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## Role of transcatheter arterial embolization for massive bleeding from gastroduodenal ulcers

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### Abstract

Intractable bleeding from gastric and duodenal ulcers is associated with significant morbidity and mortality. Aggressive treatment with early endoscopic hemostasis is essential for a favourable outcome. In as many as 12%-17% of patients, endoscopy is either not available or unsuccessful. Endovascular therapy with selective catheterization of the culprit vessel and injection of embolic material has emerged as an alternative to emergent operative intervention in high-risk patients. There has not been a systematic literature review to assess the role for embolotherapy in the treatment of acute upper gastrointestinal bleeding from gastroduodenal ulcers after failed endoscopic hemostasis. Here, we present an overview of indications, techniques, and clinical outcomes after endovascular embolization of acute peptic-ulcer bleeding. Topics of particular relevance to technical and clinical success are also discussed. Our review shows that transcatheter arterial embolization is a safe alternative to surgery for massive gastroduodenal bleeding that is refractory to endoscopic treatment, can be performed with high technical and clinical success rates, and should be considered the salvage treatment of choice in patients at high surgical risk.

### INTRODUCTION

Acute bleeding is the most common complication of peptic ulcer disease and about half the cases of upper gastrointestinal bleeding (UGI) are caused by gastric and duodenal ulcers<sup>[1,2]</sup>. First-line endoscopy achieves bleeding control in as many as 98% of patients<sup>[3,4]</sup>. Despite these measures, the mortality rate in patients with bleeding peptic ulcers remains as high as 5% to 10%<sup>[5,6]</sup> due to a combination of advanced age, multiple co-morbidities, and high transfusion requirements<sup>[7]</sup>. Current treatment algorithms for massive UGI bleeding recommend aggressive correction of coagulation disorders followed by endoscopy<sup>[8,9]</sup>. Endoscopic therapy with epinephrine injection and heat probe coagulation is the most reliable method. Re-bleeding is usually managed with a second endoscopic attempt. Severe bleeding despite conservative medical treatment or endoscopic intervention occurs in 5% of patients<sup>[10]</sup> and requires surgery or transcatheter arterial embolization. Surgery is associated with mortality rates as high as 20% to 40%<sup>[11]</sup>. Although endovascular management is not included in the treatment algorithms for UGI bleeding described in surgical textbooks, selective catheter-directed embolization has been proposed as a less hazardous alternative to surgery, especially for high-risk patients<sup>[12,13]</sup>, and is now considered in many institutions as the first-line intervention for massive gastroduodenal bleeding after failed endoscopic treatment<sup>[12-15]</sup>. The



obvious advantage of transcatheter embolization is avoidance of a laparotomy in a critically ill patient. With the advent of metallic coils, gelfoam, and surgical glue, outcomes after embolization have compared favourably with those of surgery. The purpose of this review is to review the data on the indications, safety, effectiveness, and outcomes of embolotherapy in the treatment of acute UGI from gastroduodenal ulcers.

## KEYWORD SEARCH

We searched PubMed for studies of embolization for peptic ulcer bleeding published in English from 1992 to 2009. Further studies were sought by manually searching the reference lists of articles retrieved *via* PubMed. We then selected the articles that had well-defined indications for the intervention and offered a detailed description of the outcomes, including technical and clinical success rates, re-bleeding, re-intervention, need for surgery for bleeding control, and morbidity and mortality rates. To avoid selection bias associated with a small series, we excluded studies with fewer than 10 patients and anecdotal case-reports. Studies of patients with bleeding from causes other than peptic ulcers were also excluded. The results were tabulated as absolute numbers and percentages. Mean values of the outcome variables of interest were computed. Information on indications, technique, complications, and a variety of other topics of interest is presented as a narrative, in order to provide a better understanding of the current status and controversial aspects of the endovascular treatment of UGI bleeding from peptic ulcers.

## INDICATIONS

Transcatheter arterial embolization as an alternative to surgery for the control of UGI bleeding was introduced by Rösch *et al*<sup>[16]</sup> in 1972. Since then, arterial catheterization has become a useful diagnostic and therapeutic tool in selected populations<sup>[17]</sup>. The typical candidate presents with massive bleeding (transfusion requirement of at least four units of blood per 24 h) or hemodynamic instability (hypotension with systolic pressure less than 100 mmHg and heart rate of 100/min or clinical shock secondary to blood loss) that has not responded to conservative medical treatment combining volume replacement, proton pump inhibitors, and at least one endoscopic procedure aimed at controlling the bleeding<sup>[18]</sup>. At this point, surgery is offered to low-risk patients and percutaneous embolotherapy to high-risk patients. Finally, endovascular treatment can be used if the bleeding recurs after surgery<sup>[19]</sup>.

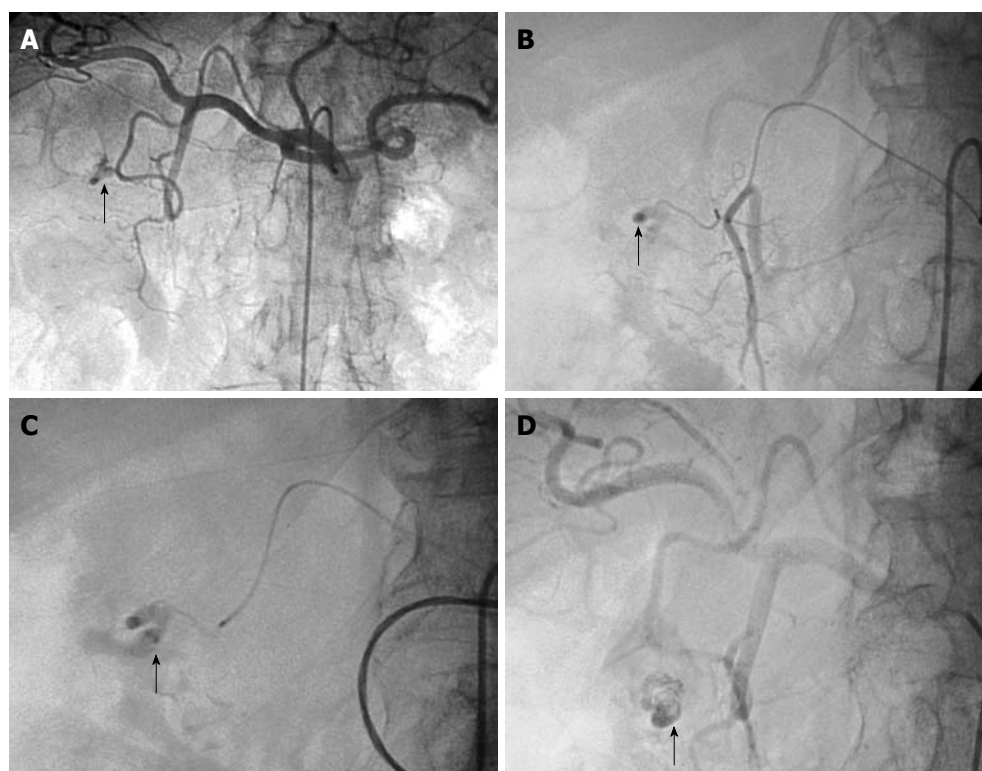
## TECHNIQUE

A transfemoral approach was used in most of the case-series retrieved by our literature search. A 5-French sheath is placed in the common femoral artery. Brachial access may be necessary when there is an acute angulation at the origin of the celiac axis. A variety of selective catheters can be used to cannulate the celiac artery

and to access the common hepatic artery. Once access is obtained, arteriography is performed to delineate the arterial anatomy and to identify contrast extravasation. If no extravasation is seen, then superselective catheterization of the gastroduodenal artery (GDA) (Figure 1), left gastric artery (Figure 2), or splenic artery (Figure 3) is performed, depending on the endoscopic evidence concerning the probable bleeding site. During this step, a microcatheter is useful but not indispensable. Arteriography after superselective cannulation might reveal extravasation that was missed during contrast injection into the main hepatic artery. When a dual supply to the bleeding area is suspected, both arterial sources must be embolized. This typically occurs with ulcers that erode the GDA: embolization in this case needs to start distally to prevent persistent “backdoor” bleeding from the right gastroepiploic and superior pancreaticoduodenal arteries, and should then move to the proximal side of the erosion. If no evidence of bleeding is found on the pre-embolization arteriogram, then blind embolization is performed, typically guided by the endoscopic findings regarding the bleeding site (Figure 4). Another useful manoeuvre in this scenario is the placement, during the pre-embolization endoscopy, of clips around the bleeding site. The clips remain in position for several hours and allow for an educated guess about the location of the bleeding arterial branch<sup>[20]</sup>. If, despite the injection of a contrast agent, no extravasation is seen, then the branches terminating at each clip are selectively catheterized using microcatheter techniques and embolized. Arteriography with multiple projections is necessary at this step to assess the relationship between each clip and the adjacent branches. Infusion of a fibrinolytic agent such as t-PA, intra-arterial anticoagulants, or vasodilators to temporarily increase the bleeding rate during angiography has been reported to facilitate the angiographic identification and localisation of the bleeding vessel<sup>[21]</sup>.

## COMPLICATIONS

Groin hematomas and contrast-related complications occur with the same frequency as during other endovascular procedures. Acute renal failure may develop as a result of multiple factors including contrast injection and intravascular volume depletion. Duodenal ischemia can result from embolization of terminal muscular branches or from embolization of the main GDA with polyvinyl alcohol (PVA) particles. Typical symptoms include persistent epigastric pain, nausea and, occasionally, vomiting. Endoscopy shows small multiple duodenal erosions consistent with healing ischemic lesions. Predisposing factors include previous abdominal surgery and/or radiation therapy. Conservative treatment with proton pump inhibitors and maintenance of NPO status is usually sufficient. Inadvertent embolization of the main hepatic artery can result in a broad spectrum of manifestations, ranging from temporary liver enzyme elevation to life-threatening hepatic failure, for which risk factors include cirrhosis and associated portal vein compromise<sup>[12]</sup>. Inadvertent placement of coils in the main branches of the



**Figure 1** Arteriogram images of bleeding from a bulbar duodenal ulcer in a 76-year-old man. A, B: Arteriogram showing contrast medium extravasated from a slender branch of the gastroduodenal artery (GDA) into the duodenum (arrows); C, D: After microcatheterization, selective glue embolization (radiopaque because of associated lipiodol (arrows)) preserving the GDA ensured control of the bleeding, with no early or late recurrences.

celiac axis has been reported. Given the rich collateral circulation, however, coils in the left gastric or splenic artery rarely produce organ-threatening ischemia<sup>[22]</sup>. Duodenal stenosis can become a serious problem after GDA embolization. It is more common after superselective embolization of terminal muscular branches with surgical glue and can occur several years after successful embolization. Balloon dilatation can be attempted initially, although surgery is required in patients with persistent symptoms of duodenal obstruction<sup>[23]</sup>.

## OUTCOMES IN CASE-SERIES

We identified 13 studies (422 patients; mean age, 69 years) on the endovascular management of intractable gastrointestinal bleeding from gastroduodenal ulcers. Endoscopy was performed unsuccessfully in 98% of these patients (Table 1). The vast majority of the patients with major comorbidities and were considered at high surgical risk. Endovascular embolization was technically successful in 392 (93%) patients. A variety of embolic materials including coils, PVA particles, blood clot, gelfoam, and cyanoacrylate glue were used. The “sandwich” technique with placement of embolic material on either side of the bleeding vessel was used in most case-series to minimize the risk of recurrent bleeding due to collaterals. Active extravasation was present at the time of embolization in 53% of patients. The other patients underwent blind embolization guided by the endoscopy findings or by clips placed around the bleeding site. In the subgroup with technically successful embolization, the rate of bleeding cessation (clinical success rate) was 81% (Table 2).

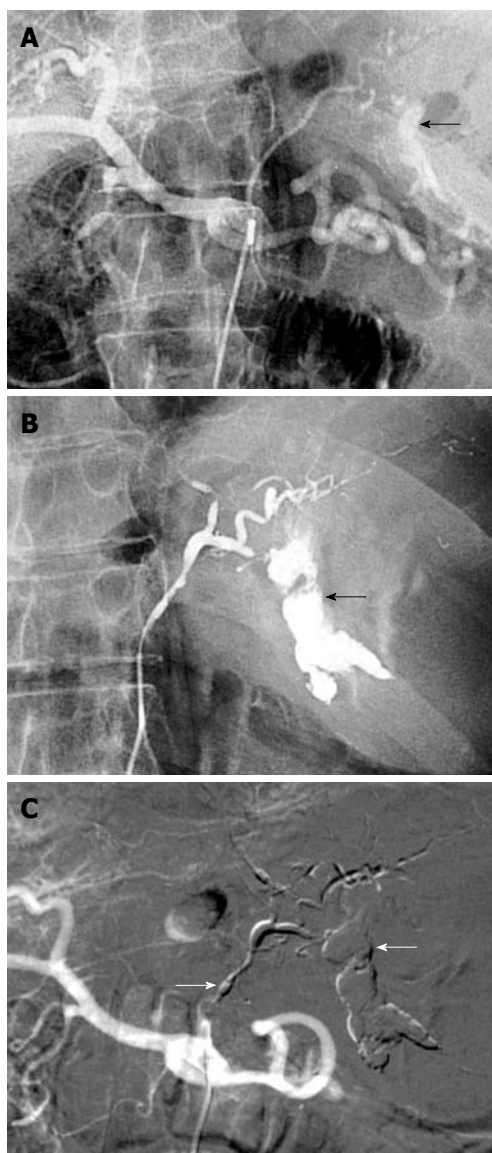
Overall, 25% (106/422) of patients had persistent bleeding. However, almost half of them responded to

**Table 1** Synopsis of the studies under review

Ref., yr	Patients (n)	Mean age (yr)	Previous endoscopy (%)	Active extravasation (%)	Technical success (%)
Lang <i>et al</i> <sup>[23]</sup> , 1992	57	52	NA	100	91
Toyoda <i>et al</i> <sup>[33]</sup> , 1995	11	65	100	54	100
Toyoda <i>et al</i> <sup>[37]</sup> , 1996	30	62	100	NA	100
Walsh <i>et al</i> <sup>[43]</sup> , 1999	50	64	100	50	92
De Wispelaere <i>et al</i> <sup>[40]</sup> , 2002	28	69	100	39	89
Ljungdahl <i>et al</i> <sup>[39]</sup> , 2002	18	78	72	50	72
Ripoll <i>et al</i> <sup>[24]</sup> , 2004	31	75	100	NA	100
Holme <i>et al</i> <sup>[25]</sup> , 2006	40	70	100	30	100
Eriksson <i>et al</i> <sup>[20]</sup> , 2006	10	75	100	10	100
Loffroy <i>et al</i> <sup>[13]</sup> , 2008	35	71	100	66	94
Larssen <i>et al</i> <sup>[15]</sup> , 2008	36	80	100	42	92
van Vugt <i>et al</i> <sup>[35]</sup> , 2009	16	71	100	75	88
Loffroy <i>et al</i> <sup>[26]</sup> , 2009	60	69	100	63	95
All studies	422	69	98	53	93

NA: Not available.

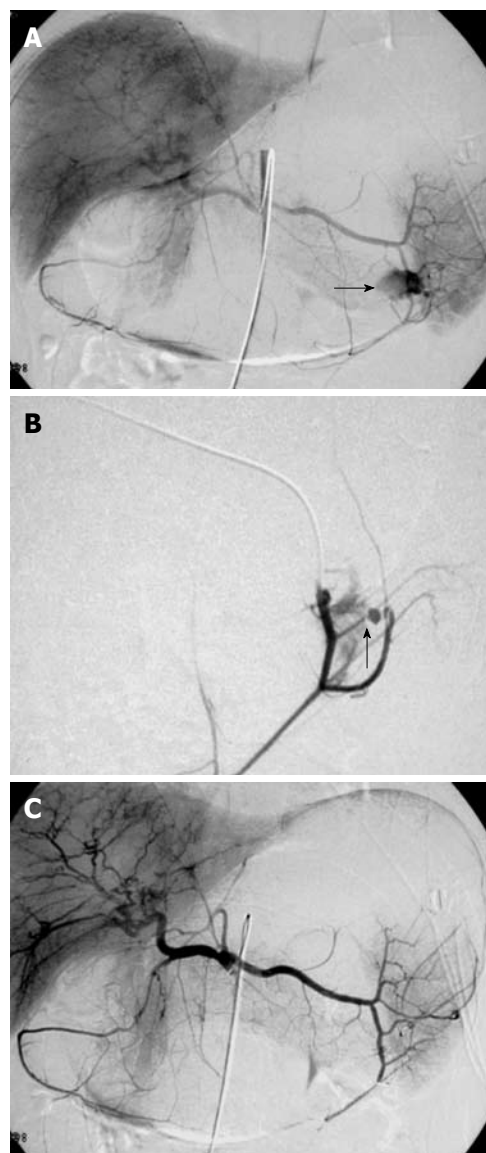
repeat embolization. Finally, 18% of patients overall underwent surgery for bleeding control (Table 2). Major and minor embolization-related complications developed in 4% of patients and included access-site complications,



**Figure 2** Bleeding Dieulafoy lesion in an 87-year-old man. A, B: Selective angiography shows contrast medium extravasation from the left gastric artery at the celiac trunk, indicating active bleeding (arrows); C: After arterial microcatheterization, bleeding was controlled after embolization of the left gastric artery using a Glubran/Lipidol mixture (1:3) (arrows).

dissection of the target vessel, and hepatic or splenic infarction. The most significant long-term complication was duodenal stenosis, particularly after glue embolization of terminal muscular branches of the GDA. Overall 30-d mortality was 25% (Table 2). The data available in the study reports did not allow us to assess the causes of death or their relationship with the result of the embolization or need for further intervention. Although the mortality rates seem as high as those in several case-series of emergent surgery for UGI bleeding, they should be interpreted with the knowledge that most of the patients treated with embolotherapy had been turned down for surgery due to major co-morbidities and advanced age.

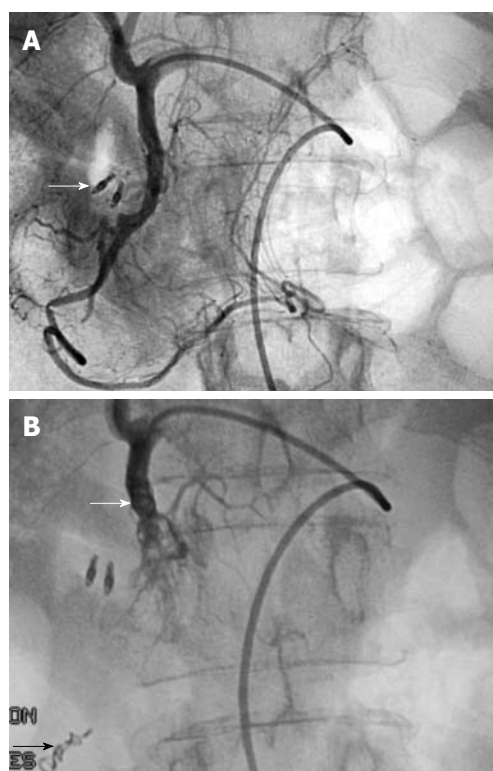
Given the variability in the way the results are reported and incomplete data on risk factors in the patient populations, we cannot draw conclusions regarding the impact of embolotherapy on mortality. Nevertheless,



**Figure 3** Digital subtraction images from a 37-year-old man with massive hematemesis. A, B: Selective angiography shows a bleeding ulcer in the fundus of the stomach. Extravasation of contrast medium from a branch of the left gastroepiploic artery is seen (arrows); C: The control angiogram after glue embolization throughout the splenic artery shows complete and selective occlusion of the bleeding branch, with no active bleeding. The patient was discharged from the hospital 4 d later.

several important points can be made. First, mortality and complication rates varied widely across the series, highlighting the influence of individual expertise and center volume on the outcomes. Second, the visualization of active extravasation followed by selective embolization was not consistently associated with a higher short-term clinical success rate. Possible explanations are the intermittent nature of gastrointestinal bleeding and the presence of bleeders missed by highly selective embolotherapy. Lastly, only 18% of the patients who initially underwent embolization finally needed surgery to control recurrent bleeding. Thus, embolotherapy considerably diminishes the need for laparotomy in patients with acute UGI bleeding from peptic ulcers.

A few of the most noteworthy case-series that raised



**Figure 4** Typical sandwich embolization in a 75-year-old woman with bleeding from a postbulbar duodenal ulcer at endoscopy. A: Angiography before embolization, guided by clip position (arrow): no evidence of active bleeding; B: Result after coil embolization of the distal and proximal GDA (with gelatine sponge in the arterial trunk), including the anterior and posterior superior pancreaticoduodenal arteries and the right gastropiploic artery, to prevent retrograde flow (arrows). No ischemic complications were reported.

interesting points are briefly summarized below. Ripoll *et al*<sup>[24]</sup> compared outcomes after embolization (31 patients) or surgery (39 patients) for bleeding from UGI peptic ulcers. Patients treated with embolotherapy were older (mean age, about 10 years older) and had higher rates of cardiovascular disease and anticoagulation treatment. Otherwise, co-morbidities were not different between the two groups. In the embolization group, the technical failure rate was 6.5% ( $n = 2$ ) and the re-bleeding rate was 29% ( $n = 9$ ). Four of the patients with persistent bleeding underwent surgical exploration. In the surgery group, the re-bleeding rate was 23.1% ( $n = 9$ ); five of these patients underwent a repeat surgical procedure for bleeding control. In addition, seven other patients required repeat surgery for complications from the initial operation. Overall, the embolization and surgery groups were not significantly different regarding the need for additional surgery (16.1% *vs* 30.8%, respectively) or survival (25.8% *vs* 20.5%, respectively). However, the re-intervention rate was considerably higher in the surgery group and the difference would perhaps have been statistically significant had the sample sizes been larger. Holme *et al*<sup>[25]</sup> reported on 40 consecutive patients who were referred for embolotherapy after unsuccessful endoscopic or surgical treatment. Long-term bleeding control was achieved in 26 (65%) patients. Of the 12 patients with active bleeding from a duodenal ulcer

**Table 2** Outcomes in case-series that included more than 10 patients treated with endovascular embolization for peptic ulcer bleeding over a 17-year period

Ref., yr	Clinical success (%)	Re-bleeding rate (%)	Need for surgery (%)	Complication rate (%)	30-d mortality (%)
Lang <i>et al</i> <sup>[23]</sup> , 1992	86	56	2	16	4
Toyoda <i>et al</i> <sup>[33]</sup> , 1995	91	18	18	0	27
Toyoda <i>et al</i> <sup>[37]</sup> , 1996	80	23	13	NA	23
Walsh <i>et al</i> <sup>[43]</sup> , 1999	52	52	37	4	40
De Wispelaere <i>et al</i> <sup>[40]</sup> , 2002	64	36	21	0	46
Ljungdahl <i>et al</i> <sup>[19]</sup> , 2002	67	8	8	0	6
Ripoll <i>et al</i> <sup>[24]</sup> , 2004	71	29	16	0	26
Holme <i>et al</i> <sup>[25]</sup> , 2006	65	28	35	0	25
Eriksson <i>et al</i> <sup>[20]</sup> , 2006	80	0	20	NA	NA
Loffroy <i>et al</i> <sup>[13]</sup> , 2008	94	17	14	6	21
Larssen <i>et al</i> <sup>[15]</sup> , 2008	72	9	30	8	17
van Vugt <i>et al</i> <sup>[35]</sup> , 2009	81	19	12	NA	38
Loffroy <i>et al</i> <sup>[26]</sup> , 2009	72	28	12	10	27
All studies	75	25	18	4	25

The table shows the rates of clinical success, recurrent bleeding after technically successful embolization, need for surgery to control the bleeding, complications, and peri-procedural mortality.

at the time of embolization, 10 (83%) had the bleeding controlled; one of these patients experienced re-bleeding, which was managed by surgery. In this subgroup, lasting hemostasis was achieved in eight (75%) patients. The 28 other patients had no signs of active bleeding on the diagnostic angiogram and underwent blind GDA coil embolization. Among them, 11 (39%) experienced re-bleeding. The inability to accurately identify and selectively embolize the culprit vessel in this subgroup is the most likely explanation for the high re-bleeding rate. The group with active bleeding on the angiogram had a higher likelihood of lasting hemostasis (75% *vs* 66%), underlining the limited effectiveness of blind coil embolization of the GDA. In this series, 10 patients died, including five as a result of continuous bleeding. Of note, this series included 13 patients who underwent duodenotomy for surgical bleeding control then experienced re-bleeding that was managed endovascularly. Eriksson *et al*<sup>[20]</sup> reported a series of 10 patients who were referred for embolotherapy after endoscopy failed to control bleeding from acute duodenal ulcers. To guide the endovascular treatment, a metallic clip was placed to mark the edge of the ulcer next to the bleeding site. Embolization ensured bleeding control in eight patients; the two remaining patients required surgery. In six patients, the clip played a crucial role in identifying the bleeding vessel,



as there was no evidence of contrast extravasation on the angiogram. The clip was particularly useful in three patients: two patients had bleeding from a supraduodenal artery without connection to the GDA, and the remaining patient had bleeding from an erosion in the inferior pancreaticoduodenal artery that arose from the superior mesenteric artery. One of the series with the best follow-up data was described by Lang *et al*<sup>[23]</sup>, who reported immediate and long-term results in 57 patients with bleeding duodenal ulcers. Control of bleeding was achieved in 52 of 57 patients. Superselective terminal muscular branch embolization was as effective as embolization of the main GDA. In eight of these patients, a second catheter-based intervention was needed to achieve complete bleeding cessation. Importantly, 29 of the 52 patients whose embolization procedure was successful experienced re-bleeding during follow-up (up to 7 years), underlining the need for aggressive long-term risk factor modification and treatment of the underlying peptic ulcer disease. Long-term bleeding control was more common in the subgroup of patients who underwent selective terminal muscular branch embolization, compared to the individuals treated with embolization of the main GDA trunk (53% *vs* 27%,  $P = 0.084$ ). Long-term success in this series was related to the embolic material used. For occlusion of the muscular branch arteries, 6-cyanoacrylate had the highest success rate, whereas for occlusion of the main GDA, an epsilon-aminocaproic acid-induced blood clot was superior over the other modalities (coils, gelatine sponge particles, or PVA particles). Together with re-bleeding, duodenal stenosis was the most troublesome complication and developed in nine patients between 8 mo and 7 years after the embolization procedure. This complication was more common after superselective embolization of terminal muscular branches. Surgical correction of the stenosis was necessary in eight patients to address persistent symptoms. Another patient required multiple balloon dilatations for duodenal stenosis. Balloon dilatations were performed in one additional patient for recurrent symptoms of duodenal obstruction after surgical resection. We reported our experience managing 60 patients with peptic ulcer bleeding<sup>[26]</sup>. The technical success rate was 95%. In 37% ( $n = 22$ ) of patients, the angiography showed no contrast extravasation and, therefore, empiric embolization was performed based on the endoscopic findings prior to the procedure. Approximately 28% ( $n = 16$ ) of these patients experienced re-bleeding, and only three underwent repeat embolization. Interestingly, the univariate analysis showed that early re-bleeding was associated with several of the study variables including coagulation disorders, a longer time from shock onset to angiography, a larger number of red-blood-cell units transfused before angiography, having two or more comorbid conditions, and being treated with coils as the only embolic agent. The multivariate analysis identified two factors that significantly predicted failure of embolization, namely, the presence of coagulation disorders ( $P = 0.027$ ) and the use of coils as the only embolic

agent ( $P = 0.022$ ). The mortality rate was not different in the patient group with clinically successful embolization and in the group with failed embolization (22% *vs* 37%)<sup>[26]</sup>.

## TOPICS OF INTEREST

### **Predictors of favourable outcome**

In surgical case-series, mortality rates in patients who have UGI bleeding from gastroduodenal ulcers and who do not respond to conservative therapy have ranged from 17% to 43%<sup>[27,28]</sup>. Factors influencing mortality include advanced age, trauma or sepsis, recent major operation, lung or liver disease, and massive blood transfusions<sup>[28,29]</sup>. After embolization in patients who are too sick to undergo surgery, mortality rates were similar, with a range of 10% to 45%. A number of factors have been identified as influencing post-embolization mortality. One of the most important and common factors is the absence of early re-bleeding, especially after selective embolization of a vessel with contrast extravasation on the initial angiography. Patients with angiographic extravasation and successful embolization have considerably lower mortality rates compared to patients who require surgery after failed embolization (38% *vs* 83%, respectively)<sup>[30]</sup>. Coagulopathy correlates closely with clinical failure and death after embolization. Thus, patients with impaired coagulation are three times more likely to re-bleed after initially successful embolization and 10 times more likely to die as a result of bleeding, compared to those with normal coagulation<sup>[2,12]</sup>. Rescue surgery after a failed embolization attempt has a very high mortality rate that exceeds even the 50% rate associated with emergent surgery<sup>[31,32]</sup>. In other series, underlying medical problems such as cirrhosis and malignancy, had major impacts on the mortality rate. Finally, in patients with multiorgan failure, clinically successful embolization appears to offer the only chance for survival. In the case-series reported by Schenker *et al*<sup>[22]</sup>, the mortality rate was 96% in patients with multiorgan failure who did not respond to embolization *vs* 31% in those who did.

### **Choice of embolic agent**

A focus of greater controversy is the influence of the type of embolic agent on the clinical outcome. There is general agreement that embolic therapy is superior over vasopressin infusion for the treatment of UGI bleeding from gastroduodenal ulcers<sup>[17]</sup>. The choice of the best embolic agent remains a matter of debate. Coils alone inserted into the GDA or super selectively in the pancreaticoduodenal arteries have been used successfully by several authors<sup>[33-35]</sup>. Lang *et al*<sup>[23]</sup> compared several embolic agents in a case-series of 57 patients. Safety and efficacy were best with autologous blood clot for proximal GDA embolization and with tissue adhesive for occlusion of the distal vessels from the GDA. These authors reported a 40% rate of duodenal stricture with tissue adhesives, a finding that may be related to the use of tissue adhesives to embolize the terminal muscular branches, and not to the nature of the embolization agent. The same group reported a high rate of re-bleeding

when PVA particles or gelfoam were used alone. Similarly, Encarnacion *et al*<sup>[2]</sup> obtained a low success rate (62%) in their case-series, which chiefly included patients embolized with gelatine sponge alone. Good results have also been reported with cyanoacrylate<sup>[36,37]</sup> and with the combination of gelatine sponge and coils<sup>[38]</sup>. Most of these series included small study populations; therefore, no statistical conclusions can be drawn. Finally, Aina *et al*<sup>[12]</sup> compared embolization with coils alone *vs* coils combined with PVA particles or gelfoam. By multivariate regression analysis, the use of coils alone was associated with re-bleeding in patients with coagulopathy, a finding that supports the use of PVA or gelfoam in combination with coils in this patient subgroup. We also found that using coils alone was significantly associated with early re-bleeding<sup>[26]</sup>. Otherwise, the nature of the embolic agent does not seem to affect the clinical response or re-bleeding rate.

### **Blind or empirical embolization**

Blind embolization, defined as embolization without angiographic proof of extravasation, is also controversial. In a study comparing several groups of patients, Dempsey *et al*<sup>[30]</sup> found that blind embolization was not helpful in achieving bleeding control. The proportion of patients who required surgery for bleeding control was similar in the patients without angiographic evidence of contrast extravasation who did not undergo embolization and in the patients who underwent blind embolization. However, endoscopy - a crucial procedure for selecting the target vessel for blind embolization - was non-diagnostic in 39% of patients in this case-series<sup>[30]</sup>. Massive bleeding is often intermittent<sup>[39]</sup>; therefore, most groups perform embolization based on the endoscopic findings, even when no extravasation is visible on the angiogram. In the case-series by Aina *et al*<sup>[12]</sup> and us<sup>[26]</sup>, outcomes were not different between patients who underwent blind embolization and those who underwent embolization after angiographic identification of a bleeding site. Other researchers also advocate endoscopy-directed blind embolization<sup>[2,33,40]</sup>. Based on the data in the literature and our own experience, we believe that blind embolization is appropriate. The GDA should be embolized using the "sandwich technique", in which both ends of the artery are filled with coils to avoid retrograde bleeding from the superior mesenteric circulation. If smaller muscular branches terminating at a clip are suspected culprits, then they should be embolized with any of the available materials.

### **Marking with a metallic clip**

Clip placement during endoscopy can help to localize the vessel feeding the bleeding ulcer, even when there is no contrast medium extravasation after injection with the catheter into the common hepatic artery or the main trunk of the GDA. Clip placement is also helpful when the bleeding artery arises separately from the proper hepatic artery or the GDA. Superselective angiography guided by clip position is more likely to visualize the extravasation, thus making blind coil

placement unnecessary, increasing the efficacy of the procedure, and decreasing the risk of coil misplacement and inadvertent hepatic embolization<sup>[20,26]</sup>. The only limitation of this technique is the need for around-the-clock availability of an experienced interventionalist and gastroenterologist, which is easy to achieve only at high-volume centres. This approach increases the likelihood of successful embolization and is now routinely used at our centre. Even when extravasation is not visualized, the clips can guide the blind embolization procedure, as pointed out previously.

### **Risk for gastrointestinal tract necrosis**

Arterial embolization in the UGI tract above the ligament of Treitz is generally considered very safe because of the rich collateral supply to the stomach and duodenum. However, the risk of significant ischemia after embolization is increased in patients with a history of surgery in the same area<sup>[41]</sup> or with embolic agents that can advance far into the vascular bed such as liquid agents (e.g. tissue adhesives such as cyanoacrylate) or very small particles (e.g. gelatine sponge powder)<sup>[41-44]</sup>. Although cases have been reported at the acute phase, post-embolization ischemia usually presents as duodenal stenosis at the chronic phase. Lang *et al*<sup>[23]</sup> reported duodenal stenosis in seven of 28 patients after embolization of terminal vessels, mostly when tissue adhesive was used. In this series, duodenal stenosis after GDA embolization was far less common and occurred in only two of 29 patients who underwent more proximal GDA occlusion. No major gastric or duodenal ischemic events occurred in our case-series; coils were the most often used single embolic agents, and gelatine sponge plugs were used instead of powder. A tissue adhesive was used only when angiographic extravasation was considered massive, and one part of cyanoacrylate was then diluted in two parts of lipiodol to ensure rapid polymerization. In addition, the mixture was injected selectively into the bleeding vessel while taking care not to fill the normal branches<sup>[12]</sup>.

### **Angiographic embolization vs surgery**

To date, there has been no controlled trial comparing angiographic embolization to surgery as a salvage procedure for failed endoscopic therapy. Two retrospective comparisons showed at least similar efficacy in terms of rates of re-bleeding, morbidity, and mortality. Ripoll *et al*<sup>[24]</sup> retrospectively assessed the outcomes of 70 patients with refractory peptic ulcer bleeding: 31 patients underwent angiographic embolization, and 39 patients were managed with surgery. Although the patients treated with angiographic embolization were 10 years older on average and more often had heart disease, there were no major differences in the rates of re-bleeding (29% *vs* 23%) or mortality (26% *vs* 21%). Another retrospective comparison, by Eriksson *et al*<sup>[45]</sup>, included 40 patients who underwent angiographic embolization and 51 patients who underwent surgery after failed endoscopic therapy. The angiographic embolization group was older and had a higher co-morbidity rate. Nevertheless, 30-d mortality

was lower in the angiographic embolization group (3% vs 14%). These results are promising, and we are eagerly awaiting the results of randomized, controlled trials.

## CONCLUSION

Massive bleeding from a peptic ulcer remains a challenge. Optimal management required a multidisciplinary team of skilled endoscopists, intensivists, experienced UGI surgeons, and interventional radiologists. Endoscopy is the first-line treatment. The role for early elective surgery or angiographic embolization in selected high-risk patients to prevent re-bleeding remains controversial. However, technological advances including lower-profile catheter systems will probably broaden the indications for endovascular treatment of UGI bleeding from gastroduodenal ulcers after failed endoscopy. Although prospective studies are needed to compare these management strategies, the available data suggest that transcatheter arterial embolization is not only a good alternative to surgery, but should now be considered the salvage treatment of choice after failed endoscopic treatment. However, only high volume centers, with experienced and skillful interventional radiologists, have the opportunity to use this technique as an alternative treatment.

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EDITORIAL

## Invasive front of colorectal cancer: Dynamic interface of pro-/anti-tumor factors

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### Abstract

Tumor-host interaction at the invasive front of colorectal cancer represents a critical interface encompassing a dynamic process of de-differentiation of colorectal carcinoma cells known as epithelial mesenchymal transition (EMT). EMT can be identified histologically by the presence of "tumor budding", a feature which can be highly specific for tumors showing an infiltrating tumor growth pattern. Importantly, tumor budding and tumor border configuration have generated considerable interest as additional prognostic factors and are also recognized as such by the International Union Against Cancer. Evidence seems to suggest that the presence of tumor budding or an infiltrating growth pattern is inversely correlated with the presence of immune and inflammatory responses at the invasive tumor front. In fact, several tumor-associated antigens such as CD3, CD4, CD8, CD20, Granzyme B, FOXP3 and other immunological or inflammatory cell types have been identified as potentially prognostic in patients with this disease. Evidence seems to suggest that the balance between pro-tumor (including budding and infiltrating growth pattern) and anti-tumor (immune response or certain inflammatory cell types) factors at the invasive front of colorectal cancer may be decisive in determining tumor progression and the clinical outcome of patients with colorectal cancer. On one hand, the infiltrating tumor border configuration and tumor budding promote progression and dissemination of tumor cells by penetrating the vascular and lymphatic vessels. On the other, the host attempts to fend off this attack by mounting an immune response to protect vascular and lymphatic channels from invasion by tumor buds. Whereas standard pathology reporting of breast and prostate cancer involves addi-

tional prognostic features, such as the BRE and Gleason scores, the ratio of pro- and anti-tumor factors could be a promising approach for the future development of a prognostic score for patients with colorectal cancer which could complement tumor node metastasis staging to improve the clinical management of patients with this disease.

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**Key words:** Colorectal cancer; Prognosis; Tumor invasive front; Tumor budding; Tumor growth pattern; Tumor infiltrating lymphocytes; Tumor immunity; Microsatellite instability

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### INTRODUCTION

The tumor node metastasis (TNM) staging system from the American Joint Committee on Cancer/International Union Against Cancer (UICC) remains the most reliable prognostic indicator for patients with colorectal cancer<sup>[1]</sup>. Overall 5-year survival rates are reported at 65% and correspond closely to disease progression; patients with stage I disease have more favourable prognoses with 5-year survival rates exceeding 80%-90%. In contrast, patients with stage II, III and IV disease experience progressively worse outcomes with varying 5-year survival rates of 70%-85%, 44%-80% and < 10%, respectively<sup>[2]</sup>. It is recognized, however, that patients with tumors of the same TNM stage may be variable both in terms of prognosis and response to therapy.

A range of other histomorphological, molecular and protein biomarkers have additionally been investigated for their prognostic value independently of TNM stage. These tumor-related factors such as venous and lymphatic invasion, tumor grade, perineural invasion, histological

type, loss of heterozygosity at 18q, mutation in p53, tumor expression of vascular endothelial growth factor and thymidylate synthase are recognized as essential, additional or new and promising prognostic factors by the UICC<sup>[3,4]</sup>. In particular, microsatellite instability (MSI) status has revealed itself not only as a significant prognostic factor but also as an attribute categorizing colorectal carcinogenesis into two major pathways: the chromosomal instability (or microsatellite stable; MSS) and MSI pathways, the latter including both sporadic and hereditary Lynch syndrome [Hereditary non-polyposis colorectal cancer (HNPCC)] patients both demonstrating mismatch repair deficiencies and high level MSI (MSI-H)<sup>[5]</sup>.

Tumor-host interaction at the invasive front of colorectal cancer represents a critical interface where tumor progression and tumor cell dissemination ensue. The invasive tumor front encompasses a dynamic process of de-differentiation of colorectal carcinoma cells, a process known as epithelial mesenchymal transition (EMT)<sup>[6]</sup>. EMT can be identified histologically by the presence of “tumor budding”, a feature which is specific for tumors showing an infiltrating growth pattern<sup>[7]</sup>. Importantly, tumor budding and tumor border configuration have generated considerable interest as additional prognostic factors and are also recognized as such by the UICC<sup>[3,4]</sup>. Evidence seems to suggest that the presence of tumor budding or an infiltrating growth pattern is inversely correlated with the presence of immune and inflammatory responses at the invasive tumor front<sup>[8,9]</sup>. In fact, several tumor-associated antigens such as CD3, CD4, CD8, CD20, Granzyme B, FOXP3 and other immunological or inflammatory cell types have been identified as potentially prognostic in patients with this disease<sup>[10-16]</sup>.

Together, evidence seems to suggest that the balance between pro-tumor (including budding and infiltrating growth pattern) and anti-tumor (immune response or certain inflammatory cell types) factors at the invasive front of colorectal cancer may be decisive in determining tumor progression and the clinical outcome of patients with colorectal cancer.

The aim of this review is to outline the evidence supporting a pro-/anti-tumor factor model of colorectal cancer progression, one which highlights the dynamic interface between tumor and host-related factors at the invasive front of colorectal cancer.

## THE INVASIVE FRONT OF COLORECTAL CANCER: PRO-TUMOR FACTORS

### *Tumor budding-migrating cancer stem cells (CSCs)?*

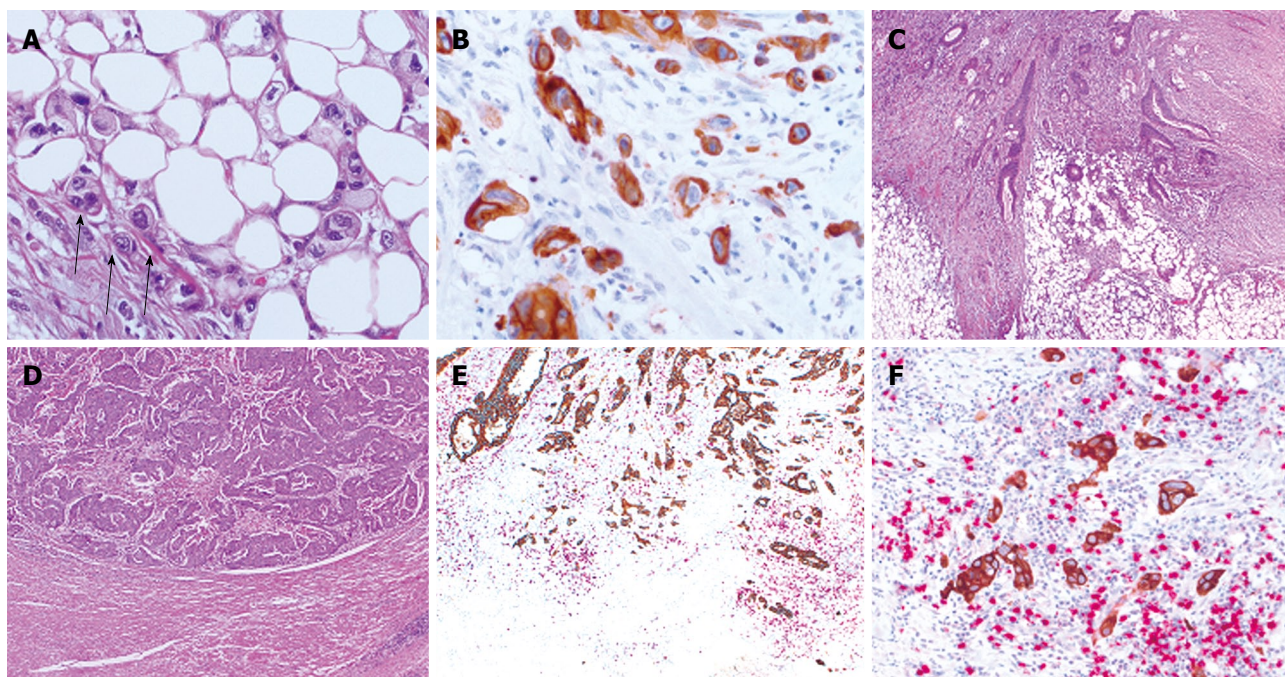
In 1985, a study of colonic adenocarcinoma in rats reported a peculiar feature at the invasive border of differentiated tumors<sup>[17,18]</sup>. Using both light and electron microscopy, Gabbert *et al.*<sup>[18]</sup> observed neoplastic glands irregularly arranged into small strands and cords. In addition, they noted discontinuous small aggregates or single tumor cells which ultrastructurally did not possess junctional complexes, often had incomplete desmosomes,

missing or rudimentary basement membranes and absent or incomplete brush borders. They determined that at the invasive tumor front of colorectal cancer, differentiated tumors could acquire an undifferentiated phenotype. Their observation is credited for pioneering the concept known as de-differentiation at the invasive margin, which today is commonly referred to as “tumor budding”.

Tumor budding is a histological feature diagnosed at high magnification and is defined as single cells or clusters of up to four or five cells at the invasive tumor front<sup>[19]</sup>. Budding cells can be spotted using standard HE-stained tissue slides and their visualization is further facilitated using pan-cytokeratin stains (Figure 1A and B)<sup>[7]</sup>. The process of tumor budding is likened to EMT observed during gastrulation in embryonic development<sup>[7]</sup>. In the early phase of EMT, epithelial cells reduce intercellular contacts and cell-matrix contacts and reorganize the cytoskeleton to form cell membrane ruffles (lamellipodia) or cytoplasmic protrusions. Migration ensues and new cell-matrix contacts are formed providing cells with an anchorage for the contraction of the cell body. In colorectal cancer, tumor budding is a highly dynamic process giving temporal heterogeneity to the tumor.

Budding cells have been credited with the properties of malignant stem cells including the potential for redifferentiation both locally and at sites of distant metastasis and marking, what appears to be, the first histological event in tumor cell migration and invasion. Supporting this hypothesis further is the presence of “pseudopodia-like” cytoplasmic protrusions in tumor buds which have been identified by both electron microscopy and recently by immunohistochemistry with pan-cytokeratins<sup>[17,20]</sup>. These podia appear to be in direct contact with the adjacent interstitial tissue suggesting their formation occurs during tumor cell migration. Moreover, Shinto *et al.*<sup>[21]</sup> recently suggested that cytoplasmic pseudo-fragments could be used as a marker for an activated budding phenotype that is associated with cell motility and increased invasiveness independent of the extent of budding. Not surprisingly, tumor buds have been shown to over-express proteins involved in extracellular matrix degradation and to under-express adhesion molecules. Previous studies on EMT and events occurring at the invasive tumor front implicate, in particular, the Wntless-INT (WNT) signaling pathway in the process of tumor budding evidenced by increased  $\beta$ -catenin immunohistochemical staining in tumor buds, a concomitant loss of E-cadherin and over-expression of laminin5 $\gamma$ 2 along with activation of transcriptional repressors SLUG, and ZEB1<sup>[22,23]</sup>. Over-expression of urokinase plasminogen activator receptor (uPAR), matrix metalloproteinase-7 and -9 (MMP7, MMP9), matrilysin, CD44, Ep-CAM, and extensive staining of  $\beta$ (III)-tubulin, a major constituent of microtubules, have all been reported<sup>[20,23-30]</sup> suggestive of the invasion and migration potential of tumor buds. Tumor buds seem to over-express CXCL12, a stromal cell-derived factor whose receptor CXCR4 is involved in chemotaxis and angiogenesis<sup>[31]</sup>. In addition, we recently documented the over-





**Figure 1** The invasive front of colorectal cancer. A: HE staining of colorectal cancer ( $\times 40$  magnification) showing tumor buds (arrows) at the invasive front; B: Pan-cytokeratin staining (CK22) highlighting tumor buds at the invasive front of colorectal cancer ( $\times 40$  magnification). Colorectal cancers with different tumor border configurations upon evaluation of HE staining at low magnification ( $\times 5$ ); C: Infiltrating tumor border configuration; D: Pushing tumor border configuration; E: Low ( $\times 5$ ); F: High ( $\times 20$ ) power magnification of double immunostaining for pan-cytokeratin (CK22) and anti-CD8 antibody highlighting "attackers" (tumor buds, brown) and "defenders" (CD8+ T-lymphocytes, red) at the invasive front of a colorectal cancer with infiltrating tumor border configuration.

expression of the putative colorectal CSC marker ABCG5 within tumor buds leading to a poorer outcome of patients including those with node-negative disease (Hostettler, *World J Gastroenterol*, in press). Whether a sub-population of tumor buds may in fact represent malignant stem cells is still an open question which necessitates further investigation.

### Prognostic impact of tumor budding

Since tumor budding appears to play a critical role in the initiation of metastasis, several authors have investigated the potential of this feature to predict dissemination of tumor cells to regional lymph nodes. A significant association between tumor budding and lymph node positivity has been consistently demonstrated correlating with tumor aggressiveness and more advanced TNM stage<sup>[32-43]</sup>. Tumor budding is frequently associated with poorly differentiated tumors, and with the presence of vascular and lymphatic invasion independently of disease extent<sup>[44-48]</sup>. Local tumor recurrence and distant metastasis to the lung and liver are also more commonly observed in patients with tumor budding<sup>[36,39,48-50]</sup> and additionally represent a reproducible prognostic factor in stage II patients<sup>[51]</sup>. Recently, Suzuki *et al*<sup>[52]</sup> found that tumor budding and venous invasion were significant predictors of local and distant metastases in patients with T1 stage colorectal cancers. Xu *et al*<sup>[53]</sup> demonstrated an increased rate of tumor budding in colorectal carcinomas with the aggressive micropapillary component. The presence of tumor budding has repeatedly been linked to poor clinical outcome, underlined by the adverse effect on overall survival independently of TNM stage<sup>[47,51,54]</sup>.

### Tumor growth pattern and prognosis

Tumor budding is closely linked to tumor growth pattern, a feature described by Jass *et al*<sup>[55]</sup> in 1987 which led to the proposal of an alternative prognostic classification system for rectal cancers<sup>[55,56]</sup>. The diagnosis of either a pushing (or expanding) or infiltrating tumor border configuration can be made at low magnification and is reproducible among pathologists thereby underlining its usefulness as a prognostic indicator (Figure 1C and D)<sup>[7]</sup>. The pushing tumor border is one in which margins are reasonably well-circumscribed and often associated with a well-developed inflammatory lamina. In contrast, the infiltrative tumor border is characterized by widespread dissection of normal tissue structures with loss of a clear boundary between tumor and host tissues.

Several studies have confirmed that an infiltrative tumor border configuration has a significant adverse prognostic impact in colorectal cancer and may predict local recurrence<sup>[57,58]</sup>. Our study group has also recently provided evidence for the improved stratification of stage II colorectal cancer patients based on the diagnosis of tumor border configuration. In particular, the 5-year survival rates for patients with stage II tumors decreased substantially from 80% in those with a pushing margin to 62.7% in patients with an infiltrating growth pattern, a survival rate similarly found in patients with stage III disease<sup>[59]</sup>. Considering that patients with stage III tumors are generally considered for adjuvant therapy<sup>[60]</sup>, the implications of these findings suggest that stage II patients with an infiltrating tumor margin should perhaps be considered for post-operative therapy. The addition of tumor border configuration to TNM stage improved

the prognostic classification of colorectal cancer patients by 17.9%. Since the presence of tumor budding can be strongly specific for an infiltrating, rather than a pushing/expanding growth pattern, it is not surprising that loss of cell-adhesion molecule E-cadherin and apoptosis activating factor-1, a pro-apoptotic molecule and over-expression of uPA and uPAR have all been reported as significant predictors of an infiltrating tumor border configuration in colorectal cancer<sup>[9,61]</sup>.

## THE INVASIVE FRONT OF COLORECTAL CANCER: ANTI-TUMOR FACTORS

Immunotherapy for patients with colorectal cancer represents a realistic alternative approach to treatment of this disease<sup>[62-64]</sup>. The last 20 years has seen a wide range of publications on tumor immunity and the prognostic impact of immune and inflammatory cell types in the microenvironment of colorectal tumors demonstrating promising results both *in vitro* and *in vivo*.

### Peritumoral inflammation

The presence of conspicuous peritumoral lymphocytic (PTL) inflammation, viewed as a distinctive “encapsulating” connective tissue mantle cap at the invasive front of colorectal cancer, is inversely correlated with the presence of tumor budding and positively associated with improved survival<sup>[19,65,66]</sup>. Jass<sup>[8]</sup> demonstrated that PTL infiltration in rectal cancer decreased with more advanced Dukes’ stage to 53%, 28% and 13% with Dukes’ A, B and C cases, respectively. In addition, the significantly worsened prognosis in patients lacking PTL inflammation at the tumor border was highlighted, while patients with moderate or pronounced infiltration performed significantly better independently of disease stage. The results have also been confirmed by other study groups<sup>[67]</sup>. However, the presence of PTL inflammation at the invasive front does not appear to be an independent prognostic factor in patients with this disease<sup>[59]</sup>. Nonetheless, PTL inflammation seems to be intimately linked with abundant CD8+ tumor infiltrating T-lymphocytes, further implicating tumor immunity in the defense against colorectal cancer.

### T-lymphocytes

Most studies to date confirm that a high rate of tumor infiltrating lymphocytes (TILs), in particular those located intra-epithelially characterized by CD4+ and CD8+ tumor-associated antigens are beneficial for patient outcome<sup>[68,69]</sup>. An abundant TIL count appears to be linked to earlier Dukes’ stage, decreased local recurrence rate following curative surgery and improved overall and disease-free survival time both in non-metastatic and metastatic patients undergoing hepatic resection<sup>[10,68-73]</sup>.

Galon *et al.*<sup>[13]</sup> evaluated by gene expression profiling and immunohistochemistry, the type, density and location (whether at the invasive margin or the tumor centre) of TILs in a large number of cases. They evaluated CD3, CD8, granzyme B and memory CD45RO T cells

and demonstrated a significant independent and positive effect of TILs on both recurrence and survival. Pages *et al.*<sup>[15]</sup> performed a comprehensive analysis of TILs focusing on early metastatic invasion. They found, by RT-PCR on 75 cases that mRNA levels of CD8, granzyme B and granzyme B and granzyme B were significantly greater in patients without vascular emboli, lymphatic and perineural invasion (collectively known as VELIPI) compared to those with these features and that CD45RO+ cells had independent prognostic value<sup>[15]</sup>. Diederichsen *et al.*<sup>[74]</sup> showed that a low CD4/CD8 ratio by flow cytometry was an independent prognostic factor for prolonged survival. In addition, Milasien *et al.*<sup>[75]</sup> evaluated inter-epithelial CD3, CD4, CD8, CD20 and CD16 and found that increased levels of all these markers, particularly of the natural killer cell marker CD16 led to significantly improved overall outcome<sup>[11,75]</sup>. Moreover, regulatory T-cells expressing FOXP3+ has been shown to correlate with improved outcome independently of TNM stage<sup>[16,76]</sup>.

### Macrophages, mast cells, neutrophils and dendritic cells

In addition to T cells in colorectal cancer, a growing number of studies have demonstrated the clinical impact of dendritic cells, mast cells, macrophages and neutrophils on survival. An improved survival time and a preventative effect of mast cells on local recurrence and distant metastasis in patients with rectal tumors with high mast cell counts have been identified<sup>[77-79]</sup>. Further, the significant benefit of mast cell number on tumor progression in colorectal cancer was highlighted by Gounaris *et al.*<sup>[80]</sup> who reported that depletion of mast cells whether by pharmacological means or through generation of chimeric mice with genetic lesions in mast cell development led to remission of existing polyps. Moreover, Halazun *et al.*<sup>[81]</sup> found that an elevated neutrophil/lymphocyte ratio led to a poorer survival time and higher rate of recurrence in colorectal cancer patients undergoing surgery for liver metastasis.

Dendritic cells are the most potent antigen-presenting cells and as such are now one of the many important tools for tumor immunotherapy. Evidence is accumulating which suggests that the presence of dendritic cells may be of significant benefit to patients with colorectal cancer<sup>[82]</sup>. Using immunohistochemistry for CD83, Suzuki *et al.*<sup>[83]</sup> described the presence of mature dendritic cells at the invasive margin of cancer stroma and demonstrated by light and electron microscopy their formation into clusters with lymphocytes, the majority of which were CD45RO+ T cells. They conclude that mature CD83+ dendritic cells at the invasive margin promote T-cell activation for the generation of tumor specific immunity. Using electron microscopy, tumor-infiltrating dendritic cells were found to make contacts among themselves, with TILs and tumor cells. The presence of dendritic cells was found predominantly in early compared to later disease stages and mostly located in tumor surrounding tissue<sup>[84]</sup>. Dadabayev *et al.*<sup>[12]</sup> demonstrated that dendritic cells were significantly correlated with intra-epithelial CD4+ and CD8+ lymphocytes. Recently, HLA-DR



expressed constitutively on antigen-presenting cells such as dendritic cells and macrophages has also been found to correlate with the presence of TILs and PTLs as well as improved patient outcome<sup>[85]</sup>.

## THE INVASIVE FRONT OF COLORECTAL CANCER: MSI

Works by Banerjee *et al*<sup>[86]</sup> clearly show that MSI status (MSS; sporadic MSI-H and hereditary Lynch syndrome-associated colorectal cancers) should be taken into consideration when discussing tumor immunity in colorectal cancer. Compared to MSS tumors, both sporadic and hereditary MSI-H cancers from patients with Lynch syndrome (hereditary non-polyposis coli; HNPCC) are characterized by prolonged survival time, significantly more frequent PTL inflammation at the invasive front and by an inherent abundance of intra-epithelial TILs<sup>[87-94]</sup>. In contrast to MSS tumors which primarily arise following disruption of WNT signalling, sporadic MSI-H tumors are linked to mutations in TGF $\beta$ RII<sup>[95,96]</sup>. Baker *et al*<sup>[97]</sup> hypothesized that retention of TILs in MSI-H cancers may be a consequence of refractoriness to normal TGF- $\beta$  signalling. In a subsequent study, these authors show in more than 1000 MSS and 223 MSI tumors that an abundant CD8+ TIL infiltrate has a beneficial effect on survival time in MSS, but not MSI cancers<sup>[71]</sup>. Other authors confirm the abundance of CD8+ TILs and granzyme-positive cells as well as improved clinical outcome in patients with MSI-H compared to MSS colorectal cancers<sup>[98-100]</sup>. In addition, a positive correlation between apoptosis rates and higher TIL number has been described, a finding which could perhaps help to explain the improved prognosis seen in patients with MSI-H compared to MSS tumors<sup>[98,101]</sup>. Studies on T-reg cells such as FOXP3 and CD25+ have recently been undertaken<sup>[102]</sup>. Drescher *et al*<sup>[102]</sup>, evaluating both MSS and MSI-H cancers found that in contrast to CD8+ T-cells which may be involved in actively preventing growth and/or metastatic in MSI-H tumors, CD25+ cell counts were similar between MSS and MSI-H tumors suggesting no active immunosuppressive mechanisms in MSS cancers. Finally, the upregulation in MSI-H cancers of a large number of genes involved in immune response and increased levels of pro-inflammatory cytokines and cytotoxic mediators indicate an activated anti-tumor immune response in these tumors<sup>[86]</sup>.

## THE INVASIVE FRONT OF COLORECTAL CANCER: A BALANCE OF PRO- AND ANTI-TUMOR FACTORS

Several observations have led to the hypothesis that tumor progression and prognosis in patients with colorectal cancer is not based solely on the presence of pro-tumor or absence of anti-tumor factors but rather on the balance between the two. First, the presence of

tumor buds is inversely correlated with the presence of PTL inflammation and intra-epithelial CD8+ TILs<sup>[9,21]</sup>. In MSI-H cancers, where intra-epithelial TILs are abundant, PTL inflammation “encapsulating” the tumor at the invasive front and pushing tumor border are commonly seen, tumor budding is virtually absent<sup>[20]</sup>. When it occurs, tumor budding in the MSI-H lacks the full budding immunophenotype and the cytoplasmic podia which give budding cells a temporal dimension<sup>[20]</sup>. In a previous study on MSS colorectal cancers, we hypothesized that an intense peritumoral inflammatory reaction at the invasive front could be “nipping colorectal cancer in the bud” by specifically targeting budding cells for destruction<sup>[9]</sup>. We recently investigated CD8+ lymphocytes directly positioned in the microenvironment of the tumor buds. We could demonstrate that the ratio of CD8+ lymphocytes to tumor buds (CD8+/tumor budding index) out-performed both tumor budding or CD8+ lymphocytes alone as independent prognostic factors in two independent cohorts<sup>[103]</sup>. Using double immunostaining for CD8+ antibody and pan-cytokeratin, a ratio of 3:1 for CD8+ lymphocytes to tumor buds was a highly favourable phenotypic constellation associated with earlier pT stage, lymph node negativity, low tumor grade and absence of vascular and lymphatic invasion in addition to conferring a prolonged clinical outcome in both stage II and stage III colorectal cancer (Figure 1E and F). Although we cannot allude to the direct functional interaction between CD8+ lymphocytes and tumor buds themselves, the strong circumstantial relationship between the ratio of tumor budding and CD8+ lymphocytes in the microenvironment of budding cells appears, nonetheless, to be a reproducible and independent prognostic factor in colorectal cancer.

## DISCUSSION

The invasive front of colorectal cancer represents a dynamic interface between pro- and anti-tumor factors, which can be visualized as a balance between “attackers” (pro-tumor) and “defenders” (anti-tumor). On the one hand, the infiltrating tumor border configuration and its “cavalry” tumor budding promote progression and dissemination of tumor cells by penetrating the vascular and lymphatic vessels. On the other, the host attempts to fend off this attack by mounting an immune response using its “infantry”, in particular cytotoxic T lymphocytes, to protect vascular and lymphatic channels from invasion by tumor buds. Although evidence shows that both pro- and anti-tumor factors contribute prognostic information in a TNM-independent manner, the ratio of attackers and defenders may better capture the interaction at the invasive front which could translate into more powerful prognostic indicators.

Whereas standard pathology reporting of breast and prostate cancer involves additional prognostic features, such as the BRE and Gleason scores, the ratio of attackers/defenders could be a promising approach for the future development of a prognostic score for patients

with colorectal cancer which could complement TNM stage to improve the clinical management of patients with this disease.

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## Colorectal cancer screening in Europe

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### Abstract

Colorectal cancer (CRC) is the second most frequent malignant disease in Europe. Every year, 412 000 people are diagnosed with this condition, and 207 000 patients die of it. In 2003, recommendations for screening programs were issued by the Council of the European Union (EU), and these currently serve as the basis for the preparation of European guidelines for CRC screening. The manner in which CRC screening is carried out varies significantly from country to country within the EU, both in terms of organization and the screening test chosen. A screening program of one sort or another has been implemented in 19 of 27 EU countries. The most frequently applied method is testing stool for occult bleeding (fecal occult blood test, FOBT). In recent years, a screening colonoscopy has been introduced, either as the only method (Poland) or the method of choice (Germany, Czech Republic).

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**Key words:** Colorectal cancer; Europe; Fecal occult blood test; Screening colonoscopy; Screening programs

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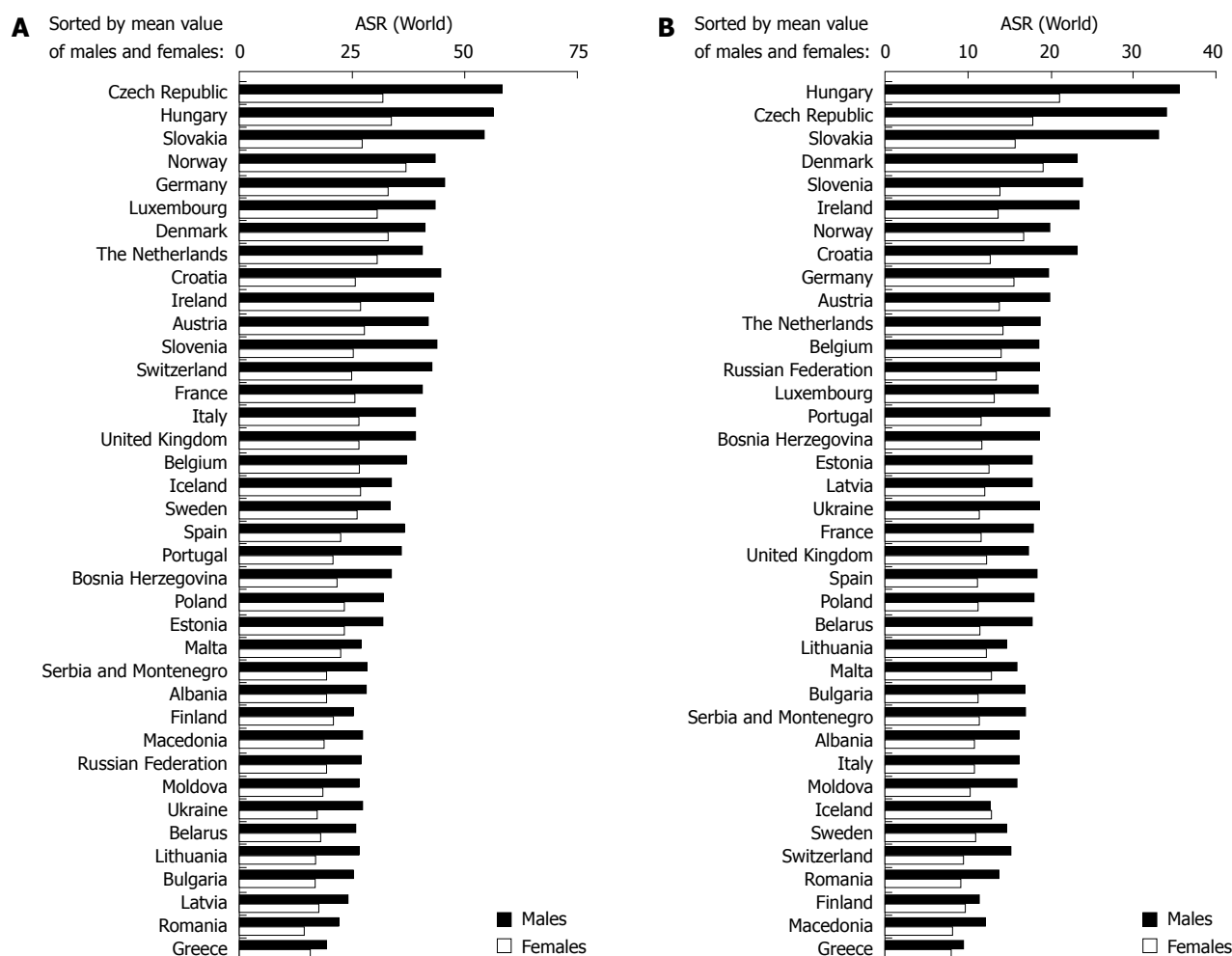
Zavoral M, Suchanek S, Zavada F, Dusek L, Muzik J, Seifert B, Fric P. Colorectal cancer screening in Europe. *World J Gastroenterol* 2009; 15(47): 5907-5915 Available from: URL: <http://www.wjgnet.com/1007-9327/15/5907.asp> DOI: <http://dx.doi.org/10.3748/wjg.15.5907>

### INTRODUCTION

Colorectal cancer (CRC) poses a serious health problem in countries with a Westernized lifestyle. Over the last decade, a whole range of new technologies have been introduced in clinical practice to diagnose and treat the disease, with therapeutic modalities extending to advanced stages of the disease. Nevertheless, prevention undoubtedly remains the key to reducing morbidity and mortality. The introduction of national or transnational population-wide screening programs is a priority for the healthcare policy of individual states, and this is also being addressed at the highest level by European Union (EU) administrators. The approach of individual countries to screening programs varies significantly because of differences in health insurance systems and budgets. This summary article focuses on a brief description and comparison of these programs.

### EPIDEMIOLOGY

CRC is the second most frequent malignant disease in developed countries. The incidence of CRC is generally higher for men, and the risk of the disease increases with age, as the majority of cases are diagnosed in patients more than 50 years of age<sup>[1]</sup>. European countries rank highest in the global statistics, both in terms of incidence and mortality. In 1998 to 2002, the incidence of CRC in the USA for men and women was 38.6 and 28.3, respectively; in Europe, it was 38.5 and 24.6 [world age standardization (ASR-W)], as calculated per 100 000 inhabitants<sup>[2]</sup>. However, mortality over the same period of time was much higher in Europe than in the US, both for men and women: in the USA, the figures were 13.5 and 9.2, respectively, while in Europe, they were 18.5 and 10.7 (ASR-W), as calculated per 100 000 inhabitants<sup>[3]</sup>. A detailed comparison of data for European countries is made difficult because of the absence of a unified data



**Figure 1 Epidemiology of colorectal cancer in European countries.** A: Incidence in international comparison-European countries; B: Mortality in international comparison-European countries. Adapted from: Ferlay J, Bray F, Pisani P, Parkin DM. GLOBOCAN 2002: Cancer incidence, mortality and prevalence worldwide. IARC Cancer Base No. 5 version 2.0. Lyon: IARC press, 2004. Available from: URL: <http://www-dep.iarc.fr/>, section C15 I-VIII (Detailed). Last accessed on August 8, 2009.

**Table 1 Colorectal cancer incidence in European countries in 2006**

Parameter	Incidence
Countries with the highest incidence	> 70/100 000 men (ASR-E): Hungary (106), Czech Republic (94.4), Slovakia (87.1), Switzerland (79.1), Germany (70.2)
	> 45/100 000 women (ASR-E): Switzerland (55.6), Norway (51.2), Hungary (50.6), Denmark (48), Czech Republic (46), Germany (45.1)
Countries with the lowest incidence	< 40/100 000 men (ASR-E): Albania (13.6), Greece (31), Bosnia Herzegovina (34.6), Republic of Moldova (38.7), Finland (39.2)
	< 26/100 000 women (ASR-E): Greece (21.3), Albania (21.4), Romania (25.1), Spain (25.4)

Adapted from: Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. *Ann Oncol* 2007; 18: 581-592. ASR-E: European age standard.

source. Not all countries maintain sophisticated population and cancer registers, and it is sometimes necessary to obtain input data by projecting aggregated data. In this outline, figures available from international studies summarizing global and European epidemiologic data have been used<sup>[4,5]</sup>. A detailed comparison of countries within Europe using the ASR-W of incidence and mortality is presented in Figure 1. Most recent epidemiologic data on CRC for 2006 recalculated to the European age standard are given in Tables 1 and 2.

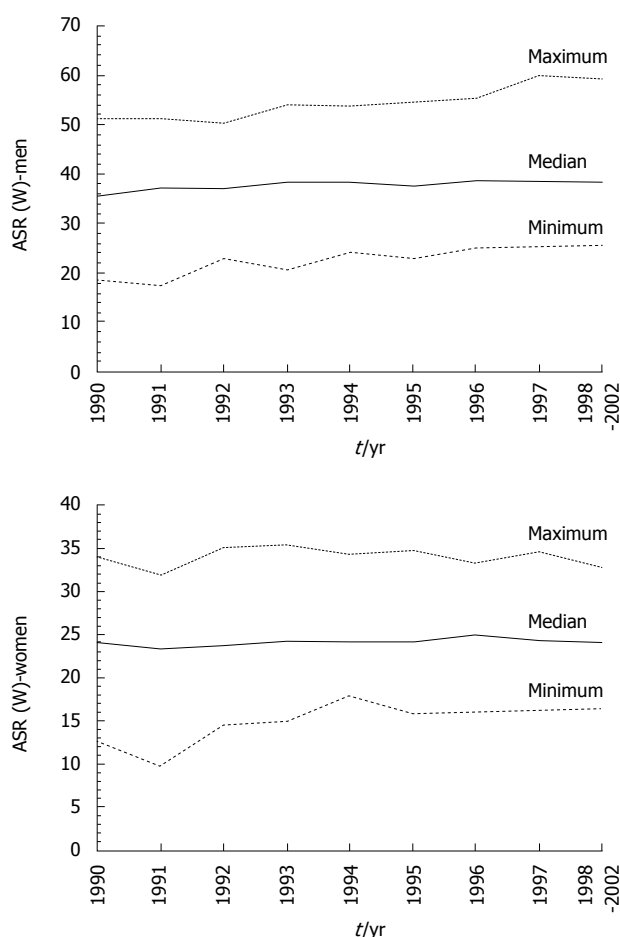
CRC comprises 12.9% of all newly-diagnosed carcinomas in the European population (men 12.8%, women 13.1%) and account for 12.2% of deaths caused by malig-

nancy. CRC is the second most frequent malignancy, after breast carcinoma (13.5% of all malignancies) and bronchogenic carcinoma (12.1% of all malignancies). It has been estimated that in 2006, 412 000 people were diagnosed with CRC in Europe, and 207 400 of them die of the disease<sup>[6]</sup>. The average incidence has shown a tendency to increase in recent years (2001-2005), with a year-on-year growth of 0.5%. Available data on time trends of CRC incidence and mortality are shown in Figures 2 and 3. A detailed analysis of individual diagnoses confirms that malignant disease of the colon is the most frequent, accounting for 57% of all cases (> 35 cases/10<sup>5</sup> inhabitants), followed by malignant diseases of the rectum

Table 2 Colorectal cancer mortality in European countries in 2006

Parameter	Mortality
Countries with the highest mortality	> 40/100 000 men (ASR-E): Hungary (54.4), Czech Republic (51), Slovakia (43.3), Croatia (40.7)
	> 20/100 000 women (ASR-E): Hungary (26.7), Slovakia (24.4), Czech Republic (24.1), Denmark (24.1), Norway (21.4)
Countries with the lowest mortality	< 20/100 000 men (ASR-E): Albania (7.3), Greece (15.5), Finland (17.9), Switzerland (19.1), Cyprus (19.3), Bosnia Herzegovina (19.5)
	< 12/100 000 women (ASR-E): Albania (9.9), Greece (10.8), Finland (11.3), Switzerland (11.6)

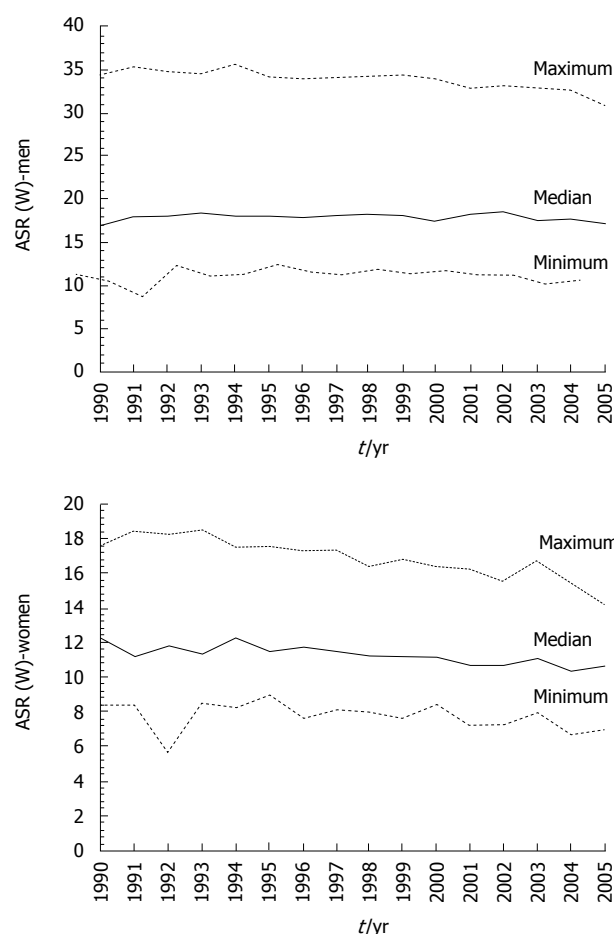
Adapted from: Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. *Ann Oncol* 2007; 18: 581-592.



**Figure 2 Incidence trends of colorectal cancer in Europe.** Thirty nine cancer registries in 1990-1996, 37 cancer registries in 1997, 96 cancer registries in 1998-2002. Adapted from: Parkin DM, Whelan SL, Ferlay J, Storm H. Cancer Incidence in Five Continents, Vol. I to VIII. IARC CancerBase No. 7, Lyon, 2005. Available from: URL: <http://www-dep.iarc.fr/>, section C15 I-VIII (Detailed). Last accessed on August 8, 2009; Curado MP, Edwards B, Shin HR, Storm H, Ferlay J, Heanue M, Boyle P, editors. Cancer Incidence in Five Continents, Vol. IX. IARC Scientific Publications No. 160, Lyon: IARC, 2007. Available from: URL: <http://www-dep.iarc.fr/> section C15 IX. Last accessed on August 8, 2009.

and rectosigmoid ( $> 22$  cases/ $10^5$  inhabitants) and tumors of the anus and anal channel ( $> 1.0$  cases/ $10^5$  inhabitants) (Table 3). According to recently published data, CRC-related mortality has stabilized or shown a slight decrease over recent years.

The most extensive population study monitoring the relative survival rate (RSR) is the EUROCARE program<sup>[7]</sup>, which takes registers of patients suffering from malignant diseases as a basis. Data have been gathered and evaluated since 1978. The most recent version, EUROCARE-4,



**Figure 3 Mortality trends of colorectal cancer in Europe.** As available in WHO database, countries with cancer registry (Cancer Incidence in Five Continents, Vol. IX). Adapted from: CancerMondial - WHO, International Agency for Research on Cancer, 2008. Available from: URL: <http://www-dep.iarc.fr/>; World Health Organization (2006), mortality database <http://www.who.int/whosis/whosis/>, United Nations, World Population Prospects, the 2006 revision. Available from: URL: <http://www-dep.iarc.fr/>. Last accessed on August 8, 2009.

uses comparative analyses of data from the year 1995 to 1999, while data are also available for the years 2000 to 2002<sup>[8]</sup>. Data from the European population carcinoma register are also used in the CONCORD study<sup>[9]</sup>, which focuses on a systematic comparison of statistical data between Europe and Northern America. Apart from these two studies, data from population registers of carcinoma have been published for some European countries. Data available regarding the 5-year RSR show high variability across European countries, with borderline values in the Czech Republic (50%) on the one hand and Germany



**Table 3** Epidemiology of colorectal cancer in the Europe (96 individual cancer registries, 1998-2002)

Parameter incidence	Sex	C18-C21	Individual diagnoses		
			C18	C19-C20	C21
Crude incidence (cases/100,000 inhabitants)	Men	63.9	37.1	26.0	0.8
	Women	53.7	34.8	17.6	1.3
	All	58.6	35.9	21.7	1.1
ASR-E	Men	58.0	33.5	23.7	0.8
	Women	36.8	23.4	12.4	1.0
	All	45.8	27.6	17.3	0.9
ASR-W	Men	38.5	22.0	15.9	0.5
	Women	24.6	15.5	8.4	0.7
	All	30.6	18.3	11.7	0.6
Mean age (yr)	Men	69.1	69.7	68.2	65.4
	Women	71.3	71.9	70.4	67.7
	All	70.1	70.8	69.1	66.8
Ratio (males: females) (based on No. of cases)		1.1:1	1.0:1	1.4:1	0.6:1

Adapted from: Curado MP, Edwards B, Shin HR, Storm H, Ferlay J, Heanue M, Boyle P, editors. Cancer Incidence in Five Continents, Vol. IX. IARC Scientific Publications No. 160, Lyon: IARC, 2007. Available from: URL: [http://www-dep.iarc.fr/section/C15 IX](http://www-dep.iarc.fr/section/C15%20IX). Last accessed on August 8, 2009. ASR-W: World age standardization.

(60%) on the other hand<sup>[7-16]</sup> (Table 4). Several studies have confirmed a favorable time trend in the 5-year RSR; however, these results have to be interpreted carefully with respect to the hidden reasons leading to such positive conclusions. Evaluation of survival rate based on clinical studies of CRC is, unfortunately, rather rare, and therefore, it is impossible to make a representative evaluation of this indicator. This fact should be seen as a challenge when improving population registers of malignant diseases.

## SCREENING METHODS

CRC screening focuses on asymptomatic individuals more than 50 years of age. Age is a low (average) risk factor for sporadic CRC, that is, carcinoma in patients with negative family or case history of CRC or chronic inflammatory bowel disease; this type of carcinoma accounts for 70 to 95% of all CRC cases. Three groups of screening methods are currently used as indicated in Table 5.

Guaiac-based fecal occult bleeding test (gFOBT) is at present the most frequently used method in screening programs. It detects the peroxidase reaction of hemoglobin, which causes the detection paper impregnated with guaiac resin to turn blue. Dietetic provisions are necessary to exclude false-positive results. A recent study showed limited sensitivity of this test for both, advanced adenomas (11%) and carcinomas (13%)<sup>[17]</sup>. With the use of gFOBT, a decrease in mortality for CRC by 15 to 33% has been proved<sup>[18]</sup>.

Immunochemical fecal occult bleeding test (iFOBT) reacts exclusively to human hemoglobin, so no dietetic restrictions are necessary. Taking and assessing the stool samples are easier than is the case with gFOBTs, which may explain a higher participation rate in the target group. A wide range of qualitative and quantitative tests is presently available, with varying levels of sensitivity

and specificity. The advantage of quantitative tests is the possibility to set cut-off limits; the most frequently used values are 75 or 100 ng/mL. The disadvantage of iFOBT is its cost; however, the price is now approaching that of gFOBT, particularly for qualitative tests<sup>[19]</sup>.

New screening methods include tests which examine the stool for the presence of abnormal DNA. Studies published to date focused on the characteristics of the test rather than the reduction in CRC incidence or mortality. Generally, these tests have higher sensitivity but lower specificity than gFOBT. The major obstacle to their implementation in screening programs is price<sup>[20]</sup>.

Flexible sigmoidoscopy (FS) is an endoscopic examination with maximum reach to the splenic flexure. On the basis of the information available, this is a promising screening test, although the studies published to date do not show sufficient statistical significance to determine reduction in CRC mortality. The recommended interval varies from 3 to 5 years. The risk of serious complications is 0% to 0.03%<sup>[21]</sup>.

Unlike FS, colonoscopy also detects lesions in the proximal colon. Its biggest advantage is the possibility of removing pathological lesions within a single examination. It is more sensitive in detecting both adenomas and carcinomas, although limited information is available on reducing CRC incidence and mortality, and on the recommended interval between examinations. The risk of serious adverse events is higher than for FS, at 3 to 5 events per 1000 colonoscopies<sup>[22]</sup>. To date, no prospective, randomized, multicenter study has been published supporting a reduction in CRC incidence and mortality with the use of screening colonoscopy. Nevertheless, its implementation in screening programs is one of the most widely discussed topics and the American College of Gastroenterology recommends screening colonoscopy as a preferred screening test<sup>[23]</sup>. On the other hand, no study addressing reductions in the incidence and mortality rates through stool analyses would have been completed without the "gold standard" of colonoscopy.

Computed tomographic colonography (CTC) shows lesions in the colorectum by reconstructing two- and three-dimensional images. To date, no studies have been published assessing reduction in CRC incidence or mortality. The majority of studies have focused on comparing the characteristics of this method with colonoscopy. For larger polyps (over 10 mm), the sensitivity of the methods is comparable; for smaller polyps (less than 5 mm), flat and depressed adenomas, the sensitivity is much higher for optical colonoscopy. Results of studies assessing the effect in terms of reduction in incidence and mortality, cost-effectiveness, and the potential risk of radiation are awaited<sup>[24]</sup>.

Double contrast barium enema shows the entire colorectum, although with significantly lower sensitivity and specificity than colonoscopy or CTC. The percentage of undetected carcinomas is up to 22%. The test is no longer widespread and available, but still has a purpose in countries lacking sufficient resources for other examinations<sup>[25]</sup>.

CRC screening is a complex process which, to function properly, requires the coexistence of a number of factors, such as a functioning invitation-reminder system,

**Table 4** Five-year relative survival rate (RSR) for colorectal cancer for selected European countries

Country	Diagnoses	Assessment period	Five-year RSR (%)	Change in time (%)	Stage-specific estimates
EUROCARE pool <sup>[7]</sup>	C18-C21	1995-1999	53.5	4.2	NA
EUROCARE pool <sup>[8]</sup>	C18-C21	2000-2002	56.2	NA	NA
England & Wales <sup>[10,11]</sup>	C18	1996-1999	47.6 M; 47.4 F	5.6 M; 5.6 F	NA
	C19-C20	1996-1999	48.7 M; 51.3 F	7.4 M; 8.1 F	NA
Germany <sup>[12]</sup>	C18-C21	2000-2002	60.8	NA	85.4 L; 58.1 R; 10.7 M
Finland <sup>[13]</sup>	C18-C20	2000-2004	57.9	2.4	NA
Norway <sup>[14]</sup>	C18-C21	2000-2004	59.2	3.6	NA
Slovenia <sup>[15]</sup>	C18-C21	2000-2004	46.9	8.0	NA
Sweden <sup>[16]</sup>	C18	2000-2002	58.1 M; 59.7 F	1.8 M; 2.6 F	NA
	C19-C21	2000-2002	57.5 M; 59.1 F	2.5 M; -1.7 F	NA

M: Estimate for males; F: Estimate for females; NA: Not available; L: Localized; R: Regional; M: Metastatic; NA: Not available. Numbers in brackets represents source of data available at references section.

**Table 5** Screening methods

Type of method	Method
Stool tests	For presence of occult blood (FOBT)
	Guaiac-based (gFOBT)
	Immunochemical (iFOBT)
	For presence of abnormal DNA
Endoscopic examinations	Flexible sigmoidoscopy (FS) colonoscopy
Radiologic examinations	Computed tomographic colonography (CTC)
	Double contrast barium enema (DCBE)

media campaigns targeted at the general public, the development of recommendations for general practitioners, patient compliance, sufficient funding, stratification of risks, and last but not least the selection of the most suitable screening test. Of the above described tests, only the fecal occult blood tests meet the WHO criteria for screening. As published recently, most CRC screening strategies lead not only to a reduction in CRC incidence and mortality, but also to better control of the costs of CRC treatment, especially with increased chemotherapy costs for advanced CRC<sup>[26]</sup>.

## GENERAL ONCOLOGY PREVENTATIVE PROGRAMS IN EUROPE

In 1985, the Europe Against Cancer program was initiated, which aimed at a reduction of 15% in the number of deaths caused by tumors (from 1 000 000 to 850 000) by 2000. The program was implemented, thanks to the cooperation of professional and lay public, charities and anti-smoking groups, healthcare media, and healthcare workers. The project focused on three major areas: prevention, screening, and education. Results published show that although the planned goal was not achieved, the mortality due to tumors was reduced by 10% in the EU. In some countries (Austria and Finland), the desired reduction of 15% was achieved, while in others (Portugal and Greece), the mortality reduction was much lower<sup>[27]</sup>. The experience gained in this program served as a basis for the Recommendations of the Council of the EU for screening programs following comprehensive European quality assurance guidelines. In December 2003, these

recommendations were unanimously approved by the health ministers of the individual EU states. European guidelines for quality assurance of breast and cervical cancer screening have been developed by experts and published by the European Commission; quality assurance guidelines for CRC screening are currently under preparation<sup>[28]</sup>.

## CRC SCREENING IN EUROPE

In 2008, the Report on the Implementation of the Council Recommendation on Cancer Screening<sup>[29]</sup>, which provides the most comprehensive available data, was published; giving the definitions of program screening as requiring public responsibility by law or official regulation and supervision in contrast to “wild” screening outside of any program. In program screening, the screening test, the examination interval and the eligible group of persons should be specified. Organized screening should generally include a regional or national team responsible for the implementation, quality assurance and reporting of results. Comprehensive guidelines, rules and a quality assurance structure should be available. Population-based screening requires the identification and personal invitation of each person in the eligible target population (by an office or special agency). According to this report CRC screening is running or being established in 19 of 27 EU countries. The target group contains approximately 136 million individuals suitable for CRC screening (aged 50 to 74 years). Of this number, 43% individuals come from 12 countries where CRC population screening is performed or being prepared on either national or regional levels; 34% come from 5 countries where national population screening has been implemented (Finland, France, Italy, Poland, and United Kingdom). In 7 EU countries, national non-population based screening is carried out, which covers 27% of the target population. In 2007, gFOBT (which in 2003 was the only test recommended by the Council of the European Union) was used as the only screening method in twelve countries (Bulgaria, Czech Republic, Finland, France, Hungary, Latvia, Portugal, Romania, Slovenia, Spain, Sweden, and United Kingdom). Colonoscopy was the only screening method used in Poland. In six countries, two types of tests were used: iFOBT and FS in Italy, and gFOBT and

Table 6 Colorectal cancer screening programs in 2007

	Program		Test type	Screening interval years or times in LT	Age eligible national population	
	Type	Status			Age (yr)	Persons (× 1000)
Austria	NonPB	Natw	FOBT	1 or 2	> 50	2210
	NonPB	Natw	CS	10	> 50	2210
Belgium	No Prog					2880
Bulgaria	NonPB	Natw	FOBT	1	> 31	2340
Cyprus	PB	Natw-plan	FOBT	1 in LT	50	10
	PB	Natw-plan	CS	1 in LT	55	10
Czech Republic	NonPB	Natw	FOBT	2	> 50	3010
Denmark	No Prog					1540
Estonia	No Prog					370
Finland	PB	Natw-roll ong	FOBT	2	60-69	570
France	PB	Natw-roll ong	FOBT	2	50-74	16600
Germany	NonPB	Natw	FOBT	1 and 2	> 50	24500
	NonPB	Natw	CS	10 (2 in LT)	55-74	18800
Greece	NonPB	Natw	FOBT	5	> 50	3180
	NonPB	Natw	CS	5	> 50	3180
Hungary	PB	Natw-pilot	FOBT	2	50-70	2630
Ireland	No Prog					940
Italy	PB	Natw-roll ong	FOBT	2	50-69 (70-75)	13800
	PB	Reg-roll ong	FS	1 in LT	58 or 60	80
Latvia	NonPB	Natw	FOBT	1	> 50	630
Lithuania	No Prog					870
Luxembourg	No Prog					120
Malta	No Prog					120
Netherlands	No Prog					4460
Poland	PB	Natw-roll ong	CS	10	50-65	7500
Portugal	PB	Natw-plan	FOBT	2	50-70	2520
Romania	PB	Natw-plan	FOBT	2	50-74	5800
Slovak Republic	NonPB	Natw	FOBT		> 50	1360
	NonPB	Natw-plan	CS	10	> 50	1360
Slovenia	PB	Natw-plan	FOBT	2	50-69	490
Spain	PB	Reg-pilot	FOBT	2	50-69	210
Sweden	PB	Reg-plan	FOBT	2	60-69	220
UK	PB	Natw-roll ong	FOBT	2	(50) 60-69 (74)	7600
Dual prog/test						-25630
Subtotal						106490
Excluded pop.						29500
Total						135990

PB: Population based; Prog: Program; Natw: Nationwide; Reg: Regional; Plan: Planning; Roll ong: Rollout ongoing; Pilot: Piloting; CS: Colonoscopy; LT: Lifetime. dual prog/test: Individuals entered twice due to screening programs of different implementation or using different screening tests. excluded pop.: Individuals excluded from national target populations due to regional or national variations in the age group targeted for screening, or due to lack of screening programs in some regions of countries with regional implementation status. Adapted from: von Karsa L, Anttila A, Ronco G, Ponti A, Malila N, Arbyn M, Segnan N, Castillo-Beltran M, Boniol M, Ferlay J, Hery C, Sauvaget C, Voti L, Autier P. Cancer screening in the European Union. Report on the implementation of the Council Recommendation on cancer screening - First Report. ISBN 978-92-79-08934-3. European Communities (publ.) Printed in Luxembourg by the services of the European Commission, 2008. Available from: URL: [http://ec.europa.eu/health/ph\\_determinants/genetics/documents/cancer\\_screening.pdf](http://ec.europa.eu/health/ph_determinants/genetics/documents/cancer_screening.pdf). Last accessed on August 4, 2009.

colonoscopy in Austria, Cyprus, Germany, Greece, and Slovak Republic. In the remaining eight states (Belgium, Denmark, Estonia, Ireland, Lithuania, Luxembourg, Malta, and the Netherlands), CRC screening has not been implemented yet. The age limit for the target population varies across EU countries (Table 6). In 2007, it was estimated that a total of 12 million individuals participated in CRC screening.

In the United Kingdom, a screening program was announced in 2004 and initiated in 2006, with the prospect of national coverage in 2009. It has been designed in two stages, with gFOBT examinations at 2-year intervals and colonoscopy for positive tests. In 2007, compliance was 52%. The program is carried out through regional centers

falling under one of five national hubs. The role of general practitioners is less significant here<sup>[30]</sup>.

In France, a screening program was initiated in 2003, based on gFOBT tests at 2-year intervals with colonoscopy for positive results. The role of general practitioners as coordinators is of crucial importance. The major advantage of the French program is its good organization, with a call-recall system comprising central management at national level and individual steps taken by centers in individual departments. Asymptomatic individuals aged from 50 to 74 are mailed gFOBT tests, with a reminder at three-monthly intervals for nonparticipants. Compliance in referred districts achieved 42%, and the overall positive test rate was 2.7%<sup>[31]</sup>.

In Italy, a nation-wide campaign was initiated in 2005; the implementation was entrusted entirely to 21 regional centers, including choice of the testing method. With state financial support, screening has been initiated in 11 regions to date, mostly in the industrial areas of northern Italy. In the Piedmont region, FS is the method of choice, in other regions immunochemical FOBT, with colonoscopy for positive tests. Compliance in iFOBT and FS programs was 44.6% and 51.4%, respectively. Positivity rate of iFOBT was 5.3% at first and 3.9% at repeat screening<sup>[32]</sup>.

In Spain, no screening program has taken place as yet. The main obstacle to its implementation is the highly heterogeneous healthcare system, in terms of organization and insurance coverage in individual self-governing units. Catalonia, for instance, considers implementation of country-wide screening in 2010, while in other regions only limited pilot studies have been held so far.

In Finland, a structured screening program was initiated in 2004. The target population, aged from 60 to 69 years (106 000 individuals), was randomized into two groups. Individuals in the screening group were mailed a gFOBT test at intervals of 2 years. The Finnish program shows a high level of compliance of the target population (70.8%), particularly for females<sup>[33]</sup>.

In the Netherlands, the optimum screening strategy is still being developed. It will be based on the results of studies currently taking place at major academic workplaces, comparing the effect of endoscopic procedures, various types of FOBTs, and fecal DNA analysis.

Poland is the only state at the moment using colonoscopy as the only screening method, without the alternative of FOBT. An opportunistic screening program was initiated in 2000, and by 2005, this had grown to 57 centers across Poland. The program is financed by the Ministry of Health, independent of the overall healthcare system. The target population (asymptomatic individuals aged 55-66 years) is recruited through general practitioners. High emphasis is placed on the quality control of colonoscopies, with complications reported for 0.1% of procedures, and no patient mortality. The advantage of the program is thorough monitoring and evaluation, including monitoring of interval cancers<sup>[34]</sup>.

Germany was the first country to introduce a population screening program (in 1976) based on annual gFOBT for individuals more than 44 years of age. Starting from 2002, it has been offering participants a choice between colonoscopy at 55 years of age and FOBT at annual intervals between 50 and 55 years of age. After 55 years of age, examinations are carried out at 2-year intervals. If the test results are positive, colonoscopy is indicated. Those who undergo a screening colonoscopy with no neoplasia detected at the initial examination are recommended reexamination in 10 years time if the first colonoscopy was carried out before they were 65 years. The positive feature of the screening and data gathering in Germany is the emphasis on staging the disease at the time of its diagnosis. Recent cost analyses have proven that this type of screening is cost-efficient<sup>[35]</sup>.

In the Czech Republic, CRC screening has many years of tradition<sup>[36,37]</sup>. The country was the second in the world to start screening nation-wide, in 2000. In the initial years, gFOBT was the first method offered to asymptomatic individuals more than 50 years of age by their general practitioners at preventative medical checks, followed by colonoscopy if tests were positive. From 2000 to 2008, 1 685 289 gFOBTs were carried out, of which 63 296 were positive (3.76%). In 2006, a central database for online data input was established. Between 2006 and 2008, 17 813 colonoscopies were carried out, indicated as a result of a positive FOBT; carcinoma was diagnosed in 1047 (5.9%) individuals, and 5362 (30.1%) adenomas were removed by endoscopic polypectomy. The participation of the target group, however, was only 20%<sup>[38]</sup>. In order to achieve a higher compliance rate, screening colonoscopy was added to current FOBT screening as an alternative method, in the same intervals as in the German program. Both, gFOBT and iFOBT are offered as well. The implementation of the newly designed program is supported by an intensive media campaign (<http://www.kolorektum.cz/index-en.php>).

The first study which focused on monitoring the effect of colonoscopy screening on reducing CRC incidence and mortality is NordICC (The Nordic-European Initiative on Colorectal Cancer), which is currently underway in northern states of Europe (Norway, Sweden, and Iceland), Poland, and the Netherlands. It will involve a minimum of 66 000 individuals aged 55 to 64 years. Individuals in the screening group will undergo a screening colonoscopy once in a lifetime. The primary objective is to compare incidence and mortality against the control group (with no screening) after 10 years<sup>[39]</sup>.

## CONCLUSION

CRC presents a serious public healthcare issue for the population of Europe. Understandably, the number of countries introducing population screening has been growing constantly. Although epidemiologic data differ in various European countries, implementation of screening programs in accordance with the principles spelled out in the Council Recommendation on Cancer Screening of 2 December 2003 may be expected to have a favorable effect on the burden of this disease in the population. Countries in the EU may benefit from unified policy, knowhow and central oncology registers, while economically less developed countries may draw on special funding for the development of preventative programs. At the same time, varying epidemiologic situations, economic conditions, and different systems of health insurance and organization of healthcare are factors that may limit the implementation of a unified screening program. Therefore, to respond to the needs of the member countries, the EU should consider adopting the recommendation of the World Gastroenterology Organization for CRC screening, possibly even in a modified form<sup>[40]</sup>. This is a cascade concept in which recommendations for individual countries are graded into six levels, depending on the resources available (financial



Table 7 Cascade concept

Level	Average risk	High risk
1	Colonoscopy in 10 years interval, from 50 years of age	Special procedure, for individual groups
2	Colonoscopy once in a lifetime, at 50 years of age	Special procedure, for individual groups
3	Flexible sigmoidoscopy in 5 years interval, from 50 years of age; colonoscopy to follow if positive	Special procedure, for individual groups
4	Flexible sigmoidoscopy once in a lifetime, at 50 years of age; colonoscopy to follow if positive	Special procedure, for individual groups
5	Flexible sigmoidoscopy once in a lifetime, at 50 years of age; colonoscopy to follow only if advanced adenoma is detected	Same as individuals with average risk, if resources are not available for colonoscopy
6	FOBT in annual interval after 50 years of age; if positively tested, colonoscopy or double contrast barium enema	Same as individuals with average risk, if resources are not available for colonoscopy

Adapted from: World Gastroenterology Organization/International Digestive Cancer Alliance. Practice Guidelines: Colorectal cancer screening. Available from: URL: <http://www.worldgastroenterology.org/colorectal-cancer-screening.html>. Last accessed on August 4, 2009.

and professional) (Table 7). In the case of lack of funds, FOBT at intervals of 1 or 2 years for individuals with average risk is a realistic possibility. This open concept best fulfils the simple recommendation by Sydney Winawer, Co-Chair of IDCA (International Digestive Cancer Alliance): “The best screening test is the one that gets done...and gets done well. Do what you can with what you have”.

In most European countries, fortunately, the majority of the population is covered by some form of health insurance, meaning that economic aspects need not critically affect the availability of screening programs. Although at the end of 2007, CRC screening was still not running or being established in 8 of 27 EU member states, some of which rank among the most developed economies of the world, additional programs are currently under development. Given the substantial burden of the disease, implementation and continuous improvement in CRC screening programs should remain high on the healthcare agenda in Europe.

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REVIEW

## Progress in researches about focal adhesion kinase in gastrointestinal tract

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### Abstract

Focal adhesion kinase (FAK) is a 125-kDa non-receptor protein tyrosine. Growth factors or the clustering of integrins facilitate the rapid phosphorylation of FAK at Tyr-397 and this in turn recruits Src-family protein tyrosine kinases, resulting in the phosphorylation of Tyr-576 and Tyr-577 in the FAK activation loop and full catalytic FAK activation. FAK plays a critical role in the biological processes of normal and cancer cells including the gastrointestinal tract. FAK also plays an important role in the restitution, cell survival and apoptosis and carcinogenesis of the gastrointestinal tract. FAK is over-expressed in cancer cells and its over-expression and elevated activities are associated with motility and invasion of cancer cells. FAK has been proposed as a potential target in cancer therapy. Small molecule inhibitors effectively inhibit the kinase activity of FAK and show a potent inhibitory effect for the proliferation and migration of tumor cells, indicating a high potential for application in cancer therapy.

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**Key words:** Focal adhesion kinase; Restitution; Survival and apoptosis; Cancer; Inhibitor

### INTRODUCTION

Focal adhesion kinase (FAK) is a 125-kDa non-receptor protein tyrosine which was originally identified in chicken embryo cells transformed by v-Src<sup>[1]</sup> and BALB/c3T3 fibroblasts<sup>[2]</sup> and was shown to localize in focal adhesions as well. FAK is a non-receptor and non-membrane associated protein tyrosine kinase (PTK), which does not contain Src homology2 (SH2) or SH3 protein interaction domains<sup>[3]</sup>. FAK contains three main domains: a centrally located catalytic kinase domain, a large N-terminal domain comprising the FERM (FAK, ezrin, radixin, moesin) region and a C-terminal domain harboring the focal adhesion targeting<sup>[3-5]</sup>. Growth factors or the clustering of integrins facilitate the rapid phosphorylation of FAK at Tyr-397 in adherent cells and this in turn recruits Src-family PTKs, resulting in the phosphorylation of Tyr-576 and Tyr-577 in the FAK activation loop and full catalytic FAK activation<sup>[3,5]</sup>; while extracellular pressure can activate the FAK in suspended cells<sup>[6,7]</sup>.

FAK is associated with gastrointestinal diseases, here we will review the progresses which have been made in the researches about FAK in the gastrointestinal tract. Research data shows that FAK plays an important role in the restitution, cell survival and apoptosis and carcinogenesis of the gastrointestinal tract. Due to the crucial role of FAK in integrin-mediated signal transduction, which affects the regulation of cell survival, proliferation, spreading and migration, FAK has been proposed to be a potential target in cancer therapy. Antisense oligonucleotides, the entire C-terminal, non-catalytic domain of FAK (FAK related non-kinase-FRNK), siRNA

and small molecule inhibitors can affect and inhibit the activities and expression of FAK in various tumor cells. Small molecule inhibitors targeting FAK have been developed as potential cancer treatment modalities. PF-573228, PF-562271 and NVP-226 (TAE226) have already shown potent inhibitory effect for tumor cell growth *in vitro* and *in vivo*.

## RESTITUTION

After intestinal superficial mucosal injuries such as erosion, ulcerations, inflammatory bowel disease and infection, the repair of epithelial injury in the gastrointestinal tract begins in a process known as restitution<sup>[8]</sup>. The restitution is established through migration of viable epithelial cells from areas adjacent to or just beneath the injured surface to cover the denuded area, independent of cell proliferation and regulated by cytokines and growth factors<sup>[9-14]</sup>. Intestinal epithelial migration, proliferation and differentiation are essential to restitution<sup>[15]</sup>. FAK has been indicated to be involved in the integrin signaling which regulates the migration, proliferation and differentiation of various normal and cancer cells<sup>[16]</sup>. Correspondingly, FAK plays an important role in the mucosal restitution of the intestine.

It is well established that intestinal epithelial cells undertake a specialized phenotype adapted to motility and mucosal healing during mucosal restitution and FAK is involved in the cell signaling which regulates the intestinal epithelial migratory phenotype<sup>[17]</sup>. The disruption of actin stress fiber formation with reduced tyrosine phosphorylation of FAK and FAK in focal adhesions can suppress the repair of gastric mucosal injury and ulcer healing<sup>[18-20]</sup>. FAK plays a critical role in lysophosphatidic acid (LPA)-induced migration, lamellipodia formation and assembly of focal adhesions in intestinal epithelial cells<sup>[21,22]</sup>.

The expression and activation levels of FAK protein are linked to phenotypic changes which affect cell differentiation, function, adhesion and migration in various tissues<sup>[23-29]</sup>. Activated FAK<sup>397</sup> levels vary with differentiation and cell migration in Caco-2 and HT-29 human colon cancer cells<sup>[30]</sup>. The expression level of activated FAK is related to gastric wound healing *in vivo*<sup>[19,31]</sup>. Intestinal epithelial cell motility regulates FAK protein abundance at the mRNA level in both human Caco-2 and rat non-transformed IEC-6 intestinal epithelial cells<sup>[32]</sup>. It has been shown that immunoreactivity to FAK is decreased in cells migrating across matrix protein compared to static Caco-2 cells<sup>[33]</sup> and immunoreactivity to FAK and FAK<sup>397</sup> were lower in epithelial cells at the migrating edge of the ulcer<sup>[34]</sup>.

FAK mediates the mitogenic response to repetitive deformation in intestinal epithelial cells. Two deformation-activated signal pathways that converge upon FAK have been proposed: one is Src- and Rac1-independent-which stimulates FAK-Tyr397 phosphorylation, and the other is Src- and Rac1-dependent, which is required to further activate FAK by phosphorylation at FAK-Tyr576 (within the FAK kinase activation loop)<sup>[28]</sup>. Repetitive deformation stimulates intestinal epithelial motility

across fibronectin, which requires both Src activation and a novel Src-independent FAK-Tyr 925-dependent pathway activating extracellular signal-related kinase (ERK)<sup>[29]</sup>. Smad3-dependent disruption of the transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling pathway impairs the healing of murine intestinal mucosal ulcers, which is followed by altering patterns of activated FAK and ERK immunoreactivity important for cell migration at the ulcer edge<sup>[15]</sup>.

Recently, the relationship between TGF- $\beta$  and FAK has been studied. TGF- $\beta$  was found to enhance FAK protein, mRNA levels and FAK promoter activity in human and rat intestinal epithelial cells<sup>[34]</sup>. TGF- $\beta$  also affected the restitution and proliferation partly mediated through its induction of FAK expression<sup>[35]</sup>. It is considered to play an essential role in embryogenesis, host response to tumors, and the repair response damaging the tissues by immune and non-immune reactions<sup>[36]</sup>.

Taken together, the interaction between inflammatory cells, the extracellular matrix, locally released cytokines and growth factors guarantee efficient ulcer healing<sup>[31]</sup>. Tissue injury and wound healing spatially and temporally activate several growth factors and extracellular matrix facilitates the rapid phosphorylation of FAK at Tyr-397 and this in turn recruits Src-family PTKs, resulting in the phosphorylation of Tyr-576 and Tyr-577 in the FAK activated loop and other focal adhesive proteins including talin,  $\alpha$ -actinin, vinculin, paxillin and p130Cas (Figure 1)<sup>[3,5,16,37]</sup>. Activated FAK and cell adhesive protein transduce the signal to the Mek/Erk to down-regulate proliferation, differentiation and migration in the process of restitution. However, the detailed mechanism for the role of FAK in the process of restitution is still unknown.

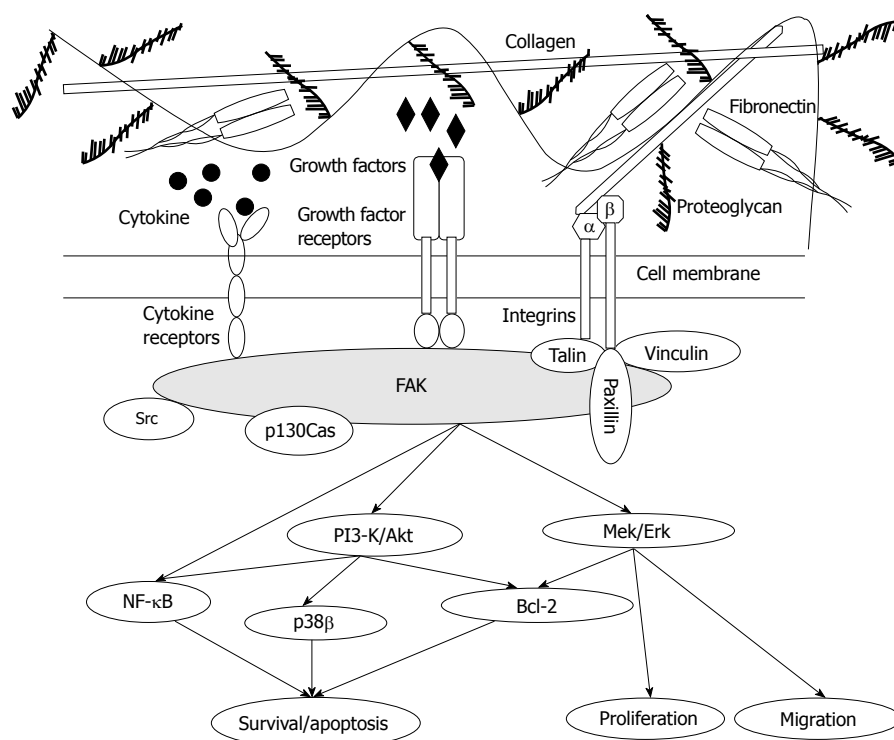
## SURVIVAL AND APOPTOSIS

Programmed cell death, or apoptosis, is a complex and tightly regulated process that executes crucial roles in tissue homeostasis and repair<sup>[38,39]</sup>. It is well established that the Bcl-2 family of proteins plays a major role in cell survival and apoptosis<sup>[38,40,41]</sup>. Extracellular signals can affect the expression and/or functions of the Bcl-2 family by signaling events to determine if a cell lives or dies<sup>[42]</sup>. FAK is the canonical mediator of such extracellular signals which originate from integrin and growth factors<sup>[5]</sup>. Thus, FAK is related to cell survival and apoptosis in the gastrointestinal epithelium.

The detachment of intestinal epithelial cells from matrix induces apoptosis through the disruption of anti-apoptotic signals transduced by integrin/FAK/Src<sup>[43]</sup>. Induced FAK suppresses apoptosis by activating nuclear factor  $\kappa$ B (NF- $\kappa$ B) signaling in intestinal epithelial cells<sup>[44]</sup>. FAK inhibition in human intestinal epithelial cells produces anoikis while FAK induction in rat intestinal IEC-6 cells suppresses apoptosis<sup>[44,45]</sup>.

Recent studies in the function of FAK in survival and apoptosis of intestinal epithelial cells have focused on integrin/Fak/Src, PI3K/Akt and MEK/Erk pathways





**Figure 1** FAK mediates the extracellular signaling to regulate the proliferation, migration and survival/apoptosis of the cells. NF- $\kappa$ B: Nuclear factor  $\kappa$ B; FAK: Focal adhesion kinase.

which are all presumed to modulate the expression and function of multiple Bcl-2 homologs<sup>[42,44-46]</sup>. Many studies showed that integrin/FAK/Src modulate the PI3K/Akt and MEK/Erk pathways individually or in combination in different cell lines<sup>[5,42,47-55]</sup>. The Bcl-2 family is the central regulator of caspase activation which executed the cell-suicide program and play an anti- or pro-apoptotic role in cell apoptosis<sup>[56]</sup>. Butyrate-induced apoptosis of Caco-2 cells might occur *via* NF- $\kappa$ B activation together with a defective  $\beta$ 1 integrin-FAK-PI3-kinase pathway signaling<sup>[47]</sup>. A study showed that integrins, FAK, PI3-K/Akt-1, MEK/Erk, and p38 isoforms play distinct roles in the regulation of HIEC-6 cell survival and/or death, accompanied by modulating individual Bcl-2 homologs<sup>[46]</sup>.  $\beta$ 1 integrins/Fak/Src signaling down-regulated PI3-K/Akt-1 and MEK/Erk pathways in the suppression of anoikis, which play a role in the survival of differentiated cells, whereas the PI3-K/Akt-1 pathway is crucial for cell survival regardless of the state of differentiation<sup>[45]</sup>.  $\beta$ 1 integrins/Fak/Src signaling translates into integrated, complex regulatory functions by PI3-K/Akt-1 and MEK/Erk in the expression/activity of Bcl-2 homologs, as well as in the specific activation of the pro-apoptotic p38 $\beta$  SAPK isoform, thus determining their own requirement (or not) in the suppression of HIEC (Human Intestinal Epithelial Crypt) apoptosis/anoikis<sup>[42]</sup>.

Extracellular/Fak/Src signaling down-regulates PI3-K/Akt and Mek/Erk and further regulates the expression and activity of Bcl-2, and finally control the survival and apoptosis. PI3-K/Akt also specifically activates the apoptosis/anoikis driving p38 $\beta$  SAPK, and regulates the survival and apoptosis. Besides, extracellular/Fak/Src signaling has a new pathway to control the survival and apoptosis *via* regulating the NF- $\kappa$ B.

## CANCER

FAK is closely associated with cancer. Many studies have shown FAK over-expression in various tumor cells and its expression correlate with increased tumor malignancy. The alteration of FAK function in normal cells causes tumor progression.

FAK has been indicated to over-express at mRNA and protein levels in various tumors including gastrointestinal tumors. As early as in 1993, researchers found increased levels of FAK in 1 of 8 adenomatous tissues, in 17 of 20 invasive tumors, and in all 15 of 15 metastatic tumors, which suggests that FAK over-expression may result in changes in the signaling pathways involved in tumor cell invasion<sup>[57]</sup>. In human colon cancer cells, increased dosage of the FAK may contribute to the elevated protein expression during conversion from adenoma to carcinoma<sup>[58]</sup>. Quantitative realtime RT-PCR of gene expression levels in all gastrointestinal stromal tumors (GIST) indicated that FAK was over-expressed in malignant GIST<sup>[59]</sup>. Immunohistochemical analysis also demonstrated that FAK is over-expressed in colorectal, esophageal, pancreatic and mammary cancers, which indicated that FAK and P-FAK are involved in the carcinogenesis of digestive organs<sup>[60,61]</sup>. Another research group got similar results *via* immunohistochemistry, which showed that high levels of FAK and Src were predictive for recurrence of colorectal cancer<sup>[62]</sup>. The FAK expression level might be a valuable marker for the carcinogenesis and progression of some types of carcinoma<sup>[63,64]</sup>.

An increased expression of FAK is associated with the invasive potential of colon and breast tumors<sup>[65]</sup>. Immunohistochemical analysis of gastric cancer and colorectal cancer showed that the expression of FAK is more significantly associated with carcinogenesis,

differentiation and metastasis, and furthermore FAK may not only be a transformation-linked enzyme but also a progression-linked enzyme<sup>[63]</sup>. FAK over-expression of esophageal squamous cell carcinoma was related to cell differentiation, tumor invasiveness, and lymph node metastasis<sup>[66]</sup>. The expression of gastrin-releasing peptide (GRP) and its cognate receptor critically mediates a GRP-dependent phase of cell motility by phosphorylating FAK at multiple specific sites in colon cancer cells<sup>[30]</sup>. Gastrin can evidently promote invasiveness of Colo320 cells *via* the gastrin-gastrin receptor-FAK signal transduction pathway<sup>[67]</sup>.

Not only the expression level but also the activities of FAK are essential for the motility and invasion of cancer cells. Colon carcinomas exhibited a marked elevation in FAK tyrosine kinase activity and phosphotyrosine content and the catalytic activity of FAK is enhanced by its phosphotyrosine content<sup>[68]</sup>. The amount of total FAK and FAK phosphorylated at Y397 and Y407 correlates closely with the differentiation of human colon cancers<sup>[69]</sup>. The migratory phenotype of colon cancer cells is controlled by the combined activities of Src and FAK, and the recruitment of FAK to adhesive sites results in its phosphorylation by Src and other peripheral tyrosine kinases<sup>[70]</sup>.

The over-expression and elevated activities of FAK are associated with motility and invasion of cancer cells, however the exact mechanism is still unknown. Integrin $\alpha$ 2/FAK/ERK/ $\mu$ -calpain signaling pathway plays a critical role in tumor cell motility and these results would cause the interruption of FAK function at the early stages of colon tumorigenesis<sup>[71]</sup>. In a colon adenocarcinoma, cell proliferation and differentiation can occur concomitantly and these deregulated processes are controlled by autocrine secretion through the ErbB1/ERK1, 2 and FAK pathways<sup>[72]</sup>. The Cholecystokinin-2 receptor regulates the invasion and motility of colon cancer cells, and supports the role of CCK2R in the progression of colon cancer through the activation of FAK<sup>[73]</sup>. EGFR pathway substrate 8 could modulate the expression of FAK *via* mTOR/STAT3, which enable the cells to proliferate and migrate<sup>[74]</sup>. The mechanism of the increasing invasion of colon cancer cells by gastrin17 is probably that gastrin17 makes FAK-Tyr397 phosphorylate and localize to lamellipodia, causing the formation of FAK-Src-p130(Cas)-Dock180 signaling complex when it is bound to its receptor CCK-2 and the activation of Rac<sup>[75]</sup>. The engagement of  $\alpha$ 1-integrins with functional molecular scaffolds using FAK/Src and p130Cas/JNK is involved in human colon cancer cell invasion through the induction and activation of the MMP-2 and MMP-9 matrix metalloproteinases<sup>[76]</sup>. A model has been proposed to indicate how the interaction of FAK and SFKs down-regulate the MAPK/Erk1/2 and PI3K/Akt pathways in the early process of cell adhesion in SW480 colon cancer cells: Integrin engagement induces quick FAK-Y397 autophosphorylation and subsequent translocation of a fraction of FAK in raft compartments, FAK interacts only with Fyn in lipid domains, while it interacts with c-Src and Fyn in non-raft fractions. In parallel, PI3K/Akt signaling

is quickly activated which is dependent on lipid domain integrity, while MAPK/Erk1/2 signaling is activated with longer kinetics which is not dependent on lipid domain integrity. Both signaling pathways contribute to the adhesive process of SW480 cells<sup>[77]</sup>.

These data show the strong relationship between the expression and activity level of FAK and the generation and progression of gastrointestinal tumors, however the exact mechanism needs further studies.

### Inhibitor

Due to the crucial role of FAK in integrin-mediated signal transduction, which affects the regulation of cell survival, proliferation, spreading and migration, FAK has been considered a potential target in cancer therapy. There are many ways to suppress the activity and expression of FAK, thereby inhibiting the growth of tumor cells. The attenuation of FAK expression *via* antisense oligonucleotides induces detachment and apoptosis in tumor cells<sup>[78]</sup>. The entire C-terminal, non-catalytic domain of FAK (FAK related non-kinase-FRNK) is autonomously expressed in some cell types, and has been used as a dominant negative mutant to elucidate FAK function<sup>[79-82]</sup>. Specific short interfering RNA is often used to reduce the expression of FAK. Knockdown of FAK protein through FAK-SiRNA significantly inhibited LPA-induced migration of both IEC-18 and IEC-6 cells<sup>[22]</sup>.

As described earlier in this article, phosphorylation of Tyr-397 at FAK is essential to the phosphorylation of Tyr-576 and Tyr-577 in the FAK activation loop, full catalytic FAK activation, the activity of other adhesive protein and its downstream molecules which all play important roles in integrins or growth factors initiated signaling pathways. So targeting the phosphorylation of FAK seems to be promising for the cancer therapy. Small molecule inhibitors targeting FAK as potential cancer therapies have been developed. Sulindac sulfide (NSAID) and the phenolic antioxidant caffeic acid phenethyl ester were used to reduce the phosphorylation of FAK and cell invasion in human colon carcinoma cells<sup>[83]</sup>. Butyrate treatment results in a significant down-regulation of c-Src and FAK in human colon cancer cells and finally inhibits tumor growth and invasion<sup>[84]</sup>. Exposure of HT-29 cells to 10 mmol/L garcinol inhibited cell invasion and decreased the dose-dependent tyrosine phosphorylation of FAK, which suggests that garcinol reduces cell invasion and survival through inhibiting the downstream signaling of FAK<sup>[85]</sup>.

Recently, compounds PF-573228, PF-562271 and NVP-226 (TAE226) have been generated by two groups. These compounds are ATP analogs and effectively inhibit the kinase activity of FAK<sup>[86,87]</sup>. PF-573228 inhibited phosphorylation of FAK and its downstream effector paxillin, and affected cell migration and adhesion turnover<sup>[86]</sup>. But PF-573228 had little inhibitory effect on the growth and apoptosis of normal and cancer cells possibly because the FAK kinase activity is not essential for cell growth-proliferation mediated through FAK FERM regulation of p53<sup>[88]</sup>.

PF-562271 is a newly developed diaminopyrimidine-type compound that inhibits FAK and Pyk2 and shows a high degree of selectivity in the inhibition of PTKs<sup>[89]</sup>. PF-562271 have inhibited the tumor growth of prostate, pancreatic, colon, glioblastoma, and H460 lung xenotropic tumor models<sup>[89]</sup> and blocked bFGF-stimulated blood vessel angiogenesis as shown in chicken chorioallantoic membrane assays. Low dosage of PF-262271 potently blocked blood vessel sprouting without detectable changes in vascular leakage<sup>[88]</sup>. The oral administration of PF-562271 suppressed the growth and local spread of intratibial tumors and restored tumor-induced bone loss<sup>[90]</sup>. The combination of PF-562271 and sunitib could effectively block the growth and recovery of human hepatocellular carcinoma in a rat xenograft model<sup>[91]</sup>. PF-562271 has since moved to clinical trials, and has shown minimal toxicity along with tumor regression<sup>[92]</sup>.

TAE226 is a novel ATP-competitive tyrosine kinase small-molecule inhibitor designed to target FAK, and can effectively prevent FAK phosphorylation, ERK, S6 ribosomal protein phosphorylation and downstream signal transduction, as determined by decreased AKT. TAE226 inhibits insulin receptor (InsR) and insulin-like growth factor-I receptor (IGF-IR), albeit, 10 fold less potently (IC<sub>50</sub> = 44 nmol/L for InsR and IC<sub>50</sub> = 140 nmol/L for IGF-IR), and is a potent inhibitor of FAK (IC<sub>50</sub> = 5.5 nmol/L)<sup>[87,93]</sup>. TAE226 was shown to induce apoptosis in breast cancer cell lines<sup>[94]</sup>. Furthermore, TAE226 can significantly prolong the survival of animals bearing intracranial glioma xenografts and ovarian tumor cells orthotopic implantation<sup>[86,95]</sup>. TAE226 also showed a potent inhibitory effect of tumor cell growth in gastrointestinal tract. When esophageal adenocarcinoma cells were treated with TAE226, cell proliferation and migration were greatly inhibited with an apparent structural change of actin fiber and a loss of cell adhesion, which suggest that TAE226, a dual tyrosine kinase inhibitor for FAK and IGF-IR, might become a new remedy for Barrett's esophageal adenocarcinoma<sup>[96]</sup>. Furthermore, TAE226 has shown significant inhibitory effects on mTOR signaling and the esophageal cancer cell growth<sup>[97]</sup>. TAE226 can effectively suppress the growth of imatinib-resistant GIST cells, indicating its potential application for treating the imatinib-resistant GISTs<sup>[98]</sup>. So the small molecule inhibitors show a significant promise for cancer therapy.

## CONCLUSION

FAK plays a critical role in the biological processes of normal and cancer cells including the gastrointestinal tract. Research data shows that FAK plays an important role in the restitution, cell survival and apoptosis and carcinogenesis of the gastrointestinal tract, however the exact mechanism needs further studies. FAK is over-expressed in cancer cells and over-expression and enhanced activities of FAK are associated with motility and invasion of cancer cells. So FAK has been proposed as a potential target in cancer therapy. Small molecule inhibitors effectively inhibit the kinase activity of FAK

and show a potent inhibitory effect in the proliferation and migration of tumor cells, indicating a high potential for future application in cancer therapy.

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ORIGINAL ARTICLE

## TRAIL-induced apoptosis of hepatocellular carcinoma cells is augmented by targeted therapies

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were treated with kinase inhibitors and chemotherapeutic drugs. Apoptosis induction and cell viability were analyzed *via* flow cytometry and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

**RESULTS:** TRAIL-R1 and -R2 were profoundly expressed on the HCC cell lines Huh7 and Hep-G2. However, treatment of Huh7 and Hep-G2 with TRAIL and agonistic antibodies only induced minor apoptosis rates. Apoptosis resistance towards TRAIL could be considerably reduced by adding the chemotherapeutic drugs 5-fluorouracil and doxorubicin as well as the kinase inhibitors LY294002 [inhibition of phosphoinositol-3-kinase (PI3K)], AG1478 (epidermal growth factor receptor kinase), PD98059 (MEK1), rapamycin (mammalian target of rapamycin) and the multi-kinase inhibitor Sorafenib. Furthermore, the antiapoptotic BCL-2 proteins MCL-1 and BCL-x<sub>L</sub> play a major role in TRAIL resistance: knock-down by RNA interference increased TRAIL-induced apoptosis of HCC cells. Additionally, knock-down of MCL-1 and BCL-x<sub>L</sub> led to a significant sensitization of HCC cells towards inhibition of both c-Jun N-terminal kinase and PI3K.

**CONCLUSION:** Our data identify the blockage of survival kinases, combination with chemotherapeutic drugs and targeting of antiapoptotic BCL-2 proteins as promising ways to overcome TRAIL resistance in HCC.

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### Abstract

**AIM:** To analyze the effect of chemotherapeutic drugs and specific kinase inhibitors, in combination with the death receptor ligand tumor necrosis factor-related apoptosis inducing ligand (TRAIL), on overcoming TRAIL resistance in hepatocellular carcinoma (HCC) and to study the efficacy of agonistic TRAIL antibodies, as well as the commitment of antiapoptotic BCL-2 proteins, in TRAIL-induced apoptosis.

**METHODS:** Surface expression of TRAIL receptors (TRAIL-R1-4) and expression levels of the antiapoptotic BCL-2 proteins MCL-1 and BCL-x<sub>L</sub> were analyzed by flow cytometry and Western blotting, respectively. Knock-down of MCL-1 and BCL-x<sub>L</sub> was performed by transfecting specific small interfering RNAs. HCC cells

**Key words:** Hepatocellular carcinoma; Apoptosis; Tumor necrosis factor-related apoptosis inducing ligand; BCL-x<sub>L</sub>; MCL-1; 5-fluorouracil; Doxorubicin; Sorafenib; Phosphoinositol-3-kinase; (Mitogen-activated protein kinase)/(extracellular signal regulated kinase) kinase; c-Jun N-terminal kinase

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide. It ranks at third place in the list of malignancies leading to death. Over the past decades the incidence of HCC has increased worldwide, especially in eastern Asia and sub-Saharan Africa<sup>[1,2]</sup>. HCC is clinically characterized by its invasiveness, poor prognosis and limited therapeutic opportunities, mostly due to the high resistance of HCC cells towards chemotherapeutic agents. Today, surgery is considered to be the only curative treatment procedure for most HCC patients<sup>[3]</sup>. However, in many patients, HCC is diagnosed at an advanced or metastasized stage. For the treatment of these patients, the Food and Drug Administration approved the multi-kinase inhibitor Sorafenib in 2007<sup>[4,5]</sup>, which highlights the fact that specific inhibition of survival pathways is an effective treatment option in HCC<sup>[6]</sup>.

Apoptosis is a genetically determined process of controlled cellular suicide<sup>[7]</sup>. Dysregulation of apoptosis is involved in the pathophysiology of liver diseases including hepatocarcinogenesis<sup>[8]</sup>. Resistance of HCC cells to apoptosis is a crucial aspect in cancer treatment because it impairs the efficacy of different therapy regimens<sup>[9]</sup>.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a promising anti-tumor agent since it is capable of killing tumor cells *via* receptor-mediated apoptosis<sup>[10,11]</sup>. TRAIL ligates two different types of receptors: (1) death receptors triggering TRAIL-induced apoptosis, and (2) decoy receptors possibly inhibiting the TRAIL death-signaling pathway. Receptors TRAIL-R1 and -R2 contain an intracellular death domain (DD) motif essential for signal transduction. In contrast, TRAIL-R3 (DcR1) and -R4 (DcR2) appear to act as “decoys”, lacking a DD. Due to this fact they are capable of binding the ligand without effecting a death signal. Under certain conditions, a relative TRAIL resistance occurs in cells expressing high levels of DcR1 or DcR2.

Binding of an agonistic ligand or mAb to TRAIL-R1 or -R2 leads to the intracellular formation of a protein complex termed death inducing signaling complex (DISC). DISC formation includes the activation of the apical activator caspase 8, representing the initial point of receptor-related apoptosis signaling.

In addition to this receptor-related extrinsic pathway, there is an intrinsic pathway of apoptosis, which is crucial as a cellular response to DNA damage and oxidative stress. Central organelles for the intrinsic pathway are mitochondria, where a delicate balance between pro- and antiapoptotic BCL-2 proteins decides cell destiny. If DNA damage or other intrinsic triggers occur, proapoptotic BCL-2 proteins and mitochondria are activated. Subsequently, a

multimeric protein complex, designated as an apoptosome, is formed. The apoptosome cleaves caspase 9, which in turn activates the downstream effector caspase 3, where intrinsic and extrinsic pathways of apoptosis converge.

Notably, receptor-mediated caspase 8 activation can promote an activation of mitochondria by cleavage and subsequent activation of the proapoptotic BCL-2 protein, BID<sup>[12]</sup>. The crosstalk between extrinsic and intrinsic apoptosis pathways amplifies a death signal mediated by TRAIL, leading to a more effective execution of apoptosis.

MCL-1 and BCL-x<sub>L</sub> are antiapoptotic members of the BCL-2 family serving as protective factors against several death stimuli. Both proteins were found to be expressed at a high level in different solid tumor entities, including HCC<sup>[13-15]</sup>. Antiapoptotic BCL-2 proteins interact with proapoptotic BCL-2 proteins BAX and BAK, thereby inhibiting the activation of mitochondria. It appears that high expression levels of MCL-1 and BCL-x<sub>L</sub> provide resistance of tumor cells to chemotherapeutic drugs and TRAIL<sup>[16,17]</sup>.

Resistance towards TRAIL can be due to failure at any step in the death signaling cascade. For example, TRAIL resistance can be located at receptor level due to an inappropriate expression or at DISC level mediated by proteins counteracting DISC formation<sup>[18-20]</sup>. Furthermore, an inability to activate mitochondria during apoptosis, due to high expression levels of antiapoptotic proteins (e.g. MCL-1), can cause resistance towards TRAIL<sup>[16,21]</sup>. Finally, antiapoptotic pathways, such as phosphoinositol-3-kinase (PI3K)/Akt signaling, are aberrantly activated in various tumor cells, thus contributing to TRAIL resistance<sup>[22,23]</sup>.

In our study, we investigated whether TRAIL resistance in HCC cells can be overcome by combining TRAIL with chemotherapeutic drugs, inhibitors of survival signaling or targeted therapies against antiapoptotic BCL-2 proteins.

## MATERIALS AND METHODS

### Reagents and cell lines

HCC cell lines, Hep-G2 and Huh7, were purchased from ECACC. Cells were cultured in DMEM (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), 1% Pen/Strep (PAA laboratories, Pasching, Austria), 1% HEPES and 1% L-Glutamine (Cambrex, Verviers, Belgium). Cells were cultivated at 37°C with a concentration of 5% CO<sub>2</sub>. Transfection experiments were performed in OPTIMEM (Invitrogen).

Reagents were purchased from the following suppliers: recombinant TRAIL (with Enhancer applied in a concentration of 1 µg/mL) and SuperKillerTRAIL (SkTRAIL) from Alexis Biochemicals (SanDiego, CA, USA), goat anti-human IgG F(ab)<sup>2</sup> from Meridian Life Science (Cincinnati, USA), 5-fluorouracil (5-FU), doxorubicin (Doxo) from Sigma (Deisenhofen, Germany), SP600125, AG1478, PD98059, LY294002 and rapamycin (RAPA) from Calbiochem (Schwalbach, Germany). LBY135 was supplied from Novartis (Basel, Switzerland), Sorafenib (BAY 43-9006) from Bayer (Leverkusen, Germany).



### Viability test

Cell viability was determined by a colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HCC cell lines were seeded onto 96-well plates. On day one after seeding, cells were treated as indicated. We added 10  $\mu$ L MTT (5 mg/mL) 48 h after treatment and incubated cells for a further 3 h at 37°C. Next, supernatant was discarded and cells were lysed by adding 100  $\mu$ L 1-propanol to each well followed by shaking plates till complete lysis. Absorbance was measured at 550 nm in a microtiter plate reader. A viability of 1 was defined as the absorbance of untreated cells.

### Coating of microtiter plates

To ease ligand-receptor interaction with the crosslinking supplement IgG F(ab)'<sub>2</sub>, 96-well plates were coated with IgG F(ab)'<sub>2</sub> before seeding cells. One hundred microliters of sterile filtered 100 nmol/L sodium bicarbonate buffer (pH 9.2) containing 5  $\mu$ g/mL IgG F(ab)'<sub>2</sub> was added to each well and incubated for 2 h at room temperature (RT). After replacement of F(ab)'<sub>2</sub> buffer by cell culture media, plates were stored at 4°C. Coated plates were stable for at least 1 wk.

### RNAi and transfection

To knock-down protein expression, we administered specific small interfering RNA (siRNA) against MCL-1 or BCL-xL. As a control we used siRNA specific for green fluorescent protein (GFP). The following siRNA sequences were applied (MWG Biotech, Ebersberg, Germany): BCL-xL, 5'-gcuuggauaaagaugcaaTT-3' (sense) and 5'-uugcaucuuuauccaagcAG-3' (antisense), MCL-1, 5'-aagauacagacguucucTT-3' (sense) and 5'-gagaacgucugugauacuuTT-3' (antisense), GFP, 5'-ggcuacguccaggagcgcacTT-3' (sense) and 5'-ggugcgcuccaggacguagccTT-3' (antisense). Here, capitals represent deoxyribonucleotides and lower case letters represent ribonucleotides. Huh7 cells were seeded onto 12-well plates and after 24 h transiently transfected in OPTIMEM with Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's protocol. Expression levels were analyzed 24, 48 and 72 h after transfection *via* Western blotting analysis.

### Detection of apoptosis

HCC cells were seeded onto 12-well plates and treated as indicated 1 d after transfection. Forty eight hours after treatment, cells were washed with cold PBS, collected and resuspended in a hypotonic buffer containing 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100, and 50  $\mu$ g/mL Propidium iodide (PI, Sigma). After 3 h incubation at 4°C, nuclei from apoptotic cells were quantified by fluorometric absorbance cell sorting according to the protocol of Nicoletti *et al*<sup>[24]</sup>.

### Cell lysis and Western blotting

Cell lysis, SDS-PAGE and Western blotting were performed as described previously<sup>[13]</sup>. Immunodetection of proteins was performed using the following antibodies:

anti-BCL-xL (Labvision/NeoMarkers, Warm Springs Blvd. Fremont, Canada), anti-MCL-1 (Santa Cruz Biotechnology, Heidelberg, Germany) and anti- $\alpha$ -tubulin (Sigma) as loading control.

### Detection of receptor expression

HCC cells were cultured as described and collected. Five hundred thousand cells for each receptor analysis were transferred to polystyrene tubes, washed twice with PBS and resuspended in PBS containing 0.5% BSA (Sigma). A specific monoclonal antibody to either TRAIL-R1, -R2, -R3, -R4 or unspecific mouse IgG1 as isotype control was applied at 5  $\mu$ g/mL. Cells were incubated for 20 min with gentle rocking at RT. Cells were washed twice in PBS and secondary fluorescein isothiocyanate-conjugated polyclonal goat antibody to mouse IgG1 (1:200 in PBS containing 0.5% BSA) was added, followed by incubation protected from light for 30 min with gentle rocking at RT. Cells were then washed and resuspended in PBS containing 0.5% BSA. Analysis of receptor expression was performed *via* flow cytometry. All antibodies were purchased from Alexis.

### Statistical analysis

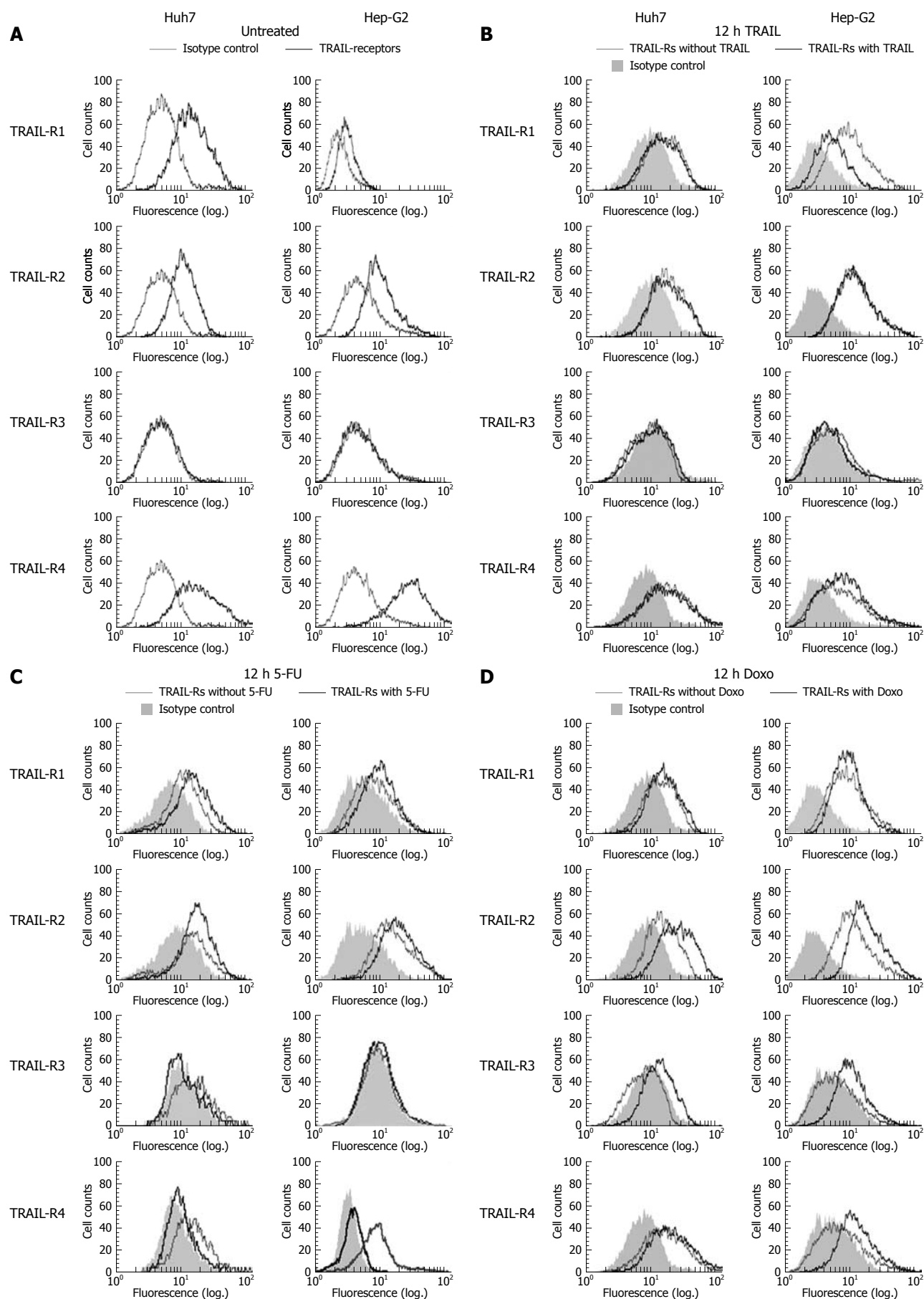
All results are expressed as mean  $\pm$  SD. Data were analyzed by students *t*-test (paired, two-sided) based on normal data distribution. *P* < 0.05 was considered significant.

## RESULTS

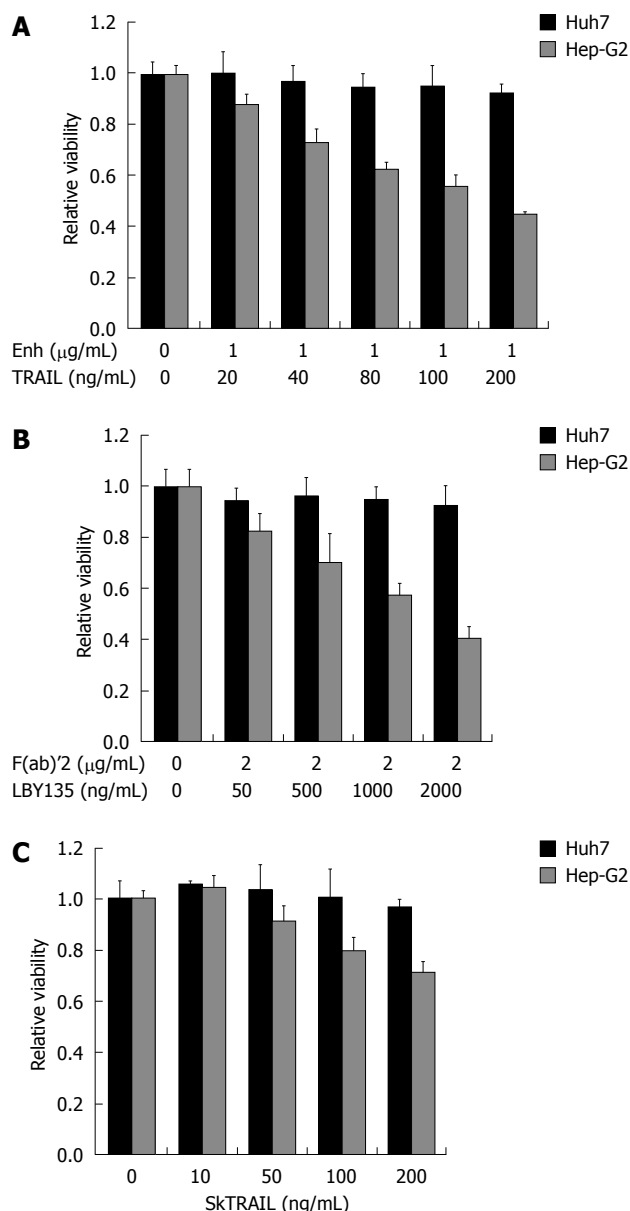
### TRAIL receptor expression in HCC cells upon treatment with TRAIL and chemotherapeutic agents

It is known that TRAIL resistance can be mediated at the receptor level, either by low expression of TRAIL-R1 and -R2 or by a comparably high expression of TRAIL-R3 and -R4<sup>[25]</sup>. Firstly, we analyzed surface receptor expression of the HCC cell lines Huh7 and Hep-G2. Except for TRAIL-R3, all receptors were found to be expressed: we detected high expression levels of TRAIL-R1, -R2 and -R4 in both cell lines (Figure 1A). Next, we analyzed the expression levels after treatment with TRAIL and consequently the possibility of TRAIL-induced regulation in a feedback manner. After 12 h-treatment with TRAIL, we observed downregulation of TRAIL-R1 and a moderate upregulation of TRAIL-R4 in Hep-G2 cells. In contrast, no changes in receptor expression were detected in Huh7 cells (Figure 1B).

In order to study the effect of chemotherapeutics on TRAIL receptor expression, we treated HCC cells with 5-FU and Doxo, both applied for transarterial chemoembolization in patients with HCC<sup>[26]</sup>. 12 h-treatment with 5-FU resulted in upregulation of TRAIL-R1 and -R2 in both cell lines. In contrast, TRAIL-R3 was downregulated in Huh7 and unaffected in Hep-G2 cells. For TRAIL-R4, we observed a significant downregulation in both Hep-G2 and Huh7 cells (Figure 1C). 12 h-treatment with Doxo resulted in a slight upregulation of TRAIL-R1 in both cell lines. Remarkably, TRAIL-R2 was considerably upregulated. TRAIL-R3 surface expression



**Figure 1** Surface expression of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptors on Huh7 and Hep-G2 cells. Flow cytometric analysis of TRAIL receptors was performed using monoclonal mouse IgG1, anti-TRAIL-R1, -R2, -R3, -R4 antibodies and secondary FITC-conjugated polyclonal goat anti mouse-IgG1 antibodies. Unspecific mouse IgG1 antibodies were used as isotype control. Receptor surface expression was analyzed in untreated Huh7 and Hep-G2 cells (A) and 12 h after treatment with 100 ng/mL rec. TRAIL + 1  $\mu$ g/mL Enhancer (B), 50  $\mu$ g/mL 5-fluorouracil (5-FU) (C) and 0.5  $\mu$ mol/L doxorubicin (D). Diagrams are representative of at least two independent experiments. Doxo: Doxorubicin.



**Figure 2** TRAIL-induced apoptosis in hepatocellular carcinoma (HCC) cells. Huh7 and Hep-G2 cells were seeded onto 96-well plates and treated on day one after seeding with different TRAIL compounds. A: Cells were treated for 48 h with rec. TRAIL + 1 μg/mL Enhancer as indicated; B: Plates were coated with crosslinker IgG F(ab)'2 for 24 h before seeding of cells. Cells were then treated for 48 h with LBY135 + 1 μg/mL F(ab)'2 as indicated; C: Cells were treated for 48 h with SkTRAIL as indicated. Cell viability was analyzed by MTT assay. Viability is shown relative to untreated controls. Assays were performed in six-fold values and are representative of three independent experiments. Values are expressed as mean ± SD. Enh: Enhancer.

was detectable in both cell lines after Doxo treatment. TRAIL-R4 was upregulated in Hep-G2 and unaffected in Huh7 cells (Figure 1D).

#### Sensitivity of HCC cells towards different TRAIL compounds

Next, we determined sensitivity of HCC cells towards TRAIL-induced apoptosis. Firstly, we analyzed the effect of recombinant TRAIL in concentrations from 20 up to 200 ng/mL (combined with Enhancer, 1 μg/mL). Hep-G2 cells were sensitive towards recombinant TRAIL in a dose-dependent manner, whereas Huh7 were resistant

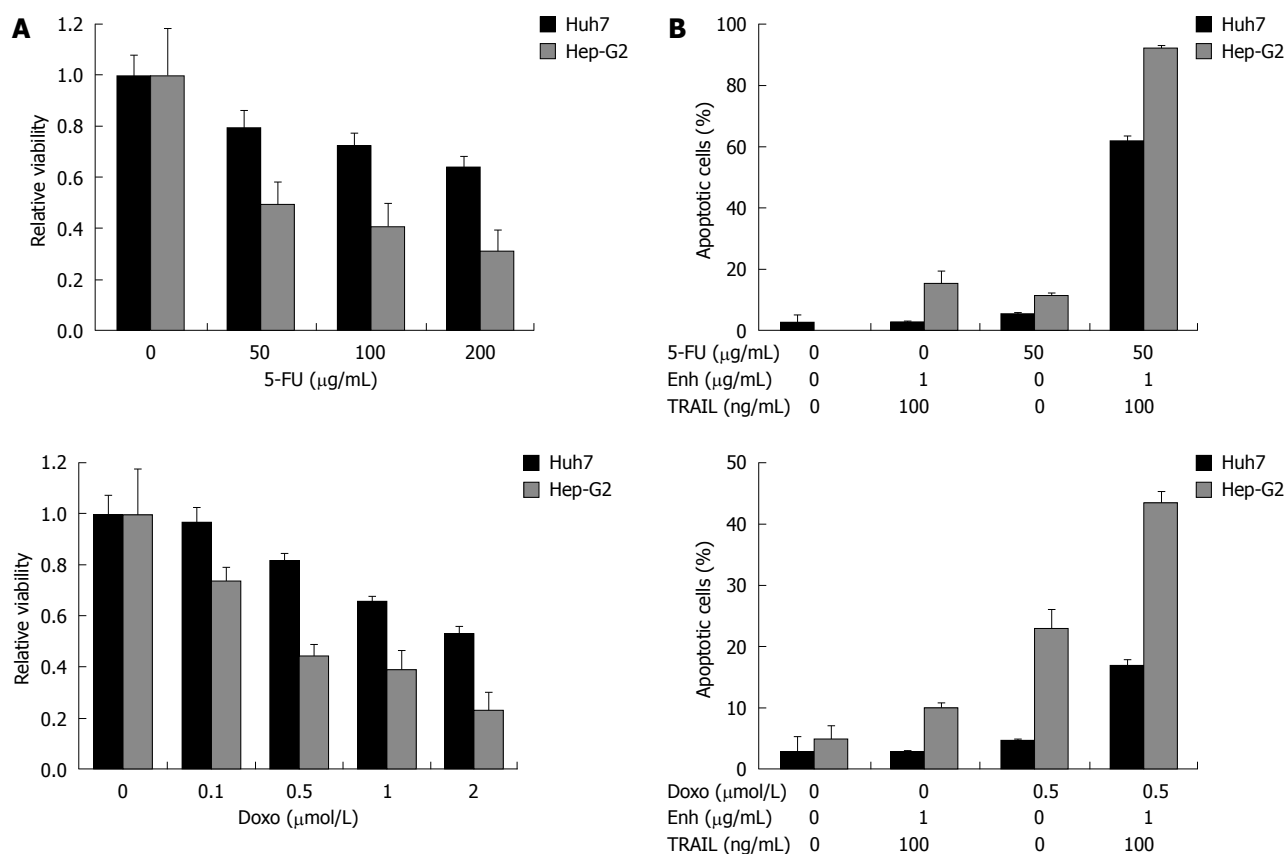
(Figure 2A). Next, we tested LBY135, a chimeric monoclonal antibody targeting TRAIL-R2, in concentrations from 50 to 2000 ng/mL, together with the cross linker F(ab)'2 (2 μg/mL). To optimize the interaction between LBY135 and F(ab)'2, we coated the plates with the F(ab)'2 -fragment before seeding the cells. Hep-G2 showed moderate sensitivity towards LBY135-induced apoptosis in a dose-dependent manner, whereas Huh7 cells were resistant (Figure 2B). To discover whether resistance was due to impaired interactions between enhancer or F(ab)'2, the ligand and the receptor, we included SkTRAIL in our study. SkTRAIL interacts effectively with TRAIL receptors without additional supplements. Again, Hep-G2 cells revealed a dose-dependent sensitivity to SkTRAIL in concentrations from 10 to 200 ng/mL. In contrast, Huh7 cells were resistant to SkTRAIL (Figure 2C).

#### Treatment of HCC cells with TRAIL in combination with chemotherapy

Next, we analyzed whether HCC cells were sensitized to TRAIL-induced apoptosis by co-treatment with the chemotherapeutic drugs 5-FU and Doxo. As a first step, we analyzed whether the chemotherapeutics induced loss of viability if applied alone: after 48 h treatment of Huh7 and Hep-G2 cells with 5-FU (50, 100 and 200 μg/mL) and Doxo (0.1, 0.5, 1 and 2 μmol/L), we observed a dose-dependent decrease of cell viability (Figure 3A). Next, we applied these agents in concentrations which exhibited less significant cytotoxic effects, in combination with recombinant TRAIL (+ Enhancer 1 μg/mL). 5-FU (50 μg/mL) or TRAIL (100 ng/mL) did not induce apoptosis in Huh7 cells when administered alone. However, combination of 5-FU and TRAIL induced apoptosis in 62% of Huh7 cells. In Hep-G2 cells, TRAIL (100 ng/mL) induced apoptosis in 15% of cells. 5-FU treatment alone triggered apoptosis of 12% of Hep-G2 cells. 5-FU and TRAIL co-treatment of Hep-G2 resulted in 93% apoptotic cells (Figure 3B, upper panel). Next, we tested the combination of Doxo (0.5 μmol/L) and TRAIL (100 ng/mL). Doxo induced apoptosis in less than 5% of Huh7 cells, whereas the combination of Doxo and TRAIL resulted in 17% apoptotic cells. Treatment of Hep-G2 with Doxo alone induced apoptosis in 24% of cells, whereas Doxo and TRAIL in combination led to apoptosis rates of 43% (Figure 3B, lower panel).

#### Treatment of HCC cells with TRAIL in combination with specific kinase inhibitors

Antiapoptotic pathways such as PI3K/Akt, epidermal growth factor receptor (EGFR), [mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) kinase] (MEK)/ERK are well known to be activated in malignant cells, thus contributing to cell cycle progression and tumor growth. Therefore, we analyzed whether inhibition of kinases involved in these pathways could overcome resistance towards TRAIL-mediated apoptosis. Firstly, we applied the multi-kinase inhibitor Sorafenib to inhibit RAF/MEK/ERK signaling, in escalating concentrations (2.5, 5 and 10 μmol/L). A dose-dependent decrease of cell viability in Huh7 and Hep-G2



**Figure 3** Treatment of HCC cells with TRAIL in combination with 5-FU and doxorubicin. Values are expressed as mean  $\pm$  SD. A: Huh7 and Hep-G2 cells were analyzed for cell viability after treatment with the chemotherapeutic agents 5-FU and doxorubicin alone. Cells were seeded onto 96-well plates and treated on day one after seeding. Cells were treated for 48 h with 5-FU (upper left panel) and doxorubicin (lower left panel) as indicated. Cell viability was analyzed by MTT assay. Viability is shown relative to untreated controls. Assays were performed in six-fold values; B: Apoptosis induction in Huh7 and Hep-G2 cells treated with 50  $\mu\text{g/mL}$  5-FU (upper right panel) and 0.5  $\mu\text{mol/L}$  doxorubicin (lower right panel) either alone or in combination with 100 ng/mL TRAIL + 1  $\mu\text{g/mL}$  Enhancer. Cells were seeded onto 12-well plates, harvested 48 h after treatment and analyzed for apoptosis induction by flow cytometry. Assays were performed in triplicate and are representative of at least two independent experiments.

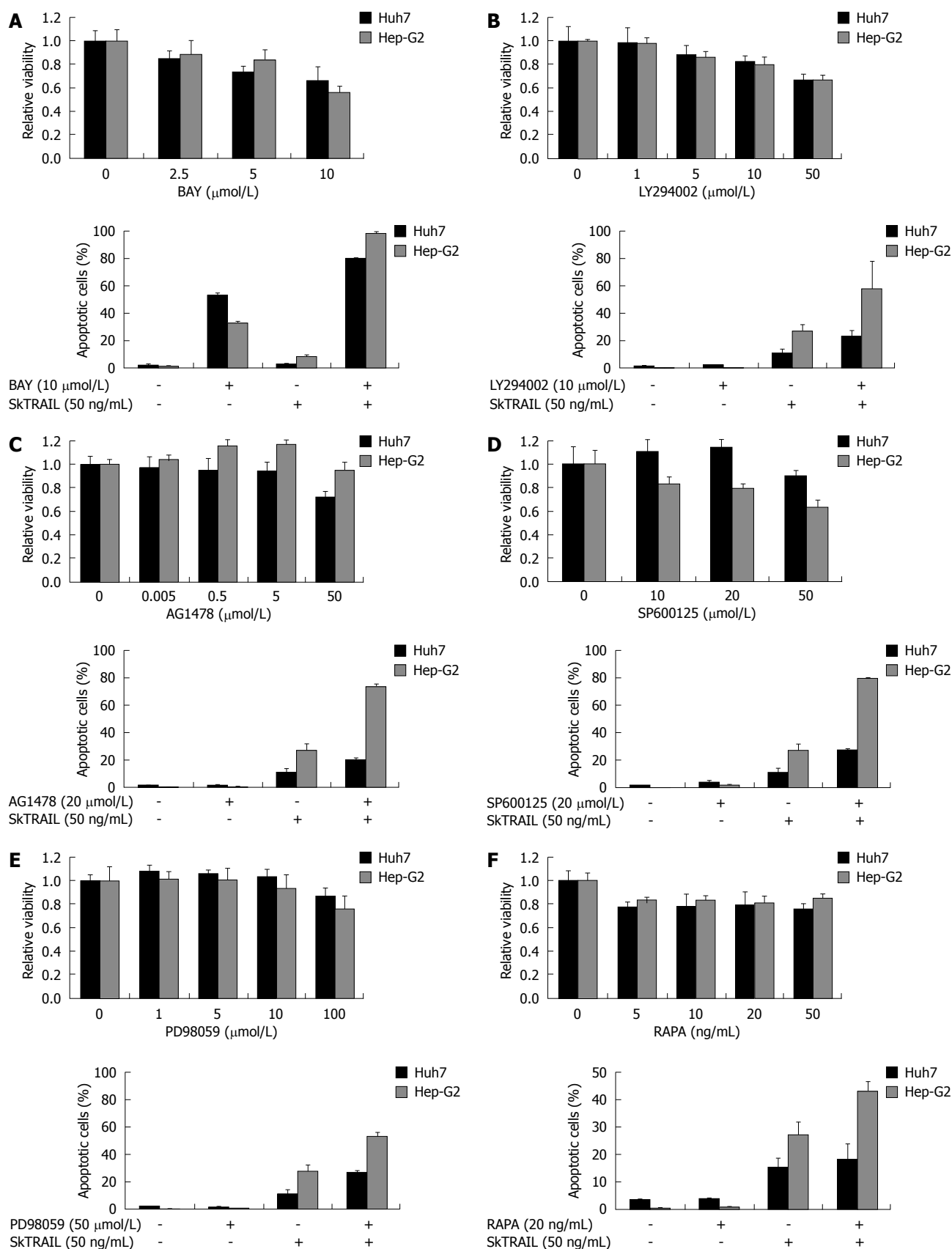
was observed (Figure 4A, upper panel). In a second step, we analyzed the impact of Sorafenib on TRAIL treatment. In Huh7 cells, Sorafenib (10  $\mu\text{mol/L}$ ) induced apoptosis rates of 50%. Strikingly, the combination of SkTRAIL (50 ng/mL) and Sorafenib (10  $\mu\text{mol/L}$ ) induced apoptosis in 80% of the cells. In Hep-G2 cells Sorafenib caused only minor apoptosis rates (33%). However, combination of TRAIL and Sorafenib led to 98% apoptotic cells (Figure 4A, lower panel).

Next, we inhibited the PI3K/Akt pathway by application of the PI3K inhibitor LY294002. A slight decrease of cell viability was observed in Huh7 and Hep-G2 after 48 h treatment in concentrations lower than 50  $\mu\text{mol/L}$  (Figure 4B, upper panel). However, combination of LY294002 (10  $\mu\text{mol/L}$ ) and SkTRAIL (50 ng/mL) doubled apoptosis rates in Hep-G2 cells to 59% compared to SkTRAIL treatment alone. In Huh7, we observed an increased rate of apoptosis after treatment with the combination of LY294002 and SkTRAIL compared to SkTRAIL alone (23% *vs* 11%, respectively. Figure 4B, lower panel). Furthermore, we used AG1478 to inhibit EGFR kinase. Interestingly, inhibition of EGFR kinase increased cell viability in Hep-G2 cells in concentrations up to 5  $\mu\text{mol/L}$ . In Huh7 cells, AG1478 caused no significant changes in cell viability when applied in low concen-

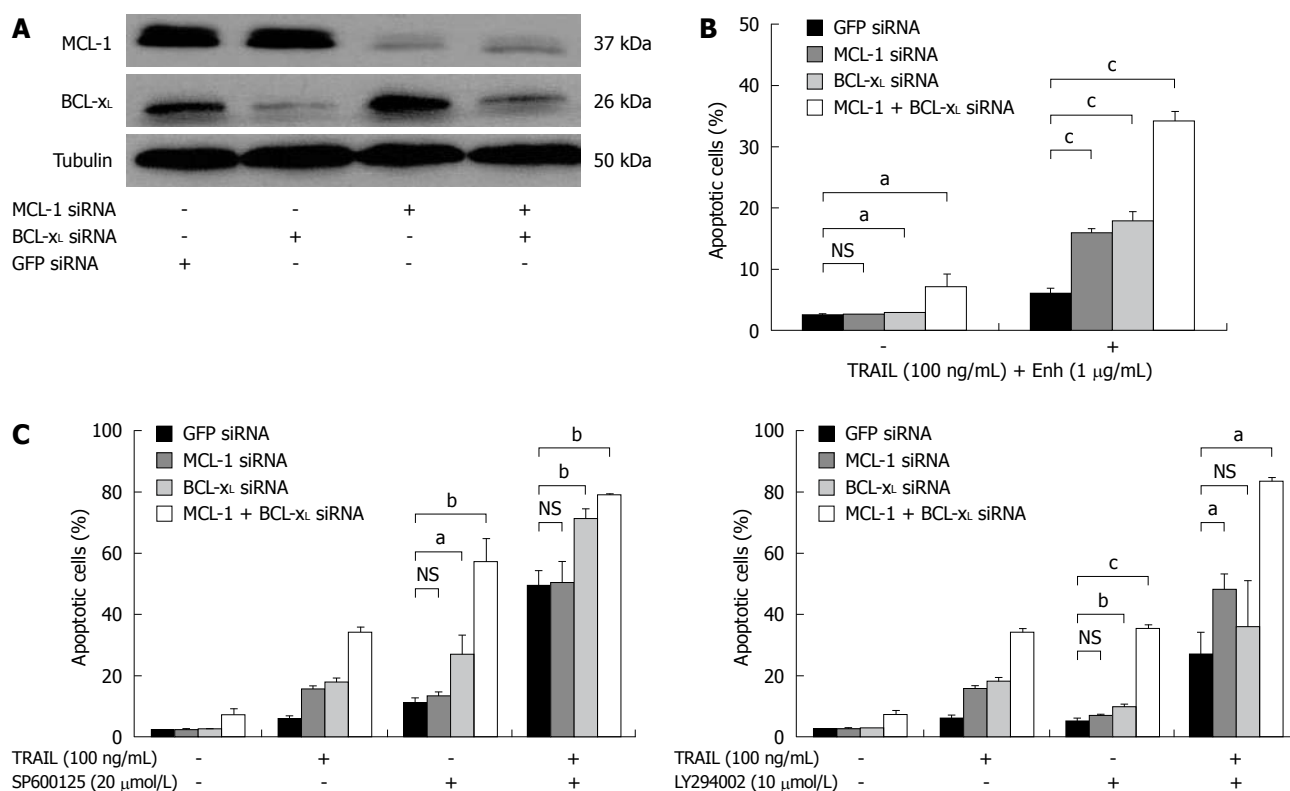
trations (Figure 4C, upper panel). AG1478 (20  $\mu\text{mol/L}$ ) and SkTRAIL (50 ng/mL) co-treatment increased the rate of apoptotic cells to 27% in Huh7 and 74% in Hep-G2 cells (Figure 4C, lower panel). Next, we inhibited the c-Jun N-terminal kinases 1 and 2 (JNK1 and JNK2) with the anthrapyrazolone inhibitor SP600125. We observed increased cell viability in Huh7 cells and a slight, dose-dependent decrease of cell viability in Hep-G2 cells after 48 h of SP600125 treatment (Figure 4D, upper panel). Notably, a high percentage of cells were arrested in the G2 phase 48 h after treatment with the JNK inhibitor (data not shown). Combined treatment with SP600125 (20  $\mu\text{mol/L}$ ) and SkTRAIL (50 ng/mL) led to 28% apoptosis of Huh7 and to 80% apoptosis of Hep-G2 cells (Figure 4D, lower panel). Next, we included a specific inhibitor of MAP kinase kinase (MEK), PD98059, in our study. Again, a death inducing effect of MEK inhibition alone was only observed when applied in high concentrations of more than 50  $\mu\text{mol/L}$  (Figure 4E, upper panel). However, in combination (50  $\mu\text{mol/L}$  PD98059 and 50 ng/mL SkTRAIL), a two-fold increase of apoptosis, compared to monotherapy with SkTRAIL, was detectable in Huh7 and Hep-G2 cells (Figure 4E, lower panel).

Finally, we inhibited mammalian target of rapamycin (mTOR) with rapamycin (Sirolimus). Rapamycin alone





**Figure 4** Treatment of HCC cells with TRAIL in combination with specific kinase inhibitors. Viability of HCC cells treated with kinase inhibitors alone (upper panels). On day one after seeding of Huh7 and Hep-G2 cells onto 96-well plates, cells were treated with multi-kinase inhibitor Sorafenib (A), PI3 kinase inhibitor LY294002 (B), EGFR kinase inhibitor AG1478 (C), JNK inhibitor SP600125 (+ 0.2% DMSO as vehicle) (D), MEK inhibitor PD98059 (E) and mTOR inhibitor rapamycin (RAPA) (F) at the indicated concentrations for 48 h. Cell viability was analyzed by MTT assay. Viability is shown relative to untreated or 0.2% DMSO treated controls, respectively. Assays were performed in six-fold values. Values are expressed as mean  $\pm$  SD. Apoptosis induction in Huh7 and Hep-G2 cells treated with 10  $\mu\text{mol/L}$  Sorafenib (A), 10  $\mu\text{mol/L}$  LY294002 (B), 20  $\mu\text{mol/L}$  AG1478 (C), 20  $\mu\text{mol/L}$  SP600125 (+ 0.2% DMSO as vehicle) (D), 50  $\mu\text{mol/L}$  PD98059 (E) and 20 ng/mL rapamycin (F) in combination with 50 ng/mL SkTRAIL (lower panels). Cells were seeded 1 d before treatment onto 12-well plates, harvested 48 h after treatment and analyzed for apoptosis induction by flow cytometry. Assays were performed in triplicate and are representative of at least two independent experiments. Values are expressed as mean  $\pm$  SD.



**Figure 5** TRAIL-induced apoptosis in Huh7 cells after targeted therapy approaches and knock-down of BCL-xL and MCL-1. **A:** Huh7 cells were transfected with siRNAs (40 nmol/L) specific for MCL-1 and BCL-xL either alone or in combination. SiGFP was used as control. Whole cell lysates were prepared 24 h after transfection. MCL-1 and BCL-xL expression was analyzed by Western blotting.  $\alpha$ -Tubulin expression was used to control equal loading; **B:** 24 h after siRNA transfection, cells were treated for 48 h with 100 ng/mL TRAIL (+ 1 µg/mL Enhancer); **C:** 20 µmol/L SP600125 (left panel) or 10 µmol/L LY294002 (+ 0.2% DMSO as vehicle, right panel), either alone or in combination with 100 ng/mL TRAIL (+ 1 µg/mL Enhancer). Cells were harvested on day two after treatment and analyzed for apoptosis induction by flow cytometry. Assays were performed in triplicate and are representative of at least two independent experiments. Values are expressed as mean  $\pm$  SD. NS: Not significant. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ .

only caused a moderate decrease of cell viability (20%) in Huh7 and Hep-G2 cells (Figure 4F, upper panel). Combination of 20 ng/mL rapamycin with 50 ng/mL SkTRAIL resulted in a slight increase of apoptosis rates in Huh7 cells (18% *vs* 15% SkTRAIL alone) and a profound increase of apoptosis in Hep-G2 cells (43% *vs* 27%, Figure 4F, lower panel).

#### Treatment of HCC cells with TRAIL after knock-down of MCL-1 and BCL-xL

The antiapoptotic BCL-2 proteins, MCL-1 and BCL-xL, are profoundly expressed in tissues of human HCC, thus contributing to apoptosis resistance of HCC cells<sup>[13,15,27]</sup>. To analyze the role of antiapoptotic BCL-2 proteins in TRAIL-induced apoptosis, we manipulated their expression in Huh7 cells *via* specific siRNA-mediated knock-down. An effective reduction of MCL-1 and BCL-xL expression levels was observed 24 h after transfection (Figure 5A).

A knock-down of BCL-xL induced significant apoptosis in comparison to mock transfected cells ( $P < 0.05$ ). Knock-down of MCL-1 did not induce significant apoptosis rates. Additionally, combined knock-down of MCL-1 and BCL-xL induced spontaneous apoptosis in 8% of Huh7 cells ( $P < 0.05$ , Figure 5B). Downregulation of either MCL-1 or BCL-xL significantly enhanced susceptibility towards TRAIL-induced apoptosis (17% *vs*

6% and 18% *vs* 6%, respectively,  $P < 0.001$ ). Remarkably, we detected 34% apoptotic cells in Huh7 lacking BCL-xL and MCL-1 expression after treatment with TRAIL ( $P < 0.001$ , Figure 5B). Furthermore, we analyzed whether lack of MCL-1 and BCL-xL expression sensitized cells towards the JNK inhibitor SP600125 and the PI3K inhibitor LY294002. Inhibition of JNK and PI3K showed significantly enhanced anti-tumoral efficacy after knock-down of BCL-xL and MCL-1. In cells lacking BCL-xL expression, apoptosis was induced in 27% *vs* 11% of control cells after treatment with SP600125 (20 µmol/L) ( $P < 0.05$ , Figure 5C). In contrast, cells lacking MCL-1 did not show increased susceptibility to JNK inhibition (14% *vs* 11%, not significant, Figure 5C). Knock-down of MCL-1 and BCL-xL increased SP600125-induced apoptosis rates to 57% ( $P < 0.005$ , Figure 5C, left panel). Additionally, single knock-down of BCL-xL ( $P < 0.001$ ) and double knock-down of MCL-1 and BCL-xL ( $P < 0.001$ ) significantly increased apoptosis after combined treatment of SP600125 with recombinant TRAIL (100 ng/mL). Single knock-down of MCL-1 did not exhibit sensitizing effects (differences not significant, Figure 5C).

Next, we analyzed the effects of MCL-1 and BCL-xL knock-down in combination with the PI3K inhibitor LY294002. We observed a significant sensitizing effect of BCL-xL knock-down on LY294002-induced apoptosis in Huh7 cells ( $P < 0.005$ , Figure 5C, right panel). Knock-

down of MCL-1 did not increase LY294002-induced apoptosis. However, in Huh7 cells lacking both MCL-1 and BCL-xL, apoptosis rates increased to 35% after LY294002 treatment ( $P < 0.001$ ). Finally, we found an increased rate of apoptosis after combined treatment of LY294002 (10  $\mu\text{mol/L}$ ) with recombinant TRAIL (100 ng/mL) in cells lacking MCL-1 (48% *vs* 27% of mock transfected Huh7,  $P < 0.05$ ). A moderate sensitizing effect in cells lacking BCL-xL was observed (not significant). Importantly, the combined knock-down of MCL-1 and BCL-xL caused apoptosis rates of 83%, if cells were treated with a combination of LY294002 and recombinant TRAIL ( $P < 0.05$ , Figure 5C, right panel).

## DISCUSSION

Amongst the various approaches to induce apoptosis in tumor cells, application of the death receptor ligand TRAIL is very promising. Preclinical studies suggest that TRAIL induces apoptosis of tumor cells *in vivo* without lethal toxicities<sup>[28,29]</sup>. A major obstacle for the clinical use of TRAIL is its limited efficacy in monotherapeutic approaches in different tumor entities. Thus, it appears worthwhile to persist in investigating ways to enhance TRAIL's capacity for apoptosis induction. Resistance towards TRAIL can be caused at receptor level by inhibitory proteins and at mitochondrial level by antiapoptotic proteins<sup>[17,18,21]</sup>. For example, a diminished membrane expression of TRAIL-R1 and -R2, as well as reduced caspase 8 levels, mediate TRAIL resistance in myeloma cells<sup>[19]</sup>. In this present study we analyzed different approaches in sensitizing HCC cells to TRAIL-induced apoptosis.

TRAIL receptor expression was similar in the HCC cell lines Huh7 and Hep-G2. After TRAIL treatment, expression patterns changed only slightly in Hep-G2 cells. Strikingly, chemotherapeutic drugs influenced the expression pattern in HCC cells. Upregulation of TRAIL-R1 and profound upregulation of TRAIL-R2 after Doxo and 5-FU treatment in both cell lines might represent a potential mechanism of chemotherapy-mediated TRAIL sensitization. Interestingly, TRAIL-R3 (DcR1) was also upregulated after chemotherapy. This could also represent a mechanism of TRAIL resistance upon chemotherapy, since TRAIL-R3 acts as a decoy receptor. Notably, upregulation of TRAIL receptors in Huh7 cells which express mutated p53 suggests that receptor regulation occurs independently from p53<sup>[30]</sup>.

Several mAbs targeting TRAIL receptors and recombinant TRAIL agonists have already entered clinical trials<sup>[31-33]</sup>. In order to analyze the efficacy of different TRAIL compounds, we included LBY135, a chimeric antibody targeting TRAIL-R2, recombinant TRAIL and SkTRAIL in our study. We demonstrate that crosslinking elements IgG F(ab)'2 are mandatory for LBY135-induced apoptosis. Consistent findings were obtained for recombinant TRAIL, where combination with an enhancer is necessary to induce apoptosis. Comparing the death-inducing capacities of LBY135, TRAIL and SkTRAIL in HCC cells, we assume that TRAIL-R2 plays

a major role, which would be in line with observations in colon and breast cancer<sup>[34]</sup>. In contrast, it has been shown that chronic leukemia cells are selectively sensitive to TRAIL-R1<sup>[35]</sup>. Taken together, it appears likely that a cell type dependency determines the efficiency of TRAIL-mediated apoptosis induction, even if both TRAIL-R1 and -R2 are expressed.

Chemotherapeutic drugs such as Doxo and 5-FU have shown limited efficacy for the treatment of HCC<sup>[26]</sup>. However, anti-tumoral effects have been described for Doxo if administered into the liver *via* chemoembolization<sup>[26,36]</sup>. In our study we aimed to discover whether the combination of TRAIL with chemotherapy exerts anti-tumoral effects in HCC. Importantly, chemotherapy with Doxo or 5-FU increased TRAIL susceptibility in Hep-G2 cells and sensitized Huh7 cells towards TRAIL, opening the possibility of a treatment regime including reduced doses of chemotherapeutic drugs in combination with TRAIL.

The multi-kinase inhibitor Sorafenib has recently been approved for the therapy of advanced HCC. Sorafenib acts by inhibition of the RAF/MEK/ERK pathway and downregulation of MCL-1, leading to a disruption of survival signals in HCC cells<sup>[4,37]</sup>. In combination with TRAIL, Sorafenib profoundly increased apoptosis induction advocating TRAIL as a potential and effective agent for HCC treatment along with Sorafenib.

There is evidence that constitutive activation of various antiapoptotic pathways is a basic principle of tumor growth, cell cycle progression and apoptosis resistance. A well described antiapoptotic pathway is the PI3K/Akt signaling pathway, found activated in several tumor entities, including HCC<sup>[38]</sup>. The PI3K inhibitor LY294002<sup>[39,40]</sup> has already been employed in preclinical studies in combination with TRAIL. Consistent with data for prostate cancer and leukemia cells, our results indicate that blockage of PI3K by LY294002 overcomes resistance towards TRAIL in HCC cells<sup>[22,23]</sup>.

The mTOR, a protein with growing clinical relevance in oncology, is located downstream of PI3K<sup>[41]</sup>. The significant sensitization towards TRAIL in Hep-G2 cells by mTOR inhibition underlines a pivotal role of PI3K/Akt signaling for the resistance of HCC towards TRAIL.

In addition, the MAPK/ERK pathway exerts antiapoptotic effects in cancer cells. The MEK is a key component downstream of Raf serine/threonine kinases<sup>[42,43]</sup>. MEK inhibitors have been described as sensitizing human cancer cells to apoptosis, e.g. after treatment with chemotherapeutic agents<sup>[44,45]</sup>. In this study, we observed no apoptosis induction and only a slight decrease of cell viability after MEK inhibition in HCC cells. However, the combination of MEK inhibition and TRAIL caused a significant increase of TRAIL-induced apoptosis. This observation suggests that an aberrantly activated Raf/MAPK/ERK pathway plays a crucial role for TRAIL resistance in HCC.

Furthermore, we focused on the EGFR, which is an upstream receptor in Ras-Raf-MEK-ERK signaling<sup>[46]</sup>. It has been shown that overexpression of EGFR represents a protective factor against apoptosis stimuli in

HCC<sup>[47,48]</sup>. The combined treatment of TRAIL with the specific EGFR kinase inhibitor AG1478 caused a significant increase of TRAIL-induced apoptosis in HCC cells. Thus, EGFR blockage is another promising approach for TRAIL sensitization of HCC cells.

Recently, it has been shown that JNK inhibition sensitizes HCC cells, but not healthy hepatocytes, towards TRAIL-induced apoptosis<sup>[49]</sup>. In contrast, other results indicate that JNK activation is not relevant for TRAIL-induced apoptosis<sup>[50]</sup>. We found a significantly increased proapoptotic effect of TRAIL if combined with the JNK inhibitor SP600125.

Aberrant activity of survival signaling pathways exerts antiapoptotic effects at least in part *via* triggering of the expression of antiapoptotic proteins, such as antiapoptotic BCL-2 proteins. Importantly, antiapoptotic BCL-2 proteins, such as MCL-1 and BCL-x<sub>L</sub>, have been described as contributing to TRAIL resistance in cancer cells<sup>[51]</sup>. MCL-1 and BCL-x<sub>L</sub> mainly act by directly inhibiting their proapoptotic relatives BAX and BAK, thereby guarding the cell from various death stimuli. In addition, expression of antiapoptotic BCL-2 proteins is a prognostic factor for various tumor entities, e.g. expression of MCL-1 in breast and gastric cancer<sup>[52,53]</sup>. In the liver, MCL-1 has been found to be a key factor for apoptosis regulation<sup>[13,54,55]</sup>. A lack of MCL-1 causes increased rates of apoptosis and a significantly higher susceptibility towards chemotherapeutic treatment in HCC<sup>[54]</sup>. In addition, it has been shown that MCL-1 acts as a key factor for resistance towards TRAIL in leukemia cells<sup>[56]</sup>. In our study, we show that knock-down of MCL-1 or Bcl-x<sub>L</sub> increased TRAIL-induced apoptosis in HCC. Taking into consideration that there is a putative functional redundancy between these two proteins, we performed a double knock-down of MCL-1 and BCL-x<sub>L</sub>. Cells lacking both MCL-1 and BCL-x<sub>L</sub> expression showed profound spontaneous apoptosis, indicating that both proteins contribute to mitochondrial integrity in HCC cells. Importantly, HCC cells lacking MCL-1, BCL-x<sub>L</sub> or both showed an increased apoptosis rate after treatment with TRAIL. In summary, our data suggest a central role of MCL-1 and Bcl-x<sub>L</sub> for the resistance of HCC cells towards TRAIL-induced apoptosis.

Additionally, recent studies have revealed a synergistic effect of PI3K/Akt signaling with MCL-1 and BCL-x<sub>L</sub>, contributing to apoptosis resistance in cancer<sup>[57-59]</sup>. In our study, we treated HCC cells with TRAIL in combination with a PI3K inhibitor and with a knock-down of MCL-1 and BCL-x<sub>L</sub> *via* RNA interference. We found that cells lacking BCL-x<sub>L</sub> or lacking both MCL-1 and BCL-x<sub>L</sub> evolve a significantly increased sensitivity to apoptosis induced by PI3K inhibition. A knock-down of MCL-1 alone did not enhance LY294002-induced apoptosis. Importantly, we observed a profound increase of apoptosis in cells lacking MCL-1 and a rather low increase in cells lacking Bcl-x<sub>L</sub> after combined treatment with PI3K inhibitors and TRAIL. Strikingly, combined knock-down of MCL-1 and BCL-x<sub>L</sub> led to profound induction of apoptosis after treatment with PI3K inhibitors and TRAIL. In summary, our results suggest a major role of PI3K/Akt signaling in resistance towards TRAIL-mediated apoptosis and

emphasize the role of antiapoptotic BCL-2 proteins for TRAIL resistance of HCC cells.

A recent study showed that JNK1 exerts antiapoptotic effects *via* stabilization of MCL-1 in hepatocytes<sup>[60]</sup>. Therefore, we analyzed the role of MCL-1 and BCL-x<sub>L</sub> expression for the apoptosis-inducing capacity of JNK inhibitors and TRAIL. We found profoundly increased apoptosis rates after JNK inhibition in cells lacking BCL-x<sub>L</sub>, whereas cells lacking MCL-1 did not exhibit sensitivity towards JNK inhibition. Strikingly, a combined treatment of a JNK inhibitor and TRAIL caused major rates of apoptosis in cells lacking MCL-1 and BCL-x<sub>L</sub>. Furthermore, we could show that inhibition of JNK is not capable of inducing apoptosis alone unless BCL-x<sub>L</sub> is effectively downregulated in HCC cells, revealing a major role of BCL-x<sub>L</sub> for the resistance of HCC cells towards apoptosis induced by JNK inhibitors.

In conclusion, the application of recombinant TRAIL, as well as that of monoclonal antibodies targeting TRAIL receptors, is an encouraging therapeutic approach in cancer patients. However, resistance to TRAIL treatment is a common phenomenon in many cancer entities. The aim of our study was to provide novel treatment options to overcome resistance of HCC cells towards TRAIL-induced apoptosis. Our results demonstrate that TRAIL is an effective treatment option in HCC if combined with the chemotherapeutic drugs Doxo and 5-FU or kinase inhibitors, such as LY294002, AG1478 and PD98059. In addition, we revealed a pivotal role of the antiapoptotic BCL-2 proteins MCL-1 and BCL-x<sub>L</sub> for HCC resistance towards TRAIL. Further studies are warranted to evaluate the potential of combined treatment approaches and clear the trail for clinical usage.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide. Numerous clinical trials have failed to establish an effective therapy regimen in patients with advanced or metastasized HCC. Thus, new strategies for these patients are mandatory. Defects in apoptosis signaling contribute to resistance of HCC cells towards the death receptor ligand tumor necrosis factor-related apoptosis inducing ligand (TRAIL), which is a promising anti-tumor agent since it is capable of killing tumor cells *via* receptor-mediated apoptosis. New combined treatment regimens have the aim of overcoming resistance towards TRAIL and to make TRAIL an effective treatment option in patients suffering from HCC.

### Research frontiers

Hyperactivation of the PI3K/Akt, EGFR and [Mitogen-activated protein kinase/extracellular signal regulated kinase (ERK) kinase] (MEK)/ERK survival pathways and decreased mitochondrial sensitivity due to overexpression of the BCL-2 proteins MCL-1 and BCL-x<sub>L</sub> are key mechanisms of TRAIL resistance in HCC. Furthermore, resistance towards TRAIL can be located at receptor level, contributing to inefficient treatment of HCC cells with TRAIL compounds.

### Innovations and breakthroughs

Previous articles have demonstrated that TRAIL is an effective treatment option in HCC in a combined setup with sensitizing agents. In this study the authors demonstrate new treatment options for the sensitization of HCC cells towards TRAIL-induced apoptosis by combination of chemotherapeutic drugs doxorubicin (Doxo) and 5-fluorouracil (5-FU) or kinase inhibitors, such as LY294002, AG1478, PD98059 and SP600125 with TRAIL. In addition, the authors reveal a pivotal role of the antiapoptotic BCL-2 proteins MCL-1 and BCL-x<sub>L</sub> for HCC resistance towards TRAIL. The importance of MCL-1 and Bcl-x<sub>L</sub> for mitochondrial integrity has been extensively studied in this study.



## Applications

TRAIL is an effective treatment option in HCC if combined with the chemotherapeutic drugs Doxo and 5-FU or kinase inhibitors, such as LY294002, AG1478, PD98059 and SP600125. These results open the possibility of a treatment regime which includes reduced doses of chemotherapeutic drugs in combination with TRAIL. The authors also revealed a pivotal role of the antiapoptotic BCL-2 proteins MCL-1 and BCL-x<sub>L</sub> for HCC resistance towards TRAIL. Thus, downregulation of these anti-apoptotic proteins alone (e.g. by application of so-called "BH3-only mimetics") is a promising approach for the treatment of HCC patients. "BH3-only mimetics" have already entered clinical trials in cancer patients.

## Terminology

Apoptosis, also described as programmed cell death, is a genetically determined process of controlled cellular suicide characterized by typical morphological changes, e.g. fragmentation of DNA. TRAIL ligates two different types of receptors: (1) death receptors triggering TRAIL-induced apoptosis and (2) decoy receptors possibly inhibiting the TRAIL death-signaling pathway. MCL-1 and BCL-x<sub>L</sub> are antiapoptotic members of the BCL-2 family serving as protective factors against several death stimuli. Antiapoptotic pathways such as PI3K/Akt, EGFR, MEK/ERK are well known as being activated in malignant cells, thus contributing to cell cycle progression and tumor growth

## Peer review

This is an interesting work, elegantly performed and with possible future clinical applications. Although the article contains a wealth of experimental details, making it a difficult reading for the uninitiated, I believe it will be of interest for both clinicians and basic science readers of the *World Journal of Gastroenterology* because of its possible clinical implications

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ORIGINAL ARTICLE

## Diarrhoea-predominant irritable bowel syndrome distinguishable by 16S rRNA gene phylotype quantification

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### Abstract

**AIM:** To study whether selected bacterial 16S ribosomal RNA (rRNA) gene phylotypes are capable of distinguishing irritable bowel syndrome (IBS).

**METHODS:** The faecal microbiota of twenty volunteers with IBS, subdivided into eight diarrhoea-predominant (IBS-D), eight constipation-predominant (IBS-C) and four mixed symptom-subtype (IBS-M) IBS patients, and fifteen control subjects, were analysed at three time-points with a set of fourteen quantitative real-time

polymerase chain reaction assays. All assays targeted 16S rRNA gene phylotypes putatively associated with IBS, based on 16S rRNA gene library sequence analysis. The target phylotypes were affiliated with *Actinobacteria*, *Bacteroidetes* and *Firmicutes*. Eight of the target phylotypes had less than 95% similarity to cultured bacterial species according to their 16S rRNA gene sequence. The data analyses were made with repeated-measures ANCOVA-type modelling of the data and principle component analysis (PCA) with linear mixed-effects models applied to the principal component scores.

**RESULTS:** Bacterial phylotypes *Clostridium cocleatum* 88%, *Clostridium thermosuccinogenes* 85%, *Coprobacillus cateniformis* 91%, *Ruminococcus bromii*-like, *Ruminococcus torques* 91%, and *R. torques* 93% were detected from all samples analysed. A multivariate analysis of the relative quantities of all 14 bacterial 16S rRNA gene phylotypes suggested that the intestinal microbiota of the IBS-D patients differed from other sample groups. The PCA on the first principal component (PC1), explaining 30.36% of the observed variation in the IBS-D patient group, was significantly altered from all other sample groups (IBS-D vs control,  $P = 0.01$ ; IBS-D vs IBS-M,  $P = 0.00$ ; IBS-D vs IBS-C,  $P = 0.05$ ). Significant differences were also observed in the levels of distinct phylotypes using relative values in proportion to the total amount of bacteria. A phylotype with 85% similarity to *C. thermosuccinogenes* was quantified in significantly different quantities among the IBS-D and control subjects ( $-4.08 \pm 0.90$  vs  $-3.33 \pm 1.16$ ,  $P = 0.04$ ) and IBS-D and IBS-M subjects ( $-4.08 \pm 0.90$  vs  $-3.08 \pm 1.38$ ,  $P = 0.05$ ). Furthermore, a phylotype with 94% similarity to *R. torques* was more prevalent in IBS-D patients' intestinal microbiota than in that of control subjects ( $-2.43 \pm 1.49$  vs  $-4.02 \pm 1.63$ ,  $P = 0.01$ ). A phylotype with 93% similarity to *R. torques* was associated with control samples when compared with IBS-M ( $-2.41 \pm 0.53$  vs  $-2.92 \pm 0.56$ ,  $P = 0.00$ ). Additionally, a *R. bromii*-like phylotype was associated with IBS-C patients in comparison to control subjects ( $-1.61 \pm 1.83$  vs  $-3.69 \pm 2.42$ ,  $P = 0.01$ ). All of the above mentioned phylotype specific alterations were independent of the effect of time.

**CONCLUSION:** Significant phylotype level alterations

in the intestinal microbiotas of IBS patients were observed, further emphasizing the possible contribution of the gastrointestinal microbiota in IBS.

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**Key words:** Irritable bowel syndrome; Diarrhoea-predominant irritable bowel syndrome; Intestinal microbiota; Quantitative real-time polymerase chain reaction; 16S ribosomal RNA

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## INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal (GI) disorder with a worldwide prevalence of 10%-20%<sup>[1]</sup>. The main symptoms include abdominal pain or discomfort, diarrhoea, constipation, abdominal bloating, and flatulence. The symptoms are associated with changes in the frequency and form of stool, improved by defecation, and they typically fluctuate with time. Although IBS does not predispose to malignancies, it essentially lowers the patients' quality of life. Multiple interacting mechanisms lie behind IBS aetiology<sup>[2,3]</sup>. These include psychological stress and disturbances, physiological features, such as altered GI motility and visceral hypersensitivity, low-grade inflammation, and bacterial gastroenteritis.

The possible role of the GI microbiota in IBS aetiology (for review, see Parkes *et al.*<sup>[4]</sup>) is supported by low-grade mucosal inflammation in the GI tract of IBS patients<sup>[5,6]</sup>, onset of GI symptoms after a gastroenteritis (generating a subset of patients diagnosed with post-infectious IBS<sup>[7,8]</sup>), and observations suggesting the presence of altered GI microbiota in IBS<sup>[9-12]</sup>. Recently, Gecse *et al.*<sup>[13]</sup> associated the elevated level of non-endogenous colonic serine protease in diarrhoea-predominant IBS patients with increased mucosal permeability and subsequent visceral hypersensitivity. The detected increase in the level of colonic serine protease was suggested to originate from intestinal bacteria. In addition, antibodies to bacterial flagellins A4-Fla2 and Fla-X associated with the *Clostridium* cluster XIVa are elevated in IBS compared to healthy controls<sup>[14]</sup>. The potential role of GI microbiota in IBS is further supported by studies where probiotics have alleviated IBS symptoms (for a review, see Spiller *et al.*<sup>[15]</sup>). In the recent study of Kajander *et al.*<sup>[16]</sup>, a multispecies probiotic was also shown to stabilize the gut microbiota, but the microbial alterations were not specified.

16S ribosomal acid (rRNA) gene based methods have identified almost 900 bacterial phylotypes in the human GI tract with, of which only 18% represent cultured species<sup>[17]</sup>. Richness estimates within an individual's colon extend to 300 phylotypes<sup>[18]</sup>, while a vast variation is introduced by disparities in the phylotype composition between individuals<sup>[18-20]</sup>. The main phyla found in 16S rRNA gene sequencing based studies are *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia*<sup>[18,21-23]</sup>.

Using culture-based techniques, the GI microbiota of IBS patients has been characterized to have less lactobacilli and bifidobacteria and an elevated amount of aerobes relative to anaerobes<sup>[24-26]</sup>. Specific divergences have been observed with quantitative real-time polymerase chain reaction (qPCR) assays targeting *Lactobacillus* spp, *Veillonella* spp, *Bifidobacterium* spp, *Clostridium coccoides*, and *Bifidobacterium catenulatum*<sup>[10]</sup>, and with 16S rRNA cloned sequence-based assays targeting phylotypes within the genera *Coproccoccus*, *Collinsella*, and *Coprobacillus*<sup>[11]</sup>. With a 16S rRNA gene-based phylogenetic microarray analysis targeting over a 1000 human intestinal phylotypes, the faecal microbiota of IBS patients and control subjects could be distinguished by hierarchical cluster analysis and stronger variation in the composition of the microbiota was seen in the IBS patients' profiles<sup>[12]</sup>. Furthermore, a higher degree of temporal instability among IBS patients has been detected with ribosomal RNA-based denaturing gradient gel electrophoresis<sup>[9]</sup>. Mucosal bacteria have also been found to be more abundant in IBS patients than in healthy controls<sup>[27]</sup>.

In this study, we applied a set of eight novel and six previously published<sup>[11]</sup> qPCR assays to the analysis of faecal samples obtained from IBS patients and healthy controls to detect possible aberrations in the GI microbiotas of IBS patients. The design of the novel qPCR assays was based on comparing the 16S rRNA clone libraries of IBS patients and healthy controls, but in this study three time-points per subject during a 6-mo survey were analysed instead of one<sup>[11]</sup>.

## MATERIALS AND METHODS

### Subjects and study design

Faecal samples were collected from 20 IBS patients and 15 healthy control subjects (Table 1) at time-points 0, 3 and 6 mo of a 6-mo follow-up period.

The IBS patients were recruited by experienced physicians and fulfilled the Rome II criteria<sup>[28]</sup>, except for three subjects who reported slightly less than 12 wk of abdominal pain during the preceding year<sup>[29]</sup>. All patients had undergone clinical investigation and endoscopy or barium enema of the GI tract less than a year prior to the study. Exclusion criteria included pregnancy, lactation, organic intestinal disease, other severe systematic disease, antimicrobial medication during the previous 2 mo, previous major or complicated abdominal surgery, severe endometriosis and dementia or otherwise inadequate cooperation capability. Patients with lactose intolerance



**Table 1** Characteristics of IBS patients and control subjects

	IBS-D	IBS-C	IBS-M	Controls
<i>n</i>	8	8	4	15
Age (yr): mean (range)	43.6 (26-60)	48.6 (24-64)	50.8 (31-62)	47 (25-64)
Gender (female/male)	4/4	9/1	3/1	10/5
Predominant bowel habit	Diarrhoea	Constipation	Mixed-type	-

IBS: Irritable bowel syndrome; IBS-D: Diarrhoea-predominant IBS; IBS-C: Constipation-predominant IBS; IBS-M: Mixed symptom-subtype IBS.

were included if they were reported to follow a low-lactose or lactose-free diet. All IBS patients were advised not to make any changes to their medication, including ongoing IBS medication (mainly commercial fibre analogues, laxatives, or antidiarrhoeals). The IBS patients formed the placebo group of a 6-mo probiotic intervention study<sup>[29]</sup>. They received daily a placebo capsule consisting of microcrystalline cellulose, magnesium stearate, and gelatine as the encapsulating material. Consumption of probiotic products was not allowed during the study.

Control subjects devoid of GI symptoms were also recruited and originally age- and gender-matched with the IBS patients as a whole<sup>[26]</sup>. Volunteers with regular intestinal disturbances, lactose intolerance, celiac disease, or antibiotic therapy during the preceding 2 mo of the study were excluded. The faecal samples of the controls and IBS subjects<sup>[9-11,26,29,30]</sup> have been studied previously. The novelty in the present study resides in the eight previously unpublished 16S rRNA phylotype targeting assays, the analysis of several time-points during the 6-mo survey, and in the in-depth statistical analysis of the results.

### Ethics

All participants gave their written informed consent and were told that they could withdraw from the study at any time. The Human Ethics Committee of the Joint Authority for the Hospital District of Helsinki and Uusimaa (HUS) approved the study protocol for the IBS patients. The ethical committee of the Technical Research Centre of Finland (VTT) approved the study protocol for the healthy controls.

### Extraction and purification of DNA from faecal samples

Faecal samples were preserved anaerobically immediately after defecation, stirred and aliquoted, and stored at -70°C within 4 h of delivery. For qPCR analysis, total DNA was isolated from 1 g of faecal material according to Apajalahti *et al*<sup>[31]</sup>, which included removing the undigested particles from the faecal material by three rounds of low-speed (200 × *g*) centrifugation and collection of the bacterial cells with high-speed centrifugation (30 000 × *g*) at 15°C for 15 min using a Beckman Avanti<sup>TM</sup> centrifuge (Fullerton, CA, USA) with the rotor JA 25.50 or JLA 16.250 rotor, respectively. The bacterial cells were lysed after centrifugation with a combination of freeze-thaw

cycles (freezing for 1 h at -70°C and thawing for 15 min in a 37°C water bath), lysozyme and vortexing with glass beads. DNA concentrations were determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop products, Wilmington, DE, USA).

### Design of qPCR assays

Divergences detected by comparing the sequence data of 16S rRNA gene clone libraries of healthy controls and symptomatically sub-grouped IBS patients (diarrhoea-predominant IBS, IBS-D; constipation-predominant IBS, IBS-C; and mixed symptom-subtype IBS, IBS-M) were used as the basis for selection of qPCR targets<sup>[11]</sup>. Prior to cloning and sequencing, the faecal microbial genomes had been profiled and fractioned on the basis of genomic guanine-plus-cytosine content<sup>[11]</sup>. Partial 16S rRNA gene sequences encompassing the variable regions V1 and V2 combined from all four sample types were aligned using either the version Beta 2003-08-22 of ARB<sup>[11,32]</sup> or ClustalW 1.83<sup>[33]</sup>. For the ARB alignment, an aligned sequence database (ssu\_jan04\_corr\_opt.arb) was downloaded from the ARB home page (<http://www.arb-home.de>) and the in-house sequences<sup>[11]</sup> were aligned using the ARB-EDIT FastAlign function, followed by manual correction of the alignments with special attention to the ends of the sequences. Finally, the sequences were imported into an existing tree file of the database (Tree-Bacteria) by filtering the data against a sequence of similar length as the imported partial 16S rRNA gene sequences. Regions of the tree where sequences derived from one subject group (healthy *vs* IBS or healthy *vs* IBS subtypes) dominated over the other groups were considered as potentially interesting. In addition, a ClustalW 1.83 alignment (FAST DNA pairwise alignment algorithm option, gap penalty 3, word size 4, number of top diagonals 1 and window size 1) was constructed covering approximately 450 bp from the 5' end of the 16S rRNA gene and visually inspected and cut from the *Escherichia coli* position 430 (universally conserved GTAAA) with BioEdit version 7.0.5.3<sup>[34]</sup>. Distance matrices were calculated from the ClustalW alignment with Phylip 3.66 Dnadist<sup>[35]</sup> using Jukes-Cantor correction. The distribution of sequences into operative taxonomic units (OTUs) was determined using DOTUR<sup>[36]</sup> by applying the furthest neighbour rule option and 98% cut-off for sequence similarity. Uneven distribution of sequences originating from the different sample types within an OTU was used as criteria for qPCR target selection.

Potential primer target sites for specific quantitative analyses were assessed manually from ClustalW 1.83 alignments. Primer 3 online interface<sup>[37]</sup> and mfold 3.3 DNA-folding servers<sup>[38]</sup> were used for optimizing the final primer sequences and secondary structure analyses. The primer specificity against publicly available prokaryotic 16S rRNA sequences was checked with FASTA<sup>[39]</sup> provided by the European Bioinformatics Institute (<http://www.ebi.ac.uk/>) and against in-house 16S rRNA clone library sequences of human faecal origin, using the blastall option of Parallel BLAST<sup>[40]</sup> with Corona hardware

(<http://corona.csc.fi>) maintained by the Finnish IT Center for Science (CSC - Scientific Computing Ltd., Finland). The qPCR primers were synthesized commercially by Oligomer Oy (Helsinki, Finland). The clone sequences used to generate the standard curve in each qPCR assay were classified using The Ribosomal Database Project II Classifier<sup>[41]</sup>. The assays were named according to the most similar 16S rRNA gene sequence of a cultured bacterial species with the similarity percentages below 98% indicated.

### qPCR optimization and conditions

For each assay, the optimal annealing temperature and MgCl<sub>2</sub> concentration were defined using the iCycler iQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA) associated with the iCycler Optical System Interface software (version 2.3; Bio-Rad). Actual samples were run as triplicates with optimized reaction conditions using SYBR Green I chemistry and 25 ng (specific phylotype targeting assays) or 0.5 ng (universal 16S rRNA gene assay) of faecal bacterial DNA. For all assays, the samples were run with different sample groups randomly mixed in the individual runs to minimize the effect of technical deviation between runs. Amplified clonal 16S rRNA genes were used as standards, ranging from 10<sup>2</sup> to 10<sup>7</sup> gene copies per reaction. The reaction mixtures consisted of a 1:75 000 dilution of SYBR Green I (Lonza biosciences, Basel, Switzerland), 10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl, 0.1 % Triton X-100, 2.5 mmol/L MgCl<sub>2</sub>, 100 µmol/L each dNTP, 0.5 µmol/L each primer, 0.024 U Dynazyme II polymerase (Finnzymes, Espoo, Finland) and 5 µL of either template or water. The amplification involved one cycle at 95°C for 5 min for initial denaturation, followed by 40 cycles of denaturation at 95°C for 20 s, primer annealing at the defined optimal temperatures for 20 s, extension at 72°C for 30 s and a fluorescence detection step at 80–89°C for 30 s. The specificity of the qPCR assays was checked with a reassociation curve analysis after amplification by slow cooling from 95°C to 60°C, with fluorescence collection at 0.3°C intervals for 10 s at each decrement. The qPCR efficiencies were calculated from the standard curves using the equation  $E = (10^{1/k}) - 1$ , where  $E$  and  $k$  stand for efficiency and slope, respectively.

### Statistical analysis

In the raw data from qPCR assays, microbe groups with low abundance were occasionally undetected (below qPCR detection limit). These values may not be truly zero or missing values, but are caused by limitations in the technical accuracy of the qPCR equipment. Therefore, for data analysis, zeros and missing values were imputed with the mean values obtained from the qPCR runs with the same primer pair applied to molecular grade water. If those too were undetected, the minimum of all the detected water runs was used.

After imputing the undetected values, the raw data was transformed to log<sub>10</sub> ratios of relative amount of 16S rRNA gene copies detected *vs* the amount of bacterial

16S rRNA gene copies detected with the universal qPCR assay. Using the ratio will, to some extent, control the sample specific variation due to lab procedures and sample handling affecting the overall bacterial concentration. All the statistical analyses were carried out with these values.

Statistical analyses were made with standard mixed-effect linear models having fixed effects for the time, and the IBS subtype, and a random effect for individual (taking into account the repeated measures from the same subject). In summary, this set up results in a repeated-measures ANOVA-type modelling of the data.

The model selection between whether to use the full model with interaction term between time and group and the age term, or the simpler model without interaction and the age was based on  $F$ -tests. The inference from the estimated models was based on the standard  $F$ -tests and  $t$ -tests.

For multivariate analysis of the data, principal component analysis (PCA) was used to visualize the data sets. Linear mixed-effects models were also applied to the first four principal component scores to quantify potential multivariate effects present in the data.

All the analyses were made with statistical programming language R 2.6.2<sup>[42]</sup> utilizing the package *lme* for mixed-effects linear models<sup>[43]</sup> and contrast for computing the contrasts.

## RESULTS

### Design and optimization of qPCR assays

A total of 14 qPCR assays were designed and optimized (Table 2) for analyzing alterations in the faecal microbiotas of IBS patients sub-grouped according to symptom subtype and healthy controls. The optimized annealing and detection temperatures ranged from 60°C to 67°C and 80°C to 89°C, respectively. For the universal assay, an annealing temperature of 50°C was used. The PCR efficiencies for the optimized qPCR reactions were above 80% with the exception of *Collinsella aerofaciens*-like, *Coprococcus eutactus* 97% and *Spiroplasma chinense* 84% assays.

Non-specific product peaks with a lower melting temperature than the desired product were observed for some of the faecal DNA samples in the reassociation analyses of several assays (*Bacteroides intestinalis*-like, *Butyrivibrio crossotus*-like, *Clostridium coccleatum* 88%, *C. eutactus* 97%, *S. chinense* 84%, *Ruminococcus torques* 91%, *R. torques* 94% and *Slackia faecicanis* 91%). The fluorescence detection temperatures in these assays were set above the melting point of the unspecific products to avoid detecting them.

### Analysis of faecal samples

The log<sub>10</sub> number of bacterial 16S rRNA gene copies detected ranged from 11.71 to 11.93 per gram of faeces (wet weight) and the average relative log<sub>10</sub> numbers of 16S rRNA gene copies detected with phylotype targeting assays in proportion to the universal bacterial assay ranged from -7.34 to -0.72 (Table 3; time-point specific averages are presented in Supplementary Table 1). Target

Table 2 Phylum level classification, primers and assay conditions of qPCR assays

qPCR assay (Phylum)	Primers (5'→3')	Standard	Target size (bp)	MgCl <sub>2</sub> (mmol/L)	Annealing T (°C)	Detection T (°C)	Average PCR efficiency ± SD
<i>Bacteroides intestinalis</i> -like (Bacteroidetes)	F: AGCATGACCTAGCAATAGGTT R: CCTTCTCGTTATACTATCCGGTAT	AM277809	124	3	63	83	87 ± 6
<i>Bifidobacterium catenulatum</i> / <i>Bifidobacterium pseudocatenulatum</i> -like <sup>[11]</sup> (Actinobacteria)	F: ACTCCTCGCATGGGGTGTC R: CCGAAGGCTTGCTCCCGAT	AM277149	275	3	68	87	90 ± 8
<i>Butyrivibrio crossotus</i> -like (Firmicutes)	F: TGCTAATACCGCATAAAACAGCAGA R: CGCTGGATCAGGCTTTTCG	AM275497	232	4	63	85	82 ± 5
<i>Clostridium cocleatum</i> 88% <sup>[11]</sup> (Firmicutes)	F: AATACATAAGTAACCTGGCRTC R: CGTAGCACTTTTCATATAGAGTT	AM276544	104	4	60	80	88 ± 10
<i>Clostridium thermosuccinogenes</i> 85% (Firmicutes)	F: ACATGCAAGTCGAACGGAAGTC R: TGCCTCAGAGTTTCTCCATTG	AM275406	373	2	62	81	88 ± 8
<i>Collinsella aerofaciens</i> -like <sup>[11]</sup> (Actinobacteria)	F: CCCGACGGGAGGGGAT R: CTCTGCGAGTACAGTCTTGAC	AM276090	260	4	67	89	75 ± 3
<i>Coprobacillus cateniformis</i> 91% (Firmicutes)	F: CGGACGCGATGCTTCT(A/G)GC R: AACATATCTCCCATGCGGTIG	AM275478	133	4	62	82	93 ± 8
<i>Coprococcus eutactus</i> 97% <sup>[11]</sup> (Firmicutes)	F: AGCTTGCTCCGGCYGATTTA R: CGGTTTTACCAGTCGTTTCCAA	AM275825	97	2	63	83	73 ± 5
<i>Ruminococcus bromii</i> -like (Firmicutes)	F: CGAACGGAAGTGTGTTTGAAGA R: CAAAACCATGTGGTTCGATAT	AM275413	156	4	62	81	97 ± 6
<i>Ruminococcus torques</i> 91% <sup>[11]</sup> (Firmicutes)	F: TGCTTAAGTATCTTCTTCGGA R: CGGTATTAGCAGTCATTTCTG	AM276624	119	5	62	82	88 ± 5
<i>R. torques</i> 93% <sup>1</sup> (Firmicutes)	F: GACTGCTTTTGAACCTGTCA R: AGGTCCGGTTAAGGA	AM275798	396	4	61	83	85 ± 4
<i>R. torques</i> 94% <sup>[11]</sup> (Firmicutes)	F: AATCTTCGGAGGAAGAGGACA R: AACTACACCATGCGGTCTT	AM275522	137	2	65	85	81 ± 6
<i>Slackia faecicanis</i> 91% (Actinobacteria)	F: GAGTAACGCGTGACCGACCTT R: CCCGGAGTACCCGGTATCA	AM276086	75	4	64	86	90 ± 5
<i>Spiroplasma chinense</i> 84% (Firmicutes)	F: ATGGCCCAGTGAAGGTIG R: CCCAACGAAAAGGTAGGTCA	AM275518	101	4	66	83	79 ± 4
Universal <sup>[61]</sup>	F: TCCTACGGGAGGCAGCAGT R: GGACTACCAGGTATCTAATCCTGTT	<i>B. longum</i> <sup>2</sup>	466	3	50	80	92 ± 6

<sup>1</sup>For the *R. torques* 93% assay the sequence AY305319<sup>[62]</sup> was used for primer design; <sup>2</sup>The *Bifidobacterium longum* DSM 20219T 16S rRNA gene was used as standard in the universal qPCR assay. qPCR: Quantitative real-time polymerase chain reaction.

Table 3 The average relative log<sub>10</sub> amount of the 16S rRNA gene copies detected with qPCR assays in proportion to the universal qPCR results

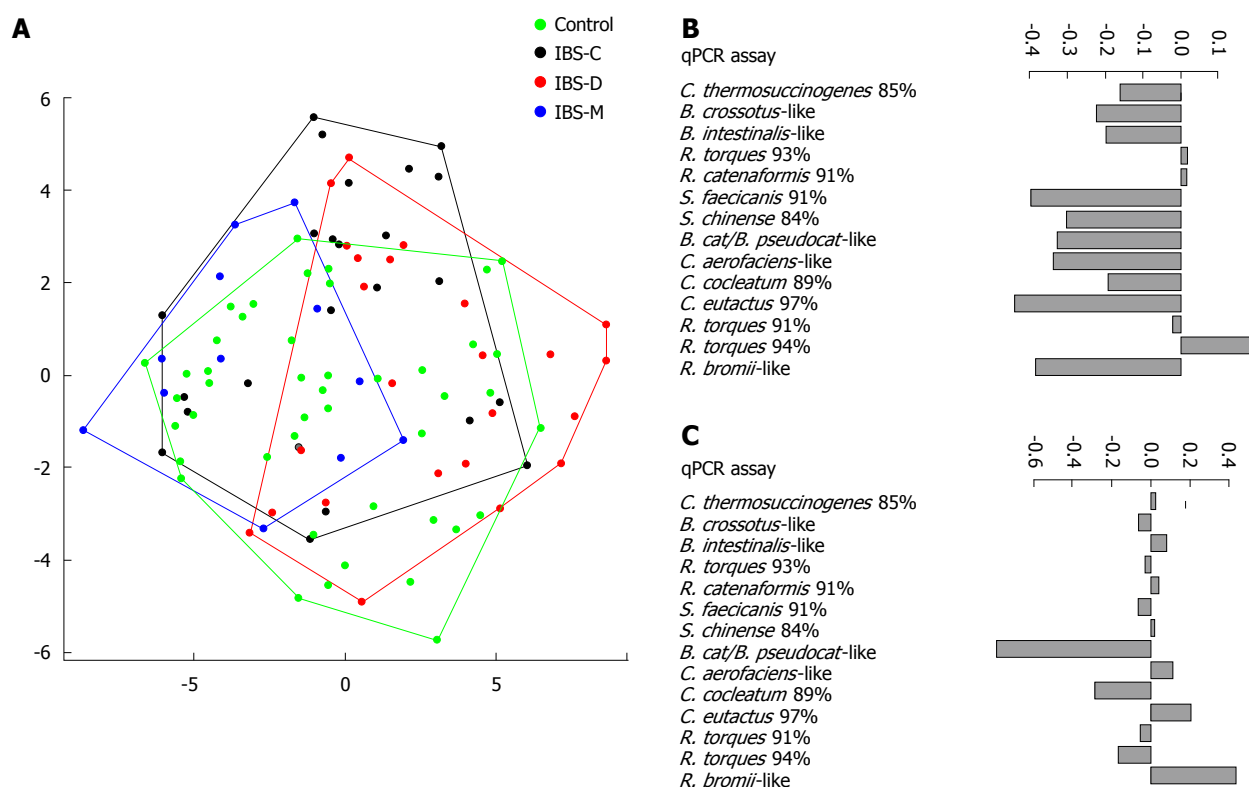
qPCR assay	Control (n = 15)	IBS-C (n = 8)	IBS-D (n = 8)	IBS-M (n = 4)
<i>Bacteroides intestinalis</i> -like	-4.85 ± 1.52 <sup>1</sup> (12)	-4.71 ± 1.42 (5)	-5.8 ± 1.32 (5)	-3.46 ± 1.26 (4)
<i>Bifidobacterium catenulatum</i> / <i>Bifidobacterium pseudocatenulatum</i> -like	-4.1 ± 2.22 (14)	-5.63 ± 2.52 (7)	-5.42 ± 2.63 (5)	-4.4 ± 2.54 (4)
<i>Butyrivibrio crossotus</i> -like	-6.2 ± 2.03 (8)	-6.5 ± 1.97 (3)	-7.34 ± 1.58 (0)	-6.04 ± 2.19 (2)
<i>Clostridium cocleatum</i> 88%	-1.7 ± 1.32 (15)	-2.36 ± 2.35 (8)	-2.69 ± 2.33 (8)	-0.72 ± 0.98 (4)
<i>Clostridium thermosuccinogenes</i> 85%	-3.33 ± 1.16 <sup>a</sup> (15)	-3.7 ± 0.84 (8)	-4.08 ± 0.90 <sup>ab</sup> (8)	-3.08 ± 1.38 <sup>b</sup> (4)
<i>Collinsella aerofaciens</i> -like	-2.45 ± 1.16 (15)	-2.9 ± 2.33 (7)	-4.63 ± 2.35 (7)	-1.73 ± 2.61 (4)
<i>Coprobacillus cateniformis</i> 91%	-4.72 ± 0.77 (15)	-4.41 ± 0.67 (8)	-4.79 ± 0.61 (8)	-4.71 ± 0.25 (4)
<i>Coprococcus eutactus</i> 97%	-5.44 ± 2.53 (9)	-5.91 ± 2.61 (3)	-6.55 ± 2.28 (2)	-4.09 ± 2.69 (3)
<i>Ruminococcus bromii</i> -like	-3.69 ± 2.42 <sup>c</sup> (15)	-1.61 ± 1.83 <sup>c</sup> (8)	-3.4 ± 2.49 (8)	-2.08 ± 1.56 (4)
<i>Ruminococcus torques</i> 91%	-3.13 ± 0.77 (15)	-2.87 ± 1.10 (8)	-2.58 ± 1.09 (8)	-2.83 ± 1.26 (4)
<i>R. torques</i> 93%	-2.41 ± 0.53 <sup>d</sup> (15)	-2.61 ± 0.72 (8)	-2.65 ± 0.59 (8)	-2.92 ± 0.56 <sup>d</sup> (4)
<i>R. torques</i> 94%	-4.02 ± 1.63 <sup>c</sup> (14)	-3.39 ± 1.40 (8)	-2.43 ± 1.49 <sup>c</sup> (8)	-3.82 ± 2.16 (3)
<i>Slackia faecicanis</i> 91%	-5.53 ± 2.26 (8)	-5.6 ± 2.33 (4)	-6.22 ± 2.16 (3)	-4.01 ± 2.28 (4)
<i>Spiroplasma chinense</i> 84%	-5.62 ± 2.04 (9)	-5.36 ± 2.20 (5)	-6.51 ± 1.98 (2)	-5.7 ± 2.22 (2)

The number of subjects with target 16S rRNA gene copies detected above the calculated threshold value in any of the three samples analysed are given in parentheses. <sup>1</sup>Values are presented as averages of log<sub>10</sub>-values ± SD from three time-points (0, 3 and 6 mo). <sup>a</sup>P = 0.04, <sup>b</sup>P = 0.05, <sup>c</sup>P = 0.01, <sup>d</sup>P = 0.00.

bacterial phylotypes were detected from all samples with the *C. cocleatum* 88%, *Coprobacillus cateniformis* 91%, *Clostridium thermosuccinogenes* 85%, *Ruminococcus bromii*-like, *R. torques* 91%, and *R. torques* 93% assays (Table 3).

### Divergences in the intestinal microbiota in IBS

In a PCA of the 14 phylotype targeting assays and three time-points (0, 3 and 6 mo), the IBS-D group differed from the control group (*P* = 0.01), IBS-M (*P* = 0.00),



**Figure 1** Principal component analysis (PCA) of fourteen 16S rRNA phylotypes quantified from faecal samples of irritable bowel syndrome (IBS) patients and healthy volunteers. **A:** The PCA plot with outermost data points within each sample group is outlined. The control samples are presented in green, the constipation-predominant IBS (IBS-C) in black, the diarrhoea-predominant IBS (IBS-D) in red and the mixed symptom-subtype IBS (IBS-M) in blue. Each time-point is presented as a separate point. To quantify the multivariate differences between the groups, linear mixed-effects models were applied to the first (x-axis) and the second (y-axis) principal component scores, which represent the dominant multivariate changes present in the data; **B:** The bars represent the relative contribution of each quantitative real-time PCR (qPCR) assay to the principal component 1 (PC1). On PC1 the IBS-D samples differed from the control ( $P \leq 0.01$ ), IBS-M ( $P \leq 0.01$ ), and IBS-C ( $P \leq 0.05$ ) samples; **C:** The bars represent the relative contribution of each qPCR assay to the principal component 2 (PC2). On PC2, the IBS-C patients diverged from the control subjects ( $P \leq 0.05$ ) and time-points. In addition, the second time-point (3 mo) diverged significantly from the first (0 mo,  $P \leq 0.01$ ) and the third (6 mo,  $P \leq 0.01$ ) time-points independent of sample group; The height of the bars in graphs in Figure 1B and C reflect the relative magnitude of the contribution and the direction the sign of the contribution (in relation to the other assays and to the axis in Figure 1A). For example, in PC1 (Figure 1B), the largest contributor is the *Coprococcus eutactus* 97% phylotype, while the samples on the right in Figure 1A (mostly IBS-D) tend to have higher concentrations of *Ruminococcus torques* 94% and lower concentrations of phylotypes with bars highly on the negative side. Similarly, on PC2 (Figure 1C) the samples with high PC2 value in the top part of the Figure 1A tend to have higher concentrations of the *Ruminococcus bromii*-like phylotype, and lower concentrations of the *Bifidobacterium catenulatum*/*Bifidobacterium pseudocatenulatum*-like phylotype. On PC2, the IBS-C patients diverged from the control subjects ( $P \leq 0.05$ ) and time-points. In addition, the second time-point (3 mo) diverged significantly from the first (0 mo,  $P \leq 0.01$ ) and the third (6 mo,  $P \leq 0.01$ ) time-points independent of sample group. qPCR: Quantitative real-time polymerase chain reaction; IBS-C: Constipation-predominant irritable bowel syndrome; IBS-D: Diarrhoea-predominant irritable bowel syndrome; IBS-M: Mixed-subtype irritable bowel syndrome.

and IBS-C ( $P = 0.05$ ) on the first principal component (PC1; Figure 1A and B). The *R. torques* 94% phylotype was unique in being more predominant in IBS-D (Figure 1A and B). On the second principal component (PC2), the IBS-C patients diverged from the control subjects ( $P = 0.03$ ; Figure 1A and C). Time-points were significantly different on PC1 and PC2 (data not shown).

Quantities of *C. thermosuccinogenes* 85%, *R. bromii*-like, *R. torques* 93%, and *R. torques* 94% phylotypes diverged between different IBS symptom subtypes and healthy subjects independent of the effect of time (Table 3). Relatively high levels of the *C. thermosuccinogenes* 85% phylotype were associated with IBS-M patients and control subjects compared with IBS-D patients. The relative amount of *C. thermosuccinogenes* 85% 16S rRNA gene copies detected in proportion to the universal assay were 0.08%, 0.05%, and  $< 0.01\%$  for the IBS-M, control, and IBS-D subjects, respectively. The *R. bromii*-like

phylotype was significantly ( $P = 0.01$ ) more abundant in the IBS-C (relative abundance 2.45%) than in the control (relative abundance 0.02%) subjects' samples and the *R. torques* 93% phylotype was significantly ( $P = 0.00$ ) more abundant in the control (relative abundance 0.39%) than in the IBS-M subjects' samples (relative abundance 0.12%). The lowest amount of *R. torques* 94% phylotypes was quantified in the control samples (relative abundance  $< 0.01\%$ ) significantly differing ( $P = 0.01$ ) from the relative amount detected among the IBS-D patients' samples (relative abundance 0.37%).

Additional time-point dependent divergences between the sample groups were also detected (Supplementary Table 1): The *B. intestinalis*-like and *C. cocleatum* 88% phylotypes were relatively abundant in the IBS-M and control samples, and were detected in lower amounts in the IBS-D patients' samples. The relative amounts of *C. aerofaciens*-like phylotype detected were lowest in samples of the IBS-D patients, whereas the relative



amounts of *R. torques* 91% phylotype were lowest in the control subjects' samples.

## DISCUSSION

The aim of this study was to test the capability of a set of qPCR assays targeting the 16S rRNA gene on a phylotype level to differentiate between IBS symptom subtypes and healthy controls. Eight novel and six previously published<sup>[11]</sup> qPCR assays were used to study faecal samples of 20 IBS patients grouped according to symptom subtype and 15 healthy controls at three time-points (0, 3 and 6 mo). None of the assays have previously been applied to samples from several time-points. The knowledge of putative alterations on phylotype level may be essential in association with health, as has been shown to be the case for *Faecalibacterium prausnitzii* in Crohn's disease<sup>[44,45]</sup>.

In our approach, the selection of faecal bacteria phylotypes for analysis was based on a comparison of clone sequence libraries of IBS patient symptom subtypes and healthy controls<sup>[11]</sup>. The amount of bacterial 16S rRNA genes detected with the universal qPCR was in accordance with previous findings<sup>[46]</sup>. Quantities relative to the amount of bacterial 16S rRNA gene copies detected with the universal bacterial qPCR assay were used in data analyses. The diarrhoea-predominant symptom subtype diverged significantly from the other IBS symptom subtypes and healthy controls in a PCA of all 14 qPCR analyses and three time-points (Figure 1). In addition, *C. thermosuccinogenes* 85%, *R. bromii*-like, *R. torques* 93%, and *R. torques* 94% phylotypes diverged between different IBS symptom subtypes and healthy controls independent of the time-point analysed (Table 3). According to the results presented here and in previous studies<sup>[10-12,47,48]</sup>, grouping of IBS patients based on their main symptom subtype is advisable in future studies.

The *C. thermosuccinogenes* 85% -phylotype represents an uncultured firmicute within the human GI microbiota. It was detected in significantly lower amounts in IBS-D patients' samples in comparison to healthy controls or IBS-C patients. The target sequence of the *C. thermosuccinogenes* 85% -assay has previously been found from human faecal samples in several studies<sup>[11,18,49]</sup> and from human mucosal biopsy samples taken from the caecum, descending and sigmoid colon, and the rectum<sup>[18]</sup>, implying that the phylotype truly represents a human intestinal bacterium. However, the closest isolated strain has negligible similarity according to the 16S rRNA sequence (85% similarity with *Ruminococcus* sp. 16442 strain 16S rRNA sequence).

The *R. bromii*-like phylotype was significantly more abundant in IBS-C patients than in healthy controls samples. *R. bromii* is a common starch degrader of the human intestinal microbiota<sup>[50]</sup>. The amounts of *R. bromii*-related phylotypes have been shown to increase with a diet high in resistant starch<sup>[51]</sup>. In the present study, the possible effect of diet could not be ruled out, but it is more likely that the slowed colonic transit in IBS-C, rather

than a dietary effect, results in a favourable environment for the *R. bromii*-like phylotype associated with IBS-C.

*Ruminococcus torques*, a resident mucin-degrading member of the human GI microbiota<sup>[52]</sup>, has been associated with the mucosa of Crohn's disease patients<sup>[53]</sup>. The specific target sequence of the *R. torques* 94% -assay applied in this study has been found from human faecal samples in several studies<sup>[19,54,55]</sup> and has also been associated with Crohn's disease<sup>[56]</sup>. In the present study, a comparatively higher abundance of *R. torques* 94% phylotype was linked with IBS-D in both the multivariate and assay specific analyses. The *R. torques* 91% phylotype was associated with IBS-D and IBS-M and the *R. torques* 93% phylotype was more abundant in IBS-M than in healthy controls. The target sequences of *R. torques* 91%, 93% and 94% are affiliated with *Lachnospiraceae* as is the 16S rRNA sequence of the strain A4 (DQ789118)<sup>[57]</sup> carrying the IBS associated flagellin Fla2<sup>[14]</sup>.

As a further support to our previous results<sup>[11]</sup>, a significantly lower abundance of the *C. aerofaciens*-like phylotype was associated with the IBS-C and IBS-D symptom subtypes at two of the time-points analysed. *Collinsella aerofaciens* (formerly *Eubacterium aerofaciens*) belongs to the order *Coriobacteriales* within the high G+C Gram-positive *Actinobacteria*. It is a prominent member of the endogenous human intestinal microbiota<sup>[58]</sup> and has previously been connected with a low risk of colon cancer<sup>[59]</sup>.

Significantly lower levels of several 16S rRNA gene phylotypes within the genus *Bacteroides* (*B. ovatus*, *B. uniformis*, and *B. vulgatus*) have previously been discovered among IBS-C patients in comparison to healthy controls, but no effect was seen with the *B. intestinalis*-like phylotype targeting probes<sup>[12]</sup>. All samples analysed in this study have previously been analysed with a *Bacteroides-Prevotella-Porphyromonas* -group and a *B. fragilis* species-specific qPCR assay<sup>[10]</sup> without detecting any significant divergences. In this study, a *B. intestinalis*-like phylotype was quantified with qPCR and found to be least abundant in the IBS-D patient group and most abundant in the IBS-M patient group at the selected time-points. The seemingly contradictory results might be due to different specificities of the probes and primers used.

The qPCR assays presented here were based on a thorough analysis of IBS associated faecal bacterial 16S rRNA gene sequence data originating from the same samples and both the previously published partial 16S rRNA gene sequences. The qPCR assays detailed here will be valuable in upcoming IBS studies. A more thorough sequencing approach using novel high-throughput sequencing technologies<sup>[23]</sup> on IBS subjects' GI microbiota would be valuable in further investigating IBS-associated alterations within the GI microbiota.

The faecal microbiota of IBS patients has been associated with less temporal stability within individuals<sup>[47]</sup> and more variation between individuals<sup>[12]</sup> compared to that of the healthy controls. Therefore, the results of this study should be further confirmed with independent sample panels including both IBS subjects and healthy

controls. In addition, analyzing mucosal samples, in addition to luminal samples, would be of interest, since the mucosal and faecal microbiotas differ from each other<sup>[60]</sup>. Previously, IBS patients have been shown to have a slightly more abundant mucosal microbiota compared to that of healthy volunteers, but the difference was not statistically significant<sup>[27]</sup>. However, obtaining mucosal samples from IBS patients would require colonoscopy, which is not a regular procedure on IBS patients.

In conclusion, we observed alterations in the GI microbiota of IBS-D subjects with a multivariate analysis and several additional statistically significant differences were detected between the intestinal microbiotas of the different IBS subtypes and healthy controls in assay-specific analyses. Recovering the target bacteria of the *C. thermosuccinogenes* 85% and *R. torques* 94% qPCR assays would be essential for further analysis of their possible role in the human GI tract and their association to IBS. In the future, biomarkers associated to the GI microbiota could aid therapeutic trial follow-up, diagnosis and treatment of IBS patients.

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## COMMENTS

### Background

Irritable bowel syndrome (IBS) is a common gastrointestinal functional disorder that can greatly affect the patient's well being. Multiple interacting mechanisms, including alterations in the intestinal microbiota, are suspected to lie behind IBS aetiology.

### Research frontiers

Alterations in the gastrointestinal microbiota in association to health and disease have become an essential field of research in gastroenterology. For instance, indications of dysbiosis have been detected in relation to Crohn's disease. In this study, assays for analyzing phylotype specific bacterial alterations in association to IBS were developed and applied.

### Innovations and breakthroughs

The authors' results support the hypothesis of intestinal bacteria having a role in IBS, as significant phylotype specific alterations between the faecal microbiotas of IBS symptom subtype groups and healthy controls were detected. Furthermore, the results emphasize the importance of subgrouping IBS patients in future studies.

### Applications

An IBS-associated 16S ribosomal RNA (rRNA) gene sequence library data was used to design the real-time polymerase chain reaction (PCR) assays capable of differentiating IBS symptom subgroups and healthy controls in the test sample panel. The detected altering phylotypes might be useful as targets in diagnostic, therapeutic and host-microbe interaction studies.

### Terminology

The bacterial 16S rRNA gene is constructed from conserved and variable regions according to its phylogenetic origin. It enables the detection and quantification of microbes from environmental samples even when the bacteria cannot be cultivated. Real-time PCR targeting the 16S rRNA gene can be used to quantify bacterial subpopulations of 0.01% from faecal DNA samples.

### Peer review

The authors examined faecal bacterial phylotypes in eight diarrhea-predominant, eight constipation-predominant, four mixed symptom subtype IBS patients, and 15 control subjects with quantitative real-time polymerase chain

reaction assays. They found significant phylotype level alterations in the intestinal microbiotas of IBS patients.

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BRIEF ARTICLE

## Ketoprofen, peginterferon 2a and ribavirin for genotype 1 chronic hepatitis C: A phase II study

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molecular study of IFN-dependent signal transduction was conducted in 9 patients from each group.

**RESULTS:** The combination of ketoprofen and PEG-IFN with or without ribavirin was safe and well tolerated. An early activation of STAT1 was observed in ketoprofen-treated patients, but this activation was less sustained over time. Conversely, ketoprofen plus PEG-IFN and ribavirin induced an early and sustained increase of 2'-5'OAS transcription starting 24 h after the first dose until the 36th wk. These data are consistent with the clinical results, showing a better sustained virological response and a lower relapse rate in patients receiving ketoprofen plus PEG-IFN and ribavirin.

**CONCLUSION:** The addition of ketoprofen to the standard therapy of chronic hepatitis C should be explored in larger randomized clinical studies.

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**Key words:** Liver; Viral hepatitis; Chronic hepatitis C; Clinical pharmacology; Non-steroidal antiinflammatory drugs

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### Abstract

**AIM:** To evaluate the safety of adding ketoprofen to pegylated-interferon (PEG-IFN) with or without ribavirin and the effect on viral kinetics, STAT1 activity and expression of 2'-5'-oligoadenylate synthetase (2'-5'OAS) in genotype 1 chronic hepatitis C in a phase II study.

**METHODS:** Forty-five patients were studied: fifteen were randomized to PEG-IFN plus ribavirin (PR), 16 to PEG-IFN plus ketoprofen and 14 to PR and ketoprofen. The

### INTRODUCTION

The treatment of chronic hepatitis C virus (HCV) infection has dramatically improved over the last 20 years. Interferon  $\alpha$  (IFN $\alpha$ ) was one of the first agents used to treat this infection, but its efficacy in terms of sustained virological response (SVR, i.e. sustained absence of HCV from serum up to 6 mo after stopping therapy)

was extremely poor<sup>[1]</sup>. Strategies to overcome this limitation have included increasing the duration of therapy or adding ribavirin (an antiviral drug) to the treatment regimen<sup>[2,3]</sup>. The introduction of a “pegylated” preparation substantially increased the antiviral activity of IFN $\alpha$ . Today, pegylated-interferon (PEG-IFN) in combination with ribavirin is the standard treatment for patients with chronic hepatitis C<sup>[4]</sup>. Large international controlled clinical trials have demonstrated that this combination therapy yields SVR rates in 54%-63% of treated HCV-infected patients<sup>[5-7]</sup>. However, patients infected with HCV genotype 1 are particularly resistant to antiviral treatment, as demonstrated by the lower SVR rates observed in this patient subset, ranging from 42% to 52%<sup>[5-7]</sup>. Thus, a substantial proportion of patients remain unresponsive to antiviral treatment.

IFN signaling pathways are activated by binding of IFN to its specific receptor, which induces autophosphorylation of protein tyrosine kinases Tyk-2 and Jak-1 on tyrosine residues, thus activating signal transducer and activator of transcription (STAT1 and STAT2) proteins. Activated STATs translocate to the nucleus where they activate the transcription of IFN-inducible genes, such as 2'-5'-oligoadenylate synthetase (2'-5'OAS)<sup>[8]</sup>. Non-steroidal antiinflammatory drugs (NSAIDs) have been demonstrated to amplify the IFN signaling pathways and to enhance the anti-viral effect of IFN<sup>[9-14]</sup>. Furthermore, it has recently been found that acetylsalicylic acid suppresses HCV expression in a hepatoma cell line containing HCV subgenomic replicon<sup>[15]</sup>. However, clinical studies evaluating the use of NSAIDs in combination with standard IFN $\alpha$  in patients with chronic HCV infection have given conflicting results<sup>[16-20]</sup>, although results with ketoprofen have generally been encouraging<sup>[17-21]</sup>.

A rational approach to combination therapies for patients with chronic HCV infection demands a detailed knowledge of how the different drugs affect viral kinetics and IFN intracellular signaling. Therefore, we conducted a pilot phase II study to evaluate the effect of ketoprofen plus PEG-IFN with or without ribavirin compared with PEG-IFN plus ribavirin (PR) on viral kinetics, STAT1 activity and expression of the IFN-dependent gene 2'-5'OAS in patients with chronic hepatitis C. We also assessed the safety and tolerability of these treatment schedules. In order to minimize the influence of HCV viral variability on our results, only patients infected with HCV genotype 1 were included.

## MATERIALS AND METHODS

### Patients

Treatment-naïve patients aged 18 to 65 years with chronic HCV infection genotype 1a or 1b were eligible for enrollment if they had: elevated alanine aminotransferase (ALT) levels within the previous 6 mo, a positive test for serum HCV-RNA and a liver biopsy specimen consistent with chronic hepatitis C obtained in the previous 12 mo. Patients were excluded if they had neutropenia (neutrophil count  $< 1.5 \times 10^9$  cells/L), thrombocytopenia (platelet count  $< 70 \times 10^9$  cells/L), anemia (hemoglobin

level  $< 12.0$  g/dL in women and  $< 13.0$  g/dL in men) or a medical condition that would be clinically worsened by anemia, serum creatinine levels more than 1.5 times the upper limit of normal, evidence of liver disease due to causes other than chronic HCV infection, human immunodeficiency virus positivity, esophageal varices, decompensated liver disease, organ transplant, severe or poorly controlled psychiatric disease (especially depression), malignant neoplastic disease, severe cardiac or chronic pulmonary disease, history of peptic disease, autoimmune disease (except controlled thyroid disease), seizure disorder, alcohol or drug dependency within 1 year of study entry, clinically significant comorbid conditions; and, if female, pregnancy or unwilling to use contraception.

### Study design

This was an open-label, randomized, phase II, pilot study. Patients eligible for the study were randomized into three groups: (1) PR group: patients received subcutaneous PEG-IFN $\alpha$ 2a 180  $\mu$ g/wk plus oral ribavirin 800 mg/d for 48 wk; (2) PEG-IFN plus ketoprofen (PK) group: patients received subcutaneous PEG-IFN $\alpha$ 2a 180  $\mu$ g/wk for 48 wk plus oral ketoprofen 200 mg twice daily for the first 4 wk and then 200 mg/d for the next 20 wk; (3) PEG-IFN plus ribavirin and ketoprofen (PRK) group: patients received subcutaneous PEG-IFN $\alpha$ 2a 180  $\mu$ g/wk plus oral ribavirin 800 mg/d for 48 wk plus oral ketoprofen 200 mg twice daily for the first 4 wk and then 200 mg/d for the next 20 wk.

After 24 wk, treatment was withdrawn in patients who did not experience a decrease from baseline in viral load of  $\geq 2 \log_{10}$  IU/mL or a negative qualitative serum HCV-RNA. A safety assessment was conducted in these patients between 4 and 8 wk after their last dose of study drug.

After the 48-wk study there was a 24-wk treatment-free follow-up period. Virological response was defined as a negative test for qualitative serum HCV-RNA. An end of treatment response (ETR) was defined as undetectable levels of HCV-RNA at the end of treatment (week 48) and SVR was defined as undetectable levels of HCV-RNA at the end of follow-up (24 wk after treatment cessation). Relapsers were defined as patients who obtained an ETR, but relapsed after completion of treatment and tested HCV-RNA positive at the end of follow-up.

PEG-IFN $\alpha$ 2a and ribavirin were kindly supplied by Roche S.p.A., Monza (MI), Italy and ketoprofen by IBI (Istituto Biochimico Italiano) S.p.A., Aprilia (LT), Italy. All subjects gave written informed consent before entering the study. The study protocol and patient-informed consent forms were approved by the Institutional Ethics Committee of Azienda Ospedaliero-Universitaria di Bologna, Policlinico S.Orsola-Malpighi, Bologna, Italy (registration number: 58/2002/U) and the study was conducted according to the ethical guidelines of the Declaration of Helsinki.

### Study procedures

All patients underwent liver biopsy within 12 mo before entry. Each liver biopsy was scored according to the

histological activity index proposed by Knodell *et al*<sup>[22]</sup>. After the screening evaluations, laboratory parameters were monitored and recorded at regular intervals throughout the 48-wk study period and a physical examination was conducted at the end of treatment. Adverse events and serious adverse events were recorded throughout the study and up to the end of follow-up period.

Serum samples were collected from each patient before treatment (on day -1), at baseline (day 0), at 24 and 48 h after the first dose and at 1, 2, 4, 12, 24, 36, 48, 60 and 72 wk after the beginning of treatment. Quantitative HCV-RNA serum levels were assessed in all samples using a branched DNA assay (Versant® RNA 3.0 assay; Siemens, Milano, Italy). Virological response was assessed on weeks 1, 2, 4, 12, 24, 36, 48 and 72 by means of Transcription Mediated Amplification technique (Versant® HCV-RNA Qualitative Assay; Siemens, Milano, Italy; low limit of detection: 6 HCV IU/mL).

### RNA extraction and real-time quantitative polymerase chain reaction (RTQ-PCR)

Peripheral blood mononuclear cells (PBMCs) were collected from patients at 0, 24 and 48 h and at 1, 2, 4 and 36 wk after the beginning of treatment. Total RNA was extracted from PBMCs using an “RNeasy Protect Mini Kit” (Qiagen, GmbH, Hilden, Germany). Full length cDNAs were synthesized from 1 µg total RNA using the “Thermoscript RT-PCR system” kit (Invitrogen), according to the manufacturer’s instructions.

Comparative RTQ-PCR was performed with the “Platinum SYBR Green qPCR SuperMix-UDG” kit (Invitrogen) and analyzed on Mx3000P apparatus from Stratagene.

A 0.2 µmol/L concentration of the following primers was used: for 2'-5'OAS forward 5'-ATTGACAGTGCT GTTAACATCATCC-3' and reverse 5'-GTGAGTTATG GAACACGACGAG-3'; for GAPDH forward 5'-GAAG GTGAAGGTCGGAGTC-3' and reverse 5'-GAAGAT GGTGATGGGATTTC-3'. RTQ-PCR conditions used to amplify 2'-5'OAS and GAPDH cDNAs were: 95°C for 5 min, followed by 40 cycles comprising 30 s at 95°C, 30 s at 60°C and 1 min at 72°C. In order to check for DNA contamination, amplification of total RNA before cDNA synthesis was performed in parallel with amplification of the cDNA. Reactions were run in triplicate, and a mean value of the three samples was calculated. 2'-5'OAS mRNA levels were expressed as the relative amount of product adjusted for the level of GAPDH, using the Mx3000P software (Stratagene) and employing a comparative Ct ( $\Delta\Delta C_t$ ) value method. Dissociation curves were generated to ensure that a single amplicon had been produced.

### Western blotting

PBMCs were lysed with Ripa buffer containing 50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 0.1% Nonidet P-40, 1 mmol/L EDTA, 2 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, 1 µg/mL of leupeptin and phosphatase inhibitors. Protein concentrations were measured

**Table 1** Baseline characteristics of enrolled patients (mean  $\pm$  SD)

	PR group (n = 15)	PK group (n = 16)	PRK group (n = 14)	P-value
Age (yr)	45 $\pm$ 12	48 $\pm$ 12	42 $\pm$ 10	NS
Sex (M/F)	9/6	9/7	9/5	NS
HCV-RNA $\times$ 10 <sup>3</sup> IU/mL	429 $\pm$ 578	1261 $\pm$ 1073	1555 $\pm$ 1322	0.01
HCV-RNA > 700 $\times$ 10 <sup>3</sup> IU/mL (%)	4 (27)	8 (50)	10 (71)	0.02
ALT (U/L)	82 $\pm$ 45	85 $\pm$ 33	87 $\pm$ 49	NS
Median total HAI <sup>1</sup> (range)	8 (3-14)	10 (6-13)	8 (3-10)	NS
Median grading (range)	7 (2-11)	9 (5-10)	7 (3-9)	NS
Median fibrosis (range)	1 (1-3)	1 (1-3)	1 (0-3)	NS

<sup>1</sup>HAI: Histological activity index according to Knodell *et al*<sup>[22]</sup>. PR: PEG-IFN $\alpha$ 2a plus ribavirin; PK: PEG-IFN $\alpha$ 2a plus ketoprofen; PRK: PEG-IFN $\alpha$ 2a plus ribavirin and ketoprofen; M: Male; F: Female; HCV: Hepatitis C virus; ALT: Alanine aminotransferase; NS: Not significant.

using a colorimetric assay (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins were electrophoresed on SDS-PAGE. Proteins were transferred to PVDF membrane (Immobilon-PVDF, Millipore) and treated with 1:300 specific primary antibody anti-STAT1 (Santa Cruz Biotechnology, Inc., CA, USA) and its activated form (Biosource). After incubation with peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology), the sheets were visualized by ECL kits (Amersham, GE Healthcare Life Science, Germany). Antibody anti-actin was purchased from Santa Cruz Biotechnology.

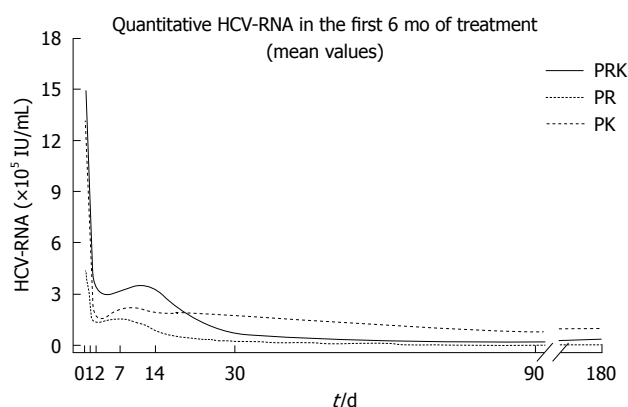
### Statistical analysis

This was a pilot study and therefore no formal sample size calculation was performed. Data were analyzed on an intention-to-treat basis. Patients who dropped out of the trial for any reason were classified as non responders. The safety population comprised all patients who received at least one dose of study drugs.  $\chi^2$  test and Mann-Whitney *U* test were used to compare quantitative and qualitative variables between the groups, respectively. Differences in virological response rates between treatment groups were analyzed using Fisher’s exact test and/or Yates corrected  $\chi^2$  test, as necessary. A *P* < 0.05 was considered to be significant. Data analysis was carried out using the SPSS for Windows version 11.0.1. Plots of the viral load were carried out using SigmaPlot version 9.0.

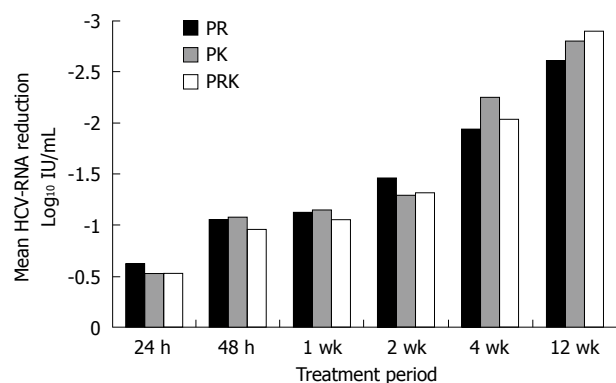
## RESULTS

Forty-five patients were enrolled: 15 were randomized to the PR group, 16 to the PK group and 14 to the PRK group.

The baseline characteristics of the three treatment groups are reported in Table 1. Patients were comparable with respect to age, gender, serum ALT levels and histological features. Overall, 4 out 15 (27%) of PR patients, 3 out 16 of PK (19%) and 2 out of 14 (14%) of PRK



**Figure 1** Mean hepatitis C virus (HCV)-RNA levels during the first 24 wk of treatment in patients with HCV genotype 1 infection receiving PEG-IFN $\alpha$ 2a plus ribavirin (PR) or PEG-IFN $\alpha$ 2a plus ketoprofen (PK) or PEG-IFN $\alpha$ 2a plus ribavirin and ketoprofen (PRK).



**Figure 2** Mean reduction from baseline in log<sub>10</sub> HCV-RNA levels in patients with HCV genotype 1 infection receiving PR or PK or PRK.

patients had a liver fibrosis score equal to 3. The viral load was significantly different between the three groups, being significantly lower in the PR group ( $P = 0.01$ ).

### Viral kinetics and antiviral activity

Mathematical modeling of the decline in HCV-RNA serum levels revealed a triphasic response in all treatment groups (Figure 1). The rapid first phase (1–2 d) was similar in the three groups, although HCV-RNA levels were consistently higher in the PRK group during this time. A second, or “shoulder” phase was observed between 2 and 7–14 d, where the decline in HCV-RNA levels was faster in the PR group compared with both PK and PRK groups. In particular, the PR group showed a slight and progressive reduction of viremia over time after week 1. In the third phase (week 2 onwards), the decrease in HCV-RNA levels was slowest in the PK group while it was more rapid in the PRK group. In this latter group, HCV-RNA levels declined to those observed in the PR group by study end.

The mean log<sub>10</sub> reduction from baseline in HCV-RNA levels over the course of the first 12 wk of treatment is shown in Figure 2. At week 4, the mean log<sub>10</sub> decrease from baseline was comparable between the three groups (–1.95 in the PR group, –2.25 in the PK group and –2.05

**Table 2** Virological response rates by study group  $n$  (%)

	PR group ( $n = 15$ )	PK group ( $n = 16$ )	PRK group ( $n = 14$ )	$P$ -value
Virological response rate				
Week 1	0	0	1 (7.1)	NS
Week 2	1 (6.7)	1 (6.3)	2 (14.3)	NS
Week 4	5 (33.3)	2 (12.5)	3 (21.4)	NS
Week 12	10 (66.7)	7 (43.8)	7 (50)	NS
Week 24	11 (73.3)	11 (68.8)	10 (71.4)	NS
ETR	11 (73.3)	11 (68.8)	10 (71.4)	NS
Relapse rate	4/11 (36.4)	6/11 (54.5)	2/10 (20)	NS
SVR	7 (46.7)	5 (31.3)	8 (57.1)	NS

ETR: End of treatment response; SVR: Sustained virological response.

**Table 3** Baseline characteristics of the patients enrolled in the molecular study (mean  $\pm$  SD)  $n$  (%)

	PR group ( $n = 9$ )	PK group ( $n = 9$ )	PRK group ( $n = 9$ )	$P$ -value
Age (yr)	42 $\pm$ 13	54 $\pm$ 9	42 $\pm$ 12	0.04
Sex (M/F)	5/4	4/5	5/4	NS
HCV-RNA $\times 10^3$ IU/mL	263 $\pm$ 232	1032 $\pm$ 878	1742 $\pm$ 1392	0.01
HCV-RNA $> 700 \times 10^3$ IU/mL	1 (27)	4 (44)	7 (78)	0.02
ALT (U/L)	70 $\pm$ 21	80 $\pm$ 27	100 $\pm$ 57	NS
ETR	7 (78)	5 (56)	6 (67)	NS
SVR	5 (56)	3 (33)	5 (56)	NS

in the PRK group). As shown in Table 2, some patients had undetectable HCV-RNA levels as early as 7–14 d after starting treatment. The PR treatment group displayed an earlier virological response when compared with the other two groups (Table 2).

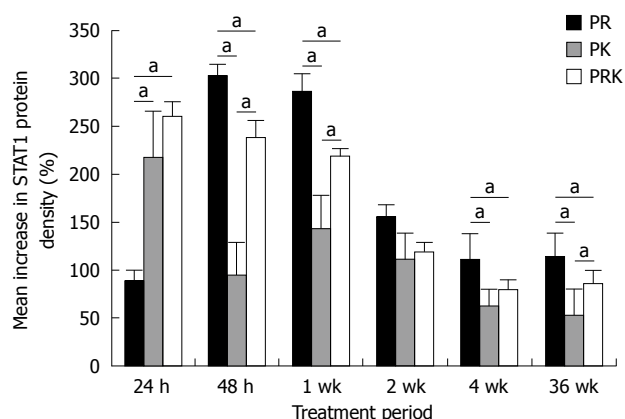
An ETR was obtained in 11/15 patients (73%) in the PR group, 11/16 (69%) in the PK group and 10/14 (71%) in the PRK group. During the treatment-free follow-up period, the relapse rate was lower in the PRK group than in both the other two groups, but the differences were not statistically significant. A SVR was obtained in 7 patients in the PR group (47%), in 5 in the PK group (31%) and in 8 in the PRK group (57%). No association was found between baseline viremia and SVR in any treatment group. However, in the subgroup of patients with high baseline viremia ( $> 700 \times 10^3$  IU/mL), 6/10 (60%) of the PRK group achieved a SVR compared to a quarter (25%) of the PR group and 3/8 (37.5%) of the PK group.

### Modulation of IFN signaling

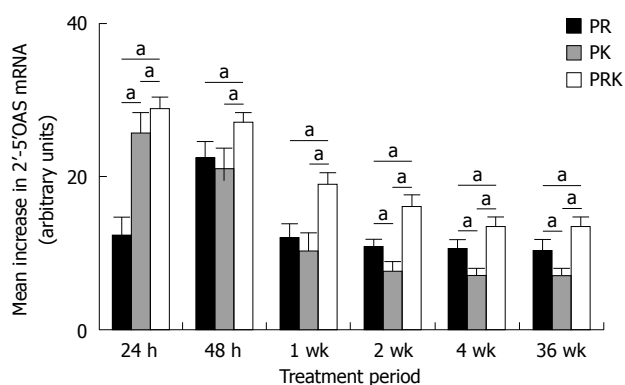
The first 9 randomized patients in each group were enrolled in the molecular study of IFN-dependent signal transduction. Their characteristics are described in Table 3.

A time course analysis of STAT1 activity over 36 wk by means of densitometric analysis was performed. STAT1 activity was consistently up-regulated, as shown by an increase from baseline in mean STAT1 density as early as 24 h after the first drug administration. Nevertheless, a difference between the three treatment groups can be seen in Figure 3. At 24 h, the increase from baseline in STAT1 density was significantly higher in both ketoprofen-





**Figure 3** Mean percentage increase from baseline in STAT1 protein density in patients with HCV genotype 1 infection receiving PR or PK or PRK. Data are presented as the mean ( $\pm$  SD) percentage increase over basal activity in STAT1, assessed by densitometric analysis.  $^aP < 0.05$ .



**Figure 4** 2'-5'-oligoadenylate synthetase (2'-5'OAS) induction in patients with HCV genotype 1 infection receiving PR or PK or PRK. Data are presented as the mean ( $\pm$  SD) fold increase over basal activity in 2'-5'OAS mRNA levels, assessed by RTQ-PCR.  $^aP < 0.05$ .

treated groups as compared with patients of the PR group. However, in PK patients STAT1 activation not only peaked earlier, but it also decreased more rapidly, while in the PRK group a strong STAT1 activation was still present after 1 wk. In contrast, in PR patients the activation of STAT1 peaked at 48 h, 24 h later than in both PK and PRK patients, but was still maintained at higher levels after 1 wk. A steady state was gained between 2 to 36 wk. Thus, the activation of STAT1 in the presence of ribavirin was slower and peaked later but was sustained longer as compared with that observed in the presence of ketoprofen. No relationship was found between STAT1 and viral load. There was no significant difference in STAT1 activity between patients with a SVR and non responders (data not shown).

The analysis of 2'-5'OAS mRNA levels by RTQ-PCR (Figure 4) showed that the presence of ketoprofen increased the transcription rate of this gene, especially early in treatment (24 h after the first dose). The addition of ketoprofen resulted in an early upregulation of 2'-5'OAS mRNA level and permitted the maintenance of this transcript at significantly higher levels in the PRK

**Table 4** Side effects occurring during 48 wk of treatment *n* (%)

	PR group ( <i>n</i> = 15)	PK group ( <i>n</i> = 16)	PRK group ( <i>n</i> = 14)	<i>P</i> -value
Anemia	8 (53)	7 (44)	11 (79)	NS
Neutropenia	9 (60)	7 (44)	10 (71)	NS
Arthro-myalgia	4 (27)	4 (25)	5 (36)	NS
Headache	3 (20)	3 (19)	2 (14)	NS
Fatigue	5 (33)	5 (31)	4 (29)	NS
Flu-like symptoms	4 (27)	4 (25)	2 (14)	NS
Psychiatric disorders	5 (33)	4 (25)	4 (29)	NS
Gastrointestinal disorders	6 (40)	3 (19)	4 (29)	NS
Pruritus	4 (27)	1 (6)	0	NS
Insomnia	2 (13)	2 (12.5)	2 (14)	NS

**Table 5** Time course evaluation of hemoglobin and creatinine serum levels during treatment (mean  $\pm$  SD)

	PR group ( <i>n</i> = 15)	PK group ( <i>n</i> = 16)	PRK group ( <i>n</i> = 14)
Hemoglobin (g/dL)			
Baseline	14.5 $\pm$ 1.3	14.2 $\pm$ 1.9	14.9 $\pm$ 1.5
Week 12	12.2 $\pm$ 1.4	12.5 $\pm$ 1.7	12.2 $\pm$ 1.6
Week 24	12.2 $\pm$ 1.6	12.6 $\pm$ 1.8	11.9 $\pm$ 1.7
Week 48	11.6 $\pm$ 1.4	13.1 $\pm$ 1.8	12.0 $\pm$ 1.6
Creatinine (mg/dL)			
Baseline	0.97 $\pm$ 0.13	0.82 $\pm$ 0.22	1.01 $\pm$ 0.07
Week 12	0.87 $\pm$ 0.11	0.82 $\pm$ 0.16	0.92 $\pm$ 0.12
Week 24	0.86 $\pm$ 0.15	0.79 $\pm$ 0.16	0.91 $\pm$ 0.15
Week 48	0.44 $\pm$ 0.15	0.80 $\pm$ 0.17	0.92 $\pm$ 0.15

group compared with the PR and PK groups, from the beginning of treatment until week 36. Finally, 2'-5'OAS mRNA levels were significantly higher in patients with a SVR than in non responders at each time point and in each group (data not shown).

### Safety

The type and frequency of adverse events were similar in the three treatment arms and are summarized in Table 4. Anemia, defined as hemoglobin level  $< 12.0$  g/dL in women and  $< 13.0$  g/dL in men, and neutropenia, defined as neutrophil count  $< 1000/\text{mm}^3$  were the most common side effects. However, only one patient in the PRK group met the hemoglobin criterion for ribavirin dose reduction (hemoglobin level  $< 10$  and  $\geq 8.5$  g/dL), while the dose of PEG-IFN was reduced because of neutropenia (neutrophil count of  $< 750$  and  $\geq 500/\text{mm}^3$ ) in one patient each in the PR and in PK group. The latter developed a pneumonia requiring antibiotic treatment. No patient experienced renal dysfunction. In particular, in no case did creatinine serum levels exceed the upper limit of normal range (1.2 mg/dL) during treatment. Time course evaluation of hemoglobin and creatinine serum levels during treatment in the three groups is reported in Table 5.

One patient each in the PR group and the PK group withdrew prematurely from the study. The patient in the PR group dropped out after 1 mo because of severe depression and the patient in the PK group dropped out after 6 mo because of poor compliance.

## DISCUSSION

The objective of this phase II study was to assess the safety of ketoprofen in combination with PEG-IFN $\alpha$ 2a with or without ribavirin in treatment-naïve patients with HCV genotype 1 infection, and the effect of these regimens on viral kinetics and IFN $\alpha$  signaling modulation. Our results showed that ketoprofen was safe and well tolerated. In particular, gastrointestinal-related adverse events were mild and did not lead to dose reduction or to premature treatment discontinuation in any patient. Given the importance of managing the tolerability of HCV antiviral treatment, our observation that the addition of ketoprofen to PEG-IFN and ribavirin is as well tolerated as the standard regimen is reassuring.

The kinetics of viral decay during treatment showed a triphasic response that was more evident in patients receiving ketoprofen, independent of the use of ribavirin. Following the first phase of rapid decline in HCV-RNA levels, patients receiving ketoprofen showed a pronounced “shoulder phase” starting 2 to 14 d after initiation of therapy. It has recently been suggested that the triphasic decline in HCV-RNA levels occurs only in patients in whom a majority of hepatocytes are infected before therapy<sup>[23]</sup>. Thus, the higher baseline viremia of both ketoprofen groups could help to explain the differences in viral kinetics between the groups receiving ketoprofen and the PR group. Interestingly, in the third and final phase, the HCV-RNA levels were lower in the two groups receiving ribavirin than in the PK group. This enhanced response to treatment in ribavirin recipients supports the hypothesis that ribavirin not only improves the anti-HCV immune response, but also had a mutagenic effect against HCV<sup>[23,24]</sup>. However, it should be pointed out that in both the PK and PRK groups, ketoprofen was administered only during the first 24 wk of treatment. Thus, the similarity between the PRK and PR groups in HCV-RNA levels during the final phase could be attributable to the absence of ketoprofen in the PRK group during this time.

As far as the IFN $\alpha$  signaling modulation is concerned, the activation of STAT1 occurred very early after treatment initiation in the two groups receiving ketoprofen, being evident after 24 h from the start of treatment. However, there was a rapid decline in STAT1 activation thereafter, particularly in the PK group. In contrast, the PR group exhibited the greatest activation after 48 h and this activation was more sustained over time compared with the ketoprofen-containing regimens. The mechanisms responsible for these differences in the control of IFN-induced responses probably include down-regulation and degradation of receptors<sup>[15]</sup>. Moreover, it has been demonstrated that both ribavirin and NSAIDs act synergistically with IFN- $\alpha$  in induction of STAT1 activation<sup>[13,25]</sup>, yet there was lower STAT1 activation in the PRK group compared with the PR group. Thus, an antagonistic effect between ketoprofen and ribavirin cannot be excluded.

On the other hand, our data demonstrated an early and sustained increase of 2'-5'OAS transcription in the PRK group compared with the PR group, suggesting that the addition of ketoprofen to the conventional combination therapy induces early activation of the IFN $\alpha$

pathway, followed by a better activation of the IFN $\alpha$ -dependent intracellular pathway.

Even if this study was not designed to assess antiviral efficacy, the clinical results are consistent with the molecular data. At baseline, the PR group had a significantly lower mean HCV viral load than both the PK and PRK groups. Thus, the proportion of patients with low viral load ( $\leq 700\,000$  IU/mL) was significantly higher in the PR group (73%) than in the PK (50%) or in the PRK group (29%). It is well known that in genotype 1 patients, baseline viral load is the best prognostic factor for response to antiviral treatment<sup>[5-7,26]</sup>. Thus, it was not surprising that patients in the PR group obtained a better virological response at week 4. Nevertheless, the SVR rate observed in the PRK group (57%) was better than that observed in PR (47%) and in PK (31%). Furthermore, among patients with high viral load ( $> 700\,000$  IU/mL), those of the PRK group obtained the better SVR (60% *vs* 25% and 38% in the PR and PK groups, respectively). It should be pointed out that our study was initiated before the optimal dose of ribavirin for patients with HCV genotype 1 (i.e. 1000 mg/d or 1200 mg/d according to body weight) was determined<sup>[7]</sup>. However, even if the dose of ribavirin utilized in this study was suboptimal and might have influenced the SVR, both groups receiving ribavirin had the same dose regimen.

In conclusion, considering that the use of ketoprofen does not add further side effects, is associated with better viral kinetics and early activation of the IFN signaling pathway, and in combination with PR improves virological response rates, this pilot study suggests the exploration of the clinical efficacy of this three-drug combination in well-designed randomized clinical trials. We conclude that such studies are warranted since, in this era of development of new drugs for HCV, the clinical use of novel compounds up till now has been hampered by toxicity issues and rapid promotion of drug-resistant HCV viruses<sup>[27]</sup>.

## COMMENTS

### Background

The current standard treatment for chronic hepatitis C with pegylated-interferon (PEG-IFN) and ribavirin is effective in approximately 50%-60% of patients, so that a substantial proportion of patients remain unresponsive. A rational approach to develop alternative therapeutic strategies for patients with chronic hepatitis C virus (HCV) infection demands a detailed knowledge of how the different drugs affect viral kinetics and IFN intracellular signaling. Non-steroidal antiinflammatory drugs (NSAIDs) have been demonstrated to amplify the IFN signaling pathways and to enhance the anti-viral effect of IFN. This phase II study evaluated the effect of ketoprofen (a NSAID) plus PEG-IFN with or without ribavirin compared with PEG-IFN plus ribavirin (PR) on viral kinetics, STAT1 activity and expression of the IFN-dependent gene, 2'-5'-oligoadenylate synthetase (2'-5'OAS), in patients with genotype 1 chronic hepatitis C.

### Research frontiers

The results of this pilot study support the proposal of an evaluation of the clinical efficacy of the addition of ketoprofen to the standard PR treatment for chronic hepatitis C in well-designed randomized clinical trials.

### Innovations and breakthroughs

This is the first study to report both molecular and clinical data about the use of ketoprofen in association with PEG-IFN $\alpha$  and ribavirin in chronic hepatitis C. The authors found that the addition of ketoprofen to the conventional combination therapy is associated with better viral kinetics and early activation of the IFN $\alpha$  signaling pathway, thus improving virological response rates.

## Applications

The results may stimulate further experimental and clinical investigations regarding the role of NSAIDs in association with IFN-based therapy in the context of HCV-related liver diseases.

## Terminology

IFN signaling pathways are activated by binding of IFN to its specific receptor, which induces autophosphorylation of protein tyrosine kinases Tyk-2 and Jak-1 on tyrosine residues, thus activating signal transducer and activator of transcription (STAT1 and STAT2) proteins. Activated STATs translocate to the nucleus where they activate the transcription of IFN-inducible genes, such as 2'-5'OAS.

## Peer review

The authors postulate that a larger trial should be done with this 3 drug combination, compared to standard of care. While a small study, there is useful data.

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# Education-based approach to addressing non-evidence-based practice in preventing NSAID-associated gastrointestinal complications

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in the education-based study that recorded data from 3728 patients. The specialists overestimated the risk of GI complications with NSAIDs, underestimated the GI safety profile of coxibs, but were aware of the risk factors and of the current prevention strategies. Proton pump inhibitors were co-prescribed with NSAIDs in > 80% of patients with and without risk factors. The educational program had little impact on prescribing habits.

**CONCLUSION:** Specialists are informed of advances in NSAID-associated adverse effects and have high rates of GI-prevention therapy. Our educational program did not alter these rates.

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**Key words:** Nonsteroidal anti-inflammatory agents; Education; Gastrointestinal diseases; Adverse effects; Cyclooxygenase 2 inhibitors; Proton pump inhibitors

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## Abstract

**AIM:** To evaluate an evidence-based educational program for improving strategies for prevention of non-steroidal anti-inflammatory drug (NSAID)-associated gastrointestinal (GI) complications.

**METHODS:** Four hundred and fifty-six specialists replied to a questionnaire that covered issues related to NSAID-induced adverse effects. They also collected data from their last five consecutive patients before and after they had attended an evidence-based seminar on GI prevention strategies.

**RESULTS:** Four hundred and forty-one of 456 specialists (96.7%) participated in the survey, and 382 (83.7%)

## INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are prescribed extensively worldwide, with at least 20% of the adult population using them for at least 1 mo per year<sup>[1]</sup>. NSAID use is associated with a wide range of side effects, the most usual being those involving the gastrointestinal (GI) tract. Research in this field has progressed considerably, especially since the commercialization of cyclooxygenase 2 inhibitors (coxibs), and the amount of information published is impressive. Recent data derived from studies of the side effects associated with coxibs and traditional NSAIDs have received a good deal of attention in scientific and non-scientific



publications. Based on existing and new information, scientific organizations, regulatory agencies and influential journals have made recommendations regarding GI prevention strategies with NSAIDs<sup>[2-4]</sup>. These indicate that patients requiring NSAIDs should be evaluated for the presence of GI and cardiovascular (CV) risk factors, and should undergo prevention therapy when found to be at risk. Patients with a high risk of CV complications are to avoid coxibs and/or NSAIDs. Patients with GI risks should receive either a coxib, or concomitant therapy with misoprostol or a proton pump inhibitor (PPI). In all cases, the minimum effective dose and the shortest possible administration time should be a joint objective. In the light of all this knowledge, it remains unclear whether or not information has been translated into clinical practice.

Recent evidence indicates that prescribing patterns are far from appropriate; a trend that has become more pronounced since the withdrawal of rofecoxib from the market<sup>[5]</sup>. Reports suggest that, in Europe, up to 80% of patients with one or two risk factors for GI complications do not receive the appropriate prevention strategies<sup>[6,7]</sup> and that, in the United States, the lack of GI protection has grown since 2004<sup>[5]</sup>. In addition, some patients who are not at risk receive unnecessary therapy or receive inadequate and ineffective drugs that compromise their health and increase the cost of NSAID treatment<sup>[8,9]</sup>. The reasons for the lack of or inappropriate use of prevention strategies are unclear, but conflicting results or variations in their interpretation and divergences in the recommendations that they support<sup>[2-4,10,11]</sup> may have contributed to the confusion.

However, the aforementioned data and conclusions have been obtained at primary care level, therefore, the present study was designed firstly to evaluate the level of knowledge regarding NSAID-associated adverse effects among specialists treating patients with rheumatic diseases, and secondly, to evaluate whether an educational program based on a review of current evidence produced an improvement in the pattern of patient management.

## MATERIALS AND METHODS

The study was approved by the Regional Institutional Review Board of Aragón and carried out during 2006 to 2007. First, we conducted a nationwide survey of 456 specialists distributed across the country, and among those who had previously participated in similar studies and had delivered good-quality data, and that represented the most frequently occurring specialties among patients suffering from musculoskeletal conditions. Physicians received a letter from one of the researchers (Lanas A) explaining the purpose of the study, voluntary nature of participation, the confidentiality of the information provided, and absence of commercial purposes of the study. Physicians were questioned about issues related to NSAIDs and their adverse effects, with a special focus on research over the previous 4-5 years that may have affected clinical practice directly. Table 1 summarizes the main questions.

The educational program was designed to answer the objective of the study, which was to evaluate the impact of an evidence-based seminar on clinical practice, and to describe current practice in the prevention of GI adverse effects in the specialized setting. Only physicians who participated in the initial survey were invited to participate in the educational program. Firstly, doctors sent data of their last five consecutive patients visited in the office (phase I). These patients had to be at least 18 years old and taking or having been prescribed NSAIDs at the time of the visit. None of the data collected revealed the identity of the subjects. The responses were transmitted anonymously to one of the researchers (Sobreviola E), who entered the information into a database without including any data that could identify physicians or patients. Between 2 and 4 mo later, these specialists attended small group seminars that lasted approximately 2 h, and were based on current evidence in the field and received literature related to NSAID prevention strategies. These educational programs reviewed the main adverse effects associated with NSAIDs and coxibs, as well as risk factors, therapy-specific risks, the pros and cons of available prevention strategies, and treatment options for the cases most frequently encountered in clinical practice. The different therapeutic options, depending on the presence/absence of GI and CV risk factors<sup>[12,13]</sup>, were also reviewed and discussed. All these seminars were given by the same two investigators (Lanas A and Esplugues JV). Between 3 and 4 mo after the seminar, the same physicians again sent data of their last five consecutive patients, with the same inclusion criteria as described above (phase II of the educational program-based study).

### Statistical analysis

Data were analyzed using SAS software v.8.02 for Windows (SAS Institute, Cary, NC, USA). For categorical variables, absolute frequencies and percentages were obtained; for continuous variables, mean  $\pm$  SD, median, percentiles 25-75, maximal and minimum values and 95% CI were obtained. Significance related to categorical variables was obtained using the  $\chi^2$  test or Fisher's exact test. Comparisons reached statistical significance at  $P < 0.05$ .

## RESULTS

### Physician survey sub-study

Of a total of 456 invited physicians, 441 (96.7%) returned valid questionnaires. Those that responded had a mean  $14 \pm 8.6$  years of professional activity. Three hundred and seventy-four (84.8%) were members of one or more scientific societies, and 189 (42.9%) were aware that their respective societies had published guidelines or recommendations for the management of NSAIDs. Two hundred and eighty (63.4%) were orthopedic surgeons, 116 (24.7%) were rheumatologists, and 45 were other types of specialists (10.2%).

Only 24 (5.7%) doctors responded that NSAID use was not associated with GI toxicity; 368 (88.2%), a substantial majority, stated that NSAID use was associated with GI, renal, CV, or liver damage. A total of 207 (50.2%)

**Table 1** A summary of the main questions (from a total of 22) assessing physicians' knowledge of current evidence in the field of NSAID use and adverse effects

NSAID use is associated with adverse effects. Which of the following do you believe is not associated with NSAID use?
What is the expected annual incidence of upper GI complications in patients taking NSAIDs, as reported in the most recent large outcome studies?
The occurrence of dyspepsia in patients who take NSAIDs has been reported to be less than 25% (true or false)
NSAIDs may induce GI complications in the lower GI tract (true or false)
Which of the following factors do you believe is/are risk factors for GI complications in patients who take NSAIDs? (list)
Which of the following NSAIDs do you believe is more toxic to the GI tract? (list)
Concerning COX-2 selective inhibitors, for each of the following, indicate whether the statement is true or false:
They are not as effective as traditional NSAIDs in the treatment of OA or RA
The use of these compounds is associated with a 50% reduction in the risk of GI complications compared to NSAIDs
The concomitant use of low-dose aspirin reduces or eliminates the GI benefit of these compounds when compared to NSAIDs
The use of these compounds has been associated with an increased risk of CV events
In high-risk patients, the combination of NSAIDs plus a PPI is safer than a coxib alone
Concerning gastroprotective agents, indicate for each of the following statements whether they are true or false:
H2-RAs are effective in the prevention of gastric ulcers, duodenal ulcers, and GI complications
PPIs are effective in the prevention of gastric ulcers, duodenal ulcers, and GI complications
Misoprostol is effective in the prevention of gastric ulcers, duodenal ulcers, and GI complications
Which of the following agents has been proved to be effective in the treatment or prevention of NSAID-induced dyspepsia? (list)

NSAID: Nonsteroidal anti-inflammatory drug; GI: Gastrointestinal; CV: Cardiovascular; PPI: Proton pump inhibitor; H2-RAs: H2 receptor antagonists.

**Table 2** Responses to the question, "Which of the following factors do you believe is/are risk factors for GI complications in patients who take NSAIDs?" *n* (%)

	Rheumatologists	Orthopedic surgeons	Others	Total
History of peptic ulcer	115 (99.1)	275 (98.2)	21 (100.0)	411 (98.6)
History of complicated peptic ulcer	116 (100.0)	275 (98.2)	21 (100.0)	412 (98.8)
Age > 65 yr	114 (98.3)	229 (81.8)	18 (90.4)	361 (86.6)
Concomitant use of low-dose aspirin for CV prevention	114 (98.3)	228 (81.4)	19 (90.4)	361 (86.6)
Concomitant use of anticoagulants	112 (96.5)	247 (88.2)	20 (95.3)	379 (90.9)
<i>Helicobacter pylori</i> infection	103 (88.8)	257 (91.8)	19 (90.4)	379 (90.9)
Smoking	87 (75.00)	223 (79.6)	13 (61.7)	323 (77.5)
Dyspepsia history	73 (62.9)	250 (89.3)	19 (90.4)	342 (82.0)
Alcohol	105 (90.5)	257 (91.8)	20 (95.3)	382 (91.6)
High dose of NSAIDs	113 (97.4)	275 (98.2)	21 (100.0)	409 (98.1)

overestimated the overall rate of upper GI complications in NSAID users, and 261 (63.0%) stated that NSAID use could lead to complications of the lower GI tract. The two symptoms that doctors considered to be the most frequently reported by patients in relation to NSAID therapy were epigastric pain (67.1%) and heartburn (54.8%). The frequency of dyspepsia as an adverse effect of NSAIDs was underestimated by 45.2% of respondents. As summarized in Table 2, most identified the risk factors for GI complications in NSAID users; there were no differences between the responses of rheumatologists and orthopedists, which were the two main specialties represented by the participants. Indomethacin (61.9%), piroxicam (34.0%), diclofenac (18.5%) and ketorolac (11.0%) were considered to be the most gastrototoxic agents, while coxibs, paracetamol and metamizol were considered to be the safest for the GI tract.

When questioned about coxibs, 93 (22.5%) of the specialists believed them to be less effective than NSAIDs, but 84.6% said they were safer for the GI than NSAIDs were. However, 43.9% of the specialists stated that coxibs were more toxic for the GI tract than a combination of NSAID + PPI. Furthermore, 211 (52.2%) reported that concomitant low-dose aspirin reduced the GI benefit of coxibs, and 394 (94.7%) considered coxibs to be toxic to

the CV system; a proportion that fell to 72.7% ( $P = 0.140$ ) when the same question was asked about NSAIDs.

Over half of the physicians (56.1%) reported that histamine H2 receptor antagonists (H2-RAs) were effective in preventing ulcers and ulcer complications in NSAID users; almost all (98.5%) reported the same effect with PPIs. Responding about GI prevention therapy habits with NSAIDs, 217 (52.4%) took this precaution on a routine basis, 45.9% only when risk factors were present, and 5.3% only when patients were receiving long-term NSAID therapy. H2-RAs (44.6%), misoprostol (41.2%) and PPIs (94%) were considered to be effective for the prevention and treatment of NSAID-induced dyspepsia.

### **Effects of the educational program on patient management**

**Demographics and characteristics of patients:** Of 456 invited participants, 382 (83.7%) submitted information regarding 3728 patients over the two phases (1732 in phase I - before the evidence-based seminar, and 1722 in phase II - after the seminar). Two hundred and seventy-four patients were excluded for the following reasons: 43 were under the age of 18 years, and 231 lacked an NSAID prescription. Table 3 summarizes the main characteristics of the patients included in the study. No statistical

**Table 3** Characteristics of patients included in the educational program of the study<sup>1</sup> *n* (%)

Variable	Phase I ( <i>n</i> = 1732)	Phase II ( <i>n</i> = 1722)
Age (mean ± SD)	61.06 ± 13.37	60.81 ± 13.89
Female	1038 (60.4)	980 (57.6)
History of ulcer	238 (13.7)	307 (17.8)
History of ulcer bleeding	61 (3.5)	69 (4.0)
ASA use	167 (9.6)	168 (9.8)
CV history	203 (11.7)	205 (11.9)
Increased blood pressure	845 (48.8)	810 (47.0)
Anticoagulant use	126 (7.3)	120 (7.0)
Corticosteroid use	162 (9.3)	190 (11.0)
History of dyspepsia	782 (45.1)	766 (44.5)

<sup>1</sup>No statistical differences were found between patients enrolled in the two phases. Phase I: Before physicians attended the evidence-based seminar; Phase II: After the seminar; ASA: Aspirin.

**Table 4** Prescription of NSAIDs to patients in each of the two study phases of the educational program *n* (%)

Drug therapy	Phase I		Phase II	
	Before visit	After visit	Before visit	After visit
No NSAID therapy	718 (41.45)	162 (9.35)	653 (37.92)	190 (11.03)
NSAID therapy	1014 (58.55)	1570 (90.65)	1069 (62.08)	1532 (88.97)
Acetofenac	146 (8.43)	248 (14.32) <sup>b</sup>	148 (8.59)	202 (11.73) <sup>b</sup>
Celecoxib	45 (2.60)	100 (5.77) <sup>b</sup>	35 (2.03)	116 (6.74) <sup>b</sup>
Diclofenac	229 (13.22)	271 (15.65)	238 (13.82)	270 (15.68)
Etoricoxib	16 (0.92)	46 (2.66) <sup>b</sup>	18 (1.05)	79 (4.58) <sup>b</sup>
Ibuprofen	281 (16.22)	432 (24.94) <sup>b</sup>	297 (17.25)	406 (23.58) <sup>b</sup>
Indomethacin	63 (3.64)	62 (3.58)	73 (4.24)	75 (4.36)
Ketorolac	15 (0.87)	25 (1.44)	28 (1.63)	31 (1.80)
Meloxicam	71 (4.10)	234 (13.51) <sup>b</sup>	101 (5.87)	215 (12.49) <sup>b</sup>
Piroxicam	74 (4.27)	75 (4.33)	64 (3.72)	64 (3.72)
Other NSAIDs (includes naproxen)	19 (1.10)	22 (1.27)	16 (0.93)	28 (1.63)
Analgesics				
Paracetamol	137 (7.91)	120 (6.93)	136 (7.90)	122 (7.08)
Metamizol	35 (2.02)	28 (1.62)	53 (3.08)	26 (1.51)
Total	1732 (100)		1722 (100)	

<sup>b</sup>*P* < 0.001 *vs* before the visit.

differences were found between patients referred to in the two phases.

**NSAID treatment:** In both phases, ibuprofen (16.2% and 17.25% in phases I and II, respectively), diclofenac (13.2% and 13.8%) and acetofenac (8.4% and 8.6%) were the three most frequently prescribed NSAIDs. Coxib prescription was low (3.5%). There was a statistically significant (*P* < 0.0001) increase in prescription rates of acetofenac, celecoxib, ibuprofen, meloxicam and etoricoxib after the visit with the specialist, but this increase was similar in both phases (Table 4). The main reasons for prescribing NSAIDs was the diagnosis of osteoarthritis [1015 (63.24%) in phase I and 987 (61.96%) in phase II] or rheumatoid arthritis [148 (9.22%) and 186 (11.68%) in phases I and II, respectively]. In phase I, NSAID therapy was terminated in 15.98% of patients following the visit to the specialist, a similar percentage

**Table 5** Risk factors (RFs) of patients reported by doctors in the educational program according to either a non-restrictive or a restrictive definition<sup>1</sup> *n* (%)

Number of RFs	Non-restrictive		Restrictive	
	Phase I <sup>2</sup>	Phase II <sup>3</sup>	Phase I <sup>2</sup>	Phase II <sup>3</sup>
0	347 (20.03)	352 (20.44)	961 (55.48)	891 (51.74)
1	660 (38.11)	598 (34.73)	558 (32.22)	573 (33.28)
2	517 (29.85)	536 (31.13)	176 (10.16)	213 (12.37)
> 2	208 (12.01)	236 (13.70)	37 (2.14)	45 (2.61)
Total	1732 (100)	1722 (100)	1732 (100)	1722 (100)

<sup>1</sup>A non-restrictive definition of risk factors for NSAID-related complications included age > 60 years, history of dyspepsia, history of either complicated or non-complicated ulcer, concomitant therapy with NSAIDs and low-dose aspirin, or anticoagulants or corticosteroids. A restrictive definition of risk factors included age ≥ 70 years, history of complicated or non-complicated ulcer, concomitant therapy with NSAIDs and low-dose aspirin, or anticoagulants or corticosteroids; <sup>2</sup>In Phase I, the specialists received an anonymous questionnaire regarding data and prescriptions for their last five consecutive patients; <sup>3</sup>In Phase II, the process was repeated 4-5 mo later after specialists had attended an evidence-based seminar that reviewed current evidence on NSAID-related issues, with a focus on GI prevention strategies in NSAID users.

to that reported in phase II (17.77%). The duration of NSAID therapy after the visit was also similar in both phases. Most treatments were prescribed for a short duration (< 30 d) (74.8% and 72.04% in phases I and II, respectively). No significant differences were found between the two phases.

**NSAID treatment and dyspepsia:** In phase I, 1129 (66%) of 1710 patients who were seen had suffered or were suffering GI symptoms prior to the visit, and 65.6% of the 1129 were receiving NSAID therapy; a higher proportion than those who did not have symptoms before the visit 256/581 (45.6%) (*P* < 0.0001). After the visit, physicians increased the prescription of NSAIDs to a similar rate (88.8% and 94%) in both groups of patients (*P* = 0.0006 *vs* before the visit). Similar percentages were observed in phase II, and no differences were observed between the phases.

Among the patients with GI symptoms, 57.1% in phase I and 60.1% in phase II were undergoing treatment for symptom relief before the visit to the specialist, and about one-third of them were being treated with a PPI. After the visit to the specialist, almost all these patients with symptoms were prescribed PPI therapy (*P* < 0.0001). No differences were found between the two phases.

**Risk factors and prevention strategies:** The number of patients with risk factors depends on the definition of these factors. The two most prevalent risk factors were age and a history of dyspepsia. We present data for a restrictive definition (age > 70 years and excluding history of dyspepsia) and for a non-restrictive definition of those risk factors (e.g. age > 60 years and history of dyspepsia; Table 5). Very few patients with risk factors were switched from a traditional NSAID to a coxib alone; 54 (3.1%) *vs* 129 (7.4%) (*P* < 0.0001) before and after the visit to the specialist in phase I, and 42 (2.4%) *vs* 155



**Table 6** Proportion of patients on NSAID therapy that received concomitant therapy with a PPI or misoprostol after the medical visit, according to the number of RFs *n* (%)

Number of RFs	Non-restrictive		Restrictive	
	Phase I	Phase II	Phase I	Phase II
0	268/347 (77.2)	283/352 (80.4)	782/961 (81.4)	728/891 (81.7)
1	536/660 (81.2)	499/598 (83.4)	471/558 (84.4)	504/573 (87.9)
2	453/517 (87.6)	456/536 (85.1)	151/176 (85.8)	168/213 (78.9)
> 2	175/208 (84.1)	201/236 (85.2)	28/37 (75.7)	39/45 (86.7)

(9.0%) in phase II ( $P < 0.0001$ ). No differences between phases I and II were observed after the visit, although we observed a trend toward an increase in coxib prescription in phase II ( $P = 0.09$ ).

The most widely used strategy for prevention of GI complications in Spain is concomitant therapy with PPIs. In both phases of the study, physicians prescribed appropriate gastroprotection therapy for over 80% of patients with risk factors, with little therapeutic benefits observed after the educational program (Table 6). The study also reveals a similar pattern of gastroprotection prescription rates among patients without GI risk factors.

A sub-analysis of data in high-risk patients (defined as those with previous ulcer bleeding or those who were being treated with anticoagulants) showed that very few of these patients were prescribed NSAIDs without PPIs [11/163 (8.4%) in phase I, and 6/158 (4.08%) in phase II].

## DISCUSSION

We found that the majority of specialists who treat patients with rheumatic disease are aware of recent evidence concerning the adverse effects associated with traditional NSAIDs and coxibs. The study also revealed that gastroprotection-related prescribing rates by the specialists among at-risk patients receiving NSAIDs were high, and that an educational program aimed at influencing prescription patterns had little impact.

The first step in the process of implementing prevention strategies in NSAID-treated patients at risk of GI complications is to be familiar with the risk factors. We observed that the specialists who treat patients with different rheumatic conditions are well informed of these factors, which may explain the high rates of preventative prescriptions observed in our study population. Other recent findings in the field, such as the increased risk of CV events with coxibs and traditional NSAIDs, are also well known and may explain the use of short-term courses of treatment with these compounds.

Previous studies have reported that most patients on NSAIDs with one or more risk factors for GI complications were not prescribed prevention-related treatment<sup>[6,7]</sup>. This was not the case in our patient population, of which a high proportion showed risk factors and received concomitant prescription of NSAIDs with gastroprotective agents,

specifically PPIs. The reasons for this discrepancy are not clear, but previous studies were based on a primary care database and not on prescribing data obtained from specialists, who may be more aware of risk factors and strategies to reduce their impact. In addition, this study provides more recent data on prescribing habits than the above-mentioned studies<sup>[6,7]</sup>, which did report a tendency towards more appropriate prescribing rates with time.

This study differs from those carried out in the United States<sup>[5]</sup>, in one aspect specifically: the most prevalent prevention strategy in the current study was the concomitant prescription of PPIs and NSAIDs, while the prescribing rate of coxibs was low, in agreement with sales data for these compounds across Europe<sup>[14]</sup>. This difference between practices in the United States and Europe<sup>[15]</sup> may reflect a widespread belief among the participants in the current study that adding a PPI to a NSAID confers greater upper GI protection than administration of a coxib alone, a belief that is not based on evidence<sup>[16]</sup>.

Also of interest is our finding that the PPI prescribing rate was high among patients whose NSAID treatment was discontinued after a visit to the specialist, and in patients with no risk factors. Even considering the non-restrictive framework for risk factors, which includes a history of dyspepsia as justification for prescribing gastroprotectants, > 20% of patients from the overall study population who had no GI risk factors were being prescribed preventative therapies. This excess of PPI concomitant therapy is not intrinsically inappropriate, given that it may reduce the risk of complications in patients with a low risk of GI, but it does significantly increase the cost of NSAID therapy by an estimated 80%<sup>[17]</sup>. Even in a market in which generics are prescribed and promoted widely, this added expense is not to be disregarded. Furthermore, although PPI treatment is considered to be relatively safe<sup>[18]</sup>, the long-term treatment with these type of drugs is associated with some adverse effects, including an increased risk of GI infections, pneumonia and even hip fracture<sup>[19-22]</sup>. Finally, according to current guidelines, implementing unnecessary prevention strategies is incorrect medical practice.

The other major finding of our study was the failure of our educational program to have any real effect on prescribing habits, although the effect achieved may have been so small because of the baseline circumstances. We observed a minor and statistically insignificant increase in prescribing rates for the safest NSAIDs, including coxibs, and for gastroprotectants among patients running the highest risk of GI adverse effects; a change that would appear to be a result of the educational program. On the other hand, we saw no effect on declining prescribing rates for gastroprotectants among patients without GI risk factors. The shift towards an evidence-based approach to practice seems a challenging task.

A recent study demonstrated that intervention consisting of a combination of education and computer alerts improved gastroprotection<sup>[23]</sup> in at risk patients prescribed NSAIDs, but still the rates were far from being optimal. On the other hand, educational programs may lead to short-term improvements in our knowledge,



but the impact on clinical practice is modest, especially in the mid- to long-term<sup>[24]</sup>. As suggested previously<sup>[25]</sup>, successful educational tools are costly because they require regular feedback and reinforcement. In any case, our study suggests that a high proportion of specialists are well informed about the latest advances in the NSAID field and implement appropriate prevention therapies in at-risk patients, which suggests that continuing medical education is the key to progress.

Our study had some limitations. The information was not obtained from a database but from the records of the participating physicians. Therefore, it was possible that their records differed from actual practice. However, high PPI prescribing rates have been observed in other studies<sup>[26]</sup> and reflect the marked decrease in national rates of upper GI bleeding over the last 5 years. In addition, the survey results concerning the degree of knowledge were in accordance with the clinical practice reported. Another limitation was that the study involved only one time point of observation after completion of the educational program, which did not allow the short- or long-term effects of the program to be analyzed. Finally, the data obtained cannot be extrapolated to clinical practice at the primary care level, which accounts for a major part of NSAID prescribing rates. In fact, the concomitant prescribing rate of gastroprotectants in NSAID users before visiting the specialist (which may well reflect practices in primary care) was much lower than that observed after the visit. Obviously, if a continued drop in GI complications among NSAID users is the goal, prevention strategies should be implemented at all levels of care. Further research should be carried out at the level of primary care to detect areas for improvement and to design improved educational programs by which GI complications in NSAID users may be prevented. Finally one potential limitation of the study is the validity of our conclusions outside Spain. While some data may be country-specific (e.g. prescription rates of PPI), we believe that other aspects of the study can probably be extrapolated (e.g. usefulness of the educational approach, awareness of the specialist on the medical advance,) and therefore be of interest in other areas.

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## COMMENTS

### Background

Advances in medical sciences may not be rapidly translated into medical practice. Also contradictory results reported in the literature may difficult appropriate medical care. Advances in the understanding of adverse effects of nonsteroidal anti-inflammatory drugs (NSAIDs) and prevention strategies have been enormous in the last 5 years.

### Research frontiers

It is not know whether specialists treating patients rheumatic diseases are aware of advances in the field of NSAID-associated adverse effects and whether these advances have been translated into medical practice. It has been tested whether specialists confronted with scientific evidence incorporate this into medical practice and modify prescription patterns to these patients.

## Innovations and breakthroughs

The study shows that specialists dealing with patients suffering from rheumatic diseases and prescribing NSAIDs in Spain are aware of the recent advances in the NSAID field, identify the main gastrointestinal risk factors and of the current available prevention strategies. The study detects inappropriate use of prevention strategies in patients being prescribed with NSAIDs. An evidence based seminar of the prevention strategies carried out with these specialist do not change their prescription patterns.

## Applications

The study was carried out in one European country and it is unclear whether the data can be extrapolated to other countries, where prescription patterns have shown to be different. Nevertheless, the study shows other aspects that can be applied to other areas and countries: (1) Knowledge of evidence by the specialist is no automatically translated into clinical practice; (2) Modification of clinical practice based on scientific evidence needs a complex intervention.

## Terminology

Evidence based clinical practice refers to medical care which is applied to patients based on studies with sufficient scientific quality that have been published in peer-review and that have been accepted by the scientific community (guidelines, expert reports, scientific societies, etc.) as appropriate.

## Peer review

This is a well presented approach to evaluation of an evidence-based educational program for improving NSAID-associated prevention strategies.

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BRIEF ARTICLE

## Hyperphosphatemia after sodium phosphate laxatives in low risk patients: Prospective study

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### Abstract

**AIM:** To establish the frequency of hyperphosphatemia following the administration of sodium phosphate laxatives in low-risk patients.

**METHODS:** One hundred consecutive ASA I-II individuals aged 35-74 years, who were undergoing colonic cleansing with oral sodium phosphate (OSP) before colonoscopy were recruited for this prospective study. Exclusion criteria: congestive heart failure, chronic kidney disease, diabetes, liver cirrhosis, intestinal obstruction, decreased bowel motility, increased bowel permeability, and hyperparathyroidism. The day before colonoscopy, all the participants entered a 24-h period of diet that consisted of 4 L of clear fluids with sugar or honey and 90 mL (60 g) of OSP in two 45-mL doses, 5 h apart. Serum phosphate was measured before and after the administration of the laxative.

**RESULTS:** The main demographic data (mean  $\pm$  SD) were: age,  $58.9 \pm 8.4$  years; height,  $163.8 \pm 8.6$  cm; weight,  $71 \pm 13$  kg; body mass index,  $26 \pm 4$ ; women, 66%. Serum phosphate increased from  $3.74 \pm 0.56$  to  $5.58 \pm 1.1$  mg/dL, which surpassed the normal value (2.5-4.5 mg/dL) in 87% of the patients. The highest serum phosphate was 9.6 mg/dL. Urea and creatinine remained within normal limits. Post-treatment OSP se-

rum phosphate concentration correlated inversely with glomerular filtration rate ( $P < 0.007$ ,  $R^2 = 0.0755$ ), total body water ( $P < 0.001$ ,  $R^2 = 0.156$ ) and weight ( $P < 0.013$ ,  $R^2 = 0.0635$ ).

**CONCLUSION:** In low-risk, well-hydrated patients, the standard dose of OSP-laxative-induced hyperphosphatemia is related to body weight.

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**Key words:** Bowel preparation; Colonic cleansing; Colonoscopy; Hyperphosphatemia; Laxatives; Sodium phosphate; Preoperative evaluation; Dehydration

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### INTRODUCTION

The widespread use of colonoscopy for early detection of colorectal pathology has increased the use of osmotic laxatives for colonic cleansing. Among these, oral sodium phosphate (OSP) is the preparation of choice because is the best tolerated given the small volume in which it is administered, and it results in better colonic cleansing<sup>[1]</sup>. Under normal conditions, phosphate is absorbed in the small intestine and eliminated by the kidney as calcium phosphate<sup>[2]</sup>. Several complications associated with the use of OSP have been reported in recent years, especially hyperphosphatemia and acute and chronic renal failure. There is evidence linking these complications to conditions that increase the absorption of phosphate or render its renal elimination difficult. Although many of these complications are facilitated by dehydration and inadequate selection of patients when indicating the laxative<sup>[3-5]</sup>, some patients without such conditions also have been reported<sup>[6]</sup>. Most of the information comes from retrospective studies or case reports.

In this prospective clinical trial, we investigated the

frequency of hyperphosphatemia in low-risk ASA I - II patients<sup>[7]</sup>, who were chosen to avoid high-risk patients. To avoid dehydration, we administered 4 L of clear liquids. The main aim was to identify the percentage of patients with hyperphosphatemia following the administration of OSP for video colonoscopy, and before anesthesia induction. A secondary objective was to establish the frequency of dehydration and hypocalcemia.

## MATERIALS AND METHODS

### Patients and methods

This study was approved by the Institutional Review Board. From May to December 2007, 100 consecutive patients who underwent elective colonoscopy were enrolled. Inclusion criteria were: 18-75 years of age, ASA I and II physical status, written informed consent, and colon cleansing with OSP.

Individuals with congestive heart failure, chronic kidney disease, diabetes, liver cirrhosis, intestinal obstruction, decreased bowel motility, increased bowel permeability (Crohn's disease, ulcerative or ischemic colitis) and hyperparathyroidism were prevented from entering this trial. These conditions were ruled out in the pre-anesthetic evaluation by medical history and anamnesis. Those patients who refused to participate were also excluded. Patients who had not undergone colonic cleansing were also excluded.

All the participants received full information regarding the study protocol and procedures in the pre-anesthetic interview and signed the informed consent to participate. Vital parameters were measured and laboratory tests, including hematocrit, hemoglobin concentration, and serum osmolality, phosphate,  $\text{Ca}^{2+}$ , electrolytes, creatinine and urea were carried out.

Forty-eight hours before the test, a fiber- and dairy-free diet (without fruit and vegetable products) was prescribed, and from 20 to 26 h before the study, 4 L of clear liquids (tea, coffee, infusions, jelly, broth and drained juices, or isotonic drinks<sup>[8]</sup>) with sugar or honey (on demand) were administered up to 2 h before the test.

The day before colonoscopy, all the participants were given 90 mL (60 g) of OSP (fosfo-dom<sup>®</sup>; Laboratorio Dominguez S.A, Buenos Aires, Argentina) diluted in 400 mL of water in two divided doses, administered 5 h apart (17:00 pm and 22:00 pm) on the day before colonoscopy. Ten micrograms metoclopramide were also administered 1 h before the laxative.

The day after colonic cleansing and immediately before starting anesthesia with propofol and sevoflurane, blood pressure and heart rate were measured and a second venous sample was drawn and sent to the laboratory to assess hematocrit, hemoglobin, and serum osmolality, phosphate,  $\text{Ca}^{2+}$ , electrolytes, creatinine and urea. The results obtained were compared with those obtained at baseline.

The following formulas were used to calculate plasma volume, total body water and glomerular filtration rate: Plasma volume (PV) (Beaumont formula)<sup>[9]</sup> %PV:

Table 1 Demographic data (mean  $\pm$  SD)

Demographic data	
Age (yr)	58.9 $\pm$ 8.4
Height (cm)	163.8 $\pm$ 8.6
Weight (kg)	71 $\pm$ 13
BMI	26 $\pm$ 4
Sex (%)	66% women, 34% men
TBW	36.8 $\pm$ 8.63
GFR	95.25 $\pm$ 21.27

BMI: Body mass index; TBW: Total body water; GFR: Glomerular filtration rate.

$100/(100 - \text{HCT1}) \times 100 (\text{HCT1} - \text{HCT2})/\text{HCT2}$ .  
HCT = hematocrit.

Total body water (TBW; L) (Watson formula)<sup>[10]</sup>:  
Male:  $\text{TBW} - \text{W} = 2.447 - (0.09156 \times \text{age}) + (0.1074 \times \text{height}) + (0.3362 \times \text{weight})$ ; Female:  $\text{TBW} - \text{W} = -2.097 + (0.1069 \times \text{height}) + (0.2466 \times \text{weight})$ .

Glomerular filtration rate (GFR; mL/min) (Cockcroft-Gault equation)<sup>[11]</sup>:  $(140 - \text{age}) \times \text{weight kg} (\times 0.85 \text{ if female})/\text{creatinine} \times 72$ .

### Statistical analysis

All data are expressed as mean  $\pm$  SD. The Student *t* test was used to analyze normally distributed variables. A univariate linear correlation model that considered post-treatment OSP serum phosphate as a dependent variable was also performed. STATA<sup>®</sup> version 8.0 (StataCorp LP, <http://www.stata.com>) statistical software was used to carry out the statistical analysis.  $P < 0.05$  was considered as statistically significant.

## RESULTS

The main demographic data (mean  $\pm$  SD) were: age, 58.9  $\pm$  8.4 years; height, 163.8  $\pm$  8.6 cm; weight, 71  $\pm$  13 kg; body mass index (BMI), 26  $\pm$  4; women, 66% (Table 1). The main laboratory data (mean  $\pm$  SD) are shown in Table 2. Serum phosphate (mg/dL; mean  $\pm$  SD) increased from a basal value of 3.74  $\pm$  0.56 to 5.58  $\pm$  1.1 after OSP ( $P = 0.001$ ). Hyperphosphatemia appeared in 87% of the patients. The highest serum phosphate was 9.6 mg/dL. Post-OSP serum phosphate had a significant inverse correlation with GFR ( $P < 0.007$ ,  $R^2 = 0.0755$ , Figure 1A), TBW ( $P < 0.001$ ,  $R^2 = 0.156$ , Figure 1B), and weight ( $P < 0.013$ ,  $R^2 = 0.0635$ , Figure 1C). No correlation was observed between post-OSP serum phosphate and creatinine, height or BMI. The prevalence of hyperphosphatemia increased in parallel and steadily with stage of chronic renal disease according to the National Kidney Foundation classification<sup>[12]</sup>, which approached 80% for stage 1, 88% for stage 2, and 100% for stage 3.

After OSP,  $\text{Ca}^{2+}$  decreased significantly ( $P = 0.001$ ), although the difference was not clinically relevant. Pre- and post-OSP urea and creatinine levels remained within normal limits.

Plasma volume decreased by 3.65% after OSP. This represents a dehydration of  $< 1.46\%$ , which was not



**Table 2** Laboratory data, creatinine values and arterial pressure

		Mean	SD	Min	Max	P
Na <sup>+</sup> (mmol/L)	Pre	139.26	2.05	135.00	146.00	NS
	Post	139.72	2.95	133.00	146.00	
Cl <sup>-</sup> (mmol/L)	Pre	104.88	2.68	98.00	111.00	NS
	Post	104.46	3.64	95.00	120.00	
K <sup>+</sup> (mmol/L)	Pre	4.46	0.39	3.50	5.80	0.001
	Post	3.62	0.46	2.40	5.10	
PO <sub>4</sub> (mg/dL)	Pre	3.74	0.56	2.60	5.70	0.001
	Post	5.58	1.10	2.50	9.60	
Ca <sup>2+</sup> (mmol/L)	Pre	1.14	0.10	0.76	1.37	0.001
	Post	1.04	0.12	0.50	1.28	
Hto (%)	Pre	40.28	3.13	31.70	48.30	0.070
	Post	41.18	4.00	27.10	50.90	
Urea (mg/dL)	Pre	32.57	9.93	15.00	68.00	0.001
	Post	21.36	7.53	6.00	43.00	
Osm (mosm/kg)	Pre	291.03	5.35	277.00	304.00	0.002
	Post	288.56	6.01	274.00	307.00	
Creatinine (mg/dL)	Pre	0.87	0.193	0.40	1.50	NS
	Post	0.87	0.190	0.50	1.40	
AP <sub>S/D</sub> (mmHg)	Pre	125/78	14/10	90/60	170/100	NS
	Post	128/74	29/12	80/40	185/100	

AP<sub>S/D</sub>: Arterial pressure systolic/diastolic; NS: Not significant.

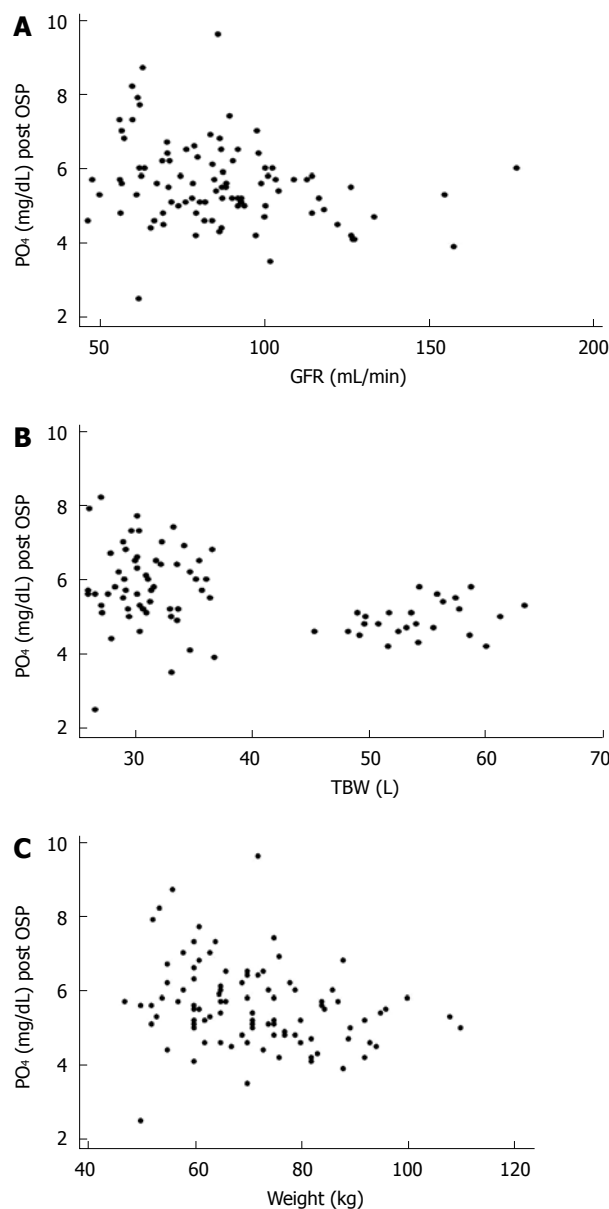
significant<sup>[13,14]</sup>. There was a decrease in serum osmolality (Tables 1 and 2). There was a low incidence (4%) of hypotension (arterial pressure reduction  $\geq 30\%$ ) after colonic cleansing (Table 2).

## DISCUSSION

The osmotic effect of OSP causes dehydration<sup>[15]</sup>; an average loss of 3-4 L of fluids is estimated during colonic cleansing with 60 g OSP<sup>[16,17]</sup>. In support of these data, increases in the concentration of hemoglobin<sup>[18]</sup>, hematocrit and serum osmolality<sup>[15]</sup> have been reported. Several authors have stated that maintaining appropriate hydration is possible to dilute the urine, and reduce its calcium and phosphate concentration<sup>[19,20]</sup>. In consequence, the risk of calcium phosphate crystalluria and precipitation in the renal tubules is diminished<sup>[16,21]</sup>. Sanders *et al*<sup>[18]</sup> have corroborated the efficiency of intravenous hydration (average 2 L) during colonic cleansing for surgery, but this requires a hospital stay and makes ambulatory procedures difficult. Markowitz *et al*<sup>[21]</sup> has suggested that patients must be encouraged to drink eight cups of fluids (1920 mL) and Rex *et al*<sup>[19]</sup> have promoted taking 3.6 L of clear fluids.

Following the 1999 American Society of Anesthesiologists recommendations for all interventions that require general anesthesia or sedation, oral fluid intake is allowed up to 2 h before colonoscopic evaluation<sup>[22]</sup>. The rationale for the preoperative fasting is to reduce the content and acidity of the stomach, thus avoiding the risk of aspiration pneumonia at induction of anesthesia<sup>[23,24]</sup>.

Since the seminal studies of Beaumont in 1833<sup>[25]</sup>, it is widely known that emptying of clear liquids is passive, without the need for gastric motility, and is completed in  $< 60$  min<sup>[26]</sup>. Clear fluids have a washing and dragging effect that allows the gastric content to move easily



**Figure 1** Correlation among phosphorus post-oral sodium phosphate (OSP) with glomerular filtration rate (GFR), total body water (TBW), and weight. A: Between phosphorus post-OSP and GFR; B: Between phosphorus post-OSP and GFR and TBW; C: Between phosphorus post-OSP and weight.

to the duodenum<sup>[27]</sup>. Patients with 2 h fasting with clear liquids (i.e. no liquid intake for 2 h before colonoscopy) had less volume and gastric acidity than those with complete 8 h fasting<sup>[28-32]</sup>. These results also have been reported in children<sup>[33-36]</sup>.

The absence of fluid intake before surgery favors the development of hypotensive reactions during anesthesia induction, as well as dehydration, hypoglycemia and a strong sensation of thirst and hunger that leads to irritability, especially in older patients and infants<sup>[8,37]</sup>. Clear liquid intake not only diminishes the risk of aspiration pneumonia and notably improves patient wellbeing, but it also facilitates adequate hydration.

To evaluate the changes produced by the administration of OSP, it was vital to avoid dehydration. We encouraged patients to freely take 4 L of clear liquids during colonic preparation, up to 2 h before the test. This did not

lead to a significant incidence of dehydration and hypotension, which was reinforced by no significant modifications in haemoglobin and hematocrit. The average reduction in PV was 3.65%, which represented dehydration of < 1.46%, which was not significant<sup>[13,14]</sup>. Besides, in contrast to Gutierrez Santiago's study<sup>[15]</sup>, we observed a decrease in the average osmolality. Only 4% of the patients developed hypotension, a degree of blood pressure reduction of 20%-30%. These results support the efficiency of this oral hydration regime for avoiding dehydration.

At the onset of our study, the suggested interval between doses was 5-10 h, and we used a 5-h interval. As 28% of the phosphate taken is retained by the body for up to 18 h<sup>[16,38]</sup>, recent studies have recommended longer intervals between doses<sup>[4]</sup>.

The maximum safe dose of sodium phosphate is 90 mL<sup>[39]</sup>. Several studies on the adverse effects of high doses of OSP have suggested that these should be avoided<sup>[2,3]</sup>, as is the case with their association with phosphate enemas<sup>[40-42]</sup>. If the recommended dose of 60 g (90 mL) is surpassed, or if the interval between doses is < 5 h, severe hyperphosphatemia could develop<sup>[2,19,39,43-46]</sup>.

Many authors make reference to the fact that administering laxatives and sodium phosphate enemas<sup>[40,41]</sup> leads to a slight though statistically significant increase in phosphorus and a decrease in calcium concentration<sup>[2,19,21,47-49]</sup>, due to intestinal absorption<sup>[2]</sup>. However, they have also suggested that well-hydrated adults who have normal renal function tolerate the amount of phosphate loading without showing significant adverse effects<sup>[6,4,49-52]</sup>. This trend was confirmed in our study, with a maximum registered plasma phosphate level of 9.6 mmol/L and a minimum calcium level of 0.5 mmol/L.

The serious electrolyte disturbances reported have appeared in patients in whom sodium phosphate was contraindicated: inflammatory colonic diseases (Crohn's disease, ulcerative colitis)<sup>[53,54]</sup>, delayed intestinal transit (megacolon, obstruction), and in conditions with intestinal vascular alteration (congestive heart failure, ischemic colitis)<sup>[49]</sup>. It also has been reported in patients with impaired renal function<sup>[55-57]</sup>, or who receive drugs that affect kidney perfusion (diuretics, angiotensin-converting enzyme inhibitors and angiotensin receptor blockers). To avoid the administration of OSP in the sub-clinical kidney disease, measurement of urea and creatinine is recommended<sup>[43]</sup>. Hyperphosphatemia has been observed in patients with dehydration, ascites<sup>[4]</sup> or vomiting<sup>[45]</sup>.

Fine *et al.*<sup>[57]</sup> have found that the mortality rate was 33%, and that the risk of death was high if serum phosphate increased beyond 32.69 mg/dL (10.56 mmol/L). Most of the deaths reported in the literature have been caused by arrhythmia or heart attack associated with electrolyte changes and dehydration<sup>[4]</sup>. Fatal cases have been observed among patients with a history of renal damage<sup>[41,43,50]</sup>, ischemic colitis<sup>[50]</sup>, cirrhosis<sup>[45]</sup>, and in elderly patients with normal renal function<sup>[42,58]</sup>. Azzam *et al.*<sup>[43]</sup> and Wexner *et al.*<sup>[5]</sup> have described high levels of phosphate and kidney damage in patients without previous kidney pathology.

Gutierrez-Santiago *et al.*<sup>[15]</sup> have found an increase in

phosphatemia in 57% of patients, while Lieberman *et al.*<sup>[51]</sup> have found it in 25%. Both studies were retrospective and they did not specify the patient's clinical condition. In our study in low-risk patients, we found an increase in phosphate in a significant percentage (87%). The average increase of serum phosphate was 1.84 mg/dL, which was less than that reported by Tan *et al.*<sup>[59]</sup> (3.09-3.18 mg/dL). The maximum plasma phosphate value registered was 9.6 mg/dL (3.1 mmol/L), which was twice the normal concentration. This result shows that OSP used as laxative is not free of complications, even in low-risk patients. These values do catch our attention because the careful selection of patients anticipated a much lower incidence. It is possible to assume that the wide hydration plan and careful selection of participants avoided reaching the values described by Fine *et al.*<sup>[57]</sup>.

All of the patients had normal urea and creatinine values before and after colonic cleansing. We linked the phosphate values with the TBW and GFR, and both showed a negative linear correlation with the increase in phosphate. We observed that the lower the GFR and TBW, the higher the chance of developing hyperphosphatemia. These parameters describe the relationship of weight with a specific function, which shows that the increase in phosphatemia has a negative linear correlation with weight. We avoided dehydration and there was no renal impairment, therefore, these findings contribute towards the concept that hyperphosphatemia is the result of an excessive dose of laxative, as suggested by Rex *et al.*<sup>[4]</sup>.

Tan *et al.*<sup>[2]</sup> have stated that the decrease in plasma calcium associated with OSP-induced hyperphosphatemia is the result of the binding of calcium to the high phosphate level, and thus, the tubular deposition that induces kidney damage. Gutierrez-Santiago *et al.*<sup>[15]</sup> have observed hypocalcemia in 36% of patients. In our study, the decrease in calcium concentration developed in 29% of the patients, but none had symptoms related to hypocalcemia.

The reported OPS-induced hypernatremia is the result of intestinal sodium absorption and can worsen due to dehydration<sup>[2,15]</sup>. We did not observe an increase of plasma sodium in our patients, which suggests that the hydration level achieved with this diet was appropriate.

The sodium and potassium exchange across the colonic epithelium can generate hypokalemia, which is accentuated by renal potassium loss induced as a consequence of the volume contraction-associated secondary aldosteronism<sup>[2,15]</sup>. The decrease in potassium in our sample coincided with that observed by Rex *et al.*<sup>[4]</sup>. It appeared in 4% of the patients and reached 2.4 mmol/L in one case.

Unlike previous studies by other investigators, we did not observe changes in plasma chloride values in our patients<sup>[4]</sup>.

The results in this study show that, in low-risk, well-hydrated patients, hyperphosphatemia following standard OSP doses is related to weight. This is the reason why we believe that, in low-weight patients, lower doses of the laxative should be administered. We consider that further studies are necessary to establish the adequate dose according to weight.

Oral hydration with 4 L of clear liquids during colonic preparation has proven its efficacy in avoiding dehydration.

The possibility of achieving high phosphate levels in low-risk, well-hydrated patients is certainly alarming, especially given the fact that few medical professionals currently take this possibility into account. These discoveries emphasize the need to carry out an adequate hydration and selection of patients to avoid administration of OSP to those individuals at risk of developing hyperphosphatemia or renal failure.

## COMMENTS

### Background

Colon cleansing is used widely for colonoscopic exploration and colonic and gynecological surgery. Oral sodium phosphate (OSP) solution is the osmotic laxative most commonly used for this purpose. It is known that OSP can induce severe hyperphosphatemia and hypocalcemia due to excessive absorption of phosphates, and there have been reports of deaths and irreversible dialysis-requiring renal insufficiency.

### Research frontiers

Hyperphosphatemia after OSP develops in patients with conditions that increase its intestinal absorption (ulcerative colitis, Crohn's disease, ischemic colitis), in conditions in which its elimination is difficult (kidney disease, dehydration, aging), or after OSP overdose (> 60 g). These findings have come from case reports and some rare retrospective studies. No prospective studies have investigated the prevalence of hyperphosphatemia in low-risk patients.

### Innovations and breakthroughs

This was a prospective study that was carried out in low-risk patients. Even though, the authors avoided the conditions that are known to facilitate hyperphosphatemia such as dehydration (inducing oral intake of 4 L of clear liquids) and the diseases described above, 87% of the patients had high serum phosphate levels. None of them developed symptoms of hypocalcemia, and there was no evidence of renal impairment. Hyperphosphatemia was related inversely to body weight. These results highlight the importance of being cautious with the administration of OSP in patients with contraindications and promoting aggressive oral hydration.

### Applications

Taking into account the results of this study, the authors recommend: performing preoperative evaluation aimed at avoiding administration of OSP laxatives to patients at risk; reducing the dose of OSP in patients with low weight; and avoiding dehydration with an adequate oral intake of clear liquids. Additional studies are necessary to establish the appropriate dose adjusted to body weight.

### Terminology

Hyperphosphatemia: serum phosphate levels above normal (2.5-4.5 mg/dL). Hypocalcemia: ionized calcium levels below normal values (1.0-1.35 mmol/L).

### Peer review

This paper presented provides reliable information on the side effects of OSP in low-risk patients. The conclusions addressed are useful for managing patients' prescribed OSP for colon cleansing.

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BRIEF ARTICLE

## Carcinoma of the middle bile duct: Is bile duct segmental resection appropriate?

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selected patients with no adjacent organ invasion and resection margin is negative.

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**Key words:** Bile duct cancer; Segmental resection; Pancreaticoduodenectomy

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### Abstract

**AIM:** To compare survival between bile duct segmental resection (BDSR) and pancreaticoduodenectomy (PD) for treating distal bile duct cancers.

**METHODS:** Retrospective analysis was conducted for 45 patients in a BDSR group and for 149 patients in a PD group.

**RESULTS:** The T-stage ( $P < 0.001$ ), lymph node invasion ( $P = 0.010$ ) and tumor differentiation ( $P = 0.005$ ) were significant prognostic factors in the BDSR group. The 3- and 5-year overall survival rates for the BDSR group and PD group were 51.7% and 36.6%, respectively and 46.0% and 38.1%, respectively ( $P = 0.099$ ). The BDSR group and PD group did not show any significant difference in survival when this was adjusted for the TNM stage. The 3- and 5-year survival rates were: stage I a [BDSR (100.0% and 100.0%) vs PD (76.9% and 68.4%)] ( $P = 0.226$ ); stage I b [BDSR (55.8% and 32.6%) vs PD (59.3% and 59.3%)] ( $P = 0.942$ ); stage II b [BDSR (19.2% and 19.2%) vs PD (31.9% and 14.2%)] ( $P = 0.669$ ).

**CONCLUSION:** BDSR can be justified as an alternative radical operation for patients with middle bile duct in

### INTRODUCTION

Extrahepatic bile duct cancer can be classified into hilar, proximal, middle and distal bile duct cancer (DBD) according to the portion of the involved bile duct. Hilar and proximal bile duct cancers (PBD-1) are classified into 5 types as described by Bismuth and Corlette<sup>[1]</sup>. This classification of extrahepatic bile duct cancers is due to the differences in the operative methods that are employed for cancers involving different portions of the bile duct. According to numerous reports, most surgeons consider bile duct resection with liver parenchyma resection to be the standard operation for hilar cholangiocarcinoma<sup>[2-4]</sup>. Pancreaticoduodenectomy (PD) is performed for DBD. Bile duct cancers confined to the middle bile duct (MBD) are rare because bile duct cancers have a tendency to spread along the bile duct wall. This is the reason why PD is usually undertaken for treating most MBD cancers. Yet there is still debate as to the appropriate operative procedure for the bile duct cancers that do not extend above the confluence or below the upper border of the pancreas. PD or bile duct segmental resection (BDSR) is performed at the surgeon's discretion. Clinicopathological studies on hilar and distal cholangiocarcinomas have been done by many authors<sup>[2-11]</sup>, and the results of BDSR for MBD have

been reported, but most of these studies involved a very limited number of cases, and the number of cases is not enough to justify BDSR as a standard treatment.

In the current study, a clinical review of patients who received BDSR (negative resection margins) with lymph node (LN) dissection for the Bismuth type I PBD-1 and also the patients with MBD was performed. We compared survival between BDSR group and PD for treating DBD.

## MATERIALS AND METHODS

Between November 1995 and May 2007, 379 patients underwent surgical procedures that were performed for treating extrahepatic cholangiocarcinomas at Samsung Medical Center. One hundred nine patients who underwent concomitant liver resection and 76 patients who underwent palliative procedures were excluded, and only the cases with bile duct adenocarcinomas that had been confirmed by pathologic assessment were included. There were 45 patients who underwent BDSR for PBD-1 or MBD (BDSR group), and 149 patients who underwent PD for DBD (PD group). A retrospective analysis was performed *via* a review of the medical records and by conducting telephone interviews. The clinicopathologic factors of the BDSR group that we analyzed were age, gender, the preoperative carbohydrate antigen 19-9 (CA19-9) level, serum bilirubin levels both at the time of admission and prior to surgery, preoperative biliary drainage, transfusion, the site of cancer, the extent of LN dissection [D1 (LN #12) or D2 (LN #12, #8, #13)], tumor size, histologic differentiation, TNM stage, LN metastasis and perineural invasion. To find the exact location of the tumor, ERCP or PTC and recently MRCP was done before surgery. Before surgery, biliary and GB CT scans were performed to see if there was invasion to adjacent tissues or organs. Angiography was used to detect any evidence of vascular invasion, but after 1999, we used the CT scan to rule out any vascular invasion. In the BDSR group, all patients underwent frozen section for both bile duct resection margins and to be confirmed as free from carcinoma intraoperatively and on permanent pathology. The tumor stage was based on the 6th edition of the American Joint Committee on Cancer cancer staging<sup>[12]</sup>. All patients were monitored at 6 mo intervals by measuring CA19-9 levels and performing a CT scan for 3 years and were checked annually at 6 mo intervals. Four patients (8.8%) in the BDSR group were lost to follow up.

### Statistical analysis

Survival of the BDSR and the PD groups was calculated by the Kaplan-Meier method, and the log-rank test was used to analyze differences. Only variables that were statistically significant on univariate analysis were included in the multivariate analysis, which was performed using a Cox proportional hazard regression model. Survival analysis of the PD group was done by the Kaplan-Meier method and comparison of survival between the BDSR group and the PD group was done by log-rank tests.  $\chi^2$  tests and a Mann-Whitney *U*-test were used for comparing

the postoperative complications and the length of the hospital stay between the 2 groups. *P* values less than 0.05 were considered statistically significant.

## RESULTS

### *Clinical characteristics of the groups of patients who underwent BDSR for PBD-1 and MBD*

Clinical characteristics of the groups of patients who underwent BDSR for PBD-1 and MBD are shown in Table 1. There were 45 patients who underwent BDSR for PBD-1 or MBD. There were 30 (66.7%) males and 15 (33.3%) females. The median age of the patients was 65 years (range: 37-76 years). BDSR with LN dissection and hepaticojejunostomy was done for all these patients, with negative proximal and distal bile duct resection margins being achieved in all 45 patients. The complications arising during the immediate postoperative period (within 1 mo) were analyzed. There were 9 patients with wound infections, pancreatitis, bile leakage and delayed gastric emptying. Dissection of the superior border of the pancreas was done due to the close distal resection margin in all 3 patients who showed postoperative pancreatitis. Of 45 patients, D2 LN dissection (LN #12, #8, #13) was done in 37 (82.2%) patients and D1 dissection (LN #12) was done in 8 (17.8%) patients. LN metastasis was present in 13 (28.8%) patients and perineural invasion was present in 32 (71.1%) patients. Nine (20%) patients had T1 lesions, 33 (73.3%) patients had T2 lesions and 3 (6.7%) patients had T3 lesions with invasion into the gallbladder without liver or pancreas invasion. Nine (20%) patients were stage I a (T1N0M0), 23 (51.1%) patients were stage I b (T2N0M0), and 13 (28.9%) patients were stage II b (T1-3N1M0). There were no stage II a (T3N0M0) patients. The median follow-up period was 25 mo (range: 4-104 mo) (Table 1).

Among the 45 patients, 3 patients (6.6%) underwent additional adjuvant treatment. Two patients with stage I b each had chemoradiation and radiation. One patient with stage II b had chemoradiation. There was no evidence of recurrence in all three patients. The recurrence rate was 44.4% (*n* = 20). The stage specific recurrence rate was as follows: 24.4% (*n* = 11) for stage I b and 20.0% (*n* = 9) for stage II b. There were 12 locoregional recurrences, 5 liver metastases and 3 peritoneal carcinomatoses. Eleven patients underwent palliative treatment and 9 patients refused to go under extra treatment. The 3- and 5-year survival rates of the BDSR group were 51.7% and 36.6%, respectively. The median survival was 25 mo (mean: 31.27 mo). Univariate analysis showed cellular differentiation (*P* = 0.005), the T stage (*P* < 0.001), the LN status (*P* = 0.010) and the TNM stage (*P* = 0.012) to be significant factors that influenced patient survival (Table 1, Figure 1). However, there were no statistically significant independent risk factors that influenced patient survival on multivariate analysis.

### *Comparison of survival between the BDSR group and the PD group*

There were 149 patients in the PD group. There were

**Table 1** Univariate analysis of the predictors for survival of the 45 patients who underwent radical BDSR for PBD-1 or MBD disease

Characteristic	Number of patients	Median survival time (mo)	Survival rate (%)		P-value
			3-yr	5-yr	
Overall	45	25	51.7	36.6	
Age (yr)					0.466
≤ 65	25	42	56.4	38.7	
> 65	20	33	45.5	34.1	
Gender					0.314
Male	30	35	47	28.2	
Female	15	42	58.2	48.5	
CA19-9 (U/mL)					0.519
≤ 35	19	42	51.6	25.8	
> 35	19	55	61.5	49.2	
No data	7				
Bilirubin at admission (mg/dL)					0.368
≤ 1.6	11	NR	53.3	53.3	
> 1.6	34	42	50.5	34.1	
Bilirubin at operation (mg/dL)					0.149
≤ 1.6	17	55	65.5	43.6	
> 1.6	28	29	43.7	31.9	
Preoperative biliary drainage					0.632
No	10	35	40	0.0	
Yes	35	42	53.8	42.3	
Location					0.547
MBD	34	42	51.4	44.1	
PBD-1	11	52	51.9	26	
LN dissection					0.997
D1	8	42	58.3	29.2	
D2	37	52	51.2	42.6	
Transfusion					0.832
No	36	42	51.2	39.4	
Yes	9	33	47.6	23.8	
Size (cm)					0.892
≤ 2.0	26	35	47	39.2	
> 2.0	19	52	56.6	33.9	
T stage					< 0.001
T1	9	NR	100	100	
T2	33	35	49.6	31.5	
T3	3	16	0.0	0.0	
LN stage					0.010
N0	32	52	63.1	42.6	
N1	13	25	19.2	19.2	
TNM stage					0.012
I a	9	NR	100	100	
I b	23	42	55.8	32.6	
II b	13	25	19.2	19.2	
Cell differentiation					0.005
Well	15	NR	87.5	57.5	
Moderate	26	33	41.6	33.3	
Poor	4	22	0.0	0.0	
Perineural invasion					0.180
No	13	NR	71.1	71.1	
Yes	32	35	47.7	29.8	

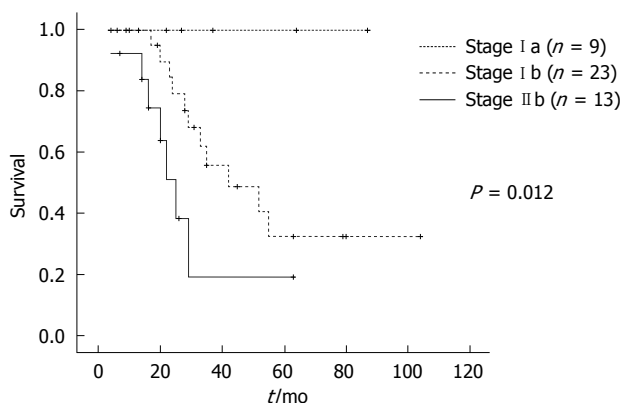
BDSR: Bile duct segmental resection; CA19-9: Carbohydrate antigen 19-9; MBD: Middle bile duct cancer; PBD-1: Proximal bile duct cancers; LN: Lymph node; NR: Not reached.

102 (68.5%) males and 47 (31.5%) females. The median age of the patients was 60 years (range: 31-78 years). Whipple's procedure was done in 97 (65.1%) patients, pylorus preserving PD was done in 45 (30.2%) patients and total pancreatectomy was done in 7 (4.7%) patients. The median follow-up was 21.9 mo (range: 0.4-108.5 mo). During the follow-up period, 88 (59.1%) patients had

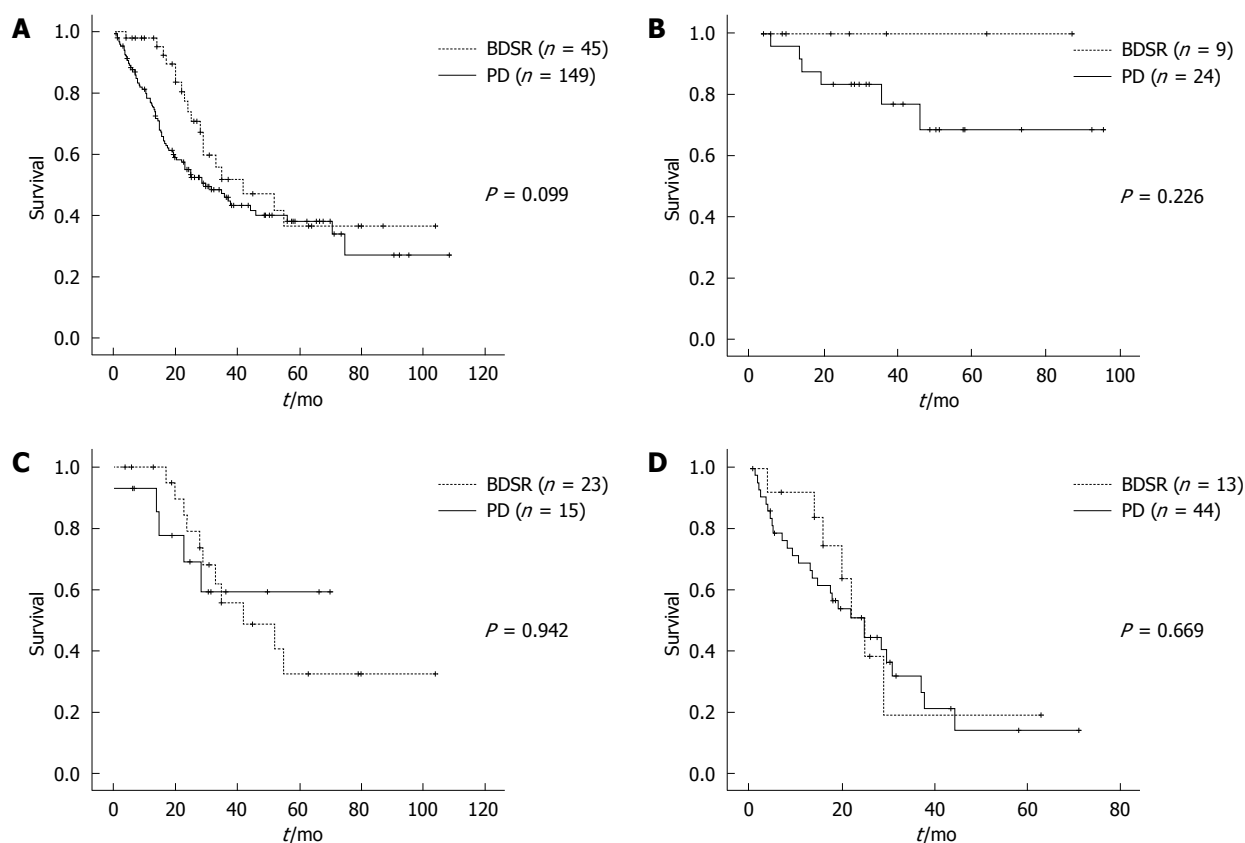
**Table 2** The clinical characteristics of the BDSR group were compared with the PD group *n* (%)

Characteristics	BDSR group	PD group
Total patients	45	149
Age (median, yr)	65 (37-76)	60 (31-78)
Gender		
Male	30 (66.7)	102 (68.5)
Female	15 (33.3)	47 (31.5)
Median follow-up (mo)	25.0 (4.0-104.0)	21.9 (0.4-108.5)
T stage		
T1	9 (20.0)	32 (21.4)
T2	33 (73.3)	20 (13.4)
T3	3 (6.7)	90 (60.5)
T4	0 (0.0)	7 (4.7)
N status		
N0	32 (71.2)	101 (67.8)
N1	13 (28.8)	48 (32.2)
TNM stage		
I a	9 (20.0)	24 (16.1)
I b	23 (51.1)	15 (10.1)
II a	0 (0.0)	58 (38.9)
II b	13 (28.9)	44 (29.5)
III	0 (0.0)	8 (5.4)

PD: Pancreaticoduodenectomy.

**Figure 1** Survival according to the TNM stage of 45 patients who underwent bile duct segmental resection (BDSR) for proximal bile duct cancers (PBD-1) or middle bile duct cancer (MBD) disease.

recurrences and 78 (52.3%) patients died of cancer-related causes. The survival of the BDSR group was compared with the group of patients who underwent PD for their DBD; these patients are known to have similar clinical characteristics as patients with PBD-1 or MBD (Table 2). The survival rate, postoperative complications and length of the postoperative hospital stay were the factors analyzed. The 3- and 5-year overall survival rates were 51.7% and 36.6% in the BDSR group, and 46.0% and 38.1% in the PD group ( $P = 0.099$ ) (Figure 2A). The stage specific survival rates were compared between the BDSR group and the PD group. The 3-year and 5-year survival rates of the patients with stage I a disease were 100.0% and 100.0%, respectively, in the BDSR group, and 76.9% and 68.4%, respectively, in the PD group ( $P = 0.226$ ) (Figure 2B). The 3-year and 5-year survival rates of the patients with stage I b disease were 55.8% and 32.6%, respectively, in the BDSR group, and 59.3% and 59.3%,



**Figure 2** Comparison of survival between the BDSR group and the pancreaticoduodenectomy (PD) group. A: The 3-year and 5-year overall survival rates were 51.7% and 36.6%, respectively, for the BDSR group and 46.0% and 38.1%, respectively, for the PD group; B: The 3-year and 5-year survival rates of the patients with stage I a disease were 100.0% and 100.0%, respectively, for the BDSR group and 76.9% and 68.4%, respectively, for the PD group; C: The 3-year and 5-year survival rates of the patients with stage I b disease were 55.8% and 32.6%, respectively, for the BDSR group and 59.3% and 59.3%, respectively, for the PD group; D: The 3-year and 5-year survival of patients with stage II b disease were 19.2% and 19.2%, respectively, for the BDSR group and 31.9% and 14.2%, respectively, for the PD group.

respectively, in the PD group ( $P = 0.942$ ) (Figure 2C). The 3-year and 5-year survival rates of patients with stage II b disease were 19.2% and 19.2%, respectively, in the BDSR group, and 31.9% and 14.2%, respectively, in the PD group ( $P = 0.669$ ) (Figure 2D). Therefore, the BDSR group and the PD group did not show any significant difference in survival when this was adjusted for the TNM stage. Sixty one (40.9%) patients in the PD group experienced postoperative complications (pancreatic fistula, bleeding, delayed gastric emptying, wound infection, intraabdominal abscess, *etc.*). There were significantly less postoperative complications in the BDSR group (20.0% *vs* 40.9%, respectively,  $P = 0.01$ ). The length of the postoperative hospital stay was significantly shorter in the BDSR group (mean: 16.62 d *vs* 28.35 d, respectively,  $P = 0.035$ ).

## DISCUSSION

Classification of extrahepatic cholangiocarcinoma according to its anatomic location (proximal, middle, distal) was first proposed by Longmire<sup>[13]</sup> in 1976. There still exists some debate about the classification of MBD carcinoma since cholangiocarcinomas are very rarely located only in this section of the bile duct without infiltration into the proximal or distal bile duct. So there have been other classifications of the cholangiocarcinomas such as

that proposed by Nakeeb *et al.*<sup>[14]</sup> which results in a simpler classification of intrahepatic, hilar and distal cholangiocarcinoma. Jarnagin *et al.*<sup>[15]</sup> also proposed a 2-category system of proximal and distal bile duct cancer, with MBD cancer being in a separate category. Such interest in the classification of cholangiocarcinomas is due to the difference in surgical treatment according the different anatomic locations. Intrahepatic and perihilar cholangiocarcinomas are usually treated by liver resection<sup>[16-18]</sup>. In contrast, distal cholangiocarcinoma requires PD for complete tumor resection. Then what about tumors confined to the MBD? Different surgical treatments are possible for MBD cancers according to the distance between the hilum or the upper border of the pancreas, and also according to the experience and preference of the surgeon. For tumors with negative bile duct margins and no infiltration into vascular structures, BDSR is sometimes undertaken, albeit for only a small proportion of these tumors.

The most important issues for BDSR for PBD-1 or MBD is the radial margin, involvement of the portal vein or hepatic artery and LN dissection<sup>[10]</sup>. T1 and T2 tumors, and T3 tumors with infiltration only into the gallbladder and without vascular invasion, were included in this study. T3 or T4 tumors with vascular, liver or pancreas invasion require extended surgery due to the fact that R0 resection is impossible with BDSR in these



cases. Sufficient radical dissection of the LNs, and especially the LNs around the superior mesenteric artery (SMA), may be another issue. According to the general rules for surgical and pathological studies on cancer of the biliary tract by the Japanese Society of Biliary Surgery, in the case of PBD-1 LNs #12 (a1, a2, b1, b2, c, p1, p2, h) are classified as group 1 and LNs #8 (a, p) and #13 are classified as group 2. For MBD, LNs #12 (b1, b2, c) are classified as group 1 and LNs #12 (a1, a2, c, p1, p2, h), #8 (a, p) and #13 (1) are classified as group 2. Since the LNs around the SMA and #17 are classified as group 3, radical resection LN dissection (D2) is possible with BDSR.

There have been occasional reports of BDSR for treating extrahepatic cholangiocarcinoma in selected patients<sup>[7,8,19-22]</sup>. In the series from the Memorial Sloan Kettering Cancer Center<sup>[7]</sup>, only 13% of patients (6 of 45) were amenable to bile duct excision alone, while this figure was 8% (3 of 34) in the Veterans Hospital study<sup>[22]</sup>. But in most of these reports, risk factor evaluation was not possible due to the limited number of BDSR cases. In this study, 28.8% of the cases had LN metastases, and the LN status was a significant risk factor that affected survival. Furthermore, the cell differentiation, the T stage and the TNM stage were significant factors that affected the outcomes. It has been reported that the frequency of LN metastasis in DBD ranged from 30% to 68%<sup>[7,8,11,14,20,23]</sup>, and negative LN metastasis was a useful predictor of a favorable outcome for patients with DBD<sup>[7,8,14,23]</sup>. However, perineural invasion did not prove to be statistically significant, unlike the other studies that reported the risk factors for survival from cholangiocarcinoma in other parts of the biliary tree<sup>[24,25]</sup>.

In the present study, 3 patients underwent BDSR due to the lesion being close to the upper border of pancreas. All 3 patients developed postoperative pancreatitis. Two of these patients developed local recurrence in the remnant distal bile duct at 9 mo and 31 mo, respectively. Although the 2 cases mentioned were categorized as recurrences, they could also have been considered remnant bile duct cancer when the slow-growth of cholangiocarcinoma was taken into account. For a tumor that is close to the upper border of pancreas and resected bile margin reveals to be free of carcinoma, PD should be considered as a treatment option since the pattern of tumor spread along the periductal tissue can be assumed and it should secure sufficient distal bile duct resection margin.

A comparison of survival would be most accurate between 2 groups that received BDSR or PD for carcinoma confined to the proximal or MBD. But this was not feasible due to the small number of patients included in this category. So a different approach was selected in this study, and comparisons were made with the group of patients who received PD for DBD, which is known to have similar clinical characteristics with its proximal counterparts. Although several authors have reported that MBD had a worse prognosis than hilar or distal bile duct cancer<sup>[26-28]</sup>, other authors did not concur<sup>[8,29]</sup>. According to previous reports, the curative resection rates for DBD

have ranged from 56% to 100%<sup>[7,8,14,19-22]</sup>. However, the 5-year survival rate for DBD is not always high, with some reports showing a range of 14%-47%<sup>[7,8,11,14,19-23]</sup>. In the present study, the overall 3-year and 5-year survival rates of the BDSR group were 51.7% and 36.6%, respectively, which is similar to other published reports<sup>[7,8,11,14,19-23]</sup>. The stage specific overall survival between BDSR group and PD group was not statistically significant. The BDSR group had a significantly shorter postoperative length of hospital stay and fewer complications compared to the PD group. This comparison may not be so meaningful when taking into consideration that BDSR is a far less extensive and complicated operative method (fewer anastomoses). Thus, BDSR can be safely applied to patients with bile duct resection margin negative PBD-1 or MBD tumor rather than performing more extensive surgery such as PD.

In conclusion, to achieve a cure, the surgeon must obtain histologically negative margins on both the proximal and distal bile ducts and all tumor-bearing nodal tissue must be removed. BDSR with LN dissection can be an alternative treatment and may be justified in preference to a more radical operation for patients with PBD-1 or MBD when there is no pancreas, liver, vascular invasion and the both bile duct resection margins are negative.

## COMMENTS

### Background

Although radical bile duct segmental resection (BDSR) is performed by many surgeons in selected cases, there are scant clinical studies on the adequacy of this procedure.

### Research frontiers

To validate the adequacy of radical BDSR, the authors compared survival between a radical BDSR group and a pancreaticoduodenectomy group for treating distal bile duct cancers.

### Innovations and breakthroughs

Clinicopathological studies on hilar and distal cholangiocarcinomas have been done by many authors, and the results of BDSR for middle bile duct (MBD) have been reported, but most of these studies involved a very limited number of cases, and the number of cases is not enough to justify BDSR as a standard treatment.

### Applications

The surgeon must obtain histologically negative margins on both the proximal and distal bile ducts and all tumor-bearing nodal tissue must be removed. BDSR with lymph node dissection can be an alternative treatment and may be justified in preference to a more radical operation for patients with MBD.

### Peer review

Even if it is a retrospective study, the data reported are interesting and well documented.

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BRIEF ARTICLE

## Percutaneous transgastric computed tomography-guided biopsy of the pancreas using large needles

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**CONCLUSION:** Pancreatic biopsy can be obtained by a transgastric route using a large needle as an alternative method, without complications of peritonitis or bleeding.

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**Key words:** Biopsy; Computed tomography; Pancreas; Stomach

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### Abstract

**AIM:** To assess the safety, yield and clinical utility of percutaneous transgastric computed tomography (CT)-guided biopsy of pancreatic tumor using large needles, in selected patients.

**METHODS:** We reviewed 34 CT-guided biopsies in patients with pancreas mass, of whom 24 (71%) had a direct path to the mass without passing through a major organ. The needle passed through the liver in one case (3%). Nine passes (26%) were made through the stomach. These nine transgastric biopsies which used a coaxial technique (i.e. a 17-gauge coaxial introducer needle and an 18-gauge biopsy needle) were the basis of this study. Immediate and late follow-up CT images to detect complications were obtained.

**RESULTS:** Tumor tissues were obtained in nine pancreatic biopsies, and histologic specimens for diagnosis were obtained in all cases. One patient, who had a rare sarcomatoid carcinoma, received a second biopsy. One patient had a complication of transient pneumoperitoneum but no subjective complaints. An immediate imaging study and clinical follow-up detected neither hemorrhage nor peritonitis. No delayed procedure-related complication was seen during the survival period of our patients.

### INTRODUCTION

The diagnosis of a pancreatic mass detected by abdominal imaging can be difficult, and therapeutic decisions are based on the ability to diagnose or exclude malignancy<sup>[1]</sup>. Although most neoplasms are ductal adenocarcinomas, imaging modalities can not reliably be used to diagnose other malignant or benign conditions which may have different treatment options and prognoses<sup>[2]</sup>. Therefore, tissue diagnosis is often needed before surgery.

Computed tomography (CT)-guided biopsy for tissue diagnosis is well established<sup>[3-5]</sup>. In a prospective analysis of 125 procedures, CT-directed biopsy for pancreatic lesions had an accuracy of 95.2%<sup>[3]</sup>. An occasional limitation of the axial CT guidance of such interventional procedures is the presence of intervening vital structures which cannot be avoided even by using a gantry tilt technique<sup>[4]</sup>. Brandt *et al*<sup>[5]</sup> stated that there were no complications with fine, 21-gauge needle passage through the gastrointestinal tract. Fine needle biopsy for pancreatic lesions had an accuracy of 85%, whereas a large needle (16-19 gauge) had an accuracy of 92%<sup>[5]</sup>. Therefore, in daily practice, large needle biopsy could reduce the repeat biopsy rate. One experimental study in rabbits reported that the transgastric route with an 18-gauge cutting needle could

be used for pancreas biopsy, without apparent peritonitis or bleeding<sup>[6]</sup>. However, only a few studies have reported on the technique of large needle, transgastric-route biopsy of pancreatic lesions in humans<sup>[7,8]</sup>.

Since the initial description in 1992, tissue diagnosis under endoscopic ultrasonography (EUS) guidance has emerged as an important modality for evaluating patients with pancreatic lesions<sup>[9-11]</sup>. EUS-guided trucut needle biopsy using a 19-gauge needle, performed through the patient's stomach, is safe and accurate<sup>[11]</sup>. This procedure, however, is limited to selected patients who are willing to undergo endoscopy. Therefore, a transgastric approach using a large needle for CT-guided biopsy of the pancreatic lesion can be an alternative method of choice.

The aim of the present study was to assess the safety, yield, and clinical utility of percutaneous transgastric CT-guided biopsy in patients with pancreatic masses.

## MATERIALS AND METHODS

We reviewed the medical records of 34 consecutive CT-guided biopsies of a solid pancreatic mass from one institution (Taipei Veterans General Hospital, Taipei, Taiwan) over a 4-year period. All the patients were unwilling or failed to undergo EUS-guided biopsy. The indication for biopsy of these pancreatic masses was to obtain a diagnosis of malignancy from tissue before the patients received adjuvant chemotherapy or radiotherapy. Those patients with a resectable mass and scheduled for surgery were not included.

We analyzed the records of these biopsy procedures, and 24 had a direct path to the mass which did not pass through a major organ (71%). In one case (3%), the needle passed through the liver. Nine passes (26%) in eight patients were made through the stomach. These nine CT-guided biopsies performed with a transgastric approach were the basis of this study. The patients were one woman and seven men, with an average age of 65 years (range, 35-78 years).

Magnetic resonance imaging (MRI) or CT images before biopsy were reviewed by two experienced radiologists in a joint meeting, and the interpretation reached consensus. The proper pass route and the biopsy site were evaluated (Figure 1A). All patients were hospitalized and fasted overnight before the procedure. Biopsies were monitored with the patient in the supine position and performed on a CT scanner (Siemens, Germany). The reference scan was obtained first, and an opaque marker placed on the patient's abdomen (Figure 1B). The opaque marker consisted of several parallel segments of angiographic catheter. On the reference image, the accessible pass route and the distance were measured. The coaxial technique was applied with a 17-gauge coaxial introducer needle (Allegiance Health Corporation, McGaw Park, IL, USA) and an 18-gauge Temno biopsy needle (Allegiance Health Corporation, McGaw Park, IL, USA). The coaxial introducer needle penetrated the stomach wall as perpendicularly as possible, with its tip stopped on the edge of the target lesion (Figure 1C). Two to four strips of tissue were obtained in each procedure (Figure 1D).

An immediate follow-up CT image was obtained in all patients to detect possible complications. After each procedure, fasting was not necessary if there were no abnormal findings in follow-up CT images or complaints by the patients. All patients were observed in hospital until their condition was stable. Any delayed procedure-related complication was recorded on the patient's chart and by follow-up images.

## RESULTS

Table 1 shows basic data on our patients who underwent CT-guided transgastric biopsy. Nine biopsies of eight pancreas masses were successfully performed. The tumor sizes ranged from 20 to 75 mm (mean 48.5 mm). The tumor locations were the pancreatic body (62%) and pancreatic head (38%). Histologic diagnoses were obtained in all nine biopsies. There were five adenocarcinomas, one squamous cell carcinoma, one poorly differentiated carcinoma, and one sarcomatoid carcinoma. Because sarcomatoid carcinoma in the pancreas is very rare, our initial biopsy specimen was classified as atypical cells. A second biopsy of the same mass was then obtained and the specimen showed similar histologic findings. Our pathologist then revised the report as a sarcomatoid carcinoma.

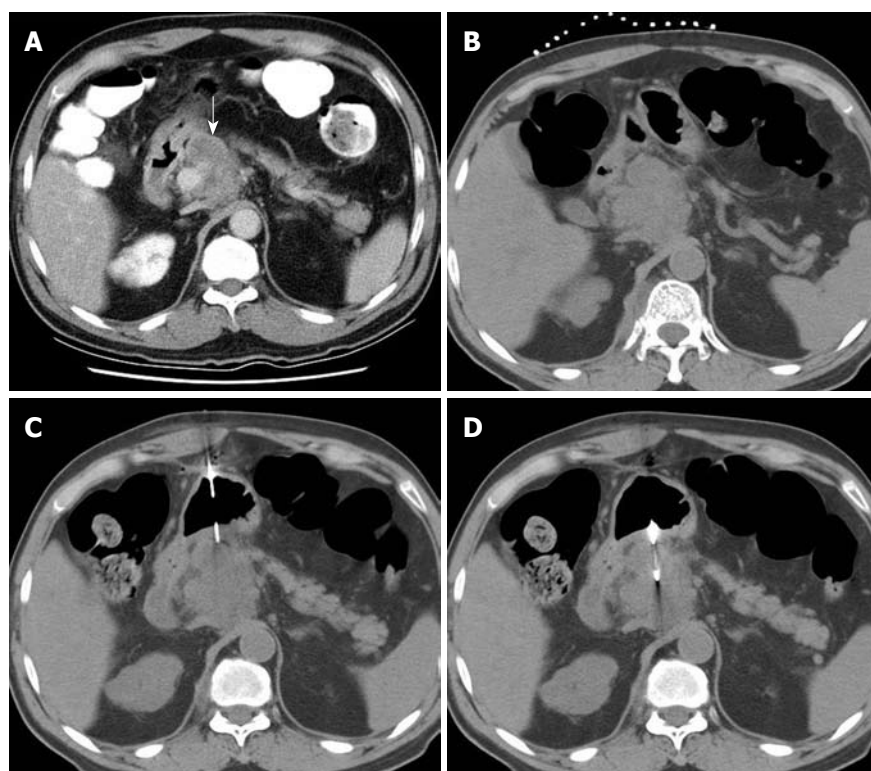
One patient had transient, minimal pneumoperitoneum, but no abdominal pain or peritoneal signs were noted. The condition resolved after the patient fasted for 1 d, and was confirmed by an erect chest film. No patient had internal hemorrhage or peritonitis, confirmed by imaging study or during clinical follow-up. There were no late complications, such as tumor spread, in the follow-up images in our patients who had a survival of 1 to 19 mo.

## DISCUSSION

In this study, percutaneous puncture of a pancreatic mass was restricted to patients having advanced disease and who were not candidates for laparotomy. Histologic diagnosis was required in all patients scheduled for chemotherapy, radiotherapy, or both. Ihse *et al*<sup>[12]</sup> suggested that biopsy is not mandatory if the clinical suspicion of cancer is high and the surgical team has documented low postoperative mortality and morbidity rates.

Although percutaneous fine needle aspiration biopsy is well established in evaluating pancreatic masses<sup>[6]</sup>, this technique requires experienced cytopathologists for tissue diagnosis. The amount of aspirated material is often suboptimal for multiple histopathologic examination or certain analyses required to detect endocrine tumors of the pancreas<sup>[13]</sup>. Recently, Li *et al*<sup>[8]</sup> reported a successful diagnosis in 69 of 80 patients (86%) suspected of having pancreatic lesions using an 18-20-gauge cutting needle automated biopsy gun, with no serious complications. In our study, pancreatic biopsies were performed with an 18-gauge biopsy gun. A final histological diagnosis from pancreatic masses can be obtained with this method. In contrast, a correct diagnosis from percutaneous fine needle aspiration with limited tissue specimen may be difficult.





**Figure 1** A 67-year-old man with a pancreatic mass who had no safe route for approaching the target lesion during biopsy. A: Contrast-enhanced axial computed tomography (CT) scan shows a mass lesion (white arrow) in the pancreatic head; B: The patient was in the supine position, with opaque catheters placed on the abdominal wall as reference lines; C: Noncontrast CT image shows a 17-gauge coaxial introducer needle perpendicularly penetrating the gastric wall and the needle tip positioned at the edge of the target lesion; D: Noncontrast CT image shows an 18-gauge biopsy needle tip in the pancreatic mass.

**Table 1** Basic data of transgastric CT-guided biopsy in eight patients with pancreatic masses

Patient	Age/Gender	Target site (Pancreas)	Size <sup>1</sup> (mm)	Histology	Complication	Follow-up
1	74/M	Body	20	Adenocarcinoma	No	Expired, 8 mo later
2	67/M	Body	25	Adenocarcinoma	No, TP	Expired, 2 mo later
3	78/M	Body	30	Adenocarcinoma	No	Expired, 2 mo later
4	76/M	Head	75	Squamous cell carcinoma	No	Expired, 6 mo later
5	77/M	Head	48	Adenocarcinoma	No	Expired, 1 mo later
6	35/M	Body	50	Poorly differentiated carcinoma	No	Expired, 6 mo later
7	48/F	Body	40	Adenocarcinoma	No	Expired, 19 mo later
8	67/M	Head	20	Sarcomatoid carcinoma <sup>2</sup>	No	Expired, 6 mo later

<sup>1</sup>Greatest diameter; <sup>2</sup>Biopsy was performed twice. TP: Transient pneumoperitoneum; M: Male; F: Female; CT: Computed tomography.

In our study, we found that 29% of consecutive biopsies had no direct route for approaching the pancreatic mass without passing through a major organ. Nine passes were made through the stomach and one through the liver. The incidence of an indirect biopsy route was lower than that of previous CT-guided biopsies (40%) and higher than that of ultrasound-guided biopsies (24%)<sup>[5]</sup>. It is generally accepted and has been clinically proved that, for pancreas biopsy, a fine needle crossing the gastrointestinal tract, rather than through the liver<sup>[5]</sup>, is safe and will not result in complications<sup>[5]</sup>. One experimental study in rabbits reported that the transgastric route with an 18-gauge cutting needle could be used without apparent peritonitis and bleeding<sup>[6]</sup>. The EUS-guided trucut needle (19-gauge) biopsy of the pancreas, all performed transgastrically, has been claimed to be safe and accurate<sup>[11]</sup> in selected patients who agreed to undergo endoscopy.

The biopsy-related complication rate using fine needle is low (0.5% to 3%) and acute pancreatitis is the most frequent complication<sup>[14,15]</sup>. Zech *et al*<sup>[7]</sup> reported only one complication in 57 patients who developed acute

pancreatitis after a large core-needle biopsy of the pancreas. In the present study, all nine transgastric biopsies which penetrated both the anterior and posterior stomach walls were performed with 17-gauge coaxial transducer needles and then an 18-gauge biopsy gun. There were no immediate complications such as peritonitis or bleeding. One patient had transient pneumoperitoneum, which resolved after overnight fasting. Late complications, including tumor spread, were not found in follow-up images. However, our study was limited by a relatively short period of follow-up during the survival period of our patients.

The hole made by a gastrostomy catheter is larger than that caused by a biopsy needle. Some authors have reported no untoward effects after the removal of a 10 or 14 French catheter used for percutaneous gastrostomy<sup>[16]</sup>. The muscular layers run in three directions- oblique, circular and longitudinal- from the inner to the outer stomach wall. This arrangement is considered to be the reason why the stomach wall can be punctured without peritoneal leakage<sup>[17]</sup>.

In our study, the stomach was usually empty and

partly collapsed after overnight fasting. It is much more difficult to penetrate the stomach wall when it is “flaccid”. There are two tricks to piercing the stomach wall. The first is to keep the biopsy needle as perpendicular as possible to the gastric wall. If the biopsy needle was tangential, it would slide over rather than penetrate the gastric wall. The second is to advance the needle forcibly and quickly when penetrating the gastric wall. If the needle was advanced slowly, it would tent the gastric wall rather than piercing it. If the stomach wall is tented and can not be punctured, the needle should be withdrawn a little and then advanced again.

We conclude that percutaneous transgastric biopsy of the pancreas in selected patients with a combination of a 17-gauge introducer needle and an 18-gauge biopsy gun can be safe and has a high successful rate.

## COMMENTS

### Background

It is reasonable to obtain a histological diagnosis before treating patients who have pancreatic masses and are unsuitable or unwilling to undergo surgery. As the pancreas is a deep seated organ surrounded by other vital structures, it is a challenge for the physician to obtain an adequate specimen for histological examination. Endoscopic ultrasound-guided biopsy of pancreatic masses has been proved to be a safe and effective method. However, if the hospital has no such facilities or patients are unwilling or intolerant of the procedure, computed tomography (CT)-guided biopsy is an alternative method. In some cases, penetration of other vital organs is unavoidable when approaching the pancreatic mass. In this article, the authors clarified the safety and efficacy of percutaneous transgastric biopsy of pancreatic masses.

### Research frontiers

The stomach has three muscle layers and can be punctured without evidence of leakage. Percutaneous gastrostomy, either by endoscopy or fluoroscopy guidance, has been widely performed for a long time. Although only the anterior wall is punctured during gastrostomy and a catheter is put in place to block the hole, the authors think it would be safe to pierce both the anterior and posterior wall of the stomach using a large biopsy needle. An experimental study in rabbits also showed that it was safe to perform transgastric biopsy.

### Innovations and breakthroughs

Eight patients received 9 CT-guided transgastric biopsies of pancreatic masses located at the pancreas head or body without passing through vital organs. All procedures went smoothly. Only one patient had transient pneumoperitoneum which completely resolved the next day. Although this is a small series, their study shows that it is feasible to perform transgastric biopsy of a pancreatic lesion using a large needle.

### Applications

Besides pancreatic lesions, there are other types of pathology in the upper abdomen, such as enlarged lymph nodes or loculated fluid. Transgastric biopsy of lymph nodes or aspiration or drainage of fluid could be performed safely.

### Terminology

CT-guided biopsy uses CT scanning to perform a biopsy. When facing a deep seated lesion, or lesion blocked by gas, such as a lung nodule, CT scanning can provide better resolution and clearly shows the biopsy needle reaching the target.

### Peer review

As the authors stated in the introduction of the manuscript, endoscopic

ultrasonography guided biopsy of the pancreas is the gold standard to obtain sample tissue for histological diagnosis of pancreatic mass. Percutaneous transgastric CT-guided biopsy for patients with pancreatic mass should be considered an alternative.

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BRIEF ARTICLE

## Clinicopathological and prognostic analysis of 429 patients with intrahepatic cholangiocarcinoma

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cirrhosis. Multivariate analysis indicated that radical resection, lymph node metastases, macroscopic tumor thrombi and size, and CA19-9 were associated with prognosis.

**CONCLUSION:** Surgical radical resection is still the most effective means to cure ICC. Certain laboratory tests (such as CA19-9) can effectively predict the survival of the patients with ICC.

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**Key words:** Intrahepatic cholangiocarcinoma; Diagnosis; Pathology; Surgery; Survival

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### Abstract

**AIM:** To understand the clinicopathological characteristics and treatment selections and improve survival and provide valuable information for patients with intrahepatic cholangiocarcinoma (ICC).

**METHODS:** We retrospectively evaluated 5311 liver cancer patients who received resection between October 1999 and December 2003. Of these, 429 (8.1%) patients were diagnosed with ICC, and their clinicopathological, surgical, and survival characteristics were analyzed.

**RESULTS:** Upper abdominal discomfort or pain (65.0%), no symptoms (12.1%), and hypodynamia (8.2%) were the major causes for medical attention. Laboratory tests showed 198 (46.4%) patients were HBsAg positive, 90 (21.3%) had  $\alpha$ -fetoprotein > 20  $\mu$ g/L, 50 (11.9%) carcinoembryonic antigen > 10  $\mu$ g/L, and 242 (57.5%) carbohydrate antigen 19-9 (CA19-9) > 37 U/mL. Survival data was available for 329 (76.7%) patients and their mean survival time was 12.4 mo. The overall survival of the patients with R0, R1 resection and punching exploration were 18.3, 6.6 and 5.6 mo, respectively. Additionally, CA19-9 > 37 U/mL was associated with lymph node metastases, but inversely associated with

### INTRODUCTION

Patients with intrahepatic cholangiocarcinoma (ICC) are typically at an advanced pathological stage at the time of diagnosis, and are therefore associated with very poor prognosis. The incidence of ICC is increasing worldwide. The cause for this increase remains unknown and may be related to predisposing genetic and environmental factors<sup>[1,2]</sup>. The incidence rate of ICC is approximately 0.5-2.0/100 000 in males and slightly lower in females. In Europe and North America, ICC accounts for 10%-25% of liver cancers in males and even a higher proportion in females<sup>[1]</sup>. The etiology and pathogenesis of ICC are not known and remain to be defined, although many potential factors may contribute to it. For example, chronic biliary tract infection is generally recognized as the most common risk factor for ICC. A multidisciplinary synthetic therapy combining surgical resection with chemotherapy is the most widely used treatment protocol. Surgical resection is the therapeutic aspect with a capacity of curing ICC, while chemotherapy is mainly used for the



patients with unresectable or recurrent disease. Moreover, no conclusion has been reached as to whether adjuvant chemotherapy is effective in the control of ICC<sup>[3]</sup>. This may be because there are no standard chemotherapeutic protocols for ICC. Recently, Gemcitabine or Gemcitabine-based treatment has been a preferable choice to treat some ICC patients. Whether the patients with unresectable and non-metastatic ICC should be given liver transplantation treatment remains controversial, although the effect of liver transplantation for these patients was much better than that of palliative treatment<sup>[4]</sup>.

ICC often shows higher malignant grades and poorer prognosis than those of hepatocellular carcinoma (HCC). The 5-year survival rate of ICC is still less than 5%<sup>[5]</sup>. As a result, improving patients' survival with early detection and more aggressive treatment of ICC has been a focus of our research. Since ICC is a relatively rare neoplasm, to date, very few large-scale studies have been reported. In the current study, we have retrospectively assessed 429 cases of ICC that have undergone surgical treatment in the Eastern Hepatobiliary Surgery Hospital in Shanghai, China. We statistically evaluated the clinical characteristics, pathology, treatment, and prognosis of these patients to determine whether these parameters could contribute to a better prediction of patient survival.

## MATERIALS AND METHODS

### Patients

The study was approved by our institutional review board, and an informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. We retrospectively surveyed a total of 5311 patients with primary liver cancer who underwent surgical treatment in our hospital between October 1999 and December 2003. The pathological diagnoses of these patients included HCC, ICC, or mixed liver neoplasm. As a result, we obtained 429 cases of ICC from the total cases (8.1%). Clinicopathological characteristics for these patients were retrieved, including age, gender, the existence of choledocholithiasis, chronic viral hepatitis, tumor size, number of lesions, existence of satellite lesions, lymph node metastases, extrahepatic metastases, cirrhosis, pathology (grade), tumor invasion, some routine tumor marker expressions [ $\alpha$ -fetoprotein (AFP), carbohydrate antigen 19-9 (CA19-9), and carcinoembryonic antigen (CEA)], surgical procedures, and survival data. For surgical procedures, R0 resection was defined as the *en bloc* resection with all margins histologically free of tumor, while R1 resection was defined as one in which the tumor mass was removed but section margins may not necessarily be tumor-free. Other patients underwent exploratory laparotomy for unresectable lesions. All patients were graded according to International Union Against Cancer (UICC) TNM classification, 1997 version. We attempted to follow all 429 patients, but only 329 were available for data analysis. The lost follow-up data in the 100 patients may be due to their death, loss of contact, or other unknown reasons.

### Statistical analysis

Statistical calculations and analyses were performed using SPSS11.0 software. Overall survival rate was plotted by the Life Table method. The univariate and multivariate predictors of prognosis were determined using univariate Cox regression analysis and the Cox proportional hazard model, respectively (Backward). The following variants were taken into account: age, gender, curative resection, lymph node metastases, number of intrahepatic lesions, satellite lesions, extrahepatic metastases, macroscopic tumor thrombi, pathology, cirrhosis, tumor size, encapsulation, microscopic tumor thrombi, tumor invasion, hepatitis B Virus (HBV) infection, AFP, CEA, and CA19-9. The Wilcoxon (Gehan) test was used to evaluate pair-wise comparisons between groups. The association between CA19-9 expression and clinicopathological parameters was analyzed using the  $\chi^2$  test and a logistic regression model.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Clinical features

The 429 ICC patients consisted of 301 men and 128 women, with ages ranging from 22 to 81 years, with a median age of 52 years. The main clinical manifestations included upper abdominal discomfort or pain (65.0%), an asymptomatic presentation (12.1%), hypodynamia (8.2%), abdominal distension (4.0%), jaundice (3.0%), nausea (2.8%), lower back pain (2.6%), abdominal mass, emaciation, and other symptoms (2.3%). Laboratory evaluations showed that 198 patients (46.4%) were HBsAg positive, 1 (1/321, 0.3%) was hepatitis C virus positive, 90 (21.3%) had AFP  $> 20 \mu\text{g/L}$ , 50 (11.9%) CEA  $> 10 \mu\text{g/L}$ , and 242 (57.5%) CA19-9  $> 37 \text{ U/mL}$ , as detected with an electrochemiluminescence immunoassay (Table 1).

Furthermore, 285 (66.4%) patients had only a single tumor mass, while additional 144 (33.6%) had multiple lesions. Tumor sizes were between 1.5 cm and 20 cm, with a mean size of  $7.1 \pm 3.8$  cm. Macroscopic satellite lesions were found in 99 cases, of these, 40 cases had  $\leq 3$  lesions and 59 cases had more than 3 lesions. In addition, there were 47 cases of macroscopic tumor thrombi with 32 intravascular thrombi (27 portal vein thrombi and 5 hepatic vein thrombi), 10 cases of bile duct thrombi, and 5 cases of concurrent thrombi. Lymph node metastases were found in 88 (20.5%) cases. Tumors metastasizing to lymph nodes at the porta hepatis and hepatoduodenal ligament accounted for 59.1% (52/88) while retroperitoneal metastases accounted for 27.2% (24/88). Extrahepatic metastases usually invaded into the diaphragm, abdominal wall, omentum, stomach, or duodenum (Table 1). TNM classifications are shown in Table 1.

### Surgical procedures and complications

All patients were preoperatively assessed and their operability was evaluated using computed tomography (CT), magnetic resonance imaging (MRI), or both. As a



**Table 1 Clinicopathological characteristics of 429 ICC patients**

	No. of cases	Total No. of cases	Percentage (%)
Gender		429	
Male	301		70.2
Female	128		29.8
Age (yr)		429	
< 53	220		51.3
≥ 53	209		48.7
Choledocholithiasis		429	
No	383		89.3
Yes	46		10.7
Pathology T		429	
T1	11		2.5
T2	159		37.1
T3	112		26.1
T4	147		34.3
Pathology N		429	
N0	341		79.5
N1	88		20.5
Pathology M		429	
M0	408		95.1
M1	21		4.9
Pathology stage		429	
I	11		2.5
II	126		29.4
III	133		31.0
IV	159		37.1
Maximum tumor diameter (cm)		429	
≤ 5	145		33.8
> 5, ≤ 10	186		43.4
> 10	76		17.7
Diffuse type	22		5.1
Macroscopic satellite lesions		429	
No	330		76.9
≤ 3	40		9.3
> 3	59		13.8
Macroscopic tumor thrombi		429	
No	382		89.0
In blood vessel	32		7.5
In bile duct	10		2.3
In both	5		1.2
Serum HBsAg and HBcAb		427	
HBsAg (+)	198		46.4
HBsAg (-) and HBcAb (+)	60		14.0
HBsAg (-) and HBcAb (-)	169		39.6
Serum AFP (μg/L)		422	
No	332		78.7
> 20, ≤ 1000	70		16.6
> 1000	20		4.7
Serum CEA (μg/L)		420	
No	370		88.1
> 10, ≤ 100	36		8.6
> 100	14		3.3
Serum CA19-9 (U/mL)		421	
No	179		42.5
> 37, ≤ 507	143		34.0
> 507	99		23.5

ICC: Intrahepatic cholangiocarcinoma; AFP: α-fetoprotein; CEA: Carcino-embryonic antigen; CA19-9: Carbohydrate antigen 19-9.

result of preoperative assessment, 319 (74.3%) received R0 liver resection, 76 (17.7%) received R1 liver resection, and 34 (7.9%) received the exploratory laparotomy. Liver resection was performed using finger fracture and clamp crushing with intermittent Pringle's maneuver under room temperature. In all 395 patients (including R0 and

R1 resections), 237 underwent partial hepatectomy (172 tumors located within two or fewer segments and 65 within three or more segments), 51 segmentectomy or bisegmentectomy, 8 trisegmentectomy, 55 left hepatectomy, 26 right hepatectomy and 18 extended hepatectomy. Fifty-four patients also received common bile duct exploration for cholelithiasis or thrombus resection, 12 patients received Roux-en-Y cholangiojejunostomy, and 19 patients received resection of invading tissues or of organs surrounding liver. Thirty-five patients underwent lymph node dissection, among them 25 patients with and 10 patients without lymph nodes metastasis. Thirty-four patients were excluded from liver resection due to intrahepatic or extrahepatic metastasis and hepatic duct system invasion by tumor metastases or metastatic lymph node.

Five (1.2%) patients died within 1 mo after surgery, 3 of them died of hepatic failure, 1 died of intraperitoneal hemorrhage, and 1 died of adult respiratory distress syndrome (ARDS). Twenty-six (6.1%) patients had surgical complications, i.e. biliary leakage (13 cases), infection of pneumonia, subphrenic or, incision infection (7 cases), bleeding (4 cases), ARDS (1 case), and intestinal obstruction (1 case).

### Pathological features

After surgery, tumors were inspected macroscopically and microscopically, and the data indicated that poorly differentiated tumors accounted for 62.0%, while moderately and well differentiated tumors accounted for 36.7% and 1.3%, respectively. Microscopic tumor thrombi were found in 34.7% of the patients, and 89.4% of tumors did not have a pseudocapsule. One hundred and forty-six patients had cirrhosis in the liver, and of these 92 cases had small-nodule liver cirrhosis. Moreover, bile duct stones were observed in 10.7% (46/429) of patients.

### Prognosis and prognostic factors

The longest follow-up period is 8 years, but only 329 (76.7%) patients were available for data analysis, the rest patients were lost to follow-up after operation. Most the reasons for the lost follow-up is unknown but may be due to lost contact, death, or unspecified causes. Among these 329 patients, the mean survival time was 12.4 mo with 1-, 3- and 5-year survival rates of 50.9%, 22.2%, and 17.4%, respectively. The overall survival period for the patients with R0 resection was 18.3 mo with 1-, 3-, and 5-year survival rates of 62.5%, 30.2%, and 23.6%, respectively. The overall survival for the patients with R1 resection and punching exploration were only 6.6 and 5.6 mo. The overall survival in patients who received R0 resection was significantly higher than those who received R1 resection or punching exploration ( $P = 0.000$ , Figure 1 and Table 2).

Furthermore, the data from the univariate analysis found that prognostic factors included radical resection, lymph node metastases, satellite lesions, extrahepatic metastasis, tumor size, number of tumor lesions, and expression of CEA and CA19-9. The multivariate analysis further confirmed that radical resection, lymph node metastases, macroscopic tumor thrombi, tumor size, and

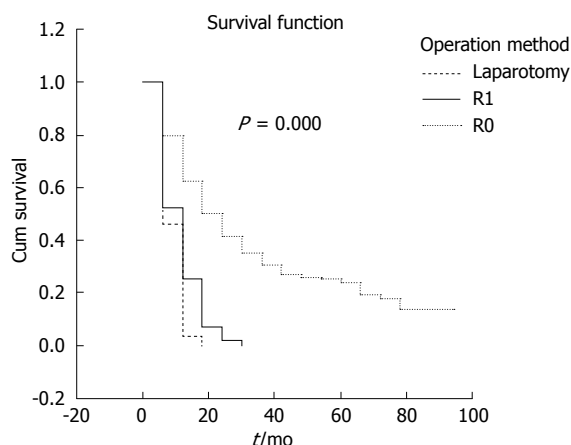


Figure 1 The overall survival of patients with intrahepatic cholangiocarcinoma (ICC) after surgery.

Table 2 Surgery selection and prognosis

Surgical procedures	n	Ratio (%)	Median survival time (mo)	Survival rate (%)			P value
				1-yr	3-yr	5-yr	
R0 <sup>a</sup>	319	74.3	18.3	62.5	30.2	23.6	0.000
R1 <sup>b</sup>	76	17.7	6.6	25.4	0	0	
Exploratory laparotomy	34	7.9	5.6	3.6	0	0	
Total	429	100	12.4	50.9	22.2	17.4	

<sup>a</sup>R0 vs R1 or exploratory laparotomy,  $P = 0.000$ ; <sup>b</sup>R1 vs exploratory laparotomy,  $P = 0.360$ .

Table 3 Multivariate analysis of patient survival

	Regression coefficient	Standard error	P value	Relative risk	95% CI
Curative resection	0.658	0.173	0.000	1.931	1.375-2.713
Lymph node metastases	0.432	0.218	0.048	1.540	1.004-2.361
Macroscopic tumor thrombi	0.455	0.206	0.027	1.576	1.053-2.360
Tumor size	0.159	0.080	0.046	1.173	1.003-1.372
CA19-9	0.191	0.085	0.024	1.210	1.025-1.428

CA19-9 were prognosis factors (Table 3). In addition, Chi-square tests showed that CA19-9 was associated with gender, age, tumor size, HBsAg positivity, and liver cirrhosis (Table 4). The logistic regression analysis revealed that CA19-9 was associated with lymph node metastases and inversely with liver cirrhosis (Table 5).

## DISCUSSION

### Risk factors of ICC

A recent review<sup>[2]</sup> showed the acknowledged risk factors in only a few cases of cholangiocarcinoma, which seem to be associated with chronic inflammation of the biliary epithelium (such as cholangiolithiasis, parasitic infection, intrahepatic biliary stones, and viral infection<sup>[6-10]</sup>). Primary sclerosing cholangitis is the most common known predisposing condition for cholangiocarcinoma in Western

Table 4 Association of CA19-9 with clinicopathological parameters of the patients

	CA19-9 expression		P value
	> 37 U/mL	≤ 37 U/mL	
Gender			0.032
Male	159	135	
Female	83	44	
Age (yr)			0.040
< 53	112	101	
≥ 53	130	78	
Surgical procedures			0.106
R0	174	139	
R1	44	32	
Exploratory laparotomy	24	8	
Lymph node metastasis			0.082
Yes	182	148	
No	57	30	
Macroscopic satellite lesions			0.229
No	184	140	
≤ 3	20	20	
> 3	38	19	
Extrahepatic metastases			0.185
Yes	227	179	
No	15	6	
Macroscopic tumor thrombi			0.440
Yes	218	157	
No	24	22	
Microscopic tumor thrombi			0.192
Yes	142	120	
No	83	53	
Tumor differentiation			0.221
Well	4	1	
Moderate	85	52	
Poorly	126	105	
Number of lesions			0.843
Single	160	120	
Multiple	82	59	
Tumor size (cm)			0.002
≤ 5	69	72	
> 5, ≤ 10	120	64	
> 10	37	39	
Diffuse type	16	4	
HBsAg			0.049
Yes	101	92	
No	141	87	
Cirrhosis			0.000
Yes	68	80	
No	174	99	

Table 5 Logistic regression analysis in relationship between CA19-9 expression, lymph node metastases, and liver cirrhosis

	Regression coefficient	Standard error	P value	Relative risk	95% CI
Lymph node metastases	0.637	0.295	0.031	1.891	1.060-3.374
Cirrhosis	-0.539	0.230	0.019	0.584	0.372-0.915
Constant	0.336	0.146	0.021	1.340	

countries<sup>[2]</sup>. In follow-up, or in examination of tissue specimens of cholangiocarcinoma, primary sclerosing cholangitis was found to account for 8%-40% of cholangiocarcinoma. In the current study, 198 patients were HBsAg-positive, accounting for 46.4% of cases, which is significantly higher than the estimated 10% HBV

carrier rate in Chinese population. This data indicate that HBV infection may be one of the risk factors for ICC. Moreover, an additional 60 patients were found to be serum positive for anti-HBc antibody, although they were negative for HBsAg, which is indicative of a past HBV infection. Combining HBsAg and anti-HBc expression, our study population had 60.4% patients with HBV or a history of HBV infection. However, it is unclear how HBV infection contributes to development of ICC. The association of cirrhosis with cholangiocarcinoma development may illuminate HBV infection as a risk factor for cholangiocarcinoma. HBV infection causes the majority of liver cirrhosis in Asian countries. Although other studies showed that hepatitis C virus infection was a risk factor for ICC<sup>[8-9]</sup>, our study did not confirm it because of very low infection rate (0.3%) in our patients.

### Diagnosis

Initial and early diagnosis of ICC could be very difficult to achieve due to the wide range of differential diagnoses. Features identified in CT or MRI evaluations are not typical for ICC, as minimal contrast may occur after enhancement. Therefore, some tumor markers, such as CA19-9, CEA and AFP, may add to the differential diagnoses or diagnostic guide for ICC, although these biomarkers may not be specific for ICC. In the current study, elevated CA19-9, CEA and AFP occurred in 57.5%, 11.9% and 21.3% of the patients, respectively, and 70.9% patients were found to express at least one of these markers. Previous studies did report expression of these biomarkers in association with ICC<sup>[11-13]</sup>; however, due to lack of a large number of patients, the exact rate of positive expression of these markers remained unrevealed until the information reported in this current study.

Nevertheless, it is well known that detection of AFP expression is routinely used for early diagnosis of HCC, and given the high infection rate of HBV in the Chinese population, HCC should be first considered in a patient with elevated AFP. In the present study, 23 patients exhibited an increased AFP ( $> 200 \mu\text{g/L}$  but  $\leq 1000 \mu\text{g/L}$ ), while highly increased AFP ( $> 1000 \mu\text{g/L}$ ) was found in 20 patients, accounting for 5.4% and 4.7% of cases, respectively. Therefore, ICC should also be taken into account for patients with elevated AFP. In addition, for patients with high levels AFP but negative in CA19-9 and CEA, ICC should also be considered before operation.

Furthermore, Positron Emission Computed Tomography (PET)/CT could be an alternative method for differential diagnoses of ICC, as it is superior to the enhanced CT in the diagnosis of extrahepatic or lymph node metastases<sup>[14]</sup>.

### Relationship between CA19-9 levels and clinical features

CA19-9 or known as sialylated Lewis antigen is a blood tumor marker and was discovered in patients with colon cancer and pancreatic cancer in 1981<sup>[15]</sup>. Previous studies found that CA19-9 expression was also prevalent in ICC<sup>[2]</sup>. In the current study, CA19-9 ( $> 37 \text{ U/mL}$ ) was found in 57.5% of ICC patients. Further analyses found

that CA19-9 positivity was significantly associated with gender, age, tumor size, cirrhosis, and HBsAg expression, while logistic regression analysis indicated that expression of CA19-9 was significantly associated with cirrhosis and lymph node metastases. ICC patients with CA19-9 ( $> 37 \text{ U/mL}$ ) presented a higher incidence of lymph node metastases. Other studies demonstrated association of positive CA19-9 and lymph node metastases of gastric and colorectal cancers<sup>[16-20]</sup>.

In addition, our study revealed that CA19-9 ( $> 37 \text{ U/mL}$ ) rate was lower in cirrhosis patients with positive HBsAg. The underlying mechanism for this remains unknown and needs further investigations. However, Schöniger-Hekele *et al*<sup>[21]</sup> reported that the combined elevation of CA19-9 and CA 125 was useful for diagnosis of the advanced fibrosis or cirrhosis. Their observation is definitely not compatible with the results of this current study.

### Surgical resection and prognosis

To date, surgical resection is still the primary and most effective means to cure ICC. Nevertheless, the selection methods used to determine a patient's suitability for surgery will directly affect the patient's chances of survival. In this study, the mean survival of patients receiving R0 resection was 18.3 mo, whereas the mean survival rate for patients with R1 resection was only 6.6 mo, indicating that radical resection is the most important factor in prolonging patient survival. Comparing R1 resection and exploratory laparotomy, the former exhibited a slightly better prognosis; however, this is not statistically significant ( $P = 0.36$ ).

Several other studies<sup>[22-27]</sup> showed that the 1-year survival rate of patients receiving R0 resection was between 61% and 83%, and the 5-year survival rate was between 22% and 63% (Table 6), indicating that their survival rates were much higher than those of our patients. Besides the different patient population and severity of the diseases, we proposed that this might be due to the different surgical methodology. For example, segmental resection is extensively used in Western countries, while non-anatomic resection is primarily used in China. The former is a more curative procedure owing to wider resection margins. The low rate of radical resection may be due to the invasion of local and portal hepatic ducts by ICC. Lymph node metastases and distant metastasis were often observed in patients with ICC.

However, it remains debatable whether extended radical operation in combination with lymph node dissection could improve survival rates. Some studies have reported that 1- and 3-year survival rates were 94% and 82%, respectively, after extended hepatectomy (including vessel resection and reconstruction) in patients with solitary tumors but without vascular invasion or extrahepatic or lymph node metastases<sup>[28]</sup>. However, rather than positive effects, increased morbidity was observed in patients with extended surgery that included anatomic hepatic resection, vessel resection and reconstruction, and extended lymph node dissection<sup>[29]</sup>.

Table 6 Comparison of post-operative survival after R0 resection

Author	No. of total	No. of R0	Ratio (%)	Survival (%)		
				1-yr	3-yr	5-yr
Ohtsuka <i>et al</i> <sup>[22]</sup> , 2003	50	34	68	61.6	37.6	22.5
Morimoto <i>et al</i> <sup>[23]</sup> , 2003 <sup>1</sup>	51	35	68.6	68.2	44.1	32.4
Nakagawa <i>et al</i> <sup>[24]</sup> , 2005	53	44	83.0	66.2	38.3	26.3
Lang <i>et al</i> <sup>[25]</sup> , 2006	54	30	55.5	83	58	48
DeOliveira <i>et al</i> <sup>[26]</sup> , 2007	44	34	77.3	NR	NR	63
Konstadoulakis <i>et al</i> <sup>[27]</sup> , 2008	72	54	75	80	49	25
Our current study	429	319	74.3	62.5	30.2	23.6

<sup>1</sup>Two cases of death were excluded. NR: Not reported.

### Prognostic factors

The present study showed that favorable prognostic factors for ICC are: radical resection, no metastasis of lymph nodes, a small tumor diameter, no macroscopic tumor thrombi, and low levels of CA19-9. Among these favorable factors, radical resection, no metastasis of lymph nodes, and a small tumor diameter are consistent with previous studies<sup>[22,24,30,31]</sup>. This study also showed that macroscopic tumor thrombi and CA19-9 expression were prognostic factors for ICC. In addition, ICC with CA19-9 (> 37 U/mL) exhibited a higher grade of malignancy and prevalence of lymph node metastases. Ohtsuka *et al*<sup>[32]</sup> also reported that CA19-9 was a prognostic factor of ICC. Other studies demonstrated that macroscopic tumor thrombus is a key factor for poor prognosis of hepatocellular carcinoma<sup>[33-35]</sup>. As the incidence of macroscopic tumor thrombus is relatively low in ICC (only 11% in our current study), it could be easily missed, especially studies with a small sample size.

### Liver transplantation

Originally, the prognosis of ICC patients who received liver transplantation treatment was not satisfactory. In particular, Pascher *et al*<sup>[36]</sup> reported that 5-year survival rate reached 29% in a study, but did not exceed 18% in other four studies. However, most recent studies showed an improving 5-year survival rate between 33% and 42%<sup>[4,37]</sup>. Multivariate analysis revealed that single tumor, tumor-free margins, no lymph node metastasis, no jaundice, or no perineural invasion, and early TNM stage were associated with better prognosis<sup>[4,38,39]</sup>. Nevertheless, due to restricted resources of liver donors and poor prognosis after liver transplantation, it is still controversial whether the patients with unresectable and non-metastasis ICC should undergo liver transplantation. Further studies are needed to determine the criteria for selecting the patients who can benefit from liver transplantation. In addition, the effectiveness of adjuvant radiotherapy and chemotherapy both before and after transplantation remains to be defined.

In conclusion, our present study demonstrated that hepatitis B infection, CA19-9, CEA, and AFP are associated with ICC development. CA19-9 levels are associated with lymph node metastases, but inversely with cirrhosis. Radical resection (R0) is the key prognostic factor for ICC. Future studies should focus on evaluation of the molecule-targeted

therapy, and whether it can efficiently control this deadly disease so as to improve the survival of the patients.

## COMMENTS

### Background

Incidence of intrahepatic cholangiocarcinoma (ICC) is increasing worldwide and its prognosis is very poor. Thus, further studies on its clinical characteristics for early detection and on surgical treatment for better prognosis are urgently needed.

### Research frontiers

Early detection of ICC could focus on defining clinical characteristics and biomarker study. Surgery with radical resection always is the key factor to improve the survival of the patients. The effectiveness of chemotherapy is currently limited and novel approaches are needed.

### Innovations and breakthroughs

This study demonstrated that carbohydrate antigen 19-9 (CA19-9) is commonly elevated in ICC and associated with lymph node metastases, but inversely associated with liver cirrhosis, indicating that CA19-9 could further be evaluated for early detection and prognosis of ICC. In addition, hepatitis B virus infection is associated with cholangiocarcinoma and increased  $\alpha$ -fetoprotein (AFP) levels may also be considered for ICC, although AFP is a routinely used biomarker for hepatocellular carcinoma.

### Applications

This study provides an initial assessment of ICC and further studies are needed to confirm the findings, which can apply to future early detection, prediction of prognosis, treatment election, and differential diagnosis of ICC.

### Peer review

This is an interesting paper, with a large number of patients involved, which might be of benefit for future studies of ICC.

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# Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis

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## Abstract

**AIM:** To compare neoadjuvant chemoradiotherapy and surgery with surgery alone for resectable esophageal carcinoma.

**METHODS:** We used MEDLINE and EMBASE databases to identify eligible studies and manual searches were done to ensure no studies were missed. Trial validity assessment was performed and a trial quality score was assigned.

**RESULTS:** Eleven randomized controlled trials (RCTs) including 1308 patients were selected. Neoadjuvant chemoradiotherapy significantly improved the overall survival compared with surgery alone. Odds ratio (OR) [95% confidence interval (CI),  $P$  value], expressed as neoadjuvant chemoradiotherapy and surgery vs surgery alone, was 1.28 (1.01-1.64,  $P = 0.05$ ) for 1-year survival, 1.78 (1.20-2.66,  $P = 0.004$ ) for 3-year survival, and 1.46 (1.07-1.99,  $P = 0.02$ ) for 5-year survival. Postoperative mortality increased in patients treated by neoadjuvant chemoradiotherapy (OR: 1.68, 95% CI: 1.03-2.73,  $P = 0.04$ ), but incidence of postoperative complications was similar in two groups (OR: 1.14, 95% CI: 0.88-1.49,  $P = 0.32$ ). Neoadjuvant chemoradiotherapy lowered the local-regional cancer recurrence (OR: 0.64, 95% CI: 0.41-0.99,  $P = 0.04$ ), but incidence of distant cancer recurrence was similar (OR: 0.94, 95% CI: 0.68-1.31,  $P = 0.73$ ). Histological subgroup analysis indicated that esophageal squamous cell carcinoma did not benefit from neoadjuvant

chemoradiotherapy, OR (95% CI,  $P$  value) was 1.16 (0.85-1.57,  $P = 0.34$ ) for 1-year survival, 1.34 (0.98-1.82,  $P = 0.07$ ) for 3-year survival and 1.41 (0.98-2.02,  $P = 0.06$ ) for 5-year survival.

**CONCLUSION:** Neoadjuvant chemoradiotherapy can raise the survival rate of patients with esophageal adenocarcinoma.

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**Key words:** Esophageal carcinoma; Neoadjuvant chemoradiotherapy; Randomized controlled trial; Meta-analysis

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## INTRODUCTION

Esophageal carcinoma (EC) is the sixth commonest cause of tumor-related death around the world<sup>[1]</sup>. It is endemic in Asia, southern and eastern Africa, and northern France<sup>[2,3]</sup>. North America and many western European countries are low-incidence regions, but the nearly 6-fold increase in the incidence of esophageal adenocarcinoma (EAC) in the past three decades and the corresponding 7-fold increase in mortality are remarkable. Surgery has always been considered as the standard treatment for patients with resectable esophageal cancer, but the effectiveness of surgery alone was unsatisfactory and the median survival of patients treated by surgery alone rarely exceeded 18 mo<sup>[4]</sup>. So clinicians always make efforts to seek for new treatment strategies to prolong the survival time of patients with

EC. Recently neoadjuvant chemoradiotherapy plus surgery has been studied widely, but opinions vary among clinicians as to the therapeutic effect of the new method, and the outcomes of randomized controlled trials (RCTs) were not consistent. Published meta-analyses did not reach a consensus, some of which was short of enough RCTs or adopted unpublished data. The current study aims to perform a meta-analysis to compare neoadjuvant chemoradiotherapy plus surgery with surgery alone for resectable EC by enough eligible published RCTs to date.

## MATERIALS AND METHODS

Computerized bibliographic and manual searches were done to identify all eligible published literature between 1980 and 2008. MEDLINE and EMBASE were the primary source of RCTs, with the following key words: esophageal cancer, surgery, radiotherapy, chemotherapy, neoadjuvant chemoradiotherapy and RCT. Manual searches were performed by reviewing articles and abstracts cited in the published meta-analysis and RCTs.

The eligible studies must meet the following inclusion criteria: (1) It must be a prospective RCT which compares neoadjuvant chemoradiotherapy plus surgery with surgery alone in the initial management of resectable EC; (2) Outcomes must include survival data; (3) There was no statistical significance in factors such as sex, age, type of pathology, tumour stage between the two groups; and (4) Studies were analyzed by intention-to-treat patients. Trials were not excluded because of cancer histology (squamous cell carcinoma or adenocarcinoma) or language of publication. Unpublished reports, abstracts and theses were excluded. This meta-analysis was performed according to the QUOROM statement<sup>[5]</sup>.

All data were abstracted by three independent researchers and the methodological qualities of all RCTs were assessed by three aspects: blinding, randomization and handling withdrawals and dropouts<sup>[6]</sup>. If researchers had discrepancies in assessing RCTs, a consensus was reached by discussion.

Outcomes including 1-year survival, 3-year survival, 5-year survival, postoperative mortality, incidence of postoperative complication, incidence of local-regional cancer recurrence and incidence of distant cancer recurrence were analyzed. In two trials<sup>[7,8]</sup> we used the Kaplan-Meier estimate of the 1-year survival and 3-year survival in the two groups and the data for the 5-year survival was obtained from another trial<sup>[9]</sup> in the same way. The remaining data were directly available in the corresponding RCTs. Evaluation of therapeutic effectiveness, including survival rate and incidence of recurrence, was performed in all patients who were enrolled in these trials, but for postoperative events, data were calculated only based on the number of patients who underwent surgery as the denominator. Sensitivity analyses were performed to identify the effect of histological subtype (squamous cell carcinoma or adenocarcinoma) and

scheduling of neoadjuvant chemoradiotherapy (concurrent or sequential) on survival.

Data were analyzed by RevMan 4.2.10.  $\chi^2$  tests were used to assess heterogeneity of study results and a planned cut-off for significance of  $P \leq 0.05$ . If  $P > 0.05$ , we used a fixed effect model, otherwise we used a random effect model. The odds ratios (OR) among the frequency of events in both neoadjuvant chemoradiotherapy plus surgery group (CRT group) and surgery alone group (S group) was calculated and these OR are presented as a point estimate with 95% confidence intervals (CI) and  $P$  values in parentheses. The significance level was set at 5%. Funnel plot analysis did not suggest publication bias against negative trials.

## RESULTS

### Features of RCTs

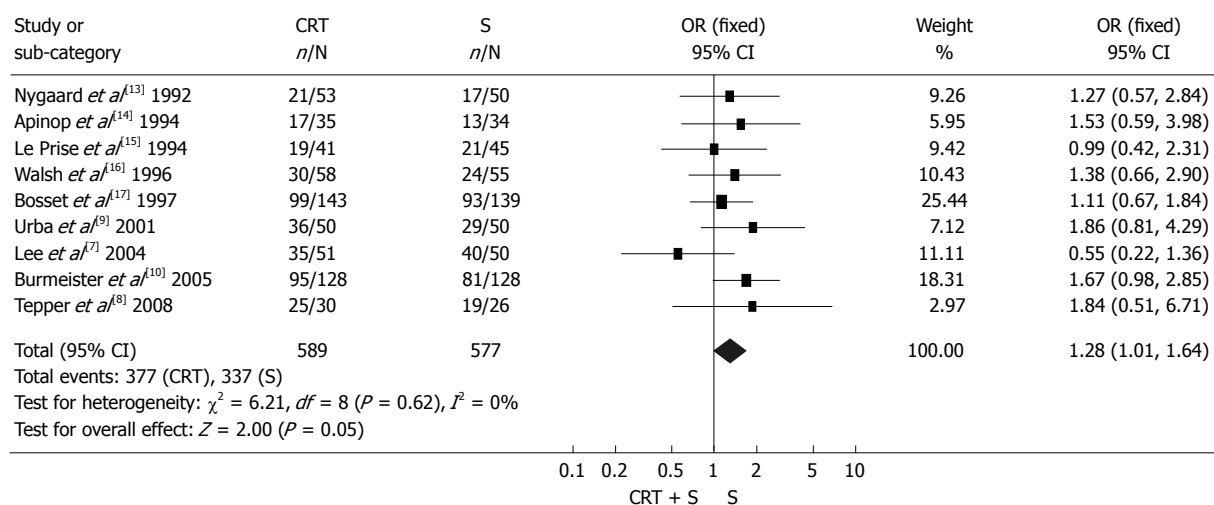
Eleven randomized studies were identified from 1980 to 2008 and the main features of the trials included in the meta-analysis are shown in Table 1<sup>[7-17]</sup>. All studies were published literature. Nine countries including Australia, United States of America, China, France, Ireland, Japan, Korea, Norway and Thailand were involved in the RCTs.

The studies were carried out from 1983 to 2002 and the literatures were published between 1992 and 2008. Because double blinding can not be performed due to the inherent difficulty of the design of the trial (e.g. chemotherapy and radiotherapy) and the method of randomization was not reported in most trials, the RCT quality scores ranged from 1 to 3 (5-point scale) and the average was 2.3<sup>[5,6]</sup>. Of these 11 studies, seven were restricted to patients with esophageal squamous cell carcinoma (ESCC) only, one was restricted to patients with EAC only, and the remaining three trials enrolled patients with either ESCC or EAC. The 11 RCTs included 1308 patients, 659 of whom received neoadjuvant chemoradiotherapy before surgery, and the remaining 649 patients received surgery alone. Nearly all the patients in the S group underwent surgery, yet there were more patients in the CRT group who had not completed the planned treatment regimen for various causes such as side effects of chemotherapy or metastasis of cancer before surgery. The tumor stage of the most patients in the 11 studies ranged from I - III (1987 UICC), but more advanced tumor stage (IVa) was also seen in two RCTs<sup>[9,11]</sup>. In addition, tumor stages were classified in the RCT by Le Prise *et al*<sup>[15]</sup> according to the 1978 American Joint Committee on Cancer, which was not a TNM staging. Finally tumor stage was not reported in the RCT by Walsh *et al*<sup>[16]</sup>.

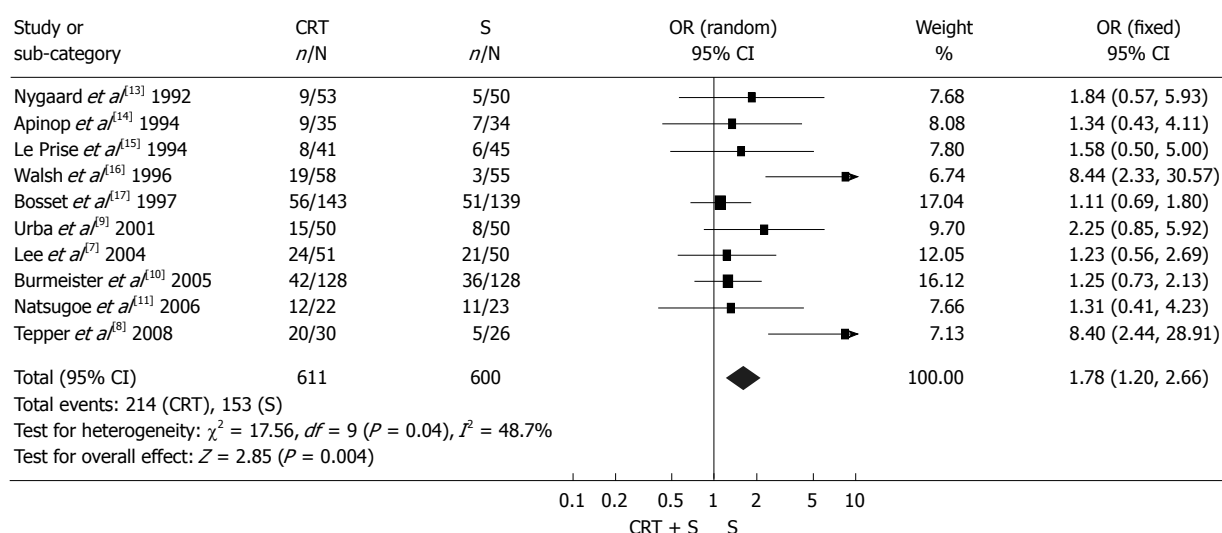
### Survival rate

The effect of neoadjuvant chemoradiotherapy on survival rate is shown in Figure 1. Obviously, there was statistical significance in survival rate between the two groups. OR (95% CI,  $P$  value), expressed as neoadjuvant chemoradiotherapy plus surgery *vs* surgery alone, was 1.28

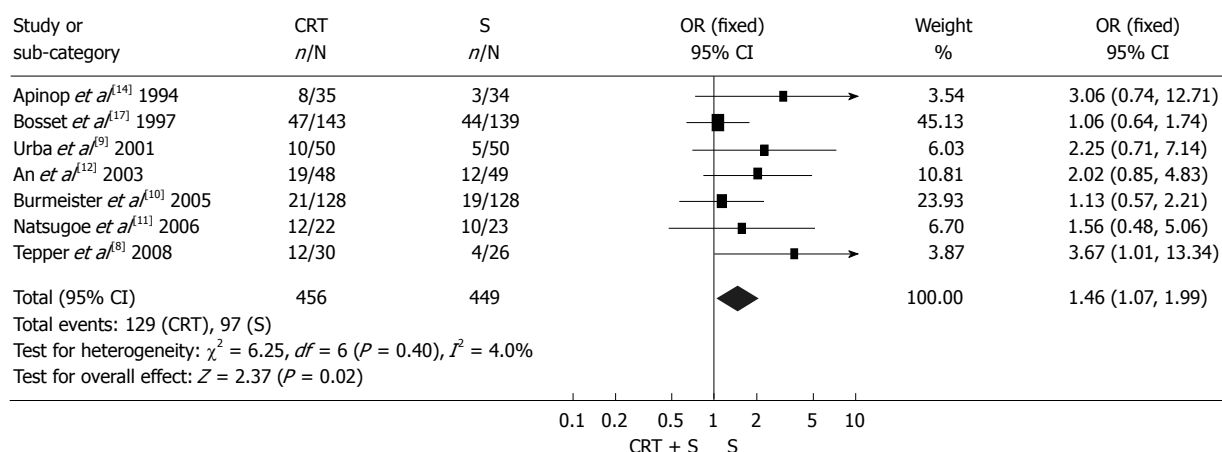
**A** Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group *vs* S group  
 Outcome: 1-yr survival



**B** Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group *vs* S group  
 Outcome: 3-yr survival



**C** Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group *vs* S group  
 Outcome: 5-yr survival



**Figure 1** Postoperative survival rate in neoadjuvant chemoradiotherapy and surgery compared with surgery alone. A: One-year survival; B: Three-year survival; C: Five-year survival. CRT + S: Neoadjuvant chemoradiotherapy and surgery; S: Surgery; OR: Odds ratio; CI: Confidence interval.



Table 1 Features of all trials included in the meta-analysis

Country	Year of RCT published	SCC or AC	Schedule of radiotherapy	Schedule of chemotherapy	Concurrent or sequential	Time of surgery
Norway	1992	SCC	35 Gy 1.75 Gy/d 5 d/wk for 4 wk	Cisplatin: 20 mg/m <sup>2</sup> D1-5, D15-19 Bleomycin: 10 mg/m <sup>2</sup> D1-5, D15-19	Sequential	Not report
Thailand	1994	SCC	40 Gy 2 Gy/d 5 d/wk for 4 wk	Cisplatin: 100 mg/m <sup>2</sup> D1, 29 FU: 1000 mg/m <sup>2</sup> D1, D29-32	Concurrent	4 wk after completion of chemotherapy
France	1994	SCC	20 Gy 2 Gy/d D8-19	Cisplatin: 100 mg/m <sup>2</sup> D1,21 FU: 600 mg/m <sup>2</sup> D2-5, D22-25	Sequential	D42
Ireland	1996	AC	40 Gy 2.67 Gy/d D1-5, 8-12, 15-19	Cisplatin: 75 mg/m <sup>2</sup> D7 FU: 15 mg/kg D1-5 Week 1 and week 6	Concurrent	8 wk after CRT
France	1997	SCC	37 Gy 3.7 Gy/d 5 d/wk for 2 wk	Cisplatin: 80 mg/m <sup>2</sup> D0-2	Sequential	2-4 wk after CRT
USA	2001	SCC and AC	45 Gy 1.5 Gy <i>bid</i> D1-5, 8-12, 15-19	Cisplatin: 20 mg/m <sup>2</sup> D1-5, 17-21 FU: 300 mg/m <sup>2</sup> D1-21 Vinblastine: 1 mg/m <sup>2</sup> D1-4, 17-20	Concurrent	D42
China	2003	SCC	36 Gy 3 Gy/d D21-24, 28-31, 35-38	Cisplatin: 25 mg/m <sup>2</sup> D2-5, D22-25 FU: 1000 mg/m <sup>2</sup> D1-5 500 mg/m <sup>2</sup> D21-25	Sequential	3 wk after CRT
Korea	2004	SCC	45.6 Gy 1.2 Gy <i>bid</i> D1-28	Cisplatin: 60 mg/m <sup>2</sup> D1, 21 FU: 1000 mg/m <sup>2</sup> D2-5	Concurrent	3-4 wk after completion of radiotherapy
Australia	2005	SCC and AC	35 Gy 2.33 Gy/d 5 d/wk for 3 wk	Cisplatin: 80 mg/m <sup>2</sup> D1 FU: 800 mg/m <sup>2</sup> D2-5	Concurrent	3-6 wk after completion of radiotherapy
Japan	2006	SCC	40 Gy 2 Gy/d 5 d/wk for 4 wk	Cisplatin: 7 mg/m <sup>2</sup> FU: 350 mg/m <sup>2</sup> 5 d/wk for 4-6 wk	Concurrent	35-40 d after CRT
USA	2008	SCC and AC	50.4 Gy 1.8 Gy/d 5 d/wk for 5.5 wk	Cisplatin: 100 mg/m <sup>2</sup> D1,29 FU: 1000 mg/m <sup>2</sup> D1-4, D29-32	Concurrent	3-8 wk after CRT

SCC: Squamous cell carcinoma; AC: Adenocarcinoma; CRT: Chemoradiotherapy.

Table 2 Survival rate estimates of patients with EC by schedule of chemoradiotherapy

Schedule of CRT	Overall survival	No. of studies	No. of patients		OR (95% CI)	P
			CRT + S	S		
Sequential	1 yr	3	237	234	1.12 (0.77, 1.64)	0.56
	3 yr	3	237	234	1.24 (0.82, 1.88)	0.31
	5 yr	2	191	188	1.24 (0.81, 1.91)	0.32
Concurrent	1 yr	6	352	343	1.41 (1.03, 1.94)	0.03
	3 yr	7	374	366	2.12 (1.20, 3.76)	0.01 <sup>1</sup>
	5 yr	5	265	261	1.72 (1.10, 2.71)	0.02

<sup>1</sup>Random effects model was used. EC: Esophageal carcinoma; CRT + S: Neoadjuvant chemoradiotherapy and surgery; S: Surgery; OR: Odds ratio; CI: Confidence interval.

(1.01-1.64,  $P = 0.05$ ) for 1-year survival, 1.78 (1.20-2.66,  $P = 0.004$ ) for 3-year survival and 1.46 (1.07-1.99,  $P = 0.02$ ) for 5-year survival. Subgroup analysis showed that there was no survival benefit from neoadjuvant chemoradiotherapy in EC patients when chemotherapy and radiotherapy were given sequentially. On the contrary, EC patients benefited from concurrent chemoradiotherapy. The corresponding OR (95% CI,  $P$  value) is shown in Table 2. Moreover, patients with ESCC did not get any survival benefit from neoadjuvant chemoradiotherapy and corresponding OR (95% CI,  $P$  value) is shown in

Table 3 Survival rate estimates of patients with ESCC for neoadjuvant chemoradiotherapy compared with surgery alone

Overall survival	No. of studies	No. of patients		OR (95% CI)	P
		CRT + S	S		
1 yr	6	368	368	1.16 (0.85, 1.57)	0.34
3 yr	7	390	391	1.34 (0.98, 1.82)	0.07
5 yr	5	293	295	1.41 (0.98, 2.02)	0.06

ESCC: Esophageal squamous cell carcinoma.

Table 3. In addition, another subgroup analysis indicated that the 3-year survival in CRT group was significantly higher than that of S group in patients of the USA and Europe, but in patients of Asia, it was a pessimistic result (Table 4).

### Morbidity after surgery

The resection rate in patients treated with surgery alone was markedly higher than that in patients treated with preoperative chemoradiotherapy (OR: 0.36, 95% CI: 0.24-0.54,  $P < 0.00001$ ), but patients treated with preoperative chemoradiotherapy were more likely to obtain a complete resection (R0 resection), which was defined as gross disease removed with negative margins (OR: 2.16, 95% CI: 1.58-2.97,  $P < 0.00001$ ) (Figure 2). Mortality

Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group vs S group  
 Outcome: R0 resection rate

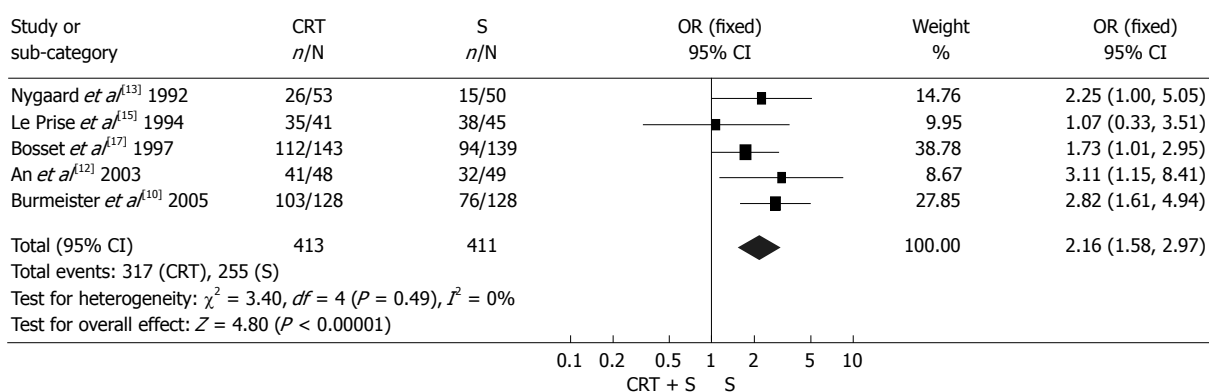


Figure 2 R0 resection rate in neoadjuvant chemoradiotherapy and surgery compared with surgery alone.

Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group vs S group  
 Outcome: Postoperative mortality

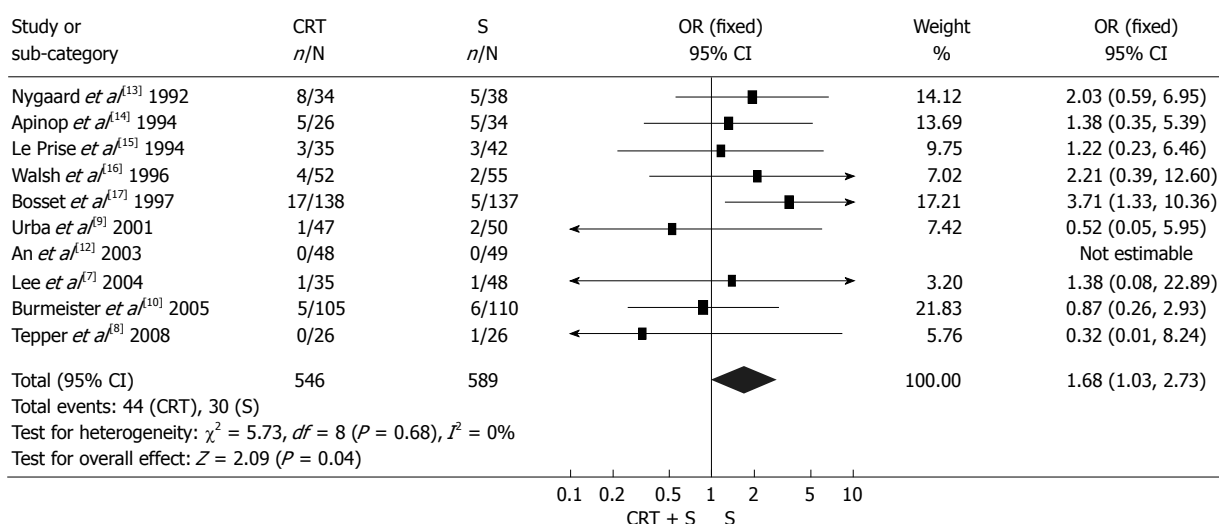


Figure 3 Postoperative mortality in neoadjuvant chemoradiotherapy and surgery compared with surgery alone.

Table 4 Three-year survival in different population

Population	No. of studies	No. of patients		OR (95% CI)	P
		CRT + S	S		
USA	2	80	76	3.74 (1.77, 7.88)	0.0005
Europe	4	295	289	1.59 (1.09, 2.33)	0.02
Asia	3	108	107	1.27 (0.72, 2.24)	0.40

after surgery varied from 0% to 23.5% in CRT group (the highest mortality was in the RCT by Nygaard *et al*<sup>[13]</sup>) and from 0% to 14.7% in S group (the highest mortality was in the RCT by Apinop *et al*<sup>[14]</sup>). Mortality after surgery in CRT group was higher than that in S group (OR: 1.68, 95% CI: 1.03-2.73,  $P = 0.04$ ) (Figure 3). But if the RCT by Nygaard *et al*<sup>[13]</sup> or the RCT by Bosset *et al*<sup>[17]</sup> were excluded, there was no significant difference between the two groups and corresponding OR (95% CI,  $P$  value) was 1.66 (0.99-2.79,  $P = 0.06$ ) and 1.30 (0.75-2.28,  $P = 0.35$ ).

The postoperative complications included nonfatal and fatal complications. There was no significant difference between the two groups (OR: 1.14, 95% CI: 0.88-1.49,  $P = 0.32$ ) (Figure 4).

### Effect on recurrence

First treatment failure was defined as unequivocal histological or radiological evidence of tumor recurrence for the first time after surgery wherever the tumor relapsed. Seven RCTs provided related data on tumor recurrence. The patients treated by preoperative chemoradiotherapy had lower incidence of local recurrence (OR: 0.64, 95% CI: 0.41-0.99,  $P = 0.04$ ) (Figure 5A), but the two groups had no significant difference in distant recurrence (OR: 0.94, 95% CI: 0.68-1.31,  $P = 0.73$ ) (Figure 5B).

## DISCUSSION

Our meta-analysis indicated that patients treated by neo-

adjuvant chemoradiotherapy had more survival benefit compared with patients treated by surgery alone, including 1-year survival, 3-year survival and 5-year survival. But subgroup analysis demonstrated patients with ESCC could not benefit from neoadjuvant chemoradiotherapy. The meta-analysis performed by Fiorica *et al*<sup>[18]</sup> suggested that neoadjuvant chemoradiotherapy plus surgery significantly lowered the 3-year mortality compared with surgery alone, but there was no statistical significance between the two groups if all RCTs including patients with EAC were excluded. Another meta-analysis performed by Gebiski *et al*<sup>[19]</sup> demonstrated that both patients with ESCC and patients with EAC benefited from neoadjuvant chemoradiotherapy and corresponding OR (95% CI, *P* value) were 0.84 (0.71-0.99, *P* = 0.04) and 0.75 (0.59-0.95, *P* = 0.02). Since the former *P* value approached 0.05, our conclusion should be cautiously done. Thus we presume that only patients with EAC could benefit from neoadjuvant chemoradiotherapy. Another subgroup indicated patients in Europe and the USA benefited from neoadjuvant chemoradiotherapy; however, patients in Asia did not. This result indicated that different population had different response to neoadjuvant chemoradiotherapy and this may be associated with ethnic difference.

Though patients receiving neoadjuvant chemoradiotherapy had higher survival than patients treated by surgery alone, our meta-analysis showed that the incidence of surgery-related death was higher in the CRT group. Moreover, some patients lost the chance of surgery for the metastasis of tumor or some patients died before surgery for the aggravation of disease. Neoadjuvant chemoradiotherapy made local tissue harder and easier to bleed and as a result fatal postoperative complications such as anastomotic leakage and respiratory insufficiency increased. This may account for the higher postoperative mortality of patients in CRT group. However, sensitivity analysis showed no significant difference between the two groups after excluding the RCT by Nygaard *et al*<sup>[13]</sup>, performed between 1983 and 1988, which was the earliest one among all the RCTs included in this meta-analysis. Probably there was no effective treatment for severe postoperative complications due to the relative undeveloped medical conditions at that time. In fact, the postoperative mortality in the RCTs published after 2000 was significantly lower than those published before 2000. In a word, it is possible that the postoperative mortality of patients receiving neoadjuvant chemoradiotherapy is not significantly higher than that of patients treated by surgery alone under present medical conditions.

Outcomes of our meta-analysis revealed that patients treated by surgery alone had more possibility to undergo the scheduled surgery, however, the rate of complete resection in CRT group was higher than that in S group. This may account for the lower incidence of local recurrence in CRT group, which was another result of this meta-analysis. In the 11 RCTs, only part of patients in CRT group can obtain clinical relief or pathological response. Two RCTs<sup>[12,14]</sup> compared the survival time

between the patients who obtained clinical relief (including complete response and partial response) and the patients who failed to obtain clinical relief (including stable disease and disease progression), and found that the former was markedly greater than the latter. So, if some biological molecules can predict the response of EC patients to neoadjuvant chemoradiotherapy, EC patients will suffer from less physical miseries. Furthermore, this could avoid waste and enhance targeted treatment. Some studies<sup>[20-23]</sup> have indicated that Hsp27, DNA-PKcs, ERCC1 and c-erbB-2 were potential biological molecules, which were related to the response of EC patients to neoadjuvant chemoradiotherapy. Studies in this field are meaningful and promising.

One study reported that the effect of sequential chemoradiotherapy was superior to that of concurrent chemoradiotherapy in treating lung cancer<sup>[24]</sup>. In EC, however, outcome was exactly opposite to those obtained from lung cancer. This meta-analysis showed that EC patients only benefited from preoperative concurrent chemoradiotherapy. Concurrent chemoradiotherapy may work by inhibiting the growth of local tumor and micrometastasis, moreover concurrent chemotherapy can increase the sensitivity of tumor to radiotherapy.

Some studies<sup>[25-27]</sup> indicated that 40%-75% of patients with resectable EC (T1-3N0-1M0) judged according to clinical examination or surgery had subclinical metastasis or tumor had already invaded the adjacent organs or tissues. Accurate tumor staging is crucial to the prognosis of EC patients receiving surgery. Therefore, further measures should be taken to improve the accuracy of tumor staging. Currently, endoscopic ultrasound (EUS) is the most accurate method for staging EC for T and N stage<sup>[28]</sup>. Although helical computed tomography still appears insensitive for the identification of T4 or metastatic involvement of celiac lymph node disease in esophageal cancer, EUS with fine needle aspiration and FDG-PET [fluorine 18-labeled fluorodeoxyglucose (FDG) positron emission tomography (PET)] can make up for this shortcoming<sup>[29]</sup>. Part of patients in RCTs<sup>[9,11]</sup> included in this meta-analysis had metastasis of non-regional lymph nodes. Investigators considered those lymph nodes could be included in the radiation port and should be resected at surgery<sup>[9]</sup>. In fact, this condition belongs to IVa according to TNM staging. We suggest that EC patients (IVa) should not give up the chance of surgery, and they will benefit from neoadjuvant chemoradiotherapy plus surgery too.

In conclusion, this meta-analysis showed that patients with ESCC did not benefit from preoperative concurrent chemoradiotherapy and patients with EAC may be the real beneficiaries of the treatment protocol. Compared with patients treated by surgery alone, patients receiving neoadjuvant chemoradiotherapy more likely obtained complete resection and had lower local cancer recurrence. Neoadjuvant chemoradiotherapy was connected with a little higher mortality after surgery. But it did not increase the incidence of postoperative complications. In addition, patients in Europe and the USA more likely benefited

Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group *vs* S group  
 Outcome: Complication after surgery

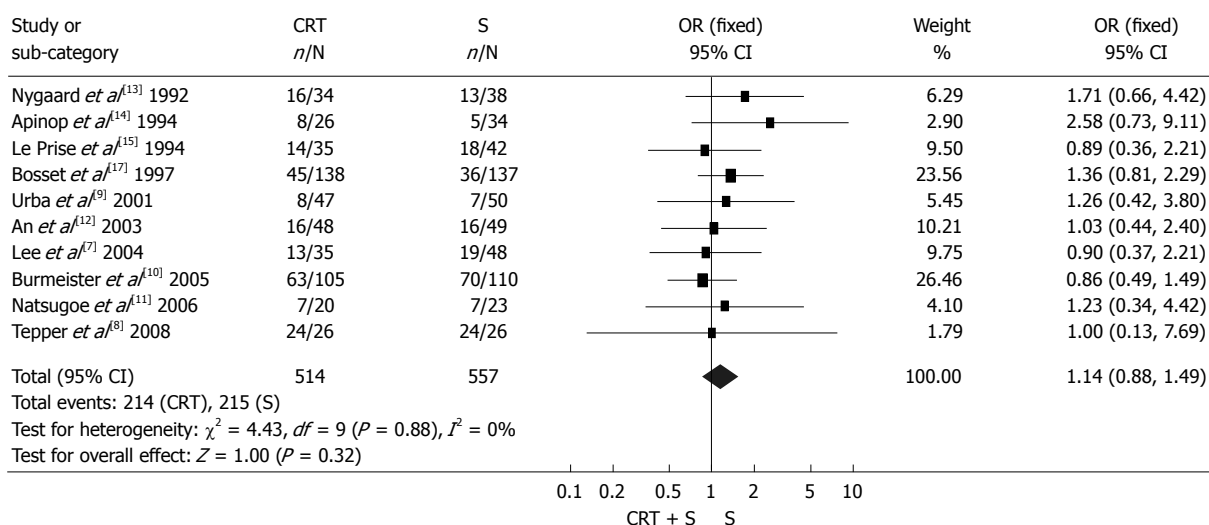
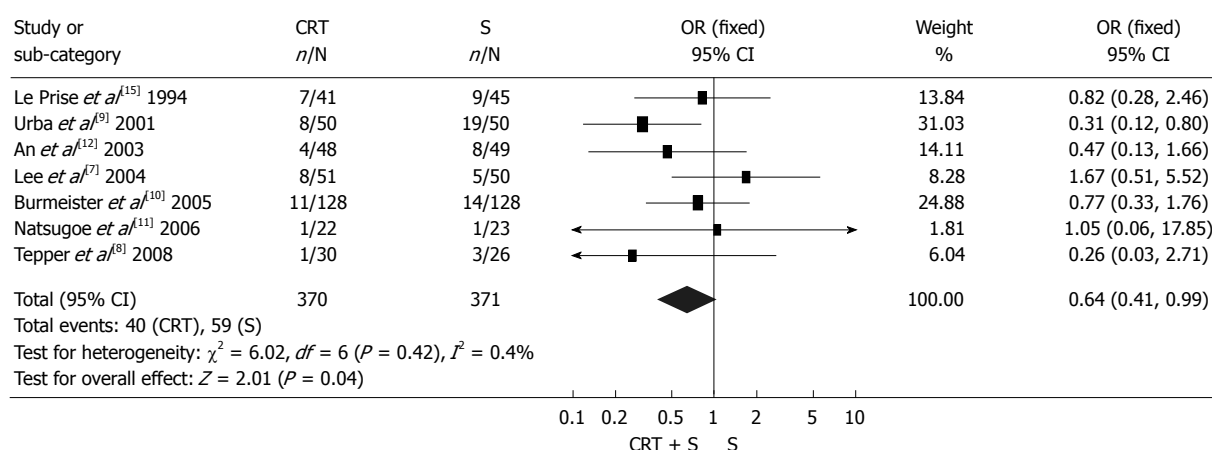


Figure 4 Incidence of postoperative complication in neoadjuvant chemoradiotherapy and surgery compared with surgery alone.

**A** Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group *vs* S group  
 Outcome: Local-regional cancer recurrence



**B** Review: Neoadjuvant chemoradiotherapy for resectable esophageal carcinoma: A meta-analysis  
 Comparison: CRT group *vs* S group  
 Outcome: Distant cancer recurrence

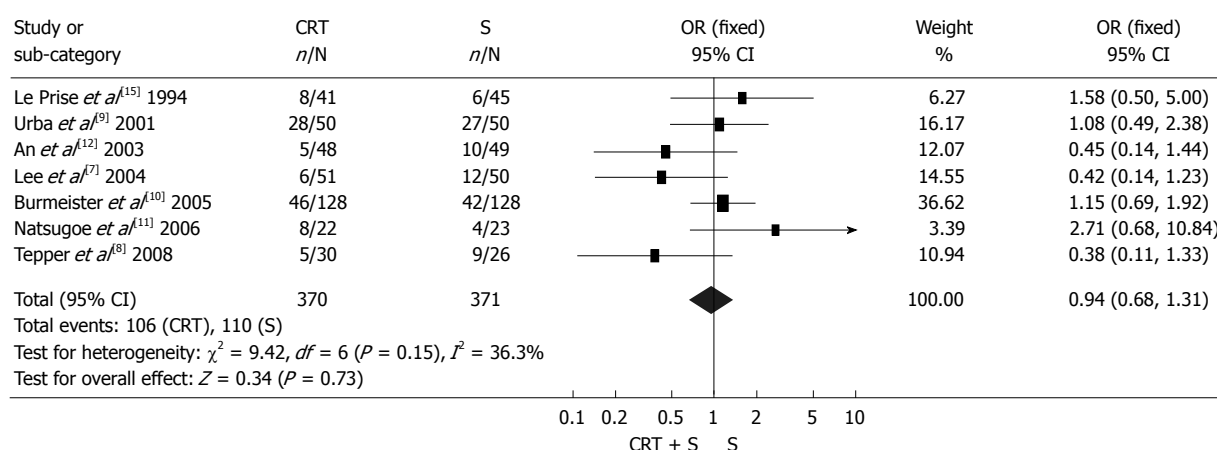


Figure 5 Cancer recurrence after surgery in neoadjuvant chemoradiotherapy and surgery compared with surgery alone. A: Incidence of local-regional cancer recurrence; B: Incidence of distant cancer recurrence.



from neoadjuvant chemoradiotherapy than those in Asia, and this is worth of further studies.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Esophageal carcinoma (EC) is one of the major malignant diseases worldwide. Surgery alone cannot obtain satisfactory effects in patients with EC.

### Research frontiers

Neoadjuvant chemoradiotherapy has been a hotspot for EC treatment research. Several related randomized controlled trials (RCTs) have been published, but opinions vary among clinicians as to the therapeutic effect of the new method. It remains uncertain whether patients with resectable EC can benefit from neoadjuvant chemoradiotherapy.

### Innovations and breakthroughs

Several meta-analyses on the neoadjuvant chemoradiotherapy for EC have been published so far, some of which lacked adequate RCTs or used unpublished data. In this study, the authors collected relatively comprehensive data and all the data were from the published literature. It was found that patients with esophageal squamous cell carcinoma did not benefit from neoadjuvant chemoradiotherapy, while patients with esophageal adenocarcinoma (EAC) were the real beneficiaries. In addition, the authors analyzed the impact of geographical differences on the efficacy of the treatment protocol and found that patients in Europe and the USA more likely benefited from neoadjuvant chemoradiotherapy than those in Asia.

### Applications

Results of this study indicate that neoadjuvant chemoradiotherapy is an effective treatment protocol, which is beneficial to patients with EAC in Europe and the USA.

### Terminology

Neoadjuvant chemoradiotherapy: Chemotherapy and radiotherapy are given to patients with cancer before surgery.

### Peer review

This work is a meta-analysis including 11 randomized prospective studies that analyze the advantages of the use of the neoadjuvant chemoradiotherapy vs surgery alone in the treatment of the EC. The results are interesting and suggest that neoadjuvant chemoradiotherapy is beneficial to patients with EAC.

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BRIEF ARTICLE

## Effect of severe acute pancreatitis on pharmacokinetics of Da-Cheng-Qi Decoction components

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sorption of DCQD components in rats and their pharmacokinetic parameters.

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**Key words:** Severe acute pancreatitis; Da-Cheng-Qi Decoction; Pharmacokinetics; Components

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### Abstract

**AIM:** To investigate the effect of severe acute pancreatitis (SAP) on pharmacokinetics of Da-Cheng-Qi Decoction (DCQD) components in rats.

**METHODS:** Rats were divided into SAP group and sham-operation group as a control group ( $n = 6$ ). Rhein, chrysophanol, rheochrysidin, magnolol, hesperidin and naringin in DCQD were quantified in rat serum by high performance liquid chromatography tandem mass spectrometry for studying their pharmacokinetics.

**RESULTS:** Early absorption of each DCQD component was tended to degrade in SAP group after treatment with DCQD by gavage. The  $C_{max}$  (chrysophanol,  $P = 0.0059$ ; rheochrysidin,  $P = 0.0288$ ; magnolol,  $P = 0.0487$ ; hesperidin,  $P = 0.0277$ ; naringin,  $P = 0.0023$ ) and AUC (rhein,  $P = 0.0186$ ; chrysophanol,  $P = 0.0013$ ; magnolol,  $P = 0.001$ ; hesperidin,  $P = 0.0081$ ; naringin,  $P = 0.0272$ ) of DCQD component were obviously lower in SAP group than in control group. The  $T_{1/2\alpha}$  of chrysophanol and rheochrysidin ( $P = 0.0467$  and  $0.0005$ , respectively) and  $T_{max}$  of chrysophanol and rheochrysidin ( $P = 0.0101$  and  $0.0037$ , respectively) lasted longer in SAP group than in control group.

**CONCLUSION:** SAP can significantly impact the ab-

### INTRODUCTION

Acute pancreatitis, occurring suddenly and usually resolving after a few days of treatment, may become life-threatening if severe complications take place. Fulminant acute pancreatitis is more dangerous<sup>[1]</sup>. Severe acute pancreatitis (SAP), characterized by intricate mechanism, variant symptoms, grave prognosis and multiple complications, seriously threatens the life of patients and brings a heavy burden to the society, families and economy. Each year, about 210 000 patients with acute pancreatitis in the United States are admitted to hospitals<sup>[2]</sup>. Additionally, neither standard treatment nor other medications are available for SAP patients at present<sup>[3]</sup>. SAP, similar to Yangming Fushi syndrome (YMFSS) according to the traditional Chinese medicine, has been treated with purgative herbals throughout China for more than three decades<sup>[4-6]</sup>.

Da-Cheng-Qi Decoction (DCQD), a famous preparation of traditional Chinese medicine used in treatment of digestive diseases, is composed of *Dabuang* (Caulis Fibraureae), *Houpu* (Cortex Magnoliae Officinalis), *Zhishi* (immature bitter orange) and *Mangxiao* (Natrii Sulphas). It has been reported that DCQD can restore gastrointestinal function by facilitating motility, relieving enteroparalysis and evacuating "dry stool"<sup>[7]</sup>, prevent bacterial translocation and counteract with endotoxin, regulate  $Ca^{2+}$ - $Mg^{2+}$ -ATPase in the pancreatic acinar cells<sup>[8]</sup>. SAP

can be treated with Chinese herbal decoctions based on the above mechanism. However, no studies are available on the pharmacokinetics of such decoctions in acute pancreatitis. According to the theory “syndrome and treatment pharmacokinetics”, YMFSS should influence the pharmacokinetics of DCQD<sup>[9]</sup>, but it has not been proved experimentally up to date.

Thus, we quantified the DCQD components absorbed in rats with SAP characterized by YMFSS and studied the influence of SAP on the pharmacokinetics of DCQD components<sup>[10]</sup>.

## MATERIALS AND METHODS

### Animals

Male clean-grade, healthy Sprague-Dawley rats, weighing  $320 \pm 25$  g, at the age of  $90 \pm 5$  d, were used in this study. The rats were handled according to the University Guidelines and the Animal Ethics Committee Guidelines of the Animal Facility of the West China Hospital, maintained in air-conditioned animal quarters at  $22 \pm 2^\circ\text{C}$  with a relative humidity of  $65\% \pm 10\%$ , acclimatized to the facilities for 10 d, and then fasted for 24 h with free access to water prior to experiments.

### Materials, chromatographic and HPLC-mass spectrometry conditions

The structures of rhein, chrysophanol, rheochrysidin, magnolol, hesperidin, naringin and ibuprofen (internal standards) are presented in Figure 1. Reference standards for these components of DCQD and the internal standard (IS) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol with a chromatographic grade was obtained from Tedia Company Inc. (USA). Acetic acid and ethyl acetate were bought from Chongqing Chemistry Co. Ltd. (Chongqing, China). Ammonium acetate, sodium hydroxide and hydrochloric acid (analysis grade) were purchased from Chengdu Kelong Chemical Reagent Factory (Chengdu, China). All aqueous solutions and buffers were prepared with deionized water from a Millipore RiosTM-16 water purifier (Millipore, Billerica, MA, USA)<sup>[11]</sup>.

High performance liquid chromatography tandem mass spectrometry (HPLC-TMS) system, consisted of a SIL-HTc autosampler and a LC-10ADvp pump, was provided by Shimadzu (Kyoto, Japan). API3000 triple-quadrupole LC-MS system was purchased from Applied Biosystems (Foster City, CA, USA). The system was controlled with Analyst 1.4.2 software. Separation was performed on a YMC-Pack ODS-A C18 column ( $5\ \mu\text{m}$ ,  $150\ \text{mm} \times 4.6\ \text{mm}$ , YMC, Kyoto, Japan) and a C<sub>18</sub> guard column ( $5\ \mu\text{m}$ ,  $4.0\ \text{mm} \times 2.0\ \text{mm}$ , Phenomenex Inc., Torrance, CA, USA). The mobile phase is consisted of methanol-water (92:8, v/v) at a flow rate of  $0.3\ \text{mL}/\text{min}$ . The column was maintained at ambient temperature and the injection volume was  $80\ \mu\text{L}$ .

A mass spectrometer was operated using an electrospray source configured to the negative ion mode and quantification was performed by multiple reaction moni-

toring (MRM). Production mass spectra of the analytes are shown in Figure 1 where  $[\text{M}-\text{H}]^-$  of each analyte was selected as the precursor ion, and the most abundant or specific fragment ion was selected as the production in MRM acquisition. Instrumental parameters were optimized for each analyte by infusing the corresponding standard solution at a flow rate of  $5\ \mu\text{L}/\text{min}$ , using a syringe pump integrated into the API 3000 mass spectrometer. Nitrogen was used as a curtain, and auxiliary gas and air were used as a nebulizer gas. Electrospray conditions for the 6 major DCQD components and IS were curtain gas ( $6.0\ \text{L}/\text{min}$ ), ion-spray voltage ( $-4500\ \text{V}$ ), nebulizer gas ( $6.0\ \text{L}/\text{min}$ ), auxiliary gas ( $7.0\ \text{L}/\text{min}$ ), turbo temperature ( $4^\circ\text{C}$ ), respectively. Optimized mass spectrometry parameters for each DCQD compound and IS are listed in Table 1<sup>[11]</sup>.

Six calibration standards were prepared by spiking  $200\ \mu\text{L}$  of blank plasma with  $100\ \mu\text{L}$  of each working solution to obtain the plasma concentrations for rhein and rheochrysidin (5000, 3750, 2500, 1250, 625, 312.5, 156, 78.13, 39.1 and  $19.53\ \text{ng}/\text{mL}$ ), and for chrysophanol, naringin, hesperidin and magnolol (879, 586, 390, 195, 97.7, 48.8, 24.4, 12.2, 6.1 and  $3.1\ \text{ng}/\text{mL}$ ). Quality control (QC) samples were prepared to obtain plasma concentrations for rhein and rheochrysidin (3750, 625, 156 and  $39.1\ \text{ng}/\text{mL}$ ) and for chrysophanol, naringin, hesperidin and magnolol (586, 97.7, 24.4 and  $6.1\ \text{ng}/\text{mL}$ ). The spiked plasma samples (standard and QC samples) were pretreated and detected in each analytical batch along with the unknown samples<sup>[11]</sup>.

### Assay validation

Blank and spiked rat plasma chromatograms were compared to evaluate the selected method (Figure 2). Calibration curves were plotted from the peak area ratio of each analyte to IS *vs* plasma concentrations using a  $1/c^2$  weighted linear least-squares regression model. The lower limit of quantification was set at the concentration of the lowest non-zero calibration standard ( $\text{S}/\text{N} \geq 10:1$ ) that could be measured with an acceptable accuracy and precision ( $\leq 20\%$  for both parameters). Intra- and inter-day precisions were determined by assessing the measured results of QC samples at low, medium and high concentrations (Table 1). Accuracy was determined as the difference in percentages between the mean and nominal concentrations detected (Table 1). Extraction recoveries of the 6 analytes were determined by comparing the peak areas obtained from rat plasma samples with those from the unextracted standard solutions at the same concentration (Table 1). Bench-top stability of the 6 analytes in rat plasma was determined by assessing the QC samples after stored for 2 and 4 h at room temperature. Freeze-thaw stability was detected after two cycles and long-term stability was determined by assessing the QC samples stored at  $-30^\circ\text{C}$  for 14 d. QC samples were prepared, injected and reinjected after the samples were maintained in the autosampler at  $8^\circ\text{C}$  for 12 d. Stability of the analytes was detected by comparing the measured results with those of freshly prepared samples at the same concentration<sup>[11]</sup> (Table 2).



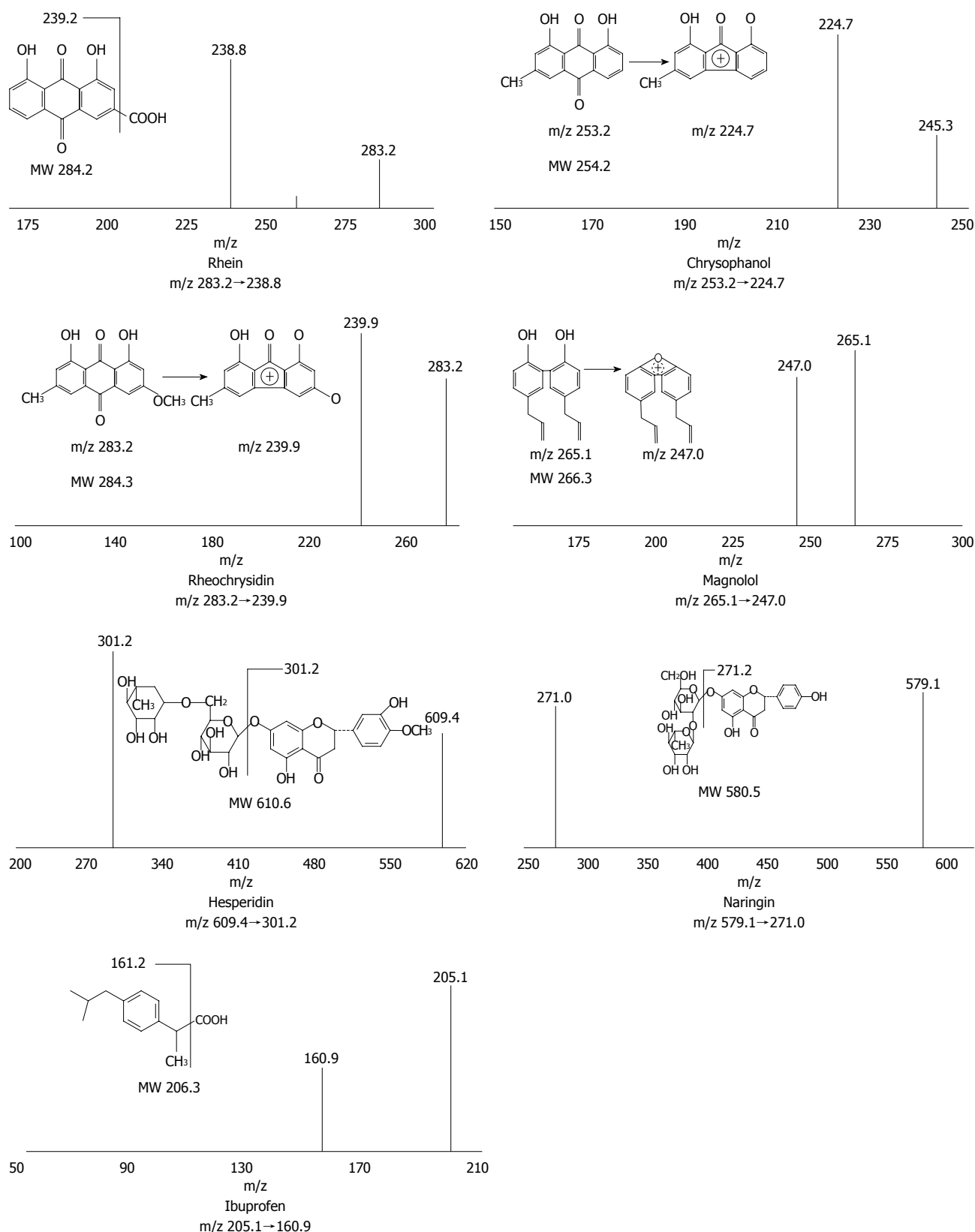


Figure 1 Product ion mass spectra (ESI-) and ion structures of the six major Da-Cheng-Qi Decoction (DCQD) components and internal standards.

### Induction of acute pancreatitis in rats

Acute pancreatitis was induced in rats. The animals were anesthetized with ethyl ether as previously described<sup>[12]</sup>.

### Preparation of Chinese drugs

*Dahuang*, *Houpu*, *Zhishi* and *Mangxiao* were purchased

from Chengdu Green Herbal Pharmaceutical Co. Ltd. (Chengdu, China) and authenticated by Professor Yang Song (Department of Pharmacognosy, Sichuan University, China). DCQD was routinely prepared with 6.0 g of *Dahuang*, 6.0 g of *Houpu*, 6.0 g of *Zhishi* and 6.0 g of *Mangxiao*. For crude drugs, the spray-dried DCQD was

Table 1 Parameters of the 6 major DCQD components in rat plasma QC samples (% ,  $n = 6$ )

	Spiked amount (ng/mL)	Intra-day		Inter-day		Extract	
		RSD	Ac	RSD	Ac	Recovery	RSD
Rhein	39.1	3.76	104.60	3.53	107.94	104.60	3.76
	156	5.55	100.32	5.52	106.87	100.32	5.55
	625	5.31	101.07	5.56	105.36	101.07	5.31
	3750	4.24	101.24	6.23	98.37	101.24	4.24
Chrysophanol	6.1	2.37	99.4	4.46	98.67	99.39	2.36
	24.4	4.38	104.17	4.88	101.14	104.17	4.37
	97.7	1.64	96.66	3.33	98.09	96.66	1.64
	586	3.78	103.98	4.44	101.13	103.98	3.78
Rheochrysidin	39.1	5.69	97.70	5.72	100.78	97.69	5.68
	156	3.33	99.89	4.13	101.14	99.89	3.33
	625	3.22	105.92	3.73	104.37	105.92	3.22
	3750	4.75	103.07	4.84	103.49	103.07	4.75
Magnolol	6.1	5.51	102.13	5.07	106.33	102.13	5.51
	24.4	2.39	104.78	5.79	99.86	104.78	2.39
	97.7	5.51	107.81	4.92	106.23	102.47	5.51
	586	3.77	105.97	5.33	105.29	105.97	3.77
Hesperidin	6.1	4.12	95.96	4.82	98.49	95.96	4.12
	24.4	5.83	99.52	5.10	100.29	99.52	5.83
	97.7	4.21	100.14	3.95	99.72	100.16	4.21
	586	2.77	97.13	4.91	98.52	97.13	2.76
Naringin	6.1	2.66	95.66	4.26	99.29	95.67	2.657
	24.4	2.82	102.46	3.95	103.37	102.45	2.82
	97.7	3.81	100.78	3.96	100.66	100.78	3.81
	586	5.24	98.27	5.59	99.32	98.27	5.24

DCQD: Da-Cheng-Qi Decoction; QC: Quality control.

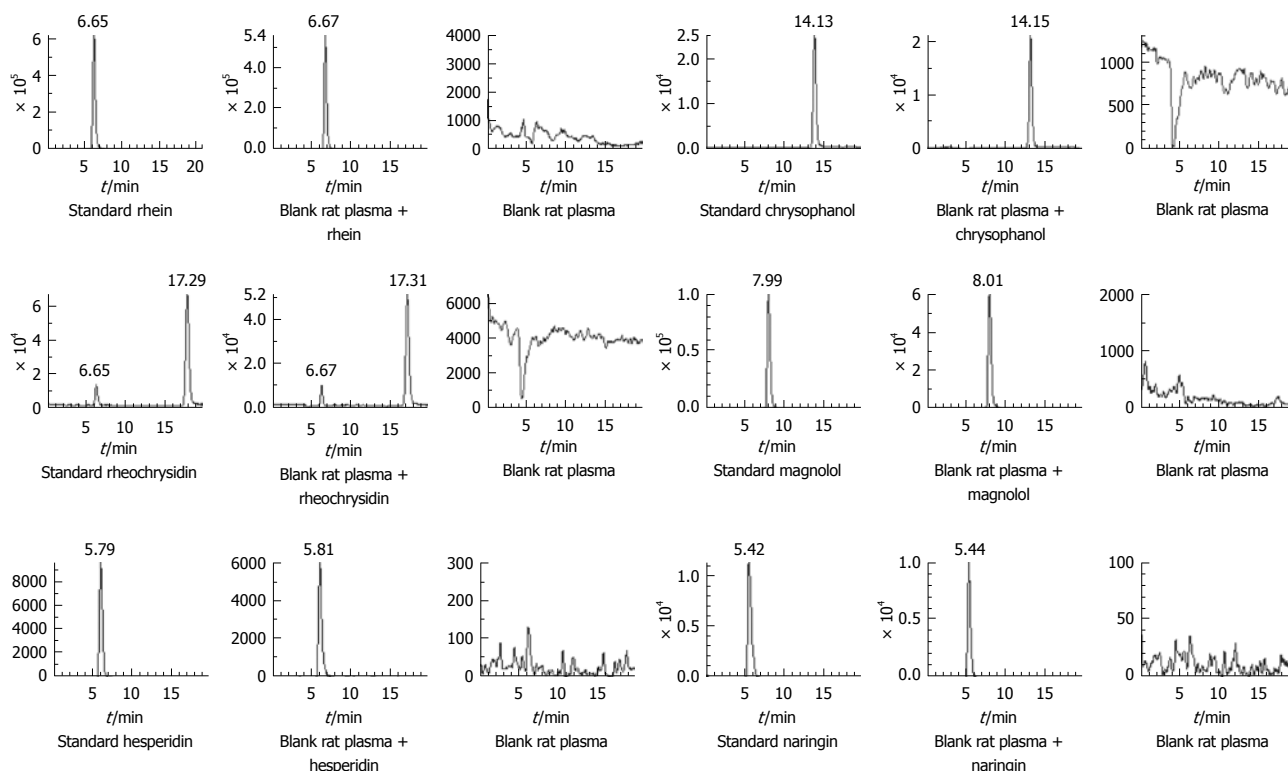


Figure 2 HPLC-TMS showing the six major DCQD components in plasma of rats in two groups.

reconstituted in water to a concentration of 1 g/mL. The contents of the six DCQD components were measured as previously described<sup>[13]</sup>. The crude DCQD preparation was administered through the duodenum of rats at a dosage of 20 g/kg. The voucher specimens were kept in our laboratory.

### *In vivo study*

Rats were randomly divided into SAP group and sham-operation group as a control group ( $n = 6$ ). Rats were given DCQD 2 h after operation. Blood sample (300  $\mu$ L) was collected into a heparinized eppendorf tube *via* the tail vein before and after (10, 15, 20, 30, 45 min and 1,

Table 2 Stability of the 6 major DCQD compositions in rat plasma QC samples (% ,  $n = 3$ )

	Spiked amount (ng/mL)	Bench-top bias		Long-term bias		Freeze-thaw bias		Extract bias		Autosampler bias	
		2 h	4 h	7 d	14 d	1	2	8 h	24 h	6 h	12 h
Rhein	39.1	-1.32	-4.57	-1.15	-4.27	-1.15	-2.54	6.06	-4.54	-5.25	-5.33
	156	-2.50	-5.39	-2.36	-0.43	-2.36	0.43	2.28	2.48	-1.29	-1.07
	625	5.28	-1.21	1.60	-2.82	1.60	-2.93	-0.90	3.40	-0.48	-0.27
	3750	2.06	5.52	-4.46	0.09	-4.46	-0.17	1.50	1.32	-1.54	-3.60
Chrysophanol	6.1	-0.67	1.00	2.42	3.54	2.42	0.90	4.73	3.19	0.73	2.36
	24.4	5.04	4.62	-2.74	-2.61	-2.74	-6.40	-2.82	-4.42	-3.39	-3.52
	97.7	-0.32	5.80	-5.39	0.27	-5.39	-1.52	-2.34	0.00	-3.41	0.00
	568.0	-0.72	-2.00	2.01	-2.13	2.01	-2.47	-2.92	-0.97	0.63	2.42
Rheochrysidin	6.1	1.59	-2.59	-4.11	-3.78	-4.11	-5.18	-3.93	-6.27	-6.33	-5.10
	24.4	4.09	-0.65	0.00	-4.03	0.00	-1.27	-0.43	3.24	-0.42	-3.18
	97.7	-0.86	-4.45	-0.38	0.59	-0.38	-2.43	-0.58	-0.32	0.38	-2.49
	568.0	2.80	-5.52	-3.40	-2.09	-3.40	-2.18	2.04	1.16	-1.66	-3.66
Magnolol	6.1	-1.58	1.33	-5.23	-4.34	-5.23	-3.09	-2.72	-2.14	-5.18	-4.60
	24.4	5.76	0.84	0.42	-2.49	0.42	0.28	-1.95	1.53	1.25	-0.83
	97.7	1.82	1.17	1.48	-1.10	1.48	0.90	4.23	1.47	-3.21	1.35
	568.0	-2.02	-3.03	-1.60	-4.43	-1.60	-2.21	0.23	-5.01	-3.82	-6.14
Hesperidin	6.1	-4.29	4.85	-0.48	-1.27	-0.48	-3.08	1.01	2.34	-3.98	-1.54
	24.4	-2.03	-3.11	0.41	0.00	0.41	-1.36	1.46	1.46	-4.21	4.76
	97.7	3.42	-1.69	0.11	0.39	0.11	1.37	2.67	2.33	2.70	5.12
	568.0	-3.00	-0.81	-4.73	-2.25	-4.73	-3.55	3.75	0.62	-2.76	0.23
Naringin	6.1	-1.65	2.70	-1.96	1.63	-1.96	-4.90	-1.73	-0.49	2.61	-2.83
	24.4	3.95	4.90	-4.56	-2.73	-4.56	-2.73	1.04	2.86	-4.69	-6.12
	97.7	0.07	2.03	-5.11	-0.96	-5.11	1.58	-4.46	-2.31	-0.45	0.93
	568.0	-4.10	1.68	-4.52	-1.45	-4.52	-4.81	1.20	-3.06	-2.08	-1.56

2, 4, 8, 12 h) DCQD was given. After centrifugation at 3000 r/min for 15 min, the plasma samples were stored at -80°C for analysis.

Rats in SAP and control groups were fed with laboratory rodent chow by gavage. Concentration of DCQD components in plasma was measured by HPLC-TMS. Concentration-time curves were plotted for various components from DCQD.

### Assay procedure

HPLC-TMS for simultaneous determination of the six components has been validated in our laboratory<sup>[11,14]</sup>. Plasma samples were spiked with the IS (ibuprofen), acidified by HPLC and extracted twice using ethyl acetate. The HPLC-TMS system was operated under MRM modes using electrospray ionization in the negative ion mode.

### Data collection and analysis

Data collection, peak integration and calibration were performed with Analyst 1.4.2 software. Calibration curves were plotted according to the peak area ratio of analytes to ISs, and the linear regression between plasma concentration and peak area ratio was weighed by  $1/x^2$ . Concentrations of QC and unknown samples were measured by interpolation from the calibration curves. Drug and statistics software programmed by the Chinese Pharmacological Society was used to process the plasma concentration data and compartment model fitting and then all the pharmacokinetic parameters were figured out. The results were expressed as mean  $\pm$  SD. The pharmacokinetic parameters of each DCQD component were compared with statistical software PEMS3.1 and

the difference was compared by sample pairing and *t*-test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Rhein in rats after a single dose of DCQD by gavage

The mean plasma concentration of rhein was obviously higher, the peak time ( $T_{max}$ ) of rhein was significantly shorter while the  $T_{1/2\alpha}$  was significantly higher, and the clearance rate (CL/F) and AUC of rhein were obviously lower in SAP group at each time point than in control group within 12 h after treatment with DCQD, suggesting that acute pancreatitis can impact the absorption, distribution and elimination of rhein in rats (Figure 3, Table 3).

### Rheochrysidin in rats after a single dose of DCQD by gavage

The mean plasma concentration of rheochrysidin was significantly higher, the  $T_{max}$  of rheochrysidin was significantly shorter, and the  $T_{1/2\alpha}$  was significantly higher in SAP group at each time point than in control group within 12 h after treatment with DCQD, demonstrating that acute pancreatitis can affect the absorption distribution and excretion of rheochrysidin in rats (Figure 3, Table 3).

### Chrysophanol in rats after a single dose of DCQD by gavage

The mean plasma concentration of chrysophanol was obviously lower, the  $T_{max}$  of chrysophanol was significantly longer, the  $C_{max}$  and AUC of chrysophanol were significantly lower, the  $T_{1/2\alpha}$  was significantly higher, and the CL/F was lower in SAP group than in control group

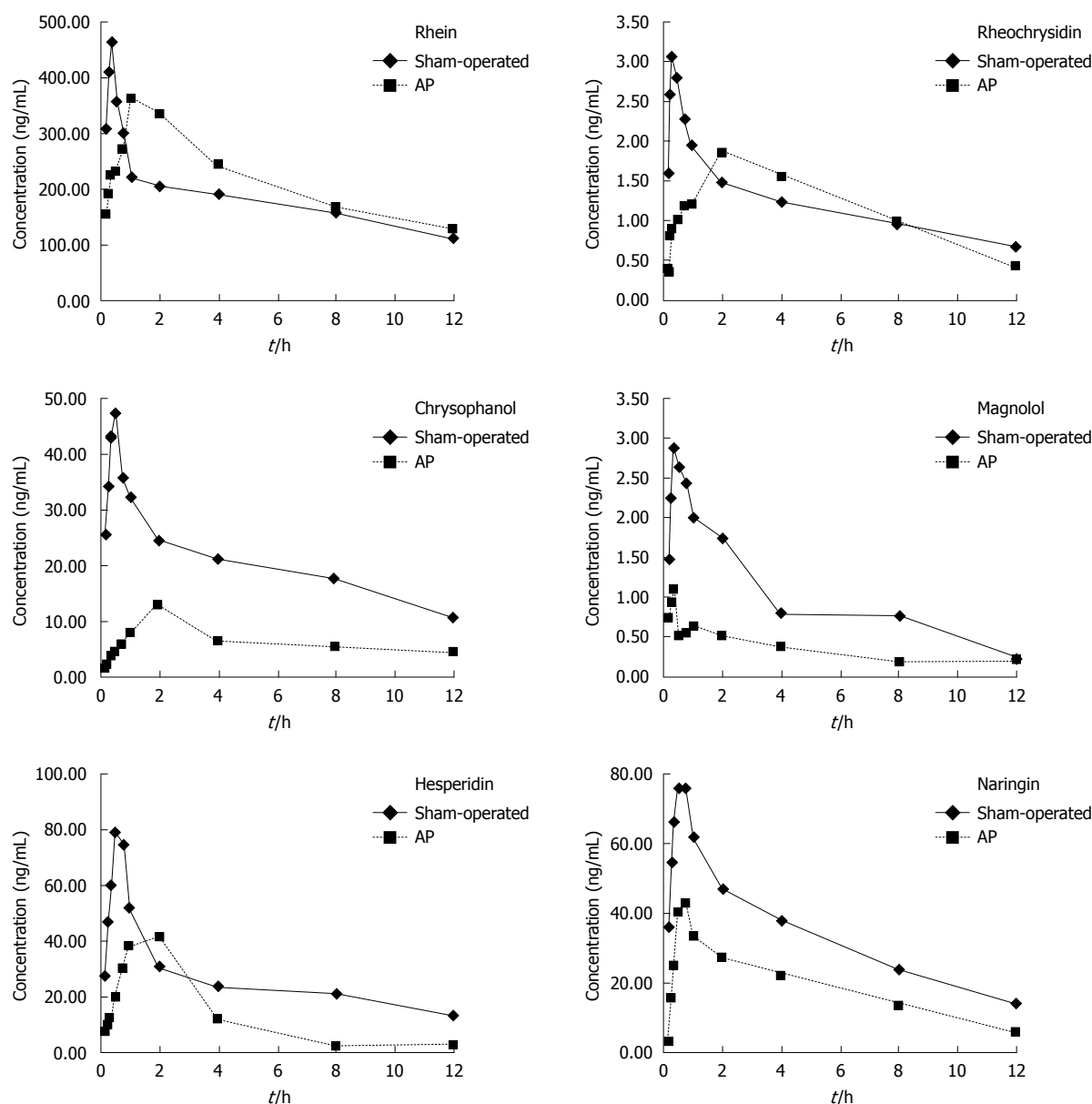


Figure 3 Plasma concentration-time curves for DCQD components in rats of the two groups ( $n = 6$ ).

with no difference in the mean retention time (MRT) between the two groups within 12 h after treatment with DCQD, suggesting that SAP can affect the absorption, distribution, and elimination of chrysophanol in rats (Figure 3, Table 3).

#### **Magnolol in rats after a single dose of DCQD by gavage**

The mean plasma concentration of magnolol was obviously lower, the  $C_{max}$  and AUC of magnolol were obviously lower while the  $T_{1/2\alpha}$ , MRT and  $T_{max}$  were similar between the two groups within 12 h after treatment with DCQD, suggesting that SAP can significantly affect the bioavailability of magnolol (Figure 3, Table 4).

#### **Hesperidin in rats after a single dose of DCQD by gavage**

The mean plasma concentration of hesperidin was obviously higher, the  $T_{max}$  of hesperidin was significantly longer, the  $C_{max}$  and AUC of hesperidin were significantly lower in

SAP group at each time point in control group within 12 h after treatment with DCQD, showing that acute pancreatitis can impact the absorption and distribution and pharmacokinetics of hesperidin in rats (Figure 3, Table 4).

#### **Naringin in rats after a single dose of DCQD by gavage**

The mean plasma concentration of naringin and the  $C_{max}$  and AUC of naringin were obviously lower in SAP group at each time point than in control group within 12 h after treatment with DCQD, revealing that acute pancreatitis can impact the absorption, distribution and bioavailability of naringin in rats (Figure 3, Table 4).

## **DISCUSSION**

In the present study, the early absorption of each DCQD component tended to degrade in SAP group, the  $C_{max}$  and AUC of DCQD components such as chrysophanol,



**Table 3** Twelve-hour pharmacokinetic parameters of DCQD components in rats of the two groups ( $n = 6$ )

	$T_{1/2\alpha}$ (h)	CL/F (L·h per kg)	AUC <sub>(0-∞)</sub> (μg/L per hour)	MRT <sub>(0-t)</sub> (h)	$T_{max}$ (h)	C <sub>max</sub> (μg/L)
Rhein						
Sham	0.33 ± 0.13	4.03 ± 1.38	4720 ± 1514	8.86 ± 0.62	0.36 ± 0.11	510 ± 283
AP	4.367 ± 2.33	0.133 ± 0.06	2870 ± 563	7.82 ± 3.37	1.75 ± 1.25	479 ± 126
<i>t</i>	4.2721	6.9106	2.8054	0.7513	2.7242	0.2448
<i>P</i>	0.0016	0	0.0186	0.4698	0.0214	0.8116
Rheochrysidin						
Sham	0.354 ± 0.302	0.356 ± 0.14	16.047 ± 6.08	4.189 ± 0.463	0.569 ± 0.26	3.86 ± 1.09
AP	1.464 ± 0.449	0.328 ± 0.109	16.63 ± 5.06	4.34 ± 0.29	1.5 ± 0.55	2.58 ± 0.58
<i>t</i>	5.0247	0.3866	0.179	0.6929	3.7597	2.5511
<i>P</i>	0.0005	0.7072	0.8615	0.5041	0.0037	0.0288
Chrysophanol						
Sham	0.38 ± 0.27	24.32 ± 9.65	461.3 ± 188.7	8.49 ± 0.93	0.59 ± 0.24	53.02 ± 21.9
AP	0.89 ± 0.48	0.095 ± 0.035	115.8 ± 34.89	10 ± 5.1	1.33 ± 0.52	17.59 ± 10.5
<i>t</i>	2.2683	6.0994	4.3965	0.7088	3.165	3.4855
<i>P</i>	0.0467	0.0001	0.0013	0.4947	0.0101	0.0059

**Table 4** Twelve-hour pharmacokinetic parameters of DCQD components in rats of the two groups ( $n = 6$ )

	$T_{1/2\alpha}$ (h)	AUC <sub>(0-∞)</sub> (μg/L per hour)	MRT <sub>(0-t)</sub> (h)	$T_{max}$ (h)	C <sub>max</sub> (μg/L)
Magnolol					
Sham	1.58 ± 1.06	24.89 ± 9.87	6.6 ± 1.85	0.71 ± 0.25	3.47 ± 2.13
AP	1.11 ± 0.48	5.739 ± 1.888	9.202 ± 2.27	0.428 ± 0.282	1.491 ± 0.596
<i>t</i>	1.0821	4.6204	2.2156	1.8329	2.2431
<i>P</i>	0.3046	0.001	0.0511	0.0967	0.0487
Hesperidin					
Sham	0.45 ± 0.25	479.39 ± 225.94	5.62 ± 2.45	0.67 ± 0.13	89.38 ± 25.02
AP	0.69 ± 0.36	162.98 ± 69.76	4.93 ± 2.233	1.25 ± 0.59	53.7 ± 22.026
<i>t</i>	1.3413	3.2953	0.5133	2.3141	2.5744
<i>P</i>	0.2095	0.0081	0.6189	0.0432	0.0277
Naringin					
Sham	1.47 ± 1.57	623.24 ± 332.55	6.43 ± 2.1	0.64 ± 0.24	88.23 ± 23.66
AP	1.1 ± 0.7	267.68 ± 53.65	7.12 ± 1.96	0.83 ± 0.13	45.13 ± 9.59
<i>t</i>	0.5272	2.5852	0.5659	1.732	4.0511
<i>P</i>	0.6095	0.0272	0.5839	0.1139	0.0023

magnolol, hesperidin and naringin were obviously lower in SAP group than in control group, suggesting that lack of an effective blood volume and a systematic inflammatory response to organ damage in SAP rats would affect the distribution, metabolism and excretion of DCQD components<sup>[15]</sup>.

No significant difference was found in  $T_{1/2\alpha}$  and  $T_{max}$  of DCQD components such as magnolol and naringin between the two groups, which may be due to the way of modeling experiments. Rats were anaesthetized with ethyl ether and recovered 10 min later with free activity. Two hours after treatment with DCQD, the rats became conscious and maintained normal physiology, indicating that influence of anesthesia on physiology and pharmacokinetics in rats can be ignored<sup>[16]</sup>.

However, the  $T_{1/2\alpha}$  and  $T_{max}$  of rhein, rheochrysidin and chrysophanol were longer in SAP group in control group. In addition, the absorption of DCQD components was greatly affected by variant molecular constitutions and lower pH of SAP rats *in vitro*.

In summary, SAP can obviously impact the absorption and pharmacokinetic parameters of DCQD containing rhein, chrysophanol, rheochrysidin, magnolol, hesperidin and naringin in rats.

## COMMENTS

### Background

Severe acute pancreatitis (SAP), characterized by intricate mechanism, variant symptoms, grave prognosis and multiple complications, seriously threatens the life of patients and brings a heavy burden to the society, families and economy. Additionally, either standard treatment or other medications for SAP is available at present. In China, clinical and experimental researches on Da-Cheng-Qi Decoction (DCQD) have shown that DCQD is a valid prescription for the treatment of SAP.

### Research frontiers

SAP, similar to Yangming Fushi Syndrome (YMFSS) according to the traditional Chinese medicine, has been treated with purgative herbals throughout China for more than three decades. However, no studies are available on the pharmacokinetics of DCQD components in rats with acute pancreatitis.

### Innovations and breakthroughs

According to the theory "syndrome and treatment pharmacokinetics" in traditional Chinese medicine, YMFSS should influence the pharmacokinetics of DCQD, which has, however, not been proved experimentally up to date. This is the first study to report the effect of acute pancreatitis on the pharmacokinetics of DCQD components in rats.

### Applications

Acute pancreatitis was found to have certain effects on the pharmacokinetics of DCQD components in rats, showing that DCQD can be used in treatment of SAP.

### Peer review

The authors investigated the effect of acute pancreatitis on the pharmacokinetics of DCQD components in rats, which may contribute to the treatment of SAP.

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CASE REPORT

## Complete response to radiation therapy of orbital metastasis from hepatocellular carcinoma

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### INTRODUCTION

According to Surveillance, Epidemiology, and End Results data, the incidence of hepatocellular carcinoma (HCC) has been steadily increasing since the mid-1980s and thus presents an increasing health problem in the United States. The average, age-adjusted incidence rates for liver and intrahepatic bile duct cancer, two-thirds of which are HCC, rose from 3.2 per 100 000 persons in 1985 to 6.4 per 100 000 persons in 2005<sup>[1,2]</sup>. The incidence is 3-4 times higher in men than in women and is highest in the Asian population<sup>[2]</sup>. The prognosis for patients diagnosed with HCC is dismal with 5-year survival rates of 3%-5%<sup>[3]</sup>.

Approximately 50%-75% of patients with HCC will develop metastases during the course of their disease<sup>[4,5]</sup>. The most common sites of metastatic disease are the regional lymph nodes and lung. Less common sites of metastases include bone, brain, adrenal glands, and skin<sup>[4,6-9]</sup>. The orbit has been reported as a site of metastasis from HCC only 14 times in the literature, and information on the palliative response of this highly symptomatic condition with radiation therapy has been very sparse. We report a case of a patient with an orbital metastasis from HCC, who achieved a complete clinical and radiographic response to intensity modulated radiation therapy (IMRT).

### CASE REPORT

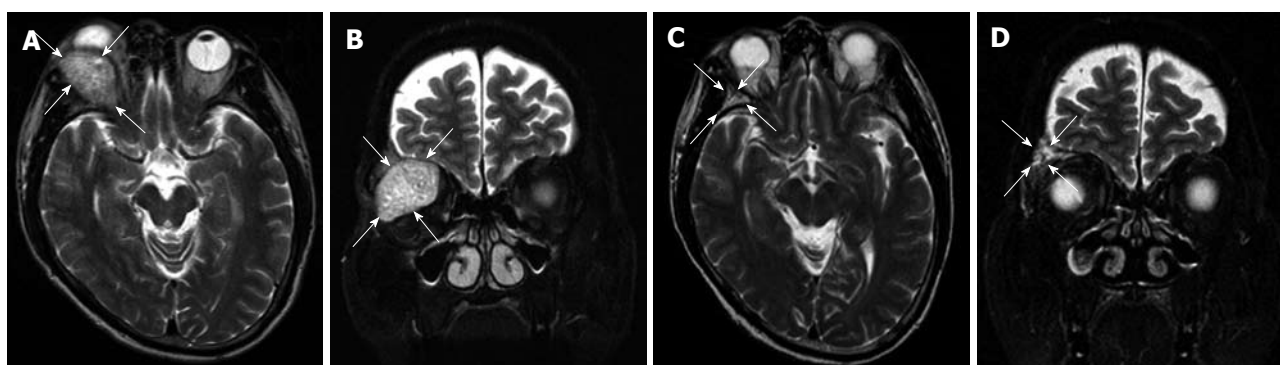
A 52-year-old Asian male with a history of hepatitis C but no known cirrhosis presented with elevated liver enzymes

### Abstract

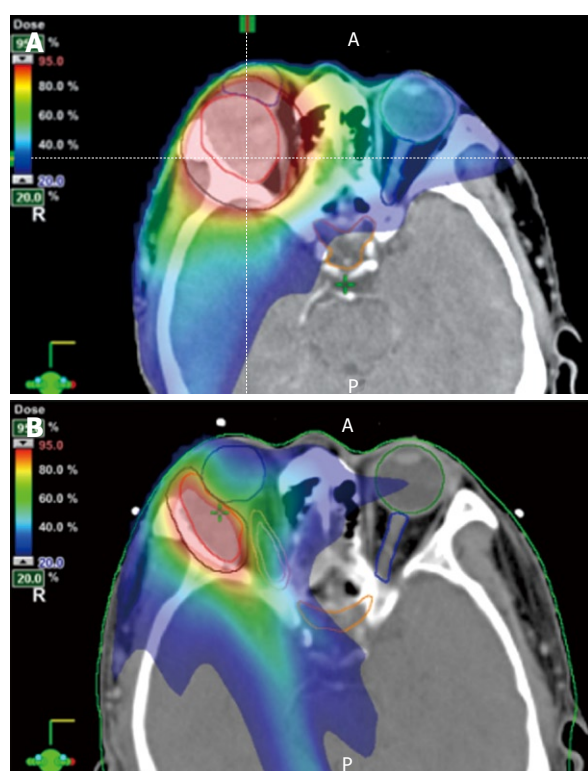
The incidence of hepatocellular carcinoma (HCC) is increasing in the United States, and 50%-75% of patients with HCC will develop metastatic disease. Orbital metastases from HCC are extremely rare. We report the case of a 52-year-old male with known metastatic HCC, who presented with severe proptosis and diplopia. An orbital mass was identified on magnetic resonance imaging (MRI) and confirmed to have hypermetabolic activity on positron emission tomography/computed tomography. He received a palliative course of external beam radiation therapy to the right orbit. Intensity modulated radiation therapy (IMRT) was used to allow sparing of critical normal tissues in close proximity to the tumor. One month after completion of IMRT to 58 Gray in 30 fractions delivered over 6 wk, the patient had a complete clinical, radiologic (MRI) and symptomatic response. The patient continues to have local control in the orbit 1.7 years after therapy completion. All critical normal structures were kept below the tolerance dose using IMRT, and no toxicities were observed.

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**Key words:** Hepatocellular carcinoma; Eye neoplasms;



**Figure 1** Axial (A) and coronal (B) T2 weighted MRI of the brain demonstrating the large soft tissue mass in the superior and lateral aspect of the right orbit (white arrows) prior to radiation treatment and resolution of mass on follow-up MRI 12 mo after treatment on axial (C) and coronal images (D).



**Figure 2** Three-dimensional contours for treatment planning demonstrate the gross target volume (GTV) encompassing the tumor (red contour) and the planning target volume (PTV, black contour) on initial (A) and boost (B) CT images. Doses from the initial 7 field intensity modulated radiation (IMRT) plan (A) are represented as color wash according to the scale shown in the figures, representing the range of 95% through 20% of dose coverage. The 7 field IMRT plan for the coned-down field (B) shows even tighter dose coverage and greater sparing of the right optic nerve (pink contour).

while being treated for polycythemia. High resolution triphasic spiral computed tomography (CT) scan of the abdomen demonstrated a well encapsulated, heterogeneous mass in the right lobe of the liver measuring 8.2 cm × 8.8 cm. Imaging characteristics were typical for HCC and  $\alpha$ -fetoprotein was 30 ng/mL. Percutaneous biopsy confirmed HCC in a background of cirrhosis. Exploratory laparotomy with intraoperative ultrasound suggested the need for extended hepatectomy for a curative approach but the volume of the future liver remnant was less than 30%. As such, surgical resection was not completed at

that time. Selective right transarterial chemoembolization, followed by portal vein embolization (PVE) of segments 4-8 was undertaken to promote atrophy of the tumor-bearing liver and hypertrophy of the future liver remnant. Four weeks following PVE, resection was again attempted but metastasis was identified in the future liver remnant. No extrahepatic disease was noted. He was subsequently treated with radioembolization using Yttrium-90.

Shortly after the Yttrium-90 therapy, he developed rapidly progressive diplopia and proptosis of the right eye. Physical examination showed severe proptosis, conjunctival hyperemia, excessive tearing, impaired vision, and limited extraocular muscle movement of the right eye. Magnetic resonance imaging (MRI) of the brain and orbits identified an extraconal 3.7 cm × 3.3 cm × 3.7 cm enhancing mass arising from the floor of the right anterior cranial fossa with extension to the right orbit, resulting in mass effect on the superior and lateral rectus muscles and globe (Figure 1). Positron emission tomography (PET)/CT confirmed a right orbital mass with a peak standardized uptake value (SUV) of 2.8.

He received a course of palliative radiation therapy to a dose of 40 Gray (Gy) in 20 fractions to the right orbit using 6 MV photons and IMRT (Figure 2A). The total gross tumor volume (GTV) was 15.8 cm<sup>3</sup>. A CT scan 10 d after treatment completion showed no response. MRI 1 mo after treatment completion showed the tumor to be decreased from the initial 3.7 cm × 3.3 cm × 3.7 cm to 3.7 cm × 2.8 cm × 3.7 cm. Clinical improvement in the proptosis was also observed. An IMRT boost to the right orbit of an additional 18 Gy in 10 fractions was then delivered (Figure 2B). The total volume of the GTV had decreased to 8.8 cm<sup>3</sup>. The total dose delivered was 58 Gy in 30 fractions over 86 d. The dose to the critical normal structures was kept below the tolerance dose and is presented in Table 1.

One month after receiving 58 Gy to the orbit, the patient reported complete resolution of the diplopia. On examination, his right extraocular muscles functioned normally and proptosis was no longer present. Follow-up PET/CT 1 mo after treatment completion showed decreased fluoro-deoxyglucose activity (peak SUV = 2.2) within the superolateral aspect of the right orbit. MRI of the brain 3, 12 and 17 mo after treatment demonstrated



**Table 1** Critical structures mean dose for original and boost IMRT plans

Structure	Original mean dose (Gy) (2 Gy/fx)	Boost mean dose (Gy) (1.8 Gy/fx)	Total dose (Gy)
Chiasm	6.98	3.55	10.52
Left eye	15.93	1.42	17.34
Left optic nerve	13.3	1.96	15.26
Right eye	30.7	3.96	34.65
Right optic nerve	34.09	6.46	40.55

IMRT: Intensity modulated radiation therapy; Gy: Gray.

complete resolution of the lesion (Figure 1). The patient is alive with controlled orbital disease 20 mo after the initial diagnosis of the orbital metastasis and 26 mo after his original HCC diagnosis. As a result of progression in the liver following Yttrium-90 therapy, he is currently receiving Sorafenib with effective systemic disease control.

## DISCUSSION

Orbital metastases are uncommon and account for 3%-7% of all orbital neoplasms<sup>[7,10,11]</sup>. The most common symptoms of orbital metastases include pain, proptosis, decreased vision or blindness, diplopia, displacement of the globe, exophthalmos, and occasionally enophthalmos. HCC very rarely metastasizes to the orbits, and only a total of 14 case reports of orbital metastases from HCC have been described (Table 2)<sup>[4-15]</sup>. Orbital metastases are usually associated with advanced disease and early mortality. The average survival after occurrence of the orbital metastases is approximately 10 mo<sup>[11]</sup>, although the prognosis ultimately depends on the systemic tumor burden<sup>[5]</sup>.

Among the 14 reported cases of orbital metastases from HCC (Table 2)<sup>[4-15]</sup>, only 4 received primary radiation as the palliative treatment in doses ranging from 30 to 54 Gy, and all showed a response. However, specific details of the radiation planning and treatment delivery in this challenging location are not available. Long-term effects of treatment are poorly understood because only one of the patients survived longer than 1 year after treatment<sup>[11,13]</sup>.

Our patient received a higher dose of radiation than used in the above case reports, and was the first to use IMRT for treatment of orbital HCC metastasis. The orbit is among the most challenging regions for radiation therapy because of the close proximity of dose-limiting critical normal structures, including the brain, ocular structures, and optic chiasm, that can all develop significant complications. IMRT enabled highly conformal treatment delivery and resulted in a durable complete radiologic response.

Our findings are in contrast to the common belief that HCC is a radioresistant tumor. Such reports frequently had to rely on older, less targeted radiation therapy techniques that were unable to spare normal tissues, thus severely limiting the deliverable radiation dose to the tumor to avoid serious toxicity to normal structures. This low tumor dose was not adequate to achieve a significant tumor response<sup>[16]</sup>. Our case shows that dose escalation to 58 Gy, enabled by IMRT, can afford effective local control in

**Table 2** Case reports of orbital metastases from hepatocellular carcinoma

Author	Gender	Age (yr)	Treatment	Survival
Gupta <i>et al</i> <sup>[10]</sup>	M	45	None	NA
Loo <i>et al</i> <sup>[8]</sup>	F	71	Transcranial orbitotomy	3 mo
Schwab <i>et al</i> <sup>[12]</sup>	M	19	Anterior orbitotomy with biopsy	2 wk (moribund state)
Wakisaka <i>et al</i> <sup>[9]</sup>	M	58	Left frontotemporal craniotomy	11 mo
Lubin <i>et al</i> <sup>[22]</sup>	M	69	3000 cGy in 2 wk	NA
Zubler <i>et al</i> <sup>[5]</sup>	M	64	4000 cGy over 8 wk + chemotherapy	3 mo
Srinivasan <i>et al</i> <sup>[4]</sup>	F	76	None	NA
Scolyer <i>et al</i> <sup>[23]</sup>	M	77	None	NA
Font <i>et al</i> <sup>[13]</sup>	F	79	Palliative RT	3 yr
Kim <i>et al</i> <sup>[6]</sup>	F	56	None	2 mo
Machado-Netto <i>et al</i> <sup>[11]</sup>	M	57	Megestrol acetate and Gemcitabine	15 mo
Hirunwiwatkul <i>et al</i> <sup>[7]</sup>	F	74	NA	2 mo
Tranfa <i>et al</i> <sup>[14]</sup>	M	85	Anterior orbitotomy with excisional biopsy	NA
Phanthumchinda <i>et al</i> <sup>[15]</sup>	F	29	5400 cGy in 4 wk	NA

NA: Not available; RT: Radiation therapy; F: Female; M: Male.

HCC. Our observations are supported by studies showing a dose-response relationship for treatment of metastases from HCC. In a retrospective review by Park *et al*<sup>[17]</sup>, 91% of patients with intraabdominal lymph node metastases from HCC treated to  $\geq 50$  Gy<sub>10</sub> had an objective response compared to 65% of patients treated to lesser doses. Recent studies have also shown excellent local control and improved survival with the use of higher doses for primary HCC, delivered with conformal radiation therapy to the partial liver, that were previously intolerable<sup>[18,19]</sup>.

The tumor response in our patient is also characterized by a protracted time course. Symptoms improved slowly, and not until a treatment break and re-imaging 1 mo after a dose of 40 Gy, was the radiologic response evident. Such a slow response pattern may also have led to the conclusion that HCC is not a radio-sensitive tumor. However, the GTV reduction after a 5 wk break following 40 Gy allowed us to further escalate the dose to the orbital tumor, while effectively sparing the sensitive normal structures, especially the right optic nerve (Figure 2B). In conjunction with the highly conformal IMRT delivery, this interval tumor reduction and dose escalation resulted in a durable complete clinical and radiologic response.

Our case also illustrates the importance of 3-dimensional (3D) volumetric analysis of tumor imaging instead of diameter-based measurements for the assessment of tumor response. In the repeat CT for boost planning after 40 Gy, 3D tumor volumetry, determined by tumor delineation on each imaging slice and computation of the volume, demonstrated a reduced tumor volume from 15.8 to 8.8 cm<sup>3</sup>, a change of 44%. However, the diagnostic brain MRI 1 wk previously showed a decrease from 23.7 to 20.1 cm<sup>3</sup> in MRI diameter-based tumor volume

calculation, a change of 15%. Other studies have also shown that diameter-based measurements overestimate tumor size during and after radiation therapy compared to 3D volumetry. This is likely related to the irregular tumor configurations and non-linear tumor shrinkage, that are not adequately assessed by current gold standard diameter-based measurements<sup>[20]</sup>. The high precision of refined 3D volumetry-based measurements, which are easily obtained from treatment planning systems, can overcome the challenge of irregular tumor configuration<sup>[21]</sup>.

In conclusion, because of the increasing incidence and improvement in systemic treatment of primary HCC, the prevalence of symptomatic metastases from HCC will likely increase. Radiation therapy is an excellent treatment option for palliation of challenging metastatic sites, including the orbit, but higher doses than the typical 30 Gy in 10 fractions may be required. With targeted radiation techniques, such as IMRT, that enable sparing of normal critical structures, and 3D volumetric assessment of the response, tumor volume-adapted dose escalation to optimal tumoricidal dose levels can provide durable effective palliation of debilitating symptoms.

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## CASE REPORT

# Superior mesenteric artery syndrome in a diabetic patient with acute weight loss

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## INTRODUCTION

Superior mesenteric artery (SMA) syndrome, also known as Wilkie's syndrome or cast syndrome, is an uncommon disease resulting from superior mesenteric artery compression of the third portion of the duodenum. The clinical manifestations include postprandial fullness or pain, nausea, vomiting, and anorexia due to duodenal obstruction<sup>[1]</sup>. However, the symptoms are similar to those of diabetic gastrointestinal complications. Therefore, SMA syndrome could be misdiagnosed as diabetic gastroparesis. The delayed diagnosis of SMA syndrome might result in malnutrition, electrolyte imbalance, dehydration, and even death.

## CASE REPORT

This 41-year-old male was diagnosed type 2 diabetes mellitus four years ago, but the disease was poor controlled. He did not take any oral antidiabetic agent or insulin therapy for one year. He visited our emergency room complaining of abdominal discomfort and vomiting 30 min after meals for 1 wk. The associated symptoms included general weakness, bilateral lower leg numbness, and a gradual bodyweight loss of 26 kg over the last 3 mo. Physical examination showed a distended abdomen and positive succussion splash sign. His glycemic control was poor and glycosylated hemoglobin (HbA1c) was 11.4%. The abnormal hematologic and biochemical findings included mild anemia (Hgb: 11.7 g/dL) and hypokalemia (K: 3.3 mEq/L). The plain abdomen revealed dilated duodenal bulb and distended stomach with air-fluid level (Figure 1). The gastroduodenoscopy showed a distended stomach with much gastric residue. Therefore, a proximal small bowel obstruction was tentatively diagnosed. To achieve the final diagnosis, we arranged a series of examinations. The upper gastrointestinal series demonstrated a sharp cut-off at the 3rd portion of the duodenum (Figure 2). Compression of the third portion of the duodenum, an

## Abstract

Superior mesenteric artery (SMA) syndrome is an uncommon disease resulting compression of the third portion of the duodenum from the superior mesenteric artery. This disease shares many common manifestations with diabetic gastroparesis, including postprandial fullness, nausea, vomiting, and bloating. Therefore, it is often overlooked in diabetic patients. Here, we report a 41-year-old man with poorly controlled diabetic mellitus who developed SMA syndrome due to rapid weight loss. The diagnosis was confirmed by computed tomography and an upper gastrointestinal series. His condition improved after parenteral nutrient, strict sugar control, and gradual weight gain.

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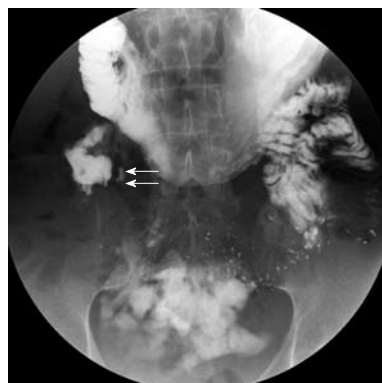
**Key words:** Diabetes mellitus; Superior mesenteric artery syndrome; Gastroparesis

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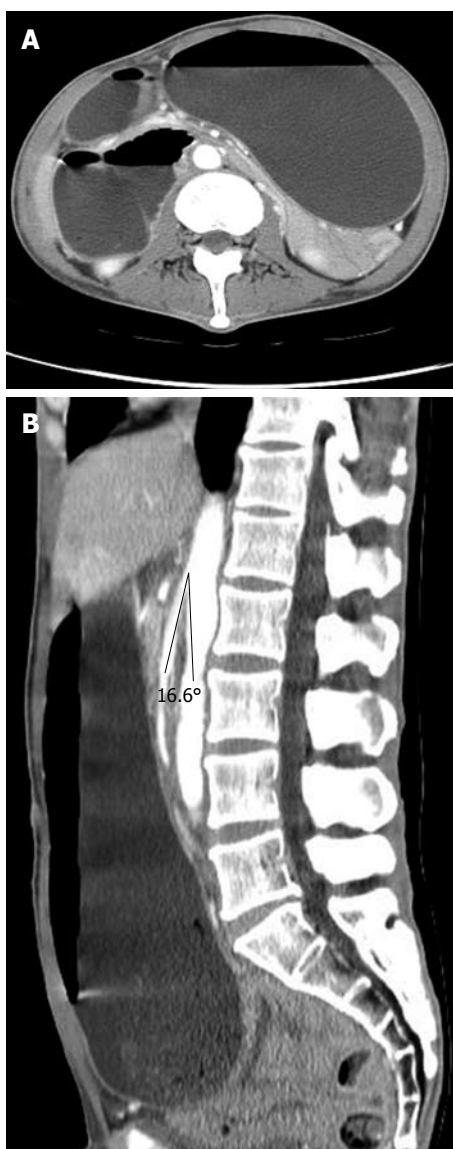




**Figure 1** Abdominal X-ray showing a distended stomach with air fluid level in the stomach and duodenal bulb. The "Double bubble sign" was consistent with high small bowel obstruction.



**Figure 3** Upper gastrointestinal series showing an abrupt cut-off (short arrows) at the third portion of the duodenum.



**Figure 2** Computed tomography (CT) scan showing distended stomach and 2nd portion of duodenum (A); The angle between aorta and superior mesenteric artery (SMA) was 16.6° (B).

aortomesenteric distance of 4.1 mm (normal: 10-28 mm) and a reduction of the aortomesenteric angle 16.6° (normal: 25°-60°) were noted by computed tomography (CT) scan<sup>[2]</sup> (Figure 3). After inserting a nasogastric tube, it drained over 3000 mL turbid, green fluid. His

symptoms improved after nasogastric tube drainage and kept the left lateral decubitus position. We gave him total parenteral nutrition as a nutrition supply and controlled his sugar with an insulin pump for 2 wk. After 2 mo, his bodyweight increased from 44 to 50 kg and he returned to oral intake without subsequent symptoms.

## DISCUSSION

Diabetes mellitus is one of the most common chronic disease of the world and the prevalence of diabetes mellitus is over 10% in Taiwan<sup>[3]</sup>. Gastroparesis is reported in 5% to 12% of diabetic patients. The cardinal symptoms include postprandial fullness, nausea, vomiting, and bloating. Treatment for gastroparesis is prokinetics including metoclopramide, domperidone, and erythromycin<sup>[4]</sup>. However, SMA syndrome can cause the same symptoms as diabetic gastroparesis.

To our knowledge, only two studies have reported diabetic patients with SMA syndrome and all of them had bodyweight loss<sup>[5,6]</sup>. The average bodyweight loss was 29.6 kg (16-50 kg). One of the reported cases received an exploratory laparotomy and the other received intravenous nutrition treatment. In our report, the patient also had a gradually weight loss of 26 kg and improved after medical treatment. The bodyweight loss is a manifestation of the new diagnosis or the poor control of the diabetic patient. It might result in the delayed or missed diagnosis of the SMA syndrome as diabetic gastroparesis.

SMA syndrome is a disease of duodenal obstruction. Weight loss results in loss of the mesenteric fat pad and the superior mesenteric artery compresses duodenum. Bodyweight loss with superior mesenteric artery syndrome, including eating disorders, cardiac cachexia, HIV patients, hereditary motor and sensory neuropathy, have been reported<sup>[7-10]</sup>. The radiographic studies used to establish diagnosis include an upper gastrointestinal series, computed tomography (CT), CT angiography, conventional angiography, abdominal sonography, and magnetic resonance angiography (MRA)<sup>[2,11-13]</sup>. The prone or left lateral decubitus position is effective in the acute status. Conservative treatment with adequate fluid and electrolyte supply is necessary after nasogastric tube placement. Enteral jejunal tube feeding and parenteral nutrition are useful to increase bodyweight. Surgery is indicated when



the conservative treatment fails<sup>[1]</sup>. Laparoscopic duodeno-jejunostomy has been successful in the cases with SMA syndrome<sup>[14]</sup>.

In conclusion, diabetic patients with gastrointestinal symptoms and bodyweight loss should be considered for SMA syndrome, despite the gastroparesis is the most common etiology. Computed tomography and upper gastrointestinal series are the reliable tools for diagnosis. Adequate nutrition supply is a useful treatment and the aim is bodyweight gain and symptom relief. Surgery is indicated when conservative treatment fails.

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## Aortoesophageal fistula: A case misdiagnosed as esophageal polyp

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### Abstract

Aortoesophageal fistula (AEF) is a rare and fatal disorder. It is also a life-threatening cause of massive upper gastrointestinal hemorrhage. Thoracic aortic aneurysm is the most common cause of AEF. Management of a patient with this disorder requires rapid diagnosis and immediate intervention, which is considered the best way to save the patient's life. We report a case of AEF misdiagnosed as esophageal polyp.

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**Key words:** Aortoesophageal fistula; Aortic aneurysm; Gastrointestinal bleeding

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### INTRODUCTION

Aortoesophageal fistula (AEF) is a rare and fatal disorder, although it was first described by Dubrueil in 1818. It is also a life-threatening cause of massive upper gastrointestinal hemorrhage. Thoracic aortic aneurysm is

the most common cause of AEF. Prompt diagnosis and emergent intervention are substantial to improve the survival of the patients. We report a case of an AEF due to a descending aorta pseudoaneurysm presenting as an esophageal polyp.

### CASE REPORT

A 62-year-old female patient was admitted to our emergency department for an episode of vomiting bright red blood, with melena and dizziness. Her initial vital sign was normal when she was in emergency. She had a history of intermittent slight chest pain for 2 years. A gastroscopy was performed which revealed a 4 cm fusiform polyp in the mid-esophagus (Figure 1). It was also diagnosed as esophageal polyp with endoscopic ultrasound. The patient was advised to take further examinations, but she refused. During the following 2 years, she received gastroscopy twice (Figure 2) which showed an orifice-like lesion on the top surface of the polyp. Barium radiography reported a 2cm filling defect in the same location (Figure 3). This patient had a history of rib fracture 20 years ago, but no hypertension or diabetes.

A subsequent enhanced computed tomography (CT) documented a descending aortic pseudoaneurysm that compressed the mid-esophagus (Figure 4). The gastroscopy taken after hospitalization documented the same result as before, without fresh blood or clot on the orifice. Twelve days later, she suddenly experienced a shock for her second hematemesis. Blood pressure dropped down to zero. Angiography was performed until her hemodynamic status was stabilized after blood transfusion and hemostasis. The contrast extravasated from the descending aorta rupture with a diameter of about 0.5 cm (Figure 5), which established a diagnosis as descending aortic pseudoaneurysm. A covered endovascular stent grafting was placed immediately. After confirmed to be in good condition by CT, she was discharged on the 10th postoperative day and has remained healthy since then.

### DISCUSSION

AEF, which constitutes approximately 10% of aorto-enteric fistulas, is associated with a high morbidity and mortality. Thoracic aortic aneurysm is the most common cause of AEF, other causes include carcinoma, trauma (including iatrogenic trauma), foreign body ingestion

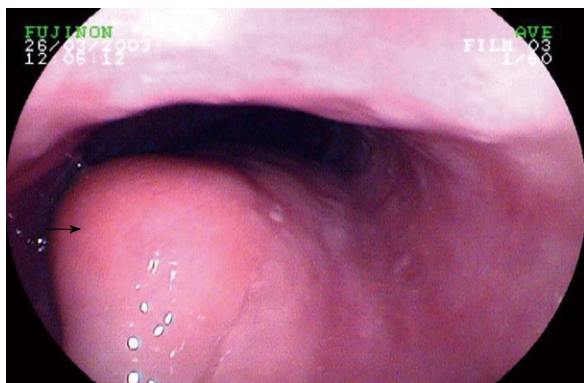


Figure 1 Gastroscopy showing a 4 cm fusiform polyp (black arrow) in mid-esophagus.

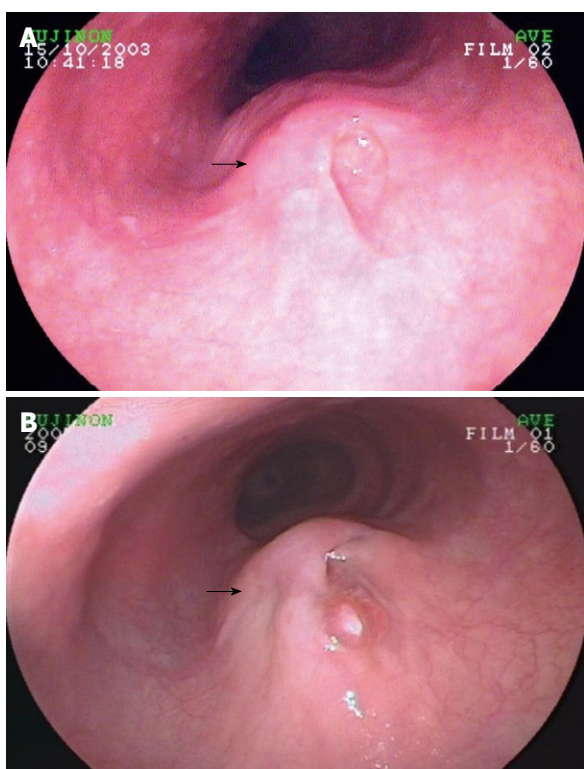


Figure 2 Gastroscopy showing an orifice-like lesion on the top surface of the polyp (black arrows). A: 1st; B: 2nd.

and tuberculous aortitis. Prompt diagnosis and emergent intervention are substantial to improve the survival of the patients. Its classical symptoms, named Chiari's triad, include dysphagia, mid-thoracic pain and sentinel minor hematemesis followed by exsanguination, as did our patient. Mid-thoracic pain may be caused by distension and dissection of the aortic wall, esophageal perforation, or tumor invasion. Spontaneous cessation of the sentinel minor hemorrhage may be caused by temporary occlusion of the fistula due to spasm of the arterial wall and/or periaortic hematoma, which is later digested by infection or gastrointestinal contents. Fifty-nine percent of the AEF patients had mid-thoracic pain, 45% experienced dysphagia, 65% had sentinel minor hematemesis, and 45% showed Chiari's triad<sup>[1]</sup>. Thus, intensive inquiry



Figure 3 Barium radiography reported a 2 cm filling defect (black arrow) in the mid-esophagus.



Figure 4 A subsequent enhanced computed tomography (CT) showing a descending aortic pseudoaneurysm (black arrow).



Figure 5 Aortography. The contrast extravasated from the descending aorta rupture with a diameter of about 0.5 cm (black arrow).

of patient's history and careful physical examination are necessary if AEF is suspected.

Early diagnosis is the key point to decrease the mortality from AEF. Endoscopy is always the first examination to be chosen. Endoscopy usually reveals esophageal compression in the mid-upper esophagus or a submucosal hematoma with bluish grey mucosa indicating the aorta wall. A pulsating mass covered with blood clots or a fistula opening is seldom seen. In our case, one of the causes for misdiagnosis is that gastroscopy showed a polyp with an orifice, without bluish grey mucosa or blood clot. However, whether gastroscopy is safe enough to AEF is still controversial. In our opinion, gastroscopy can be performed carefully for upper gastrointestinal

hemorrhage, except the definitive AEF is confirmed by CT or aortography<sup>[2,3]</sup>.

Subsequent CT or computed tomographic angiography, which is an accurate and non-invasive method for diagnosing AEF, can demonstrate the location of an aneurysm and its surrounding structures, especially esophagus. Barium radiography may display thoracic aortic aneurysm as an extrinsic compression<sup>[3]</sup>. Aortography is used for diagnosing the aneurysm, but rarely shows a fistula itself because of lacking blood. Moreover, aortography may provide more opportunities for endovascular aortic stenting in patients who are not actively bleeding<sup>[4,5]</sup>.

After definitive diagnosis, immediate repair is mandatory as surgical repairs offer the only chance to cure the patients with aneurysm and esophageal erosion. Surgery includes thoracic aorta replacement with a synthetic graft, use of cryopreserved arterial allografts and extra-anatomic bypass. Dacron graft is the most commonly used one. Primary repair and esophageal resection should also be done to avoid the complications of infection. In recent years, endovascular stent grafting has been used as an alternative to surgical treatment, but it has some limitations such as AEF remaining as it is, insufficient debridement or drain of mediastinum. Therefore, some authors suggested that an endovascular stent graft should only be used in patients with a high risk of open surgery<sup>[2,6]</sup>. Since 2005, Pirard *et al*<sup>[7,8]</sup> have attempted to combine endovascular with open surgical approach, which brings more hope to decrease the mortality of AEF.

In conclusion, AEF is an uncommon and life-threatening cause of upper gastrointestinal bleeding. The classical clinical triad, such as mid-thoracic pain or dysphagia,

a sentinel minor hematemesis followed by exsanguinations and gastroscopy will give a hint to suspected AEF, and thoracic CT scan or aortography can confirm this diagnosis. Surgery or endovascular stent grafting should be chosen individually. However, rapid diagnosis and immediate intervention are considered the best ways to save the patient's life.

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LETTERS TO THE EDITOR

## Frequency of alcohol and smoking cessation counseling in hepatitis C patients among internists and gastroenterologists

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### TO THE EDITOR

We read with interest the article by Scognamiglio *et al*<sup>[1]</sup> "Impact of hepatitis C virus infection on lifestyle", *World J Gastroenterol* 2007; 13(19): 2722-2726, which reveals that a greater number of patients with hepatitis C virus (HCV) infection could modify their behavior and lifestyle habits with regard to alcohol consumption when compared to tobacco use after the diagnosis of HCV infection. The authors also emphasized the importance of counseling patients about the effects of both tobacco and alcohol use on the liver in patients with HCV infection. Tobacco use and alcohol consumption alone have been shown to accelerate the progression of hepatitis C toward chronic hepatitis<sup>[1-3]</sup>. Furthermore, multiple studies have shown a synergistic effect of alcohol and tobacco use on the progression of hepatitis C to hepatocellular carcinoma (HCC)<sup>[1,4]</sup>. Independently, tobacco use is also associated with a decreased response to interferon treatment<sup>[1,3]</sup>. Given the above evidence of lifestyle factors on progression of hepatitis C toward chronic hepatitis or HCC, it is imperative that patients with HCV infection not only receive counseling on alcohol consumption but also on tobacco use. We evaluated the frequency of alcohol and smoking counseling of patients with HCV infection by their primary care internists and gastroenterologists.

A retrospective medical record review of patients with HCV infection who were referred by internists to gastroenterologists for management of their liver disease was conducted. The records were evaluated for documentation of alcohol consumption, tobacco use and physician counseling during an evaluation by internists or during consultation by gastroenterologists. One hundred

### Abstract

Given the overwhelming evidence that both alcohol consumption and smoking accelerate the progression of hepatitis C virus (HCV)-induced liver disease, we evaluated the frequency of alcohol and smoking counseling of patients with HCV-induced liver disease by their primary care internists and gastroenterologists. One hundred and twenty-three medical records of consecutive patients with HCV-induced liver disease referred by an internist to a gastroenterologist for its management were reviewed. Patient gender, race, history of and counseling against alcohol and tobacco use by a physician and a gastroenterologist were obtained. A database was created using Microsoft Excel. There were 105 African-Americans, 12 Caucasians and six patients of other races/ethnicities. Forty-six (37%) patients were daily tobacco users and 34 (28%) patients were daily alcohol consumers. There was a statistically significant difference in the frequencies of alcohol ( $P = 0.0002$ ) and smoking cessation ( $P = 0.0022$ ) between gastroenterologists and internists. This study reveals that internists and gastroenterologists, alike, inadequately counsel patients with hepatitis C about tobacco and alcohol use.

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**Key words:** Alcohol; Hepatitis C virus; Counseling; Smoking; Hepatocellular carcinoma

and twenty three records (75 females, 48 males) were reviewed. There were 105 African-Americans, 12 Caucasians and six patients of other races. Of the 123 patients, 36 (29%) were admitted to smoking, 24 (20%) reported daily alcohol consumption, and 10 (8%) were using both tobacco and alcohol. Ten of the 36 patients who were admitted due to tobacco use were counseled by their primary care internist about the dangers of smoking and were offered assistance in cessation. None of the patients who used tobacco were counseled by their gastroenterologist on the effects of smoking. There was a statistically significant difference ( $P = 0.0022$ ) between the internists' and gastroenterologists' frequencies of consultation on the effects of smoking. Of the 24 patients who drank alcohol daily, 14 (17%) were counseled about the effects of alcohol on the liver by gastroenterologists. Only one of the daily alcohol consumers was counseled about alcohol use by their internists ( $P = 0.0002$ ). There was a statistically significant difference in the frequency of alcohol counseling between gastroenterologists and internists.

It is essential that physicians counsel patients on the effects of both tobacco and alcohol use in the setting of HCV infection<sup>[1]</sup>. Additionally, the discrepancy between the frequencies of addressing smoking and alcohol cessation in patients with HCV infection by internists and gastroenterologists is interesting. This study is important because it reveals that physicians inadequately counsel patients with HCV infection about tobacco and alcohol use despite the overwhelming evidence that these factors accelerate the progression of HCV-induced liver disease toward chronic hepatitis or HCC. The potential fragmentation of counseling may be due to a presumed transfer of responsibility of alcohol counseling by the internist to the gastroenterologist. This can result in decreased counseling by internists about alcohol cessation. Similarly,

gastroenterologists may presume that smoking cessation counseling is the internist's responsibility. It is crucial that efforts are made to ensure that all physicians counsel patients about the effects of alcohol and tobacco use. It is uncertain whether the lapse in counseling is a result of a lack of knowledge about the synergistic effect of tobacco and alcohol use on the progression of HCV-induced liver disease toward chronic hepatitis or HCC, or whether there is simply a failure of both specialties to document their counseling practice.

Although this study is small, it may offer a partial explanation on the findings in study by Scognamiglio *et al*<sup>[1]</sup> on the decreased incidence of smoking modification *vs* alcohol modification in hepatitis C patients. Since hepatitis C treatment is often managed by specialty physicians, alcohol cessation may be emphasized rather than tobacco cessation. Further studies investigating counseling barriers on lifestyle modifications in patients with HCV infection, in both primary care and gastroenterology offices, are necessary to prevent progression of hepatitis C toward an already increasing incidence of HCC.

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## Meetings

### Events Calendar 2009

January 12-15, 2009  
 Hyatt Regency San Francisco, San Francisco, CA  
 Mouse Models of Cancer

January 21-24, 2009  
 Westin San Diego Hotel, San Diego, CA  
 Advances in Prostate Cancer Research

February 3-6, 2009  
 Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)  
 Second AACR Conference  
 The Science of Cancer Health  
 Disparities in Racial/Ethnic Minorities  
 and the Medically Underserved

February 7-10, 2009  
 Hyatt Regency Boston, Boston, MA  
 Translation of the Cancer Genome

February 8-11, 2009  
 Westin New Orleans Canal Place, New Orleans, LA  
 Chemistry in Cancer Research: A  
 Vital Partnership in Cancer Drug  
 Discovery and Development

February 13-16, 2009  
 Hong Kong Convention and  
 Exhibition Centre, Hong Kong, China  
 19th Conference of the APASL  
<http://www.apasl2009hongkong.org/en/home.aspx>

February 27-28, 2009  
 Orlando, Florida  
 AGAI/AASLD/ASGE/ACG Training  
 Directors' Workshop

February 27-Mar 1, 2009  
 Vienna, Austria  
 EASL/AASLD Monothematic:  
 Nuclear Receptors and Liver Disease  
[www.easl.ch/vienna2009](http://www.easl.ch/vienna2009)

March 13-14, 2009  
 Phoenix, Arizona  
 AGAI/AASLD Academic Skills  
 Workshop

March 20-24, 2009  
 Marriott Wardman Park Hotel  
 Washington, DC  
 13th International Symposium on  
 Viral Hepatitis and Liver Disease

March 23-26, 2009  
 Glasgow, Scotland  
 British Society of Gastroenterology  
 (BSG) Annual Meeting  
 Email: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

April 8-9, 2009  
 Silver Spring, Maryland  
 2009 Hepatotoxicity Special Interest  
 Group Meeting

April 18-22, 2009  
 Colorado Convention Center,  
 Denver, CO  
 AACR 100th Annual Meeting 2009

April 22-26, 2009  
 Copenhagen, Denmark  
 the 44th Annual Meeting of the  
 European Association for the Study  
 of the Liver (EASL)  
<http://www.easl.ch/>

May 17-20, 2009  
 Denver, Colorado, USA  
 Digestive Disease Week 2009

May 29-June 2, 2009  
 Orange County Convention Center  
 Orlando, Florida  
 45th ASCO Annual Meeting  
[www.asco.org/annualmeeting](http://www.asco.org/annualmeeting)

May 30, 2009  
 Chicago, Illinois  
 Endpoints Workshop: NASH

May 30-June 4, 2009  
 McCormick Place, Chicago, IL  
 DDW 2009  
<http://www.ddw.org>

June 17-19, 2009  
 North Bethesda, MD  
 Accelerating Anticancer Agent  
 Development

June 20-26, 2009  
 Flims, Switzerland  
 Methods in Clinical Cancer Research  
 (Europe)

June 24-27 2009  
 Barcelona, Spain  
 ESMO Conference: 11th World  
 Congress on Gastrointestinal Cancer  
[www.worldgicancer.com](http://www.worldgicancer.com)

June 25-28, 2009  
 Beijing International Convention  
 Center (BICC), Beijing, China  
 World Conference on Interventional  
 Oncology  
<http://www.chinamed.com.cn/wcio2009/>

July 5-12, 2009  
 Snowmass, CO, United States  
 Pathobiology of Cancer: The Edward  
 A. Smuckler Memorial Workshop

July 17-24, 2009  
 Aspen, CO, United States  
 Molecular Biology in Clinical  
 Oncology

August 1-7, 2009  
 Vail Marriott Mountain Resort, Vail,  
 CO, United States  
 Methods in Clinical Cancer Research

August 14-16, 2009  
 Bell Harbor Conference Center,  
 Seattle, Washington, United States  
 Practical Solutions for Successful  
 Management  
<http://www.asge.org/index.aspx?id=5040>

September 23-26, 2009  
 Beijing International Convention  
 Center (BICC), Beijing, China  
 19th World Congress of the Interna-  
 tional Association of Surgeons,  
 Gastroenterologists and Oncologists  
 (IASGO)  
<http://iasgo2009.org/en/index.shtml>

September 27-30, 2009  
 Taipei, China  
 Asian Pacific Digestive Week  
<http://www.apdwcongress.org/2009/index.shtml>

October 7-11, 2009  
 Boston Park Plaza Hotel and Towers,  
 Boston, MA, United States  
 Frontiers in Basic Cancer Research

October 13-16, 2009  
 Hyatt Regency Mission Bay Spa and  
 Marina, San Diego, CA,  
 United States  
 Advances in Breast Cancer Research:  
 Genetics, Biology, and Clinical  
 Applications

October 20-24, 2009  
 Versailles, France  
 Fifth International Conference on  
 Tumor Microenvironment: Progre-  
 ssion, Therapy, and Prevention

October 30-November 3, 2009  
 Boston, MA, United States  
 The Liver Meeting

November 15-19, 2009  
 John B. Hynes Veterans Memorial  
 Convention Center, Boston, MA,  
 United States  
 AACR-NCI-EORTC Molecular  
 Targets and Cancer Therapeutics

November 21-25, 2009  
 London, UK  
 Gastro 2009 UEGW/World Congress  
 of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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Data that are not statistically significant should not be noted.  $^aP < 0.05$ ,  $^bP < 0.01$  should be noted ( $P > 0.05$  should not be noted). If there are other series of *P* values,  $^cP < 0.05$  and  $^dP < 0.01$  are used. A third series of *P* values can be expressed as  $^eP < 0.05$  and  $^fP < 0.01$ . Other notes in tables or under illustrations should be expressed as  $^1F$ ,  $^2F$ ,  $^3F$ ; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, etc., in a certain sequence.

### Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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### Format

#### Journals

*English journal article (list all authors and include the PMID where applicable)*

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

*Chinese journal article (list all authors and include the PMID where applicable)*

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of

balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

#### Organization as author

- 4 **Diabetes Prevention Program Research Group.** Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

#### Both personal authors and an organization as author

- 5 **Vallancien G,** Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

#### No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

#### Volume with supplement

- 7 **Geraud G,** Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

#### Issue with no volume

- 8 **Banit DM,** Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

#### No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

#### Books

##### Personal author(s)

- 10 **Sherlock S,** Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

##### Chapter in a book (list all authors)

- 11 **Lam SK.** Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

##### Author(s) and editor(s)

- 12 **Breedlove GK,** Schorfheide AM. Adolescent pregnancy. 2nd ed. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

##### Conference proceedings

- 13 **Harnden P,** Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

##### Conference paper

- 14 **Christensen S,** Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

#### Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

#### Patent (list all authors)

- 16 **Pagedas AC,** inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

#### Statistical data

Write as mean  $\pm$  SD or mean  $\pm$  SE.

#### Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

#### Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration, *p* (CEA) =  $8.6 \pm 24.5$   $\mu$ g/L; CO<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub>, not 5% CO<sub>2</sub>; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantums can be found at <http://www.wjgnet.com/wjg/help/14.doc>.

#### Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

#### Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kho I*, *Kpn I*, etc.

Biology: *H. pylori*, *E. coli*, etc.

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