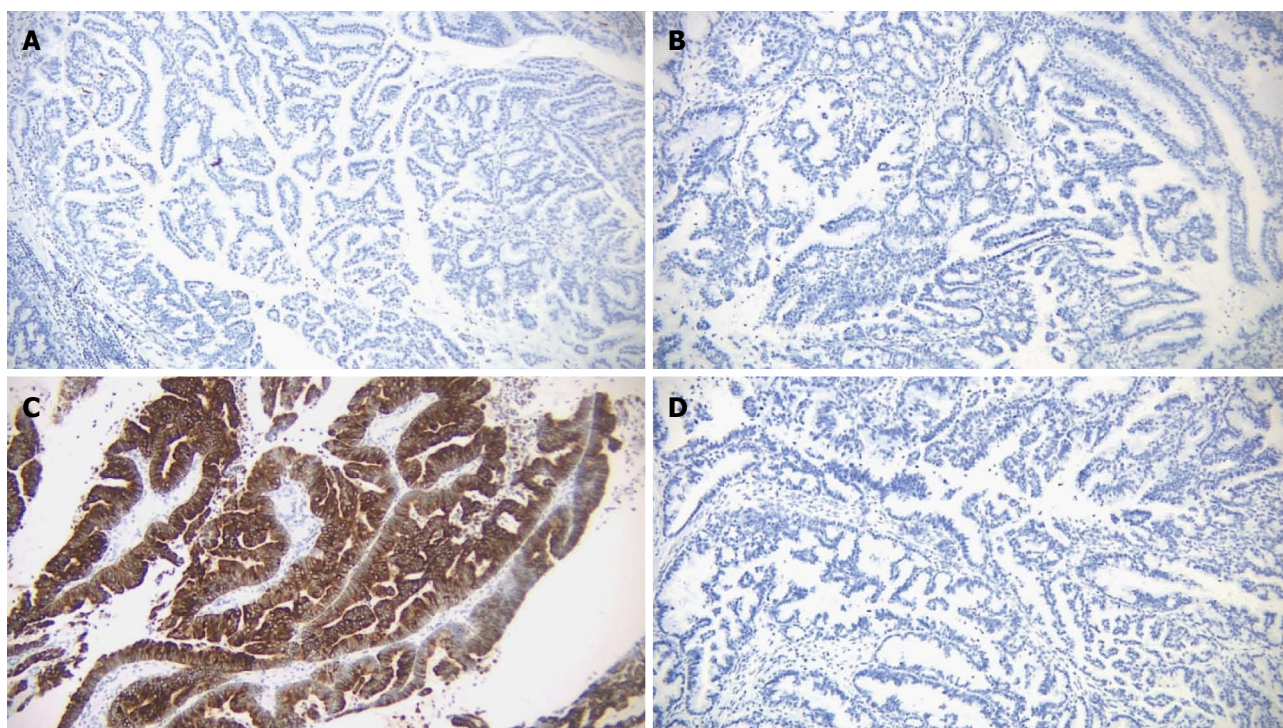
and adjacent to infiltrative adenocarcinoma (Figure 3A). The papillary fronds had fine vascular cores and were lined by foveolar-type epithelial cells with mucinous cytoplasm and different degrees of dysplasia (Figure 3B). Mucin production was evident (Figure 3C). The hepatic masses showed similar morphology with infiltrative features (Figure 3D). There was focal necrosis and sclerosis (5%) due to chemotherapy. Ovarian type stroma was not identified. By immunohistochemistry, the tumor was positive for mucin core protein (MUC)5 and negative for MUC1, MUC2 and CDX2, which is consistent with gastric subtype (Figure 4). The hepatic resection margins were negative for tumor, however, the tumor was present at less than 1 mm from the margin.

Postoperatively, the patient did well. Her CA19-9 dropped to 37.4 UI/mL one month after the surgery. She received postoperative adjuvant chemotherapy with FOLFOX for 3 mo followed by concurrent chemo-radiation. However, in May, 2014, she was found to have left periaortic and gastrohepatic lymphadenopathy and biopsy confirmed metastatic carcinoma, morphologically similar to the invasive IPNB. Since then the patient has been receiving





**Figure 3** Microscopic features of the tumor. A: Intraductal papillary mass with adjacent invasive adenocarcinoma; B: The papillary mass with fine vascular cores was lined by foveolar type epithelium; C: Some areas showed mucin production; D: The metastatic masses in the liver showed infiltrative features.



**Figure 4** By immunohistochemistry, the tumor cells were negative for MUC1 (A), MUC2 (B) and CDX2 (D), and positive for MUC5 (C).



palliative chemotherapy with FOLFIRI and has been stable.

## DISCUSSION

IPNB is a rare tumor, initially described in 1976 as multicentric biliary papillomatosis associated with invasive adenocarcinoma. In 2006, Zen *et al.*<sup>[5]</sup> reported ten cases of papillary biliary tumors, described the histopathologic features and classified the tumor cells into three subtypes including pancreatobiliary, intestinal and gastric subtype. Oncocytic type was believed to be a variant of the pancreatobiliary type. In this article the name of intraductal papillary neoplasm of bile duct (IPNB) was given for the first time to this distinct new entity which included biliary papilloma, papillomatosis and papillary adenocarcinoma. In 2010, IPNB was included in the WHO classification of the bile duct tumors.

In a search of PubMed database, we collected 22 articles including case reports and clinical studies on IPNB patients<sup>[2,6-26]</sup>. In total 354 patients were reported. Most of the patients (52.8%) were from Japan; 19.5% of these patients were from other far eastern countries including China, Korea and Taiwan; 27.7% were from western countries including the United Kingdom (4.0%), the United States (11.0%) and Germany (12.7%). The age of the patients ranged from 35 to 80 years old with an average of 64.6. Male patients were predominant with a male/female ratio of 1.5. Twenty patients had choledocholithiasis or clonorchiasis and all of them were from far eastern countries.

Similar to other bile duct tumors, most IPNB patients present with right hypochondria (35%-88.5%), recurrent acute cholangitis (5%-59%) and obstructive jaundice (20%-36%). Up to 5% of the patients can be asymptomatic<sup>[2,3]</sup>. IPNB can be found in the extrahepatic and intrahepatic bile ducts<sup>[6]</sup>. Of the 354 patients we analyzed, 57.5% of the tumors were in the left lobe, 29.5% were in the right lobe and 13.4% were in the other locations including the common hepatic duct.

Grossly, most IPNBs are papillary or solid intraductal tumors with grape-like appearance. Cystic lesions and papillary mural nodules are common<sup>[6]</sup>. Histologically, there are four subtypes: pancreatobiliary, intestinal, gastric and oncocytic. An invasive component is most often present in the pancreatobiliary and gastric subtypes. As seen in Table 1, mucin core proteins are characteristic markers for the IPNB subtypes. In the reported 354 patients, the average size of the tumors were 4.2 cm. Pancreatobiliary subtype represented 41.8%, intestinal subtype 28.0%, oncocytic subtype 16% and gastric subtype 13.5%. An invasive component was present in 36.4% of all cases.

The pathogenesis of IPNB is still not clear. Most of the studies, especially the largest studies, exclusively

enrolled Asian patients, a considerable proportion of them with hepatolithiasis or clonorchiasis infection. In western countries, this association has not been identified<sup>[2]</sup>. Schlitter *et al.*<sup>[6]</sup> investigated the common molecular pathways in the development of IPNB in 45 patients from European countries. As shown in Table 1, while MUC1, MUC2 and MUC5AC have been very consistent with the histologic subtype, MUC6 and CDX2 were seen in all the subtypes with a predominant expression of MUC6 in pancreatobiliary subtype and CDX2 in intestinal subtype. Genetic tests of these patients suggested a stepwise progression from low-grade intraductal papillary dysplasia at its beginning to invasive adenocarcinoma at its end. Mutated KRAS, overexpression of TP53 and loss of p16 are most commonly involved in this process, whereas loss of SMAD4 was found in late phases of tumor development. Alterations of HER2, EGFR,  $\beta$ -catenin and GNAS were rare events<sup>[6]</sup>. To interpret these genetic and molecular changes and to investigate the pathogenesis of IPNB, more clinical studies are needed.

The differential diagnosis of IPNB includes two entities with different histomorphology and prognosis: hepatic mucinous cystic neoplasm (HMCN) and cholangiocarcinoma (CCA). HMCN is defined as a cyst-forming epithelial neoplasm with typical ovarian-type stroma but with no communication with the bile ducts. Zen *et al.*<sup>[7]</sup> studied 29 HMCNs and found that all HMCNs were seen in female and younger patients with an age ranging from 21 to 69 (median: 45). The masses were larger and predominantly located in segment IV. They showed multilocular cysts with septation or a cyst-in-cyst appearance. Microscopically, the single layered epithelium was benign biliary-type with occasional mucin-containing cells and minimal atypia. They were negative for cytokeratin 20, MUC2, MUC5AC and MUC6. The prognosis was excellent. All the patients had resection and had no recurrence up to 132 mo.

Cholangiocarcinoma patients are older with a median age of 67 and a male predominance (male: female ratio is 3:1)<sup>[6]</sup>. CCA arises from biliary epithelial cells or hepatic progenitor cells and is classified anatomically as intrahepatic CCA, perihilar CCA and distal CCA. IPNB is the precursor lesion of dCCA. Grossly, the tumor shows solitary, multinodular or diffuse small nodules. Microscopically, the tumor is moderate to well-differentiated adenocarcinoma with glandular and tubular structures, mucin production and dense desmoplasia. The prognosis is poor; the patients have a median survival of 24 mo after diagnosis.

For IPNB patients without metastasis, surgical intervention is still the first choice of treatment including pancreaticoduodenectomy (31%), hemihepatectomy (28%), bile duct resection (18%), segmental liver resection (15%) and liver transplant (5%)<sup>[6]</sup>. When major surgery is not indicated, palliative treatments including chemotherapy, percutaneous transhepatic



biliary drainage, percutaneous cholangioscopic laser ablation and iridium-192 intraluminal therapy are recommended<sup>[3]</sup>. The overall survival of IPNB patients is better than CCA patient with a median survival of 62 mo after diagnosis. The survival is related to the percentage and depth of the invasive component, lymphovascular invasion and cellular atypia. Sex, age, location and the epithelial subtype are not associated to the survival<sup>[2]</sup>.

Despite recent advances in diagnosis, such as improved imaging, serology, cytology and molecular techniques including FISH, diagnosis of HMCN, IPNB and CCA is still challenging. However, accurate distinction of IPNB is needed to recognize patients with better prognosis and to further investigate the natural history of this precursor of cholangiocarcinoma.

In summary, we are reporting an IPNB in a very young patient, who is one of the youngest reported. The patient is a Hispanic female and was found to have choledocholithiasis. She underwent hepatic lobectomy after chemotherapy. Histology of the tumor shows foveolar appearance with MUC5AC positive immunostain, consistent with a gastric subtype. Postoperatively, she was found to have metastatic carcinoma to the left periaortic and gastrohepatic lymph nodes, morphologically similar to the invasive IPNB. Now she is receiving palliative chemotherapy and has been stable.

## COMMENTS

### Case characteristics

Thirty-eight-year-old female presented with one-week of acholic stools, jaundice, pruritus and dark colored urine.

### Clinical diagnosis

Clinical impression was obstructive jaundice.

### Differential diagnosis

Differential diagnosis includes hepatic mucinous cystic neoplasm and cholangiocarcinoma which can be differentiated from Intraductal papillary neoplasm of the bile ducts (IPNBs) by the presence of bile duct communication and ovarian-type stroma.

### Laboratory diagnosis

At the time of presenting at our hospital, the patient's laboratory tests demonstrated a total bilirubin 2.0 mg/dL, direct bilirubin 1.5 mg/dL, alkaline phosphatase 195 U/L, AST 83 U/L, ALT 187 U/L, and CA19-9 37.2 U/mL.

### Imaging diagnosis

Computed tomography and magnetic resonance imaging were performed at the time of presenting at our hospital and revealed an ill-defined soft tissue fullness at the bifurcation of the common hepatic duct involving the left and right hepatic ducts as well as three intrahepatic masses which were suspicious for metastasis.

### Pathological diagnosis

By macro- and microscopic examination the pathological diagnosis was invasive intraductal papillary neoplasm (adenocarcinoma) of bile ducts, 3 cm, gastric subtype, with multiple intrahepatic metastases up to 6.0 cm.

## Treatment

The patient underwent a left hepatic lobectomy with cholecystectomy and hepatojejunostomy followed by postoperative adjuvant chemotherapy with FOLFOX for months and then concurrent chemo-radiation.

## Related reports

This patient has a reported history of choledocholithiasis and underwent stone extraction through endoscopic retrograde cholangiopancreatogram at an outside hospital. Postoperatively, although she was receiving chemo-radiation, she was found to have left periaortic and gastrohepatic lymphadenopathy and biopsy confirmed metastatic carcinoma, morphologically similar to the invasive IPNB.

## Experiences and lessons

IPNB is very rare in the United States and caution is needed in order to differentiate this entity from other bile duct tumors and hepatic tumors, especially cholangiocarcinoma and hepatic mucinous cystic neoplasm since they have similar morphology but different in prognosis and treatment.

## Peer-review

Authors try to present a rare patient with intraductal papillary neoplasm of bile duct which was commonly combined with hepatolithiasis in Asian countries. After reviewing this manuscript, positive information of this article is worth to the readers.

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## Cholestasis, ascites and pancytopenia in an immunocompetent adult with severe cytomegalovirus hepatitis

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**Author contributions:** Qian JY and Bai XY contributed equally to this work; Qian JY, Bai XY and Feng YL conceived and designed the study, and analyzed and interpreted data; Qian JY and Bai XY drafted the article; Zhu WJ and Li F performed imaging studies; Yao F, Li JN, Yang AM, Qian JM and Feng YL revised the article; and all the authors have read and approved the final version to be published.

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### Abstract

Human cytomegalovirus (CMV) is a herpesvirus, which establishes lifelong latency after primary infection and leads to severe disease in immunocompromised patients. However, CMV infection in immunocompetent patients is usually asymptomatic and severe organ damage is rarely reported. We report a case of severe CMV hepatitis in an immunocompetent patient presenting with cholestasis, portal hypertension-related ascites and pancytopenia. The patient was asymptomatic with normal liver function and negative CMV DNA after two weeks of antiviral therapy. This case is an example of a common infection with an uncommon presentation, and suggests that testing for CMV should be carried out, even in patients with normal immune status, presenting with severe liver damage or cholestasis.

**Key words:** Cytomegalovirus hepatitis; Cholestasis; Ascites; Pancytopenia; Immunocompetent adult

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**Core tip:** Human cytomegalovirus (CMV) can establish

lifelong latency after primary infection. It also leads to severe disease in immunocompromised patients. However, CMV infection in immunocompetent patients is usually asymptomatic and severe organ damage is rarely reported. Here, we report an immunocompetent patient with a unique presentation of severe CMV hepatitis manifested by cholestasis, ascites and pancytopenia. The patient was asymptomatic with normal liver function and negative CMV DNA after two weeks of antiviral therapy. It is suggested that testing for CMV should be carried out, even in patients with normal immune status, presenting with severe liver damage or cholestasis.

Qian JY, Bai XY, Feng YL, Zhu WJ, Yao F, Li JN, Yang AM, Li F, Qian JM. Cholestasis, ascites and pancytopenia in an immunocompetent adult with severe cytomegalovirus hepatitis. *World J Gastroenterol* 2015; 21(43): 12505-12509 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v21/i43/12505.htm> DOI: <http://dx.doi.org/10.3748/wjg.v21.i43.12505>

## INTRODUCTION

Human cytomegalovirus (CMV), a herpesvirus, is a double-stranded DNA virus which can establish lifelong latency after primary infection. The infection can be transmitted by body fluid contact, placental transfer and transplantation<sup>[1]</sup>. The primary CMV infection may arise at an early age and the seroprevalence in developed countries is reported to be 30%-70%<sup>[2]</sup>. In immunocompetent patients, primary and long-term immunity are induced after the initial infection, preventing reactivation of the virus; while in immunocompromised patients, such as transplant recipients and patients with acquired immune deficiency syndrome, viral replication cannot be controlled and therefore can cause severe diseases. This explains the significant morbidity and mortality in CMV infected immunocompromised patients<sup>[3,4]</sup>. CMV infection in immunocompetent hosts is commonly asymptomatic, or in some cases, presents as mononucleosis syndrome. Severe organ involvement after CMV infection in immunocompetent adults is rare<sup>[5]</sup>. We report a unique case of severe CMV hepatitis in an immunocompetent patient presenting with cholestasis, portal hypertension-related ascites and pancytopenia.

## CASE REPORT

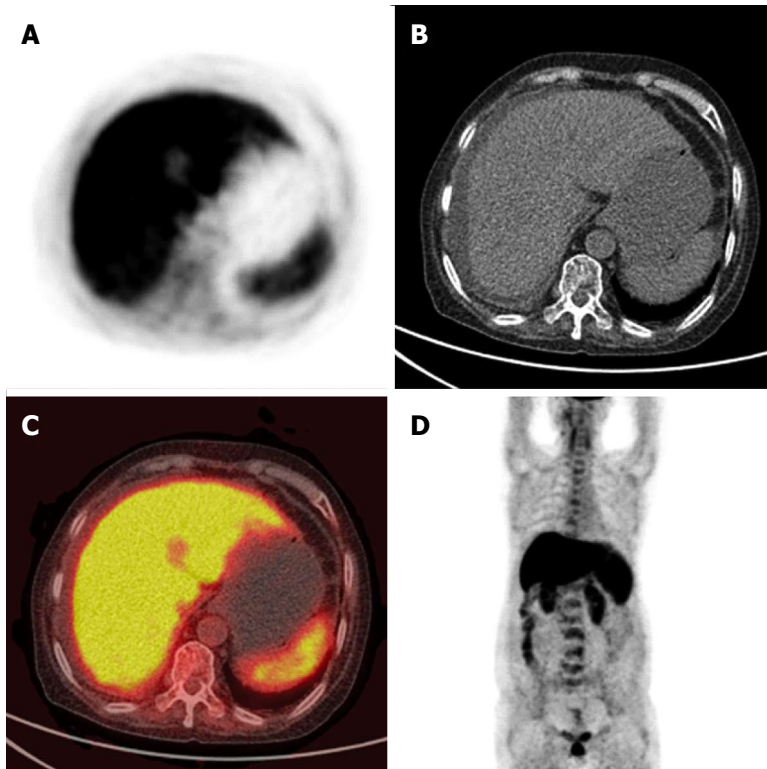
A previously healthy 66-year-old female presented with a one-month history of yellow coloration of the eyes and skin, abdominal distension, dark colored urine and pale stool. Fever, abdominal pain, nausea, vomiting and diarrhea were absent. The patient denied alcohol consumption, toxic exposure and a medication history of glucocorticoids, immunosuppressive agents or herbal medicine. No significant history of traveling

and animal contact or similar cases in the community were reported. Moderate mucocutaneous jaundice and shifting dullness were revealed on physical examination which was otherwise unremarkable.

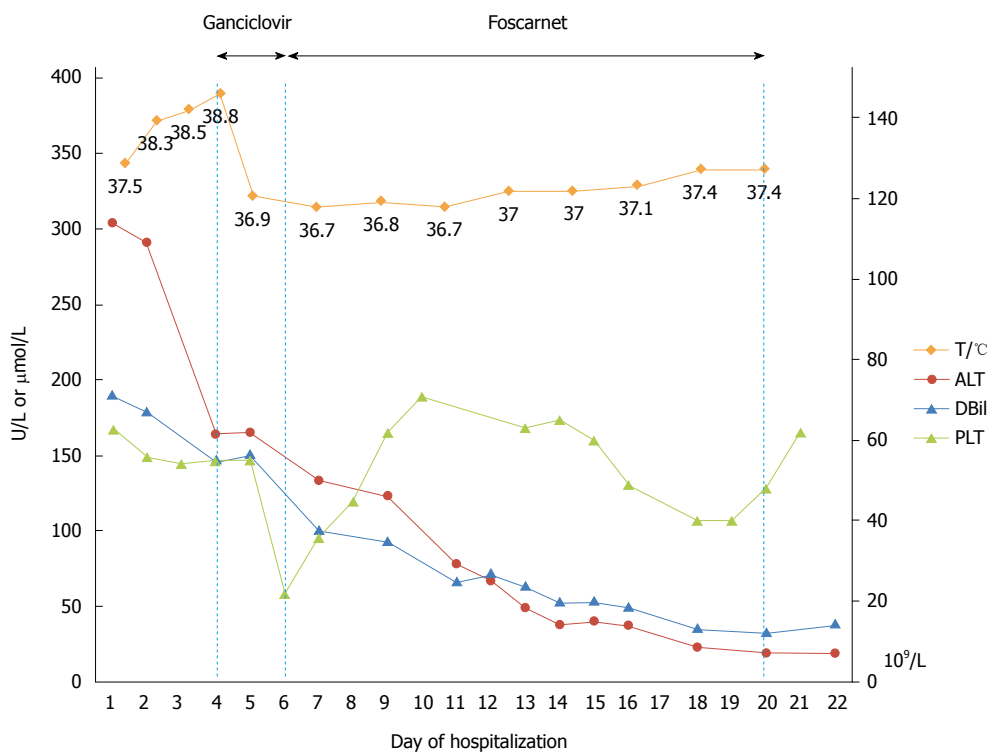
Complete blood count (CBC) showed progressive pancytopenia (hemoglobin of 113→95 g/L, platelet count of 154→54 × 10<sup>9</sup>/L and white blood cell count of 6.74→4.48 × 10<sup>9</sup>/L), and bone marrow biopsy revealed infrequent hemopoietic precursors and increased adipose tissue. Liver function tests were abnormal with alanine aminotransferase 164 U/L (normal value 7-40 U/L), aspartate aminotransferase 191 U/L (normal value 13-35 U/L), gamma glutamyl transpeptidase 83 U/L (normal value 7-45 U/L), alkaline phosphatase 420 U/L (normal value 50-135 U/L), total bilirubin 179.5 μmol/L (normal value 5.1-22.2 μmol/L) and direct bilirubin 145.6 μmol/L (normal value 0-6.8 μmol/L). Coagulation tests showed elevated prothrombin time and partial thromboplastin time of 15.7 s (normal value 10.4-12.6 s) and 65.5 s (normal value 22.7-31.8 s), respectively. Acute phase reactants and immunoglobulin A/G/M were normal. Autoantibodies including antinuclear antibodies, antimitochondrial antibodies, anti-smooth muscle antibodies, anti-liver-kidney microsome-1 antibodies, and anti-liver cytosol antibody-1 indicated that autoimmune hepatitis and primary biliary cirrhosis were negative. Abdominal paracentesis was performed and the results suggested portal hypertension-related ascites which was confirmed by a serum-ascites albumin gradient of 26 g/L. No malignant cells were found in the ascites. Contrast-enhanced computed tomography (CT) revealed homogeneous low density of the liver and slight dilatation of the intrahepatic bile ducts. No dilatation of the extrahepatic bile duct, cirrhosis or splenomegaly was found. Positron emission tomography (PET)/CT showed diffusely increased fluorodeoxyglucose uptake in the liver (Figure 1). Three-dimensional CT angiography revealed no evidence of portal vein thrombosis. A lymphocyte subset test showed a CD4<sup>+</sup> cell to CD8<sup>+</sup> cell ratio of 1.2 (normal 0.95-2.13). Activation of functional CD8<sup>+</sup> T cells was shown by CD8<sup>+</sup>HLA-DR<sup>+</sup>/CD8<sup>+</sup>% of 60.2% (normal 6.3%-23.8%) and CD8<sup>+</sup>CD38<sup>+</sup>/CD8<sup>+</sup>% of 90.2% (normal 32.4%-57.4%). Tests for hepatitis A, hepatitis B, hepatitis C, hepatitis E, Epstein-Barr virus and human herpes virus were negative, however, quantitative PCR for CMV DNA showed 7800 copies/mL (normal < 500 copies/mL). Liver biopsy was not performed due to coagulopathy.

Following admission, the patient developed recurrent episodes of fever (Tmax of 38.7 °C) without other associated symptoms. CMV hepatitis was considered to be the most likely diagnosis in this case, although liver biopsy could not be carried out, given the fact that other causes of liver damage including alcohol, drug, toxicant, tumor, and other common causes of viral hepatitis had been reasonably excluded. Anti-CMV treatment with intravenous ganciclovir was





**Figure 1 Findings on imaging.** PET (A), transverse CT (B), PET/CT fusion image (C) and maximum intensity projection (MIP, D) image of the whole body using fluorodeoxyglucose PET/CT (FDG PET/CT) demonstrate a large hypermetabolic deposit in the liver (standardized uptake value - SUVmax = 8.3 cm<sup>2</sup>/mL). PET: Positron emission tomography; CT: Computed tomography.



**Figure 2 Biomedical profile during hospitalization.** The graph shows the changing pattern of body temperature (T), alanine aminotransferase (ALT), direct bilirubin (DBil) and platelet count (PLT) before and after antiviral therapy.

commenced due to progressive deterioration of her conditions, especially pancytopenia and coagulopathy. Her body temperature normalized within a day indicating a positive response to the antiviral therapy, however, the platelet count progressively decreased to  $22 \times 10^9/L$ . As thrombocytopenia may have been an adverse effect of ganciclovir, intravenous foscarnet was started instead. Jaundice, ascites, liver function tests and CBC gradually improved (Figure 2). After two weeks of antiviral therapy, the patient became asymptomatic and discharged with normal liver function and negative CMV DNA.

## DISCUSSION

Single or multi-organ disorders as complications of CMV infection in immunocompetent hosts are rarely reported. Of these complications, gastrointestinal manifestations are thought to be the most common. Other complications include hepatic, neurological, pulmonary, ocular, and cardiovascular. Asymptomatic elevation of transaminases is the most common hepatic manifestation in immunocompetent patients, while hepatic dysfunction with significant symptoms and signs is extremely rare<sup>[5]</sup>. In the present report, an immunocompetent adult presenting with features consistent with portal hypertension and pancytopenia was found to have a significantly increased CMV DNA level. The diagnosis of severe CMV hepatitis was made after other possible causes were excluded, although liver biopsy was not performed due to coagulopathy. Fever and liver function improved with initial ganciclovir treatment, however, a decrease in the platelet count was observed. We considered the worsening of thrombocytopenia to be a side effect of ganciclovir in this case as other symptoms associated with CMV primary infection improved. It has been reported that cancer chemotherapy, decreased creatinine clearance ( $< 20$  mL/min) and high ganciclovir dose ( $\geq 12$  mg/kg daily) were independent risk factors for thrombocytopenia in CMV patients receiving ganciclovir<sup>[6]</sup>. Two weeks after switching from ganciclovir to foscarnet, the patient's liver function was back to normal. This case is an example of a common infection with an uncommon presentation, and suggests that testing for CMV is necessary in patients presenting with severe liver damage, even if they are immunocompetent. The decision to perform a liver biopsy should be based on the condition of the patient, even though it is essential to achieve a definitive diagnosis. Antiviral therapy is beneficial in severe and life-threatening conditions, but should be administered after weighing the benefits and risks.

## ACKNOWLEDGMENTS

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## COMMENTS

### Case characteristics

A 66-year-old female presented with a one-month history of yellow coloration of the eyes and skin, abdominal distension, dark colored urine and pale stool.

### Clinical diagnosis

Physical examination showed moderate mucocutaneous jaundice and shifting dullness.

### Differential diagnosis

Virus infection [hepatitis A, hepatitis B, hepatitis C, hepatitis E, Epstein-Barr virus, cytomegalovirus (CMV) and human herpes virus], autoimmune hepatitis, primary biliary cirrhosis and periampullary carcinoma.

### Laboratory diagnosis

Laboratory tests revealed severe liver injury, pancytopenia, coagulopathy, and quantitative PCR showed CMV DNA of 7800 copies/mL.

### Imaging diagnosis

Positron emission tomography-computed tomography showed diffusely increased fluorodeoxyglucose uptake in the liver.

### Treatment

As thrombocytopenia may be an adverse effect of ganciclovir, treatment was switched to intravenous foscarnet.

### Related reports

Single or multi-organ disorders as complications of CMV infection in immunocompetent hosts are rarely reported.

### Experiences and lessons

This case is an example of a common infection with an uncommon presentation, and suggests that testing for CMV should be carried out, even in patients with normal immune status who have severe liver damage or cholestasis.

### Peer-review

The authors presented the common infection of CMV in an immunocompetent patient with a unique presentation of severe cytomegalovirus hepatitis manifested with cholestasis, ascites and pancytopenia.

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## Hepatitis C eradication: A long way to go

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### Abstract

Hepatitis C virus (HCV) is a major global health problem with high morbidity and mortality. About 185 million people are living with HCV, of which 80% are living in low and middle income countries. With the development of new highly effective treatments for HCV, it is considered that the eradication of HCV may only be one step away. The major problem with new treatment options is its high price. The price of sofosbuvir-based treatment for one patient in the United States is US\$85000-110000, while the actual

production cost of a 12 wk direct-acting antiviral regimen is less than US\$250. Another major hindrance in HCV eradication is the lack of quality management of blood transfusion screens. Due to the lack of HCV screening, 75% of people in the United States with HCV infection are unaware of their positive HCV status. The control of massive HCV pandemic will require a significant financial investment, political will, and support from medical, pharmaceutical, and civil organizations around the globe.

**Key words:** Hepatitis C virus; Treatment; Diagnostics; Screening; Transfusion

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**Core tip:** With the availability of new direct-acting antivirals for hepatitis C virus (HCV), some people think that eradication of HCV may be only one step away. There are a number of issues that need to be overcome to win the fight against HCV. Although the cost of HCV treatment is reduced for certain countries, cost remains a big problem for low and middle income countries for the treatment of a large number of patients. In low income countries, 47% of blood transfusions are from laboratories with no quality management in place. There is a lack of knowledge and awareness about HCV among healthcare providers, policy makers, general public, and at risk populations.

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### TO THE EDITOR

Hepatitis C virus (HCV) is a major health problem around the globe. About 185 million people are



living with HCV, of which 80% are living in low-income and middle-income countries<sup>[1]</sup>. One third of chronically infected HCV patients develop cirrhosis and hepatocellular carcinoma. It is estimated by the World Health Organization (WHO) that the annual number of deaths caused by HCV related liver diseases ranges from 350000 to 500000<sup>[2]</sup>. Treatment with pegylated interferon and ribavirin is the treatment of choice in several countries. The therapy has limited response with a number of side effects. The cost of this therapy is US\$ 4000 for one patient<sup>[3,4]</sup>.

With the availability of new highly effective HCV treatments, it is considered that the HCV elimination may be only one step away<sup>[5]</sup>. We think that the elimination of hepatitis C is not so much easy even with the development of new highly effective therapies, but there are many issues to address before achieving the global eradication of HCV.

The new generation of direct acting antiviral (DAA) drugs has demonstrated high treatment response with minimal adverse effects. The major problem with new drugs is their affordability. The price of sofosbuvir based treatment for one HCV patient in the United States is US\$85000-110000<sup>[6]</sup>, while the actual production cost of a 12 wk regimen of DAA is less than US\$250<sup>[7]</sup>. Several European countries are negotiating the price for new HCV drugs. The French health minister warned that the high price of treatment will impose high burden on the social security system. The high treatment response of new DAA has been linked with adherence to proper treatment regimen. In clinical trials, sofosbuvir (Sovaldi) showed a discontinuation rate of 2%.

Seventy two percent of the world's poorest people live in middle income countries, and 90% of these patients pay for medications out of pocket. Some countries have an insurance scheme in place, but these do not always cover the cost of HCV diagnostics and treatment<sup>[1]</sup>. Middle income countries are an attractive market for pharmaceutical companies. Gilead has a licensing agreement with seven Indian companies to manufacture generic HCV medicine at a reduced price for 91 developing countries. Egypt and Pakistan have 11 million and 10 million people living with HCV, respectively. It will cost approximately US\$5 billion, even with the reduced price of sofosbuvir, to treat half the patients in either country. This price does not include the expenses used for laboratory monitoring, hospital visits, and medications to manage adverse events. There is dire need to make HCV treatment affordable for a large number of HCV patients. According to the Centers for Disease Control (CDC), one premature death is prevented for every three virological cures<sup>[8]</sup>. We can learn drug affordability from the example established for the affordable treatment of HIV. About 10 million people receive antiretroviral therapy for HIV at a cost of \$100 per person annually<sup>[9]</sup>.

The major route of transmission of HCV is blood

and blood products<sup>[10]</sup>. It was reported by WHO that 47% of blood donations in low income countries are from laboratories with no quality assurance<sup>[11]</sup>. Large numbers of labs are using rapid tests and third generation enzyme immunoassays (EIA) for HCV detection. The window period for HCV detection by third generation EIA test is 66 d compared with 4 d for HCV detection by nucleic acid amplification technology (NAT)<sup>[12]</sup>. CDC recommends the use of highly sensitive EIA or rapid test for HCV screening and use of another assay to confirm further positive results<sup>[13]</sup>. Efforts are needed to establish cost effective NAT laboratories for effective HCV diagnosis.

People with HCV infection remain asymptomatic for long periods during which infection may be transmitted to other persons. Seventy five percent of people in the United States with HCV infection are unaware of their infection<sup>[14]</sup>. There is a need for HCV screening programs for both general and at risk populations, including intravenous drug users (IDUs), people with a history of using blood products or unsafe injections, those with piercings and tattoos, prisoners, and homeless people. There is also a need to provide proper prevention, diagnosis, and treatment facilities to the detained population.

In a multinational study to forecast the prevalence of HCV by 2030, it was observed that HCV associated morbidity and mortality can be greatly reduced by increasing diagnosis and the number of individuals getting higher efficacy treatment<sup>[15]</sup>.

Lack of knowledge and awareness about HCV are observed among healthcare providers, policy makers, the general public, and at risk populations. Forty percent of global HCV infections are due to unsafe injections and improperly sterilized medical equipment<sup>[16]</sup>. Health care providers need education and training to reduce the risk of disease transmission by malpractice. It was observed in a recent study that only 5.5% of HCV positive IDUs of India were aware of their status and only 1.4% of HCV positive IDUs had received treatment for HCV<sup>[17]</sup>. Massive awareness programs are needed to decrease the future burden of HCV on society.

There is insufficient understanding about the seriousness of this public health problem, so inadequate public resources are allocated for the prevention and control of HCV. There is a need to develop a global strategy for HCV eradication. More than 10 million people are on their feet due to the global polio eradication initiative, and the global incidence of polio has been reduced by 99%<sup>[18]</sup>. The control of massive HCV pandemic requires financial investment, political will, and support from medical, pharmaceutical, and civil organizations around the globe.

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