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Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
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EDITORIAL

# Emerging role of obeticholic acid in the management of nonalcoholic fatty liver disease

Evangelia Makri, Evangelos Cholongitas, Konstantinos Tziomalos

Evangelia Makri, Konstantinos Tziomalos, First Propedeutic Department of Internal Medicine, Medical School, Aristotle University of Thessaloniki, AHEPA Hospital, 54636 Thessaloniki, Greece

Evangelos Cholongitas, Fourth Department of Internal Medicine, Medical School, Aristotle University of Thessaloniki, Hippokration Hospital, 54642 Thessaloniki, Greece

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Correspondence to: Evangelos Cholongitas, MD, PhD, Assistant Professor of Internal Medicine, Fourth Department of Internal Medicine, Medical School, Aristotle University of Thessaloniki, Hippokration Hospital, 49 Konstantinoupoleos street, 54642 Thessaloniki, Greece. cholongitas@yahoo.gr
Telephone: +30-6936-378903
Fax: +30-2310-992940

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## Abstract

Nonalcoholic fatty liver disease (NAFLD) is the commonest chronic liver disease and its prevalence is increasing driven by the pandemic of obesity and type 2 diabetes mellitus. NAFLD can progress to cirrhosis and is associated with increased risk for cardiovascular disease and hepatocellular cancer. Diet and exercise are limited by suboptimal long-term adherence in patients with NAFLD. On the other hand, current pharmacological treatment of NAFLD has limited efficacy and unfavorable safety profile. In this context, obeticholic acid (OCA), a selective agonist of the farnesoid X receptors, might represent a useful option in these patients. Preclinical studies suggest that OCA improves hepatic steatosis, inflammation and fibrosis. A proof-of-concept study and the randomized, placebo-controlled Farnesoid X Receptor Ligand Obeticholic Acid in non-alcoholic steatohepatitis Treatment (FLINT) trial also showed improvements in liver histology in patients with NAFLD who received OCA. Weight loss and reduction in blood pressure were also observed. However, the effects of OCA on insulin resistance are conflicting and the lipid profile is adversely affected by this agent. In addition, pruritus is frequently observed during treatment with OCA and might lead to treatment discontinuation. However, given the limitations of existing treatments for NAFLD, OCA might represent a useful therapeutic option in selected patients with NAFLD.

**Key words:** Nonalcoholic fatty liver disease; Obeticholic acid; Farnesoid X receptors; Insulin resistance; Fibrosis; Dyslipidemia; Steatosis; Hepatocellular cancer



**Core tip:** Nonalcoholic fatty liver disease (NAFLD) is the commonest chronic liver disease in Western coun-

tries, can progress to cirrhosis and is associated with increased all-cause and cardiovascular disease mortality risk. Current pharmacological treatment of NAFLD has limited efficacy and therefore, there is a pressing need to develop more effective and safe agents for this common and life-threatening disease. Obeticholic acid (OCA), a selective agonist of the farnesoid X receptors, might be a useful agent in the management of NAFLD. In the Farnesoid X Receptor Ligand Obeticholic Acid in non-alcoholic steatohepatitis (NASH) Treatment (FLINT) trial in patients with NASH, OCA administration was associated with improvements in liver histology, while weight loss and reduction in blood pressure were also observed. Although its adverse effects on the lipid profile and insulin sensitivity are worrisome, given the increased cardiovascular risk of this population, OCA might be considered in selected patients with NAFLD/NASH, particularly in those with adequately controlled glucose and lipid levels.

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is defined as hepatic steatosis in the absence of secondary hepatic fat accumulation such as significant alcohol consumption, use of steatogenic medication or hereditary disorders. NAFLD is asymptomatic in most patients but can progress to cirrhosis and hepatocellular carcinoma. The prevalence of NAFLD is steadily increasing and is currently 20%-30% in Western countries and 5%-18% in Asia[1-3]. NAFLD is the commonest cause of elevated liver enzymes and is even more prevalent in patients with metabolic diseases such as obesity and type 2 diabetes mellitus (T2DM)[4]. Consequently, its prevalence is expected to increase in the near future as an aftermath of the increasing adoption of a sedentary lifestyle and an unhealthy diet[3,4].

Patients with NAFLD have higher all-cause mortality risk than general population[5]. Moreover, cardiovascular disease represents the leading cause of death in these patients, whereas liver-related mortality is less frequent[5,6]. Lifestyle modifications, including diet and exercise, are imperative for achieving weight loss and reducing insulin resistance and hepatic steatosis /inflammation in patients with NAFLD[7,8]. Despite the short-term effectiveness of such measures, adherence to lifestyle changes wanes with time underlining the need for pharmacological therapy[7,9]. The therapeutic interventions that are used for the treatment of NAFLD aim at the underpinning pathophysiologic mechanisms of the disease but are hampered by suboptimal efficacy and safety[1,10]. Therefore, there is a pressing need to develop more effective and safe agents for this common and life-threatening disease.

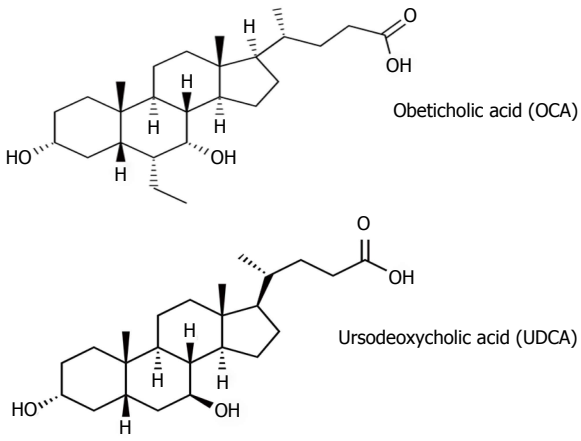


**Figure 1 Molecular structures of obeticholic acid and ursodeoxycholic acid.**

## OBETICHOLIC ACID IN NAFLD

Farnesoid X receptors (FXRs) represent an attractive target for the management of NAFLD. FXRs are nuclear receptors that are abundantly expressed and play several roles including regulation of bilirubin, carbohydrate and lipid metabolism and modulation of liver growth[11-14]. Activation of FXRs ameliorates hyperlipidemia, glucose intolerance and insulin resistance, protects against cholestasis-induced liver injury and induces hepatocyte regeneration[11-14]. Bile acids are the natural ligands of the FXRs. Obeticholic acid (OCA, 6α-ethyl-chenodeoxycholic acid) is a semi-synthetic bile acid analogue of chenodeoxycholic acid (CDCA) with a 100-fold higher affinity for FXR, compared to CDCA, and represents the first selective FXR agonist to be used in human studies[15-17]. Similarly to ursodeoxycholic acid (UDCA), OCA has anti-apoptotic action and increases bile flow and the concentrations of bile acid transport proteins, such as multidrug resistance-associated protein 2. However, UDCA is a very weak FXR agonist[13,15-17] (Figure 1).

Accumulating data suggest that OCA might be a useful agent in the management of NAFLD[18,19]. In a rabbit model of NAFLD, OCA induced weight loss and improved glucose tolerance[20]. In a rat model of NAFLD [Zucker (fa/fa) rats], in which a loss of function mutation leads to T2DM, visceral adiposity and hepatic steatosis[21,22], OCA ameliorated these consequences by reducing hepatic expression of genes involved in fatty acid synthesis, lipogenesis and gluconeogenesis[21]. In other animal models of NAFLD, OCA exerted anti-inflammatory and antifibrotic effects[23-26]. However, opposite effects have also been reported; indeed, OCA induced hepatic steatosis, ballooning and inflammation

in an animal study[27]. The effects of OCA on liver carcinogenesis are also controversial. In animal study, activation of FXR inhibited hepatocarcinogenesis by regulating nuclear factor κB-mediated hepatic inflammatory reactions[28]. In contrast, in another recent report using 3 mouse models, loss of FXR was associated with liver carcinogenesis in diabetic animals[29].

Regarding human studies, OCA in patients with NAFLD was first evaluated in a double-blind, placebo-controlled, proof-of-concept study[19]: patients with NAFLD and T2DM were randomly assigned to receive 25 mg OCA ($n$ = 20), 50 mg OCA ($n$ = 21) or placebo ($n$ = 23) for 6 weeks[19]. Insulin resistance was evaluated using hyperinsulinemic-euglycemic clamp[19]. Insulin sensitivity improved by 28.0% and 20.1% in patients treated with 25 and 50 mg OCA, respectively, whereas it worsened in the placebo group (a 5.5% reduction)[19]. A dose-dependent weight loss was observed in patients treated with OCA[19]. Moreover, alanine transaminase (ALT) and γ-glutamyltransferase (γGT) levels declined in both OCA groups[19]. The Enhanced Liver Fibrosis test, a non-invasive marker of hepatic fibrosis, improved in patients treated with 25 mg OCA and remained stable in patients treated with 50 mg OCA[19]. On the other hand, aspartate transaminase (AST) levels remained stable and alkaline phosphatase (ALP) levels increased in both OCA groups[19]. In addition, serum low-density lipoprotein cholesterol (LDL-C) levels increased with both OCA doses and serum high-density lipoprotein cholesterol (HDL-C) decreased in patients under 50 mg OCA[19]. Serum triglyceride levels also decreased in the 50 mg OCA group[19]. These lipid effects have also been reported in animal studies and were attributed to inhibition of conversion of cholesterol to bile acids and to reduction of intestinal cholesterol absorption[30]. Regarding OCA safety, adverse reactions were comparable in all groups[19].

More recently, the Farnesoid X Receptor Ligand Obeticholic Acid in NASH Treatment (FLINT) trial, a multicentre, double-blind, placebo-controlled clinical trial in patients with non-alcoholic steatohepatitis (NASH) but without cirrhosis, was published[31]. In this trial, 283 patients were randomized to receive OCA 25 mg daily or placebo for 72 wk[31]. The primary outcome was a decrease in NAFLD activity score by at least 2 points without deterioration of fibrosis. Fifty of 110 patients in the OCA group (45%) met the primary endpoint at 72 weeks compared with 23 of 109 patients in the placebo group (21%; $P$ = 0.0002)[31]. These results did not change after pre-specified sensitivity analyses with adjustment for confounders, including weight loss[31]. Moreover, 35% of patients treated with OCA had reduction in fibrosis, compared with 19% of patients treated with placebo ($P$ = 0.04)[31]. However, the rates of resolution of NASH did not differ between the two groups (22% *vs* 13%, respectively, $P$ = 0.08)[31]. Treatment with OCA resulted in a reduction in AST, ALT and γGT levels but increased ALP levels[31]. OCA induced weight loss and lowered systolic blood pressure but increased glucose levels and insulin resistance[31]. Its contrasting effects on insulin resistance in the FLINT trial and in the earlier proof-of-concept study[19] might be due to differences in the study population (only patients with T2DM in the latter study[19], patients with and without T2DM in FLINT trial[31]). Notable, insulin resistance was evaluated using homeostasis model in the FLINT trial, which is less accurate than hyperinsulinemic-euglycemic clamp[31]. Regarding the effects on lipid profile, OCA increased serum LDL-C and reduced HDL-C levels whereas TG levels did not change at 72 wk[31]. A study in healthy subjects also reported that OCA treatment for 14-20 d increased LDL-C and decreased HDL-C levels regardless of dose (5, 10 or 25 mg daily)[32]. After treatment discontinuation, differences in liver function tests, lipid profile and insulin resistance between groups were no longer apparent[31]. Finally, side effects were non-severe and occurred at similar rates, but a higher frequency of pruritus was observed in OCA group, compared to placebo group (23% *vs* 6%)[31]. These high rates of pruritus have also been reported in patients using OCA for other liver diseases, including primary biliary cirrhosis[33].

In light of these findings, how does OCA fit into the management of NAFLD? According to current guidelines, pharmacological treatment is recommended only in non-diabetic patients with biopsy-proven NASH[8]. Vitamin E is recommended as first-line agent whereas pioglitazone could also be used[8]. These recommendations are primarily based on the results of Pioglitazone versus Vitamin E versus Placebo for the Treatment of Nondiabetic Patients with Nonalcoholic Steatohepatitis (PIVENS) trial[34], which randomized 247 non-diabetic patients with NASH to receive vitamin E, pioglitazone or placebo. Both vitamin E and pioglitazone reduced hepatic steatosis and inflammation but not fibrosis[34]. In contrast, OCA improved all histological features of NAFLD (steatosis, inflammation and fibrosis)[31]. Moreover, FLINT trial included patients with T2DM (52% of the study population, $n$ = 149) and OCA was equally effective in both patients with and without T2DM[31]. On the other hand, vitamin E has no substantial metabolic effects whereas pioglitazone reduces insulin resistance and improves the lipid profile but induces weight gain[34]. OCA appears to reduce body weight but might aggravate insulin resistance and dyslipidemia[32]. However, adverse lipid effects of OCA can be mitigated using statins, which are frequently required for the management of dyslipidemia in patients with NAFLD and appear to be safe and to reduce cardiovascular morbidity in this population[8,35]. Finally, long-term studies in other populations suggest an increased risk for all-cause mortality in patients treated with vitamin E[36] and an increased risk for edema, heart failure and bone fractures in patients treated with

pioglitazone[37,38]. On the other hand, the long-term safety of treatment with OCA is unknown.

## CONCLUSION

OCA appears to represent a promising treatment for patients with NAFLD, since it improves fibrosis, induces weight loss and appears to be effective in patients with T2DM. On the other hand, the adverse effects on the lipid profile and insulin sensitivity are worrisome, given the increased cardiovascular risk of this population. Therefore, and given the suboptimal efficacy and safety of other pharmacotherapies in NAFLD, lifestyle changes should be recommended as first-line management in these patients whereas OCA might be considered in selected patients, particularly those with T2DM and adequately controlled glucose and lipid levels.

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REVIEW

# Concise review: Interferon-free treatment of hepatitis C virus-associated cirrhosis and liver graft infection

Nina Weiler, Stefan Zeuzem, Martin-Walter Welker

Nina Weiler, Stefan Zeuzem, Martin-Walter Welker, Universitätsklinikum Frankfurt, Medizinische Klinik 1, 60590 Frankfurt, Germany

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Correspondence to: Martin-Walter Welker, MD, Universitätsklinikum Frankfurt, Medizinische Klinik 1, Theodor-Stern-Kai 7, 60590 Frankfurt,
Germany. welker@med.uni-frankfurt.de
Telephone: +49-69-63016557
Fax: +49-69-63015716

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## Abstract

Chronic hepatitis C is a major reason for development of cirrhosis and hepatocellular carcinoma and a leading cause for liver transplantation. The development of direct-acting antiviral agents lead to (pegylated) interferon-alfa free antiviral therapy regimens with a remarkable increase in sustained virologic response (SVR) rates and opened therapeutic options for patients with advanced cirrhosis and liver graft recipients. This concise review gives an overview about most current prospective trials and cohort analyses for treatment of patients with liver cirrhosis and liver graft recipients. In patients with compensated cirrhosis Child-Pugh-Turcotte (CTP) class A, all approved agents are safe and SVR rates do not significantly differ from patients without cirrhosis in general. In patients with decompensated cirrhosis CTP class B or C, daclastasvir, ledipasvir, velpatasvir, and sofosbuvir are approved, and SVR rates higher than 90% can be achieved. Especially for patients with a model of end stage liver disease score higher than 15 and therefore eligible for liver transplantation, data is scarce. Reported SVR rates in patients with cirrhosis CTP class C are lower compared to patients with a less severe liver disease. In liver transplant recipients with a maximum of CTP class A, SVR rates are comparable to patients without LT. Patients with decompensated graft cirrhosis should be treated on an individual basis.

**Key words:** Hepatitis C; Cirrhosis; Liver transplantation; Direct antiviral agents; Interferon-free antiviral treatment



**Core tip:** Chronic hepatitis C is a major reason for development of cirrhosis and a leading cause for liver

transplantation. The development of direct-acting antiviral agents (DAA) offered new therapeutic options for patients with advanced cirrhosis and liver graft recipients. This review gives a high topical summary of most current therapeutic options of DAA-based antiviral therapy in patients with hepatitis C virus associated cirrhosis before and after liver transplantation.

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## INTRODUCTION

The World Health Organization estimates that approximately 150 million people worldwide are currently infected with the hepatitis C virus (HCV)[1]. Chronic hepatitis C is a major reason for development of cirrhosis and hepatocellular carcinoma (HCC) in Eastern Asia, Europe, and North America and a leading cause for liver transplantation (LT)[2-4]. In patients with HCV associated cirrhosis, the annual incidence of HCC ranges from 1% to 7%[5-7].

Sustained virologic response to antiviral therapy, defined as undetectable HCV-RNA 12 wk ($SVR_{12}$) after end of treatment (EOT), is associated with improved survival and reduced risk of HCC development in patients without and with cirrhosis[3,8,9]. The former standard treatment for HCV infection based on the combination of (pegylated) interferon-alfa [(peg)-IFN] and ribavirin (RBV) for 12 to 72 wk was associated with limited SVR rates and burdened by therapy associated adverse events[10,11]. In patients with HCV associated cirrhosis registered for liver transplantation (LT), SVR rates after (peg)-IFN ± RBV range between 0% and 50%, depending inter alia on HCV genotype and severity of liver disease[12]. In patients with decompensated cirrhosis, (peg)-IFN based antiviral therapy is contraindicated[12]. In liver transplant recipients, SVR rates to (peg)-IFN based antiviral therapy are low, and interferon-alfa-associated, immune-mediated graft dysfunction is a major concern[12,13].

The development of direct-acting antiviral agents (DAA) for (peg)-IFN free antiviral therapy regimens lead to a remarkable increase in SVR rates and opened therapeutic options for patients with contraindications or low SVR rates to (peg)-IFN based antiviral therapy regimens[14,15]. Currently available DAA can be classified according to the viral target protein as NS3/4A protease inhibitors (PI), NS5B polymerase inhibitors (including non-nucleoside and nucleoside inhibitors) and NS5A-inhibitors[16]. Antiviral regimens containing elbasvir (EBR)/grazoprevir (GZR), simeprevir (SMV), and ombitasvir (OMV)/paritaprevir/r (PTV/r) - with or without dasabuvir (DSV) - are approved by the European Medicine Agency (EMA) and/or the Federal Drug Administration (FDA) with or without RBV for treatment of patients without and with cirrhosis maximum Child-Pugh-Turcotte (CPT) class A. Regimens containing daclatasvir (DCV), ledipasvir (LDV), velpatasvir, and sofosbuvir (SOF) are approved with or without ribavirin (RBV) for patients with all stages of liver disease including cirrhosis CPT class B and C (Table 1). As SOF is not approved for patients with severe renal impairment, there is still no interferon-free therapy regimen approved for patients with decompensated cirrhosis and severe kidney disease. This article gives a concise review on current data of DAA-based antiviral therapy in patients with advanced HCV associated liver disease.

## SYSTEMATICAL LITERATURE DATABASE RESEARCH

A PubMed database research using the terms "hepatitis C", "cirrhosis" and "direct acting antiviral" was performed by the date of 21$^{th}$ of February 2016 to identify relevant clinical studies as well as national and international guidelines. The systematic research resulted in 226 hits, from those we identified 16 original articles, 10 case reports/case series, 137 reviews, 3 national guidelines, 8 articles presenting data from DAA trials without participation of cirrhotic patients, and 51 articles investigating other topics than DAA therapy. One article was listed twice. The PubMed research was amended by studies not fully published but known to the authors, and references listed in systematically identified articles. Totally, 27 trials were identified, which reported safety and efficacy of DAA-based antiviral therapy in patients with compensated and decompensated cirrhosis (Table 2)[17-40].

Additionally, a second PubMed database research using the terms "hepatitis C", "liver transplantation" and "direct acting antiviral" was performed to identify relevant clinical studies as well as national and international guidelines dealing with patients in the liver transplant setting. This systematic PubMed research revealed 72 publications, from those we identified 2 original articles, 3 case reports/series, 45 reviews, one national guideline and 21 articles investigating other topics than DAA therapy or animal model studies. The PubMed research was amended by studies not fully published but known to the authors, and references listed in systematically identified articles. In total, 6 trials were identified including also studies known to the authors as congress proceedings and not yet fully published.

## DAA-BASED ANTIVIRAL THERAPY IN HCV-ASSOCIATED CIRRHOSIS

The majority of prospective phase Ⅱ and Ⅲ trials

**Table 1 Currently approved[1] direct-acting antivirals combination regimens for hepatitis C virus associated cirrhosis**

| Drug class | Name | Combination DAA partner | Genotype | Ribavirin | Therapy duration (wk) | Approved cirrhosis class (CPT) |
|---|---|---|---|---|---|---|
| NS3/4A protease inhibitors | Simeprevir | Sofosbuvir | 1, 4 | No | 12 | A |
| | Paritaprevir | In fixed combination with ritonavir and ombitasvir | 1, 4 | No/Yes | 12-24 | A |
| | Grazoprevir | In fixed combination with elbasvir | 1, 4 | No/Yes | 12-16 | A |
| NS5A inhibitors | Daclatasvir | Sofosbuvir | 1, 3, 4 | No/Yes | 12-24 | A, B, C |
| | Ledipasvir | In fixed combination with sofosbuvir | 1, 3, 4, 5, 6 | No/Yes | 12-24 | A, B, C[2] |
| | Ombitasvir | In fixed combination with paritaprevir and ritonavir | 1, 4 | No/Yes | 12-24 | A |
| | Elbasvir | In fixed combination with grazoprevir | 1, 4 | No/Yes | 12-16 | A |
| | Velpatasvir | In fixed combination with sofosbuvir | 1, 2, 3, 4, 5, 6 | No/Yes | 12 | A, B, C |
| NS5B non-nucleoside analog polymerase inhibitors | Dasabuvir | Ombitasvir/paritaprevir/ritonavir | 1 | No/Yes | 12-24 | A |
| NS5B nucleoside analog polymerase inhibitors | Sofosbuvir | Daclatasvir<br>Simeprevir<br>Ledipasvir in fixed combination<br>Velpatasvir in fixed combination | 1, 2, 3, 4, 5, 6[3] | No/Yes | 12-24 | A, B, C |

[1]Approved by EMA and/or FDA by October 2016. Approval details may differ with respect to region, genotype, and combination partner; [2]Genotype 3, only CPT A; [3]Different approvals for SOF/RBV, and SOF/LDV. Adapted from[96]. CPT: Child-Pugh-Turcotte; DAA: Direct-acting antivirals; EMA: European Medicines Agency; FDA: Food and Drug Administration; LDV: Ledipasvir; RBV: Ribavirin; SOF: Sofosbuvir.

included only a limited number of patients with cirrhosis[17,22,24-26,28,29,32-34,37-40], and only few trials investigated especially patients with (decompensated) cirrhosis[18-20,23,27,30,31,35]. Data of patient subgroups with cirrhosis were not reported discretely in the majority of studies, including, but not focusing on cirrhotic patients. Additionally to prospective, controlled trials, safety and efficacy of DAA regimens were recorded in "real life" cohort studies and compassionate use or early access programs[41-51]. Data obtained from early access and compassionate use programs must be interpreted with caution, because treatment duration and regimens, *e.g.,* use of RBV, are mostly not controlled by respective protocols. Nevertheless, patients with decompensated cirrhosis or high MELD score ($\geqslant$ 16) were enrolled in a substantial number in these trials, and therefore, these data are of interest and will be presented in the respective sections of this review.

## APPROVED DAA-BASED TREATMENTS IN PATIENTS WITH COMPENSATED CIRRHOSIS

All currently approved agents can be administered safely in patients with CPT class A cirrhosis in interferon-free antiviral regimens[18,31,35,52-60]. However, with respect to genotype and DAA regimen, some details have to be considered.

### *Genotype 1*

The combination of LDV/SOF with and without RBV was prospectively evaluated in patients with compensated cirrhosis in the ELECTRON (NCT01260350), ELECTRON-2 (NCT01826981), LONESTAR (NCT01329978), ION-1 (NCT01701401), ION-2 (NCT01768286), GS-334-0113 (NCT01975675), and SIRIUS (NCT01965535) trials. The SIRIUS multicentre, double-blinded phase Ⅱ study evaluated LDV/SOF with RBV for 12 in comparison to LDV/SOF without RBV for 24 wk in 155 GT 1 treatment experienced patients with compensated cirrhosis[18]. Median MELD score was 7 in each study arm (range 6-16 for both arms). Overall, $SVR_{12}$ rates were 96% in patients treated with LDV/SOF with RBV for 12 wk, and 97% in patients treated with LDV/SOF without RBV for 24 wk. Adverse events were mild, namely asthenia, headache, pruritus and fatigue being the most common. To overcome the problem, that each of the respective approval trials for LDV and SOF included only a limited number of patients with cirrhosis, an integrated safety and efficacy analysis of these patients across the mentioned studies was performed[42,61]. In a meta-analysis of pooled data from the ELECTRON (NCT01260350), ELECTRON-2 (NCT01826981), LONESTAR (NCT01329978), ION-1 (NCT01701401), ION-2 (NCT01768286), GS-334-0113 (NCT01975675), and SIRIUS (NCT01965535) studies, 513 patients treated with LDV/SOF with and without RBV for 12 or 24 wk were included. The overall $SVR_{12}$ rate was 96%, and SVR was not associated with prior treatment (47% had previously received a protease-inhibitor-containing regimen), treatment duration or use of RBV. Nevertheless, previously treated patients receiving 12 wk of treatment with LDV and SOF without RBV achieved $SVR_{12}$ in only 90%. Of note, no significant safety issue was observed[42]. In conclusion, 12 wk of LDV/SOF without RBV are considered sufficient for treatment-naive patients with compensated cirrhosis and genotype 1 infection, while the addition of RBV is recommended in treatment-experienced patients with liver cirrhosis[62,63].

The combination of SMV and SOF was evaluated in the OPTIMIST-2 phase Ⅲ, open-label, single-arm

**Table 2 Efficacy of direct-acting antivirals based, (peg)-interferon-free antiviral therapy in patients with hepatitis C virus-associated (de-) compensated cirrhosis in controlled, prospective trials**

| Ref. | Therapy regimen | Treatment duration (wk) | Genotype | *n* (all) | *n* (cirrhotic patients) | *n* (MELD > 16) | $SVR_{12}$ % (all patients) |
|---|---|---|---|---|---|---|---|
| Abergel *et al*[17] | LDV/SOF | 12 | 5 | 41 | 9 | Not specified | 39/41 (95%) |
| Afdhal *et al*[53] (ION-1) | LDV/SOF ± RBV | 12-24 | 1 | 865 | 136 | Not specified | 849/865 (98%) |
| Afdhal *et al*[54] (ION-2) | LDV/SOF ± RBV | 12-24 | 1 | 440 | 88 | Not specified | 427/440 (97%) |
| Bouliere *et al*[18] (SIRIUS) | LDV/SOF ± RBV | 12-24 | 1 | 155 | 155 | Not specified | 149/154 (97%) |
| Charlton *et al*[19] (SOLAR-1) | LDV/SOF/RBV | 12-24 | 1, 4 | 337 | 108 | 27 | 89/108 (82%)[1] |
| Curry *et al*[20] (ASTRAL-4) | VEL/SOF ± RBV | 12-24 | 1, 2, 3, 4, 6 | 267 | 267 | 13 | 234/267 (88%) |
| Curry *et al*[21] | SOF/RBV | Up to 48 | 1, 2, 3, 4 | 61 | 61 | None | 30/43 (70%) |
| Feld *et al*[22] (ASTRAL-1) | VEL/SOF | 12 | 1, 2, 4, 5, 6 | 741[2] | 142 | Not specified | 618/624 (99%)[3] |
| Feld *et al*[23] (TURQUOISE-Ⅲ) | OBV/PTV/r + DSV | 12 | 1b | 60 | 60 | Not specified | 60/60 (100%) |
| Forns *et al*[24] (C-SALVAGE) | Grazoprevir/Elbasvir/RBV | 12 | 1 | 79 | 34 | Not specified | 76/79 (96%) |
| Foster *et al*[25] (ASTRAL-2/-3) | VEL/SOF *vs* SOF/RBV | 12 | 2, 3 | 818 | 201 | Not specified | 742/818 (91%) |
| Foster *et al*[68] (BOSON) | SOF/RBV ± IFN | 12-24 | 2, 3 | 592 | 219 | Not specified | 494/592 (83%) |
| Kumada *et al*[26] (GIFT-1) | OBV/PTV/r | 12 | 1b | 363 | 42 | Not specified | 346/363 (95%) |
| Lawitz *et al*[27] (OPTIMIST-2) | SMV/SOF | 12 | 1 | 103 | 103 | Not specified | 86/103 (83%) |
| Lawitz *et al*[28] (C-WORTHY) | Grazoprevir/Elbasvir ± RBV | 12-18 | 1 | 253 | 170 | Not specified | 240/253 (95%) |
| Lawitz *et al*[29] (PEARL-I) | OBV/PTV/r + DSV | 12-24 | 1 | 181 | 99 | Not specified | 172/181 (95%) |
| Leroy *et al*[30] (ALLY-3+) | DCV/SOF/RBV | 12-16 | 3 | 50 | 50 | Not specified | 45/50 (90%) |
| Manns *et al*[31] (SOLAR-2) | LDV/SOF/RBV | 12 | 1, 4 | 328 | 160[4] | 41 | 121/140 (86%) |
| Mizokami *et al*[32] | LDV/SOF ± RBV | 12 | 1 | 341 | 76 | Not specified | 338/341 (99%) |
| Nelson *et al*[33] (ALLY-3) | DCV/SOF | 12 | 3 | 152 | 32 | Not specified | 135/152 (89%) |
| Omata *et al*[34] | SOF/RBV | 12 | 2 | 153 | 17 | Not specified | 148/153 (97%) |
| Poordad *et al*[35] (TURQUOISE-Ⅱ) | OBV/PTV/r + DSV/RBV | 12-24 | 1 | 380 | 380 | Not specified[5] | 356/380 (94%) |
| Poordad *et al*[36] (ALLY-1) | DCV/SOF/RBV | 12 | 1, 2, 3, 4, 6 | 113[6] | 60 | Not specified (CPT C 16) | 100/113 (89%)[7] |
| Poordad *et al*[37] (QUARTZ-I) | OBV/PTV/r + DSV + SOF + RBV | 12-24 | 1 | 22 | 7 | Not specified | 14/15 (93%)[8] |
| Wyles *et al*[38] | LDV/SOF/RBV | 12 | 1 | 51 | 14 | Not specified | 50/51 (98%) |
| Zeuzem *et al*[39] (VALENCE) | SOF/RBV | 12-24 | 2, 3 | 419 | 90 | Not specified | 302/334 (90%)[9] |
| Zeuzem *et al*[40] (C-EDGE) | Grazoprevir/elbasvir | 12 | 1, 4, 6 | 421 | 92 | Not specified | 299/316 (95%)[10] |

[1]Only pretransplant cohort; [2]116 patients received placebo; [3]SVR in patients with compensated cirrhosis 99%; [4]Patients with CPT class B or C cirrhosis pre- and posttranplant, additionally CPT class A patients posttransplant participated in this trial, the number was not specified; [5]Only patients with CPT class A cirrhosis included; [6]Only patients who had undetectable HCV-RNA at transplant were included in efficacy analysis; [7]83% in the advanced cirrhosis cohort; [8]Not all patients completed follow up until conference; [9]85 patients received placebo; [10]105 patients had deferred therapy. CPT: Child-Pugh-Turcotte; DAA: Direct-acting antivirals; DCV: Daclatasvir; DSV: Dasabuvir; LDV: Ledipasvir; RBV: Ribavirin; SMV: Simeprevir; SOF: Sofosbuvir; PTV/r: Paritaprevir/ritonavir; VEL: Velpatasvir; $SVR_{12}$: Sustained virologic response 12 wk after end of treatment.

study, including 103 GT 1 patients with cirrhosis[27]. The overall $SVR_{12}$ rate was 83%, in detail, 88% and 79% for treatment-naive and treatment-experienced patients, respectively. In patients with baseline albumin levels higher than 40 g/L, $SVR_{12}$ rates were higher than in patients with albumin levels lower than 40 g/L (94% versus 74%).

The combination of OBV/PTV/r and DSV (3D-regimen) was evaluated in two studies in patients with compensated cirrhosis. The TURQUOISE-Ⅱ trial, an open-label phase Ⅲ trial investigated the 3D-regimen in combination with RBV for 12 wk *vs* 24 wk in 380 patients with HCV associated cirrhosis CPT class A[35]. The overall $SVR_{12}$ rate was 93.7%, 91.8% (191/208) in the 12 wk compared to 95.9% (165/172) in the 24 wk group, respectively. A significant reduction of the relapse rate in the longer treatment arm was only observed in patients with HCV subtype 1a infection and one or more specific negative predictors (alfa-fetoprotein > 20 ng/mL, platelet count < 90 × $10^9$/L, albumin level < 35 g/L). Negative predictors of SVR in general were IL28B T/T polymorphism, prior null-response to (peg)-IFN/RBV therapy, and genotype 1a infection[35]. The TURQUOISE-Ⅲ phase Ⅲb, open-label study investigated whether RBV could be dispensed without SVR decline in 60 patients with HCV genotype 1b associated cirrhosis treated with the 3D-regimen[23]. Nineteen (32%) patients had clinical signs of portal hypertension (thrombocytopenia, esophageal varices) alone or in combination with reduced serum albumin levels or hepatic coagulopathia. However, all patients included had a CPT score not higher than 6 with no

evidence of hepatic decompensation. Treatment with the 3D-regimen without RBV was safe and efficacious in patients with compensated cirrhosis and genotype 1b infection, as $SVR_{12}$ was achieved in 100%.

The combination of DCV und SOF was evaluated in the ALLY-1 trial, which included patients with compensated and decompensated cirrhosis[36]. Respective results are discussed below.

The therapeutic spectrum for patients with genotype 1 (and 4 or 6) infection was currently widened with the FDA approval of EZR in fixed combination with GZR[64]. A treatment course of 12 wk in patients with mainly genotype 1 infection resulted in an overall $SVR_{12}$ rate of 95% (299/316)[40]. The study included 92 (22%) patients with compensated cirrhosis, and $SVR_{12}$ rates did not differ between treatment naïve patients without or with cirrhosis. Severe adverse events occurred in 9 (2.8%) patients receiving the investigational drugs and 3 (2.9%) patients of the placebo group, but no event was considered drug related[40]. However, HCV subtype (1a *vs* 1b), resistant associated variants, and prior treatment status has to be taken into account according to the FDA approved label of these fixed-combination therapy.

### *Genotype 2*

The currently approved DAA regimens were investigated in a moderate number of patients with cirrhosis and genotype 2 infection[34,39,46,65-67]. According to the European Association for the Study of the Liver (EASL) guidelines, patients with GT 2 and cirrhosis should be treated with SOF/RBV for a prolonged treatment duration of 16 to 20 wk, especially in patients with treatment experience[62].

### *Genotype 3*

Studies investigating DAA based antiviral therapy in patients with HCV genotype 3 associated cirrhosis have shown lower SVR rates than in genotype 1 infected patients for SOF/RBV for 12 to 24 wk and DCV/SOF for 12 wk[33,39]. The ALLY-3+ trial investigated whether the addition of RBV to DCV/SOF and a prolongation of treatment duration from 12 to 16 wk were associated with enhanced SVR rates in 50 GT 3 patients with advanced fibrosis (14/50, 28%) or cirrhosis (36/50, 72%)[30]. The overall $SVR_{12}$ rate was 90%. The SVR rates did not differ significantly between both groups, with 88% (91% observed, excluding a patients who died due to causes not related to study) in the 12 wk group and 92% in the 16 wk group. The subgroup analysis of patients with cirrhosis reported an overall $SVR_{12}$ rate of 86%, with 83% (88% observed) in the 12 wk group compared to 89% in the 16 wk group. Cirrhosis stage or MELD score were not specified. Of note, DCV is approved in combination with SOF and RBV for 12 (FDA) or 24 (EMA) wk in patients with cirrhosis and genotype 3 infection.

The large BOSON trial investigated SOF/RBV for 16 or 24 wk *vs* SOF/RBV ± peg-IFN for 12 wk in treatment-experienced patients with cirrhosis and genotype 2 infection and patients with genotype 3 infection of any treatment status with and without cirrhosis[68]. In HCV genotype 2 infection, the SVR rate was not significantly different in the three treatment arms (87%, 100%, and 94%). In patients with genotype 3 infection, SOF/RBV ± peg-IFN was superior to 16 or 24 wk of SOF/RBV (93% *vs* 71%, 84%). The same pattern with lowest $SVR_{12}$ rate in the 16 wk group was found in the subgroup of patients with genotype 3 infection and cirrhosis.

### *Genotype 4-6*

Patients with HCV genotype 4, 5, or 6 infection and compensated cirrhosis were included in limited numbers only in the respective trials. Current guidelines recommend LDV/SOF with RBV for 12 wk, LDV/SOF without RBV for 24 wk or DCV/SOF with RBV for 12 and without RBV for 24 wk. Of note, DCV/SOF with or without RBV is not approved by EMA for HCV genotype 5 or 6 infection. Additional options for patients with genotype 4 infection are the combination of SMV/SOF with RBV for 12 and without RBV for 24 wk or OBV/PTV/r for 24 wk with RBV[62].

### *Pan-genotype treatment (genotype 1-6)*

The combination of VEL and SOF has been evaluated for 12 wk in phase Ⅲ studies including patients with compensated cirrhosis[22,25]. Among all genotypes, the overall $SVR_{12}$ rates ranged from 95% to 99%. A subgroup analysis was performed for patients with genotype 3 infection, and $SVR_{12}$ did not significantly differ in patients with our without cirrhosis[25]. The fixed drug combination was approved by the EMA and the FDA in 2016 for patients with compensated cirrhosis for a 12-wk treatment without RBV. Addition of RBV may be considered for patients in compensated cirrhosis and genotype 3 infection (EMA).

## APPROVED DAA-BASED TREATMENT IN PATIENTS WITH DECOMPENSATED CIRRHOSIS

Randomized, prospective trials of approved DAA regimens were performed in patients with advanced liver disease [(decompensated) cirrhosis, after LT] and mainly HCV genotype 1, 3, and 4 infection. Although some studies investigated patients with "decompensated" cirrhosis, data are scarce in patients with a MELD score higher than 15[69]. From the limited data may be concluded that patients with CPT class C cirrhosis have lower $SVR_{12}$ rates than patients without or with compensated cirrhosis. Improvements of MELD score as well as albumin levels indicate an improvement in liver function in patients with DAA induced SVR[31,70]. However, liver function stayed

unchanged in a significant proportion of patients or even worsened despite of SVR. Moreover, serious adverse events including fatal courses have been reported in patients with decompensated cirrhosis and DAA based antiviral therapy ranging from 18% to 34%[19,20]. Most trials did not report a benefit in SVR rates for 24 wk of treatment over 12-wk duration[18,22]. However, some data indicate similar $SVR_{12}$ rates for a treatment of 12 or 24 wk only with additional RBV in the 12 wk arm[20]. The relevant studies are discussed in detail below.

The randomized open-label phase Ⅱ studies SOLAR-1 and SOLAR-2 evaluated the combined treatment with LDV/SOF and RBV for 12 or 24 wk in patients with HCV GT 1 or 4 and advanced cirrhosis or post liver transplantation[19,31]. Totally 337 patients were enrolled in the SOLAR-1 study, 89% GT 1 and 11% GT 4. Data of 108 patients with decompensated cirrhosis have been reported supplementary prior to the final publication[19,70]. Patients had CPT class B ($n$ = 55) or C ($n$ = 53) cirrhosis, and 26% of patients (28) had a MELD score > 15. At therapy baseline, 96% of patients with cirrhosis CPT class C had ascites. The overall $SVR_{12}$ rate was 87%, and did not differ significantly in patients with CPT class B cirrhosis between the 12 wk (SVR, 87%) and the 24 wk (89%) duration arm. In patients with cirrhosis CPT class C, $SVR_{12}$ rates were 86% and 87% in the respective study arms. Of note, 4 patients with cirrhosis CPT class B/C received a liver graft, 5 patients discontinued treatment because of adverse events, and one patient died. The transplant cohorts of the SOLAR-1 study and the SOLAR-2 study are discussed in the respective section below.

The combination of DCV/SOF and RBV for 12 wk was evaluated in the open label, phase Ⅲ ALLY-1 study in patients with advanced cirrhosis or after LT[36]. Although all genotypes were allowed, mainly patients with genotype 1 infection were included in the cirrhosis cohort (45/60, 75%). Patients transplanted during treatment were eligible for additional 12 wk of treatment immediately post-transplant. In the cirrhosis cohort, CPT class distribution was 20% class A, 53% class B, and 27% class C, and MELD score ranged between 8 and 27. The overall $SVR_{12}$ rate in the cirrhosis cohort was 83% (50/60 patients with all GT) and did not differ with respect to prior treatment status or general baseline characteristics. However, SVR rates were higher in patients with CPT class A (10/11 GT 1 patients) or B (22/24 GT 1 patients) than in patients with class C (5/10 GT 1 patients, for other GT not separately displayed).

The combination of VEL/SOF has also been evaluated in 267 patients with decompensated cirrhosis in the phase Ⅲ, open-label ASTRAL-4 study[20]. Although designed as a pan-genotype study, mainly patients with genotype 1 (78%) and 3 (15%) infection were enrolled, while patients with genotypes 2, 4, 5, and 6 were included only to a low percentage of 4%, 3%, 0%, and < 1%, respectively. Patients were randomized to 12 wk of SOF/VEL with or without RBV or 24 wk with SOF/VEL without RBV. Enrolled patients had a median baseline CPT score of 8 (range 5 to 10) and a median baseline MELD score of 10 (range 6 to 24). However, 95% of patients had a baseline MELD score of 15 or less. At screening all patients had CPT class B, but 7% of patients had CPT class A at treatment baseline. This has to be kept in mind, when transferring scientific data to clinical practice in patients with "truly" decompensated cirrhosis. Overall $SVR_{12}$ rates were 83% for 12 wk of SOF/VEL, 94% for 12 wk of SOF/VEL/RBV and 86% for 24 wk of SOF/VEL. $SVR_{12}$ rates for CPT class or subgroups were not reported separately. An improvement in CPT class was achieved in 47% of patients, 42% had no change and 11% patients showed a worsened CPT score. Overall improvement of MELD score was observed. This effect was stronger in patients with an initial MELD score > 15. In detail, 51% of patients with a baseline MELD score ⩽ 15 showed an improvement of MELD score, while MELD score stayed unchanged or worsened in 22% and 27% of patients, respectively. Patients with a baseline MELD > 15 showed an improved MELD score in 81%, while MELD score stayed unchanged or worsened in 11% and 7%, respectively. Although these are encouraging data, it has to be considered, that (severity of) portal hypertension, a major risk factor of death in patients with cirrhosis, is not adequately reflected by MELD. The fixed combination of VEL/SOF is approved by the EMA and the FDA for 12 wk in combination with RBV.

As mentioned above, data from early access and compassionate use programs, as well as data from "real life" cohorts are useful supplements to prospective controlled trials, because inclusion criteria often allowed to include a substantial number of patients with advanced liver disease, *e.g.,* decompensated cirrhosis. Final data are currently available from the National Health Service England Expanded Access Programme, which included 467 patients with hepatic decompensation or life-threatening extrahepatic manifestations[49]. In this prospective, observational cohort study, patients were treated for a maximum of 12 wk with DCV/SOF ± RBV or LDV/SOF ± RBV by clinician's discretion. The majority (409/467, 88%) of patients had past or current symptoms of hepatic decompensation, defined by presence of ascites, variceal bleeding or encephalopathy. At baseline, 319 patients were classified as CPT class B, and 43 as CPT class C, and median (range) MELD score was 11 (6-32). Overall $SVR_{12}$ was 82% (381/467); 4% (17/467) of patients died and 3% (16/467) of patients were lost to follow-up. In detail, $SVR_{12}$ rates were 85% (39/46) for patients with genotype 1 infection treated with DCV/SOF ± RBV and 92% (170/185) for patients treated with LDV/SOF ± RBV, respectively. Patients with genotype 3 infection achieved $SVR_{12}$ in 73% (91/125) when treated with DCV/SOF ± RBV and in

61% (41/67) treated with LDV/SOF ± RBV[49]. Of note, DCV is currently approved in patients with cirrhosis and HCV genotype 3 infection in combination with SOF and RBV by EMA for 24 wk and by FDA for 12 wk treatment[62,63].

It is unquestionable, that efficacy and safety has improved with DAA based IFN-free therapies compared to (peg)-IFN based therapy regimen in patients with decompensated cirrhosis. For a subgroup of patients with decompensated cirrhosis and DAA induced SVR, an improvement in liver function has been reported. Nevertheless, severe adverse events were reported in a substantial percentage of patients, and ascites or hepatic encephalopathy did not resolve completely in all patients[43,44,49,71]. Currently, no biomarkers are available to predict the individual clinical course in patients with decompensated cirrhosis to decide whether treatment should be initiated before or after LT[49,72].

## FURTHER OPTIONS OF DAA-BASED TREATMENT IN PATIENTS WITH DECOMPENSATED CIRRHOSIS

SMV is not approved by the EMA or the FDA for patients with cirrhosis CPT class B or C. Nevertheless, treatment of patients with advanced cirrhosis with SMV/SOF with or without RBV has been reported[43,44]. Saxena *et al*[43] presented data of a multicenter cohort of 106 GT 1 patients with cirrhosis treated with SMV/SOF with or without RBV for 12 wk. Patients were analyzed according to CPT class A versus CPT class B/C and compared to matched untreated controls. The median (range) baseline MELD score in the overall study population was 9 (8-11), and cirrhosis was classified as CPT class A in 64% and CPT class B/C in 35%. Overall, SVR rates were 91% in patients with CPT class A and 73% in patients with CPT class B/C cirrhosis, respectively[43]. Two deaths occurred, one in each group, one liver related and one not. Shiffman *et al*[44] reported retrospectively analyzed data from 120 patients with cirrhosis and HCV genotype 1 infection treated with SMV/SOF. From those 67%, 21% and 12% had CPT class A, B, and C cirrhosis, respectively. Hepatic decompensation or portal hypertension was present in 30% of patients. The overall $SVR_{12}$ rate was 87%, 77%, and 67% in CPT class A, B, and C patients, respectively. Serious adverse events were reported in 11% of patients, sepsis ($n$ = 2, 1/2 fatal outcome), variceal bleeding ($n$ = 2), hepatocellular carcinoma ($n$ = 2), and increase in bilirubin ($n$ = 8). Although these data indicate that SMV might be safe in patients with decompensated cirrhosis, there is general concern because the simeprevir mean steady state area under the curve is increased in patients with CPT class B and C cirrhosis by 2.4-fold and 5.2-fold, respectively (summary of product characteristics).

## DAA THERAPY PERI-TRANSPLANT

Curry *et al*[21] conducted an open-label phase Ⅱ study for patients on the waiting list to LT for HCC of any GT. Endpoint of the study was undetectable HCV RNA 12 wk after LT. Patients received SOF and RBV up to 48 wk before LT, 61 patients with CPT ⩽ 7 were included. LT was performed in 46 patients, 43 from those had undetectable HCV RNA at transplant and were included in efficacy analysis. Outcome of those 43 patients was: 30 patients (70%) had SVR12 after LT, 10 patients (23%) suffered from relapse, 3 patients (7%) died (primary graft non-function, $n$ = 2; hepatic artery thrombosis, $n$ = 1). Overall SVR post LT from all 61 patients was 49%. The risk of HCV graft infection was negatively correlated with the time interval before LT, when HCV RNA was undetectable. Safety and efficacy of LDV/SOF are currently investigated in patients with genotype 1 or 4 infection in a peri-transplant setting[73].

## ANTIVIRAL THERAPY IN HCV LIVER GRAFT INFECTION

In patients with detectable HCV RNA at transplantation, HCV graft infection is almost inevitable, and HCV infection of the liver graft often shows an aggravated course with development of graft cirrhosis in up to 30% percent of patients within 5 years after transplantation[74,75]. In a minority of patients, HCV graft infection presents as fibrosing cholestatic hepatitis (FCH), a severe form of hepatitis C, leading to graft loss and death within months up to 2 years in the majority of patients[76]. Therefore, HCV graft infection is associated with a decrease in patient and graft survival[77].

Patients with fibrosis or cirrhosis after LT have a poor tolerance and low efficacy to (peg)-IFN based antiviral therapy[78]. Moreover, plasma cell hepatitis is a rare but feared complication of (peg)-IFN therapy[76,79]. First generation PI - in combination with (peg)-IFN and RBV - were associated with a slight increase in SVR rates, but a high rate of serious adverse events[80,81]. The introduction of DAA-based IFN-free antiviral therapy widened therapeutic options in patients after liver transplantation and a proof of concept study using SOF with RBV for 24 wk resulted in an overall SVR rate of 70%[82]. Currently safety and efficacy data of DAA based antiviral therapy in liver graft recipients are available from prospective trials[19,31,36,83,84] and cohort studies[77,85-90]. Table 3 summarizes available prospective and controlled trials. The most important prospective trials are the SOLAR-1 and -2 studies (LDV/SOF/RBV), the CORAL-I study (3D/RBV), and the ALLY-1 trial (DCV/SOF/RBV)[19,31,36,84].

The combination of LDV and SOF with or without RBV was investigated in several studies[19,31,83]. Reddy *et al*[83] performed a prospective, randomized multicenter study to evaluate treatment with LDV/SOF

**Table 3 Efficacy of direct-acting antivirals based, (peg-)-interferon-free antiviral therapy in patients with hepatitis C virus liver graft infection in controlled, prospective trials**

| Ref. | Therapy regime | Treatment duration (wk) | Genotype[1] | *n* | SVR$_{12}$ |
|---|---|---|---|---|---|
| Charlton *et al*[19] (SOLAR-1)[1] | LDV/RBV + RBV | 12-24 | 1, 4 | 229[2] | 214/229 (93%) |
| [1]Charlton *et al*[82] | SOF + RBV | 24 | 1, 3, 4 | 40 | 28/40 (70%) |
| Kwo *et al*[84] (CORAL-1) | DSV/OMV/PTV/r + RBV | 24 | 1 | 34 | 33/34 (97%) |
| Manns *et al*[31,72] (SOLAR-2) | LDV/SOF +RBV | 12 or 24 | 1, 4 | 168[3] | 146/151 (97%) |
| Poordad *et al*[36] (ALLY-1)[1] | DCV/SOF + RBV | 12 | 1, 2, 3, 4, 6[4] | 53[2] | 50/53 (94%) |
| Reddy *et al*[83] | SOF/LDV + RBV | 12-24 | 1, 4 | 223 | 120/129 (93%)[5] |

[1]Mainly patients with genotype 1; [2]The complete study included patients prior and after liver transplantation; [3]Preliminary results with regard to publication status or completed SVR12 (SVR not available in all patients enrolled into the study); [4]Only one patient had GT 2, 4 or 6; [5]Interims SVR4 results. LDV: Ledipasvir; RBV: Ribavirin; SMV: Simeprevir; SOF: Sofosbuvir; OMV: Ombitasvir; PTV/r: Paritaprevir/ritonavir; SVR12: Sustained virologic response 12 wk after end of treatment.

with RBV for 12 wk *vs* 24 wk in patients with GT1 und GT4 in liver graft recipients at median (range) 4.4 (0.4-23.3) years after LT. Two-hundred-twenty-three patients were enrolled, 83% had prior HCV treatment, 47% of all patients were PI treatment experienced. Fifty percent of patients had F0-F3 fibrosis, while 23%, 22% and 4% had CPT class A, B, and C cirrhosis, respectively. SVR rates were high in patients without (12 wk, 96%; 24 wk, 94%) as well as in patients with graft cirrhosis (12 wk, 92%; 24 wk, 84%). Overall safety was excellent, however, 5 patients with graft cirrhosis died during the study period due to gastrointestinal bleeding, multiorgan failure, intestinal perforation, cardiac problems, complications of cirrhosis and progressive multifocal leukoencephalitis. These early results implicated no difference between 12 and 24 wk of LDV/ SOF plus RBV.

The SOLAR-1 and -2 studies demonstrated high efficacy and an excellent safety profile of LDV/SOF for 12 or 24 wk in patients without and with graft cirrhosis. Both studies have to be highlighted, because cirrhosis stage was well classified, and all stages of severe liver disease after LT namely graft cirrhosis CPT class C and FCH were included. The overall SVR$_{12}$ rates were > 90%. A longer treatment of 24 wk was not superior over 12 wk with respect to SVR in both studies. Nevertheless, lower SVR rates were observed in patients with decompensated graft cirrhosis[19,31].

Thirty-four patients after liver transplantation without and with mild fibrosis (≤ F2) were enrolled in an open label, phase Ⅱ study (CORAL-I) by Kwo *et al*[84] receiving OBV/PTV/r/DSV plus RBV for 24 wk. Patients had a calcineurin inhibitor based immunosuppression. The overall efficacy assessed by SVR$_{12}$ was high with 33/34 (97%). Reported adverse events were mostly mild, fatigue, namely headache, and cough. Of note, substantial dosage modifications of immunosuppressants were required to maintain respective therapeutic levels.

The post-transplant cohort of the ALLY-1 open label, phase Ⅲ study included 53 HCV-infected patients of any genotype to the treatment of 12 wk with DCV/SOF and RBV[36]. Seventy-seven percent of patients had HCV genotype 1 infection. No patients with FCH or decompensated graft cirrhosis were included. The efficacy was high with an overall SVR$_{12}$ rate of 94%. In the compassionate use program of DCV/SOF also patients with FCH or decompensated graft cirrhosis were included, however, the total number (*n* = 12) of respective patients included was low[87]. In this program, patients were treated with DCV/SOF in equal parts with or without RBV for 24 wk. Nine of twelve completed 24 wk of treatment, while three patients died before end of treatment. Preliminary post-treatment data were available for five patients, and so far no viral relapse was reported. Of note, dose adjustment of immunosuppressants was not necessary during treatment.

Robust data for the use of SMV/SOF in liver graft recipients are available from cohort studies, only[77,86,90]. The largest study enrolled 132 patients with HCV genotype 1 infection at median (range) 32 (2-317) months after LT, who were treated with SMF/SOF with and without RBV for 12 wk[86]. Overall, 60% of patients were infected with genotype 1a, 30% had METAVIR F3-F4, 4% had decompensated graft cirrhosis, and 11% had FCH. Furthermore, 7% of patients had also a kidney transplant, and 82% had previously failed (peg)-IFN/RBV-based regimens. Immunosuppression contained tacrolimus in 91%. Overall SVR$_{12}$ rate was 90%. However, patients with genotype 1a infection and advanced fibrosis (METAVIR F3-F4) had significantly lower SVR rates (71%) than those with F0-F2 (91%). Twenty-five patients received RBV (20%) with no significant impact on SVR. However, 72% patients developed clinical relevant anemia. One death - possibly due to drug induced lung injury - occurred, all other adverse events were classified mild. Minimal dose adjustments in immunosuppression were necessary[86]. A further, but smaller study (*n* = 42) is of interest, because 14% of patients with decompensated graft cirrhosis were included. Overall 95% of patients achieved SVR$_{12}$, 97% of patients without and 88% of patients with cirrhosis, respectively (*P* = NS).

## DRUG-DRUG INTERACTION BETWEEN DAA'S AND IMMUNOSUPPRESSION

The calcineurin inhibitors ciclosporin and tacrolimus are substrates of CYP3A[91-93]. Clinical significant interactions with ciclosporin and tacrolimus have been described for SMV and OMV/PTV/r. For co-administration of SMV and tacrolimus monitoring of tacrolimus trough blood levels is recommended, while again the co-administration of SMV and ciclosporin is not recommended, because SMV levels may raise (summary of product characteristics). Dose reduction and respective drug monitoring of ciclosporin (20% of daily dose) and tacrolimus (fixed 0.5 mg weekly dose) is recommended for co-administration with OBV/PTV/r ± DSV. Clinical significant drug-drug interactions with DAA have not been reported for mycophenolate mofetil. However, persistent anemia with need of blood cell transfusions was reported due to combined treatment with mycophenolate mofetil and peg-IFN/RBV/SMV[94].

## CONCLUSION

There are increasing data reporting DAA based, (peg)-IFN free treatment of patients with HCV associated cirrhosis. In patients with compensated cirrhosis CPT class A, all approved agents are safe and SVR rates do not significantly differ from patients without cirrhosis[95]. In patients with decompensated CPT class B/C cirrhosis, DCV, LDV, VEL and SOF alone or in combination with RBV are safe, and SVR rates > 90% can be achieved. For most patients, a treatment course of 12 wk with or without RBV is considered sufficient. In patients with severest cirrhosis (CPT class C, MELD > 15), data from randomized trials are scarce. However, SVR rates seem lower compared to patients with less severe liver disease, and yet no (bio)markers are available to predict the further clinical outcome.

In patients with HCV graft infection after LT mostly open label trials and cohort analyses, and only few randomized trials are available. Data are conclusive that SVR rates are not different to patients without LT and maximum CPT class A cirrhosis. Patients with decompensated graft cirrhosis should be treated on an individual basis. Moreover, DAA based therapy is relatively safe in patients after LT, and therapy discontinuations due to therapy side effects are rare. Nevertheless, some challenges are to overcome. Potential drug-drug interactions - especially with immunosuppression - and concomitant impaired renal function have to be considered. A cautious surveillance during antiviral therapy is advisable to identify infections and immediately administer antibiotic treatment.

In summary, while all approved agents are eligible for patients with CPT class A cirrhosis, only 4 agents - DCV, LDV, VEL and SOF - are currently approved for patients with all severity of liver disease including CPT class B and C cirrhosis. Liver graft recipients with compensated liver disease can be treated according to patients without prior LT. The standard treatment duration for the majority of patients is 12 wk.

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REVIEW

# Mesenchymal stromal cell-based therapy: Regulatory and translational aspects in gastroenterology

Giovanni Dothel, Emanuel Raschi, Roberto Rimondini, Fabrizio De Ponti

Giovanni Dothel, Emanuel Raschi, Roberto Rimondini, Fabrizio De Ponti, Pharmacology Unit, Department of Medical and Surgical Sciences, Alma Mater Studiorum, University of Bologna, I-40126 Bologna, Italy

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Correspondence to: Fabrizio De Ponti, MD, PhD, Pharmacology Unit, Department of Medical and Surgical Sciences, Alma Mater Studiorum, University of Bologna, Via Irnerio, 48, I-40126 Bologna, Italy. fabrizio.deponti@unibo.it
Telephone: +39-51-2091805
Fax: +39-51-2091780

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## Abstract

The past decade has witnessed an outstanding scientific production focused towards the possible clinical applications of mesenchymal stromal cells (MSCs) in autoimmune and chronic inflammatory diseases. This raised the need of novel standards to adequately address quality, efficacy and safety issues of this advanced therapy. The development of a streamlined regulation is currently hampered by the complexity of analyzing dynamic biological entities rather than chemicals. Although numerous pieces of evidence show efficacy in reducing intestinal inflammation, some inconsistencies between the mechanisms of action of rodent *vs* human MSCs suggest caution before assigning translational value to preclinical studies. Preliminary evidence from clinical trials showed efficacy of MSCs in the treatment of fistulizing Crohn's disease (CD), and preparations of heterologous MSCs for CD treatment are currently tested in ongoing clinical trials. However, safety issues, especially in long-term treatment, still require solid clinical data. In this regard, standardized guidelines for appropriate dosing and methods of infusion could enhance the likelihood to predict more accurately the number of responders and the duration of remission periods. In addition, elucidating MSC mechanisms of action could lead to novel and more reliable formulations such as those derived from the MSCs themselves (*e.g.*, supernatants).

**Key words:** Mesenchymal stromal cells; Mesenchymal stem cells; Inflammatory bowel diseases; Intestinal disorders; Translational medicine



**Core tip:** Mesenchymal stromal cells (MSCs) release immunomodulatory mediators upon inflammatory *stimuli*. This behavior is attractive for the development of advanced therapeutic strategies applied to several intestinal disorders where inflammation is a key pathophysiological feature. In order to assess quality, efficacy and safety of MSC-based therapy, a novel

approach to pharmacokinetics/pharmacodynamics (PK/PD) is mandatory. This must rely on careful assessment of cell phenotype, signaling and homing mechanisms. In this regard, experimental models must take advantage of the most updated knowledge in order to reflect the PK/PD mechanisms in humans. Finally, an alternative approach to the "whole-cell treatment" applies MSC-derived mediators alone in order to avoid the hypothesized serious adverse events deriving from a biological entity mostly acting systemically.

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## INTRODUCTION

Mesenchymal stromal cells (MSCs) were first isolated from bone marrow as a population of adherent cells, characterized by a non-phagocytic fibroblast-like morphology[1]. Later, MSCs were described in several different tissues, (bone marrow-derived MSCs): cartilage, adipose tissue (adipose-derived stem cells - ASC), tendon and muscle.

The International Society for Cellular Therapy established 3 minimum criteria that MSCs must fulfill *in vitro*: (1) adherence to plastic; (2) expression of specific surface antigens (CD73+, CD90+, CD105+, CD34-, CD45-, CD11b-, CD14-, CD19-, CD79a, HLA-DR-); and (3) differentiation potential (osteogenic, chondrogenic and adipogenic lineages)[1]. Considering this multi-lineage differentiation capacity, MSCs were first considered as a therapeutic tool in bone and cartilage diseases, aiming at tissue regeneration. Later, hematopoietic stem cells (HSCs) transplantation was tested in facilitating engraftment and treating steroid-resistant acute-graft-versus-host disease.

More recently, the ability of MSCs to home and promote tissue repair and counteract inflammatory status prompted their investigation in a variety of inflammatory immune-mediated disorders, with about 250 clinical studies already registered[2].

Therefore, it is not surprising that MSCs are being extensively assessed as a possible therapy of intestinal disorders where inflammation represents a key pathophysiological feature, especially considering the epidemiologic burden of inflammatory bowel diseases (IBDs). There are three intriguing aspects that attract interest and warrant further investigation to fully exploit the potential therapeutic properties of MSCs.

First, it is widely accepted that MSCs do not *per se* inhibit inflammation, but require activation by an inflammatory environment in the host to produce their immunoregulatory effect. The presence of an inflammatory environment activates MSCs, which in turn shift into an immune-suppressive phenotype, whereas, conversely, the lack of inflammatory stimuli prompts MSCs to adopt a pro-inflammatory phenotype[3]. In fact, Duijvestein *et al*[4] found that administration of IFN-$\gamma$ in MSC culture medium increases their therapeutic potential in a model of trinitrobenzene sulfonate (TNBS)-induced colitis.

Second, healing properties of MSCs appear largely dependent on the release of soluble factors and chemokines produced by the cells themselves and/or by local microenvironment, while their survival does not seem necessary for clinical benefit[5]: the detection of biologically active compounds derived from MSCs implies that a "cell-free" therapy could be an alternative[6-8].

Third, homing and migration are still incompletely characterized, but are likely to be influenced by multiple factors such as age and number of passages, culture conditions and the delivery method[9]. Notably, different studies indicated that intravenous-injected MSCs are trapped in the lungs upon first passage[10].

## REGULATORY STATUS

From a regulatory standpoint, stem cells are regulated both in Europe and United States under specific legislation. In Europe, stem cells can only be used under two regulatory frameworks: approved clinical trial or compassionate use, according to the Regulation 1394/2007 of the European Parliament and of the Council on advanced therapy medicinal products and amending Directive 2001/83/EC.

Recently, the European Commission launched a public stakeholder consultation on the draft "Guidelines on Good Manufacturing Practice for Advanced Therapy Medicinal Products (ATMPs)" (ending on 26 September 2016). The main purpose is to provide all the requirements to assure identity/consistency, quality, safety and efficacy in a way that takes advantage of what has been learnt in the development of quality standards of medicinal products of chemical origin, taking into account the specific requirements of the ATMP. Importantly, internal audit personnel, formally designated as "quality personnel", must certify several steps of quality control and batch release. Special provisions regard investigational ATMPs which may be developed in academic or hospitals and cannot ensure all the information required in conventional procedures (*e.g.,* potency). However, risk-based approach and the application of good manufacturing processes (GMP) are mandatory regardless the site of production. Among other indications, blinding of the cell characterization procedure is suggested in case of investigational ATMPs, and special provisions are in place for automated productions[11].

**Table 1 Across species comparison of the anti-inflammatory action of mesenchymal stromal cell-derived mediators and their assumed validity as clinical biomarker**

| Bioactive molecule | Mouse | Rat | G.pig | Pig | Human | Clinical biomarker | Major mechanisms | Ref. |
|---|---|---|---|---|---|---|---|---|
| TSG-6 | X | X | X | NA | X | X | Reduction of IL-6, IFN-γ, and TNF-α, induction of T-reg lymphocytes | [5,18,19,27,72-76] |
| IDO | | | NA | X | X | X | Apoptosis of cytolytic lymphocytes, IL-10 induction T-reg proliferation | [13,15,16,52,77] |
| iNOS | X | X | X | | | | Inhibition of effector lymphocytes through IL-10 | [13,17] |
| PGE2 | X | X | | | X | | Macrophage conversion to M2 phenotype, NK cell inhibition, IDO induction | [12,78-80] |
| IL-10 | X | X | X | X | X | X | T-reg lymphocyte Induction | [12,13,15,17,19,54,78] |
| TGF-β | X | X | X | | X | | T-reg induction | [81-83] |
| PD-L1 | X | X | X | | X | | CD8+ lymphocyte inhibition | [12] |

Crosses: Major action/validity; NA: Not assessed; TSG6: Tumor necrosis factor-inducible gene 6 protein; IDO: Indoleamine 2,3-dioxygenase; iNOS: Nitric oxide synthase; PGE2: Prostaglandin E2; TGF-β: Transforming growth factor-β; PD-L1: Programmed death-ligand 1; T-reg: Regulatory T lymphocytes.

## PHARMACODYNAMIC ASPECTS - BIOLOGICAL EFFECTS

The multifaceted immuno-modulating properties of MSCs, capable of interacting with both the adaptive and innate immune system, make them an attractive source to restore immune homeostasis in the gut and even coordinate tissue remodeling during the healing process.

MSCs were demonstrated to inhibit *in vitro* differentiation of T lymphocytes into Th1 and Th17 cells, suppress cytotoxic T cells proliferation through secretion of anti-proliferative soluble factors such as hepatocyte growth factor (HGF), TGF-β, prostaglandin E2, indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO) and heme-oxigenase-1 (HO-1). In addition, MSCs possess the ability to polarize T cells towards a regulatory phenotype, interfere with differentiation and maturation of monocytes towards dendritic cells, induce dendritic cells to acquire a tolerogenic phenotype, and down-regulate NK activation (Table 1).

Taken together, these findings document the *in vitro* properties of MSCs and strongly support their anti-inflammatory mechanisms involved in IBDs[12]. Importantly, MSC production of IDO depends on IFNγ or TNFα in combination with IL-1β. The latter in turn catabolizes tryptophan producing kynurenins, a potent T-reg lymphocyte inducer[13,14]. MSC-dependent induction of IDO correlates with disease rating in humans[15,16] and, to a lesser extent, in mice where MSC anti-inflammatory action is mostly mediated by iNOS[13,17] (Table 1). Notably, IDO or iNOS-mediated MSC activity was recently shown to correlate with a specific phylogenetic tree (Figure 1)[13].

Tumor necrosis factor-inducible gene 6 protein (TSG-6) mediates MSC action in both mice and humans[18,19]. Originally, this molecule was extensively studied for its anti-inflammatory properties associated with extracellular matrix rearrangement[20] and, more recently, as an effector of MSCs[18]. MSC-secreted TSG-6 decreases NF-κB in macrophages, which in turn secretes PGE-2[21] (Figure 1). Moreover, MSCs knocked down for TSG-6 do not exert any therapeutic action.

Unfortunately, TSG-6 production *in vitro* is scarcely achieved because of its tendency to form complexes with hyaluronans. This finding is in line with the observations of Sala and colleagues, who detected the formation of intra-peritoneal TSG-6 mediated aggregates of MSCs in a mouse model secreting IL-10. As a consequence, the authors question the relevance of MSC engraftment as a therapeutic mechanism[19].

## PHARMACOKINETICS ASPECTS - BIODISTRIBUTION

Whether the anti-inflammatory properties of MSCs are local or depend on the release of soluble mediators from a distance is a matter of debate[22,23].

Intravenous injection of MSCs has been thoroughly investigated to test the claim that it can restore tissue function after myocardial infarction[24-26]. During the expansion phase *in vitro,* the size of cell bodies tends to increase and this favors their entrapment in microvessels and capillaries once they are infused[23,27,28]. This phenomenon could explain the extremely high percentage of MSCs localized in the lung after intravenous injection[18,26,29]. Recently, mouse models have confirmed the pulmonary localization of MSCs[30-33]. Furthermore, infusion of MSCs led to pulmonary parenchymal edema and hemorrhage at the highest dose tested[34]. Wang and colleagues proposed an *in silico* approach to predict the time course of the localization of MSCs after intravenous injection *in vivo*[33]. Notably, this analysis allowed to calculate changes in MSC distribution occurring in disease states entailing loss of function of specific

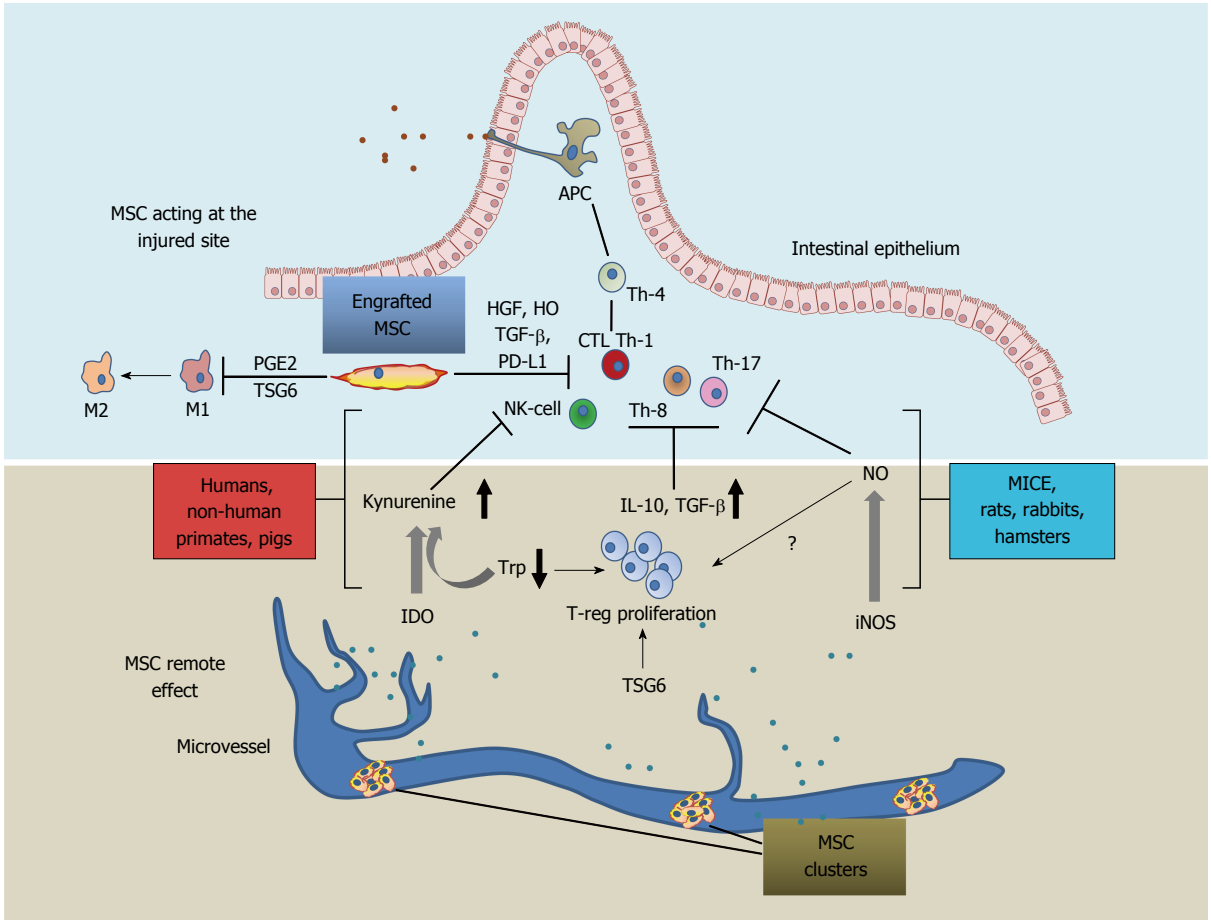


**Figure 1 Differences in mesenchymal stromal cell mechanisms of action across species: scheme representing engrafted mesenchymal stromal cell acting at the injured site (upper side) and mesenchymal stromal cell acting at a distance as a reservoir of anti-inflammatory mediators (lower side).** Several MSC-driven mechanisms concur to orientate activated lymphocytes toward the T-regulatory phenotype. TSG-6 enhances proliferation of T-reg lymphocytes and inhibits secretion of pro-inflammatory cytokines by macrophages, the latter undergoing a phenotype shift toward the anti-inflammatory one (from M1 to M2) driven by MSC-secreted PGE2. PD-L1 mediate an anti-inflammatory action by cell-cell contact. MSC immunomodulation, acts primarily through the IDO-mediated mechanism in humans, non-human primates, and pigs; while iNOS acts in mice, rats, hamsters and rabbits, inducing the T-reg proliferation through a mechanism that is still unclear; TSG-6 is a common effector across species which is assumed to mediate both in situ and remote effects. MSC: Mesenchymal stromal cells; TSG6: Tumor necrosis factor-inducible gene 6 protein; IDO: Indoleamine 2,3-dioxygenase; iNOS: Nitric oxide synthase; Trp: Tryptophan; PGE2: Prostaglandin E2; TGF-β: Transforming growth factor-β; PD-L1: Programmed death-ligand 1; HO: Heme-oxigenase-1; HGF: Hepatocyte growth factor; APC: Antigen-presenting cell; T-reg: Regulatory T lymphocytes; macr: Macrophage; M1-M2 macr. shift: Macrophage shift from M1 pro-inflammatory phenotype to M2 anti-inflammatory phenotype.

organs (*e.g.,* heart failure). In line with other studies, supply through the hepatic artery was then suggested as an alternative route of administration to avoid lung overload[23,33].

Intraperitoneal injection of autologous and xenogenic (human) MSCs provoked the formation of clusters, which remained outside the site of injury without impeding the antiinflammatory potential[19,30]. Migration and homing are particularly elusive events, either because their detection relies on *in situ* analysis of functional chemokines and because they depend on a phenotype which can be modified by the initial phases of cell expansion[23]. Importantly, compared to normal tissue inflamed/injured tissues showed an increased tendency to attract circulating MSCs[7,35]. Cell engraftment was reported to be mediated by VCAM-1 and p-selectin[36], whereas diapedesis and extravasion are still unclear, but certainly they are far from resembling lymphocyte's homing capacity[36].

In light of these findings, the real contribution of MSC homing capacity to the overall immunomodulatory effect is questionable since also pulmonary localization could achieve similar effect. However, ongoing experimental strategies aim at increasing the percentage of MSCs which successfully evade pulmonary and kidney capillary entrapment. As an example, homing receptor CXC-R4 was induced in MSCs by specific culture conditions[37] or viral transfection[38].

Certainly, *in situ* action of MSCs in large numbers may represent an advantage in those therapies aimed at tissue restitution after inflammation-induced injury. In particular, local MSC administration was particularly efficacious in the treatment of perianal fistula, a complication of Crohn's disease (CD)[39]. In addition, inhibition of pro-inflammatory cytokines and patients activated T-cell apoptosis was described as a cell-to-

cell mediated mechanism of MSCs[16].

## METHODS FOR SORTING, EXPANSION AND STORAGE: APPLICATION OF GMP

Differently from murine MSCs, which undergo rapid senescence, human MSCs can be maintained and expanded *in vitro* after multiple passages[40]. Number of cells plated, serum and number of passages can markedly influence MSC phenotype and immunomodulatory activity, the latter causing cellular senescence by different mechanisms, the most important of which is shortening of telomeres. A careful control of MSC phenotype during the expansion phase can avoid development of transformed and oncogenic MSC populations[41].

Cryogenic preservation represents an important aspect to be taken into account to obtain positive outcomes in clinical trials and reproducibility of preclinical studies. Enhanced immunomodulation was reported to be linked to "fresh" MSC preparations compared to cryo-conserved MSC batches[42]. In contrast, Luetzkendorf and colleagues extensively demonstrated an intact phenotype after MSC thawing under GMP conditions and a similar activity in the leukocyte proliferation assay[43,44]. Higher rate of MSC proliferation can be achieved with hypoxic conditions in culture, so as to reproduce the niche environment, whereas IFNγ/IFNγ + IL1-β pretreatment enhance IDO-mediated T-reg expansion[16,43,44].

Recent efforts were focused on innovative methods for MSC expansions, which apply microcarriers to increase the surface for cell adhesion in bioreactors. This apparatus allows the large-scale production needed for the formulation of commercial products[45-48]. The application of this novel technology highlighted the need for further standard in-culture conditions aimed at stabilizing MSC phenotype. These include medium flow rate, sheer tension[49] and cell-microcarrier interactions[50].

Finally, since MSC cultures are generally prone to be contaminated by leukocytes and because of the markedly unstable phenotype of MSCs under different lab methodologies, a number of recommendations has been released on the use of MSCs for therapeutic purposes[44,51].

## BIOMARKERS IN THE DEVELOPMENT OF MSC-BASED THERAPY

Due to the multiplicity of ligand-receptors systems and indirect mechanisms of action entailed in cell-based therapies, classical pharmacological standards are hardly applicable.

Considering systemic treatments, IDO-mediated serological shift of T-cells toward a regulatory phenotype has been indicated as a reliable mechanism in clinical studies; therefore the rate of tryptophan catabolism by increased concentration of kynurenin might be of value as a biomarker, or in alternative, serum concentration of IDO (Table 1).

On the contrary, clinical studies on TSG-6 serum concentrations after infusion of MSCs are still lacking. Concomitant observations by different research groups showed inconsistent levels of MSC-secreted TSG-6, which could depend on different culture conditions[18]. Therefore, this could lead to consider TSG-6 as a biomarker of efficacy of MSC preparation rather than a clinical biomarker, together with IDO or even a novel bioactive compound *per se*[16,52].

## MSC APPLICATION IN INTESTINAL DISEASES

Animal models of colitis transferred with human MSCs or autologous MSCs show marked improvement of bowel wall architecture and the overall immunological parameters[7,19,31,32,40,53-56]. However, given the afore-mentioned species-specific differences with rodents, shared biomarkers such as TSG-6 should be evaluated for further proof of concept studies. Moreover, the dinitrobenzene (DNBS) or TNBS model should be preferred over the dextran sodium sulphate (DSS) model for investigational studies on acquired immunity, since the former mimics more closely features of human chronic inflammation[57].

## CLINICAL EVIDENCE: A CRITICAL APPRAISAL

Inflammatory bowel diseases (IBD) are characterized by chronic recurrent intestinal inflammatory episodes and an exaggerated immune response to luminal antigens. IBD include ulcerative colitis, where inflammation is localized to the colonic mucosa, and CD, where inflammation extends to the entire intestinal tract with focal mucosal inflammation[58]. Complications of CD include transmural inflammation, fistula, bowel wall thickening/strictures and extra-intestinal inflammatory manifestations. As IBD entails aberrant cell-mediated immune response, MSC-based therapy is increasingly considered as a potentially valuable therapeutic strategy.

At present, HSCs and MSCs have been tested in several clinical trials, although with unpredictable and partially conflicting results[59,60]. Clinical experience on the use of HSCs is limited, with transient benefit in severe refractory CD, and hampered by toxicities, thus suggesting that this procedure has to be performed in highly experienced centers[61]. A recent first-in-human safety trial (single infusion of autologous bone marrow-derived mesenchymal stromal cells in 12 subjects with

CD using three doses in the range of 2-10 millions of cells/kg BW) was partly reassuring on feasibility and safety aspects. Only two patients experienced serious events that were possibly related to MSC infusion (appendicitis and *C. difficile* colitis). Five patients required hospitalizations likely due to the moderate to severe nature of their underlying CD and not the MSC infusions[52].

Two major instances of MSC-based therapies must be faced in future clinical trials: safety and efficacy of allogeneic MSC preparations, which would avoid the time-consuming phase of cell expansion before injection, and the possible unwanted interactions with the patients' ongoing therapies, *i.e.,* biological agents and other immunomodulators. Indeed, previous trials on perianal fistulas in CD enrolled patients refractory to standard therapies. Initial trials required discontinuation of immunomodulators, whereas the latest studies allowed continuation of therapy if the dose was maintained stable for several months. Some authors have envisioned an adjuvant role of MSCs to control residual fistulas[62]. In this regard, encouraging results come from the positive outcomes of a recent phase Ⅲ trial on efficacy and safety of allogeneic ASC treatment of patients with refractory CD and complex perianal fistula. Local injection of allogeneic ASC shortened time to remission over placebo. Importantly, the study also addressed efficacy and safety parameters with concomitant anti-TNFα therapy, immunomodulators (*i.e.,* azathioprine, 6-mercaptopurine, methotrexate) or antibiotics (*i.e.* ciprofloxacin or metronidazole)[63] (Table 2). Another ongoing trial generating much expectation is the phase Ⅲ placebo-controlled double-blind study of Prochymal® (intra-venous injection of allogeneic BM-MSCs) in CD, which plans to enroll 330 patients receiving four infusions over 2 wk of 600-1.2 million cells: results are expected by 2018[64].

The body of evidence on the potential of MSCs is remarkable, but with conflicting results in terms of efficacy, especially for systemic administration in luminal IBD[39]. In this context, there are some unresolved clinical issues.

First, safety is still a matter of debate, especially in the long term. The primary concern is the potential malignant transformation of the administered cells. However, a recent meta-analysis partly reassured the scientific community by showing that malignancies occurred only in patients with previous or current malignancies, with no formation of *de novo* tumors[65]. Table 2 provides a synopsis of published clinical studies using MSCs in refractory CD or perianal fistulizing CD, with a focus on safety aspects.

Second, MSC administration, in terms of route, dose and type, deserves optimization. In particular, dose selection is crucial to find the right balance between efficacy and safety. Initial open-label trials used doses up to $3 \times 10^7$ cells. Subsequently, the amount of cells increased aiming at improving outcomes on the basis of the portion of intestinal tract to be treated ($10^7$ cells). Notably, in the study by Molendijk *et al*[66], patients were randomized to receive 3 different doses ($1 \times 10^7$, $3 \times 10^7$, $9 \times 10^7$) or placebo, with those assigned to the $3 \times 10^7$ arm experiencing the best response rate. This further emphasizes the complex nature of MSCs as biotechnological products, which do not strictly follow the general pharmacokinetic rules in terms of dose-response. In addition, there is an urgent need to share uniform protocols and increase reproducibility and consistency of data.

## FUTURE PERSPECTIVES AND CONCLUDING REMARKS

The purpose of investigating the extracellular products of MSCs is twofold: to clarify their mechanism of action and, more importantly, to evaluate the efficacy of MSC mediators *per se*, so as to avoid whole-cell infusion. Apart from the importance of deepening the knowledge on MSC nature, an emerging interest surrounds MSC-secreted micro-mRNA (miRNA)[67] as a possible therapeutic option in pulmonary hypertension[68]. The same molecules regulate toll-like receptors (TLR) expression in MSC stimulated with bacterial derived lipopolysaccharide[69]. Further studies are warranted to clarify mechanisms of TLR4 expression of MSCs[70], especially in light of its role in the tolerogenic pathways of the intestinal immune homeostasis.

The use of MSC exosomes/extracellular vesicles is increasingly under study. These were proven to be effective in reducing NF-κB activity and the level of pro-inflammatory cytokines in a TNBS rat model[6]. Furthermore, MSC supernatants evoked an anti-inflammatory response and an overall improvement of bowel wall architecture in both DSS and TNBS rat models[71]. The same study suggested the intraperitoneal route as more effective, and a panel of candidate bioactive compounds derived from MSCs.

In conclusion, MSC-based therapies can make a step forward through strategies that: (1) enhance immunomodulatory phenotypes and cellular yields for large-scale production (for heterologous MSC-based therapy); (2) use animal models showing phylogenetic consistency for proof of concept studies on MSC mechanisms of action; (3) prefer a route of administration with no pulmonary or kidney MSC retention; (4) enhance MSC engraftment at the intestinal injured site, especially for those pathological conditions requiring cell replacement and mucosal/whole tissue healing; and (5); maintain standard cell markers favoring application of heterologous therapy. Finally, deepening the knowledge on MSC physiology could pave the way for novel pharmacological strategies based on MSC mediators.

**Table 2 Synopsis of published clinical studies using mesenchymal stromal cells in perianal fistulizing and/or refractory Crohn's disease**

| Ref. | Study design | Patients | Disease duration and characteristics | Assessment and follow-up | Source of cells | Dose | Safety outcomes (terminology) | Key safety results |
|---|---|---|---|---|---|---|---|---|
| García-Olmo *et al*[84] (2005) | Phase 1 | 9 patients aged 32-46 | Diagnosis of CD at least 5 years before, unresponsive to medical treatment and unsuccessfully treated by classic surgery at least twice | Weekly follow-up for the first 8 wk, then monthly follow-up up max 30 mo | Autologous ASC | 3-30 × $10^6$ | Not specified | No immediate adverse reactions (*e.g.*, anaphylaxis, allergic reactions) were observed in any of the cases studied |
| Garcia-Olmo *et al*[85] (2009) | Open-label, multicenter, phase 2 | 24 patients with mean age = 52 received ASC+fibrin glue | Complex perianal fistula (either of cryptoglandular origin or associated with CD). In patients with CD, immunomodulators were used continuously for at least six months and stable for more than eight weeks | Week 8, 16 and then at 3-mo interval up to 12 months | Autologous ASC | 2-4 × $10^7$ | Incidence of adverse events and serious adverse events | 11 adverse events (at week 8), of which two were SAEs, but unrelated to ASC administration. In the following phase, 9 adverse event (perianal sepsis), unrelated to ASC administration |
| Ciccocioppo *et al*[86] (2011) | Prospective study | 12 patients aged 16-44 (two drop outs) | Patients with CD unresponsive to or unsuitable for all previous medical treatment including biological agents or unsuccessfully treated by surgery | 3, 6 and 12 mo | Autologous BM-MSC | Median 20 × $10^6$ | Changes in vital signs and adverse reactions during the first 6 h after each cellular treatment, and during the following 12-month follow-up | No changes in vital signs and no adverse events were recorded during the procedure and up to the end of the 6-h observation time, or during the 12-mo follow-up period |
| Guadalajara *et al*[87] (2012) | Retrospective follow up of phase 2 | 24 patients with mean age = 42 | Patients enrolled in previous phase 2 study receiving at least one ASC administration | 8 wk, 1 yr | Autologous ASC | Not specified | Number of adverse events since the final visit of the phase II study (serious or not, severity, causality) | Ischiorectal abscess (patient treated with fibrin glue alone) and a perianal abscess (patient treated with ASCs plus fibrin glue), both toxicity grade I and unrelated to the study treatment. These events occurred at 13 and 21 mo after the original treatment, respectively |
| Herreros *et al*[88] (2012) | Multicenter randomized single-blind Phase 3 + observational | 200 patients with mean age = 50 | Cryptoglandular complex fistula-in-ano (without CD) | 1, 4 and 12, 14 24, 26, 48 wk | Autologous ASC | 2 × $10^7$ (± fibrin glue) | Incidence of adverse events and SAEs | Approximately 85%-90% of patients experienced an adverse event, but most of these were nonserious. There were 17 different AEs reported in more than 5% of the cases. The most frequent AEs were proctalgia (43.7%), abscess drainage (22.4%), pain (13.7%), perianal abscess (13.1%), pyrexia (0.3%), swelling (6.6%), and pruritus (6.6%). There were no statistically significant differences within groups. There were 37 SAEs in 30 patients. All but 4 SAEs were unrelated to study treatment |
| Lee *et al*[89] (2013) | Open-label phase 2 | 43 patients with mean age = 26 | Perianal fistulae with CD in patients not treated with infliximab within 3 mo prior to ASC | 4, 6 and 8 wk, 10 mo | Autologous ASC | Depending on the fistula (mean from 15 to 19 × $10^7$) | Systemic tolerance, adverse events and SAEs | Post-operative pain (60%), anal pain (19%) and anal bleeding (7%), unrelated to ASC administration. One hospitalization for vitamin B12 deficit; two grade 3/4 events (exacerbation of disease and peritonitis), unrelated to ASC administration |
| de la Portilla *et al*[90] (2013) | Open-label phase 1/2a | 24 patients with mean age = 36 | Diagnosis of CD at least 12 mo before, presence of persistent and active complex perianal fistula with less than three fistulous tracts and/or external opening, non-active luminal CD; no treatment with infliximab or any other anti-TNF agent in the previous 8 wk or tacrolimus or cyclosporine in the previous 4 wk | 10, 12, 22, 24 wk | Allogeneic ASC | 2 × $10^7$ (up to 4 × $10^7$ if no effect) | Incidence of treatment emergent adverse events | 32 treatment-emergent adverse events during the study, the majority of which were of mild to moderate intensity. Five treatment-related AEs were reported: "anal abscess" (3 patients), "pyrexia" (1), and "uterine leiomyoma" (1). Only two SAEs: "pyrexia" and "perianal abscess", considered to be possibly related to the study treatment and both patients were withdrawn from the study |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Ciccocioppo *et al*[91] (2015) | 5-year follow up of an open-label phase 2 | 8 patients with median age = 37 | Refractory CD or inability to undergo standard therapies | 12 mo until 5 yr | Autologous BM-MSC | Not specified | Systemic tolerance, adverse events and SAEs, as specified in the Medical Dictionary for Regulatory Activities terminology | 23 adverse events, mainly consisting of abdominal pain, headache, anal inflammation, diarrhea, erythema, nausea, and fever. All AEs were consistent with exacerbation of the primary disease, but cholecystectomy due to the presence of gallstones. None was attributed to MSC therapy, and no evidence of tumor development or opportunistic infection |
| Cho *et al*[92] (2013) | Open-label, multicenter, dose-escalation phase 1 | 10 patients with mean age = 26 | Perianal fistula associated with CD | Weeks 8, months 4,6 and 8 | Autologous ASC | 1, 2 and 4 × $10^7$ to 40 × $10^7$ | Adverse events reported after injection with ASCs, serious adverse events during study period, and laboratory toxicity observed after injection with ASCs. (CTCAE version 3.0) | 13 adverse events were reported in seven patients (70%). The adverse events, which were mild or moderate in severity, were not related to study drug. There were no grade 3 or 4 adverse events and no laboratory toxicity greater than grade 3 in this study. Adverse events reported in two or more patients included pain ($n$ = 3) and diarrhea ($n$ = 2). During the study period, two patients reported three SAEs of grade 2 (enterocolitis, seton application, and infliximab administration for new fistulas unrelated to the target fistula) requiring hospitalization |
| Cho *et al*[93] (2015) | Retrospective analysis of 1-year follow up phase 2 | 42 patients with mean age = 26 | Average duration of CD of 58 mo | 2 yr | Autologous ASC | Average 16.4 × $10^7$ | Systemic tolerance, adverse events, SAEs | 53 adverse events were reported in 30 patients (73.2%), the most common being abdominal pain (17.1%); eczema and exacerbation of disease (9.8%) and anal inflammation, diarrhea, and fever (7.3%). None was related to MSC administration |
| Garcia-Olmo *et al*[62] (2015) | Observational | 28-76 | Recurrent perianal fistulae who previously undergone at least three surgical interventions | Week 8 and year 1 | Autologous ASC | Not specified | Not specified | No adverse reactions or complications related to MSC administration |
| Molendijk *et al*[66] (2015) | Phase 2, double-blind, placebo-controlled, randomized study | > 18 | Actively draining perianal fistulizing CD (diagnosis at least 3 months before enrollment) refractory to conventional therapies | 6, 12, and 24 wk | Allogeneic BM-MSC | 1, 3 and 9 × $10^7$ | Primary endpoint: incidence of serious adverse events at week 12. (CTCAE, version 3.0). Secondary end point: incidence of surgical intervention and infections at week 12 and 24 | No infusion reactions; one patient 2 developed fever 6 h after surgery. One patient in each group developed a perianal abscess that required surgical drainage. Reported adverse events: $n$ = 17 (group 1, $n$ = 5), 9 (group 2, $n$ = 5), 10 (group 3, $n$ = 5), 14 (placebo, $n$ = 5). One patient (1 × $10^7$) developed an adenocarcinoma of the cecum with peritoneal carcinomatosis > 15 mo after the surgical intervention |
| Dhere *et al*[52] (2016) | Phase 1 safety trial | 18-52 | Established CD for at least 3 mo refractory to conventional therapies (lack of response to immunomodulators and/or biologics for at least 3 mo) | 1, 5 and 9 wk after infusion | Autologous BM-MSC | 2, 5 and 10 × $10^6$ | Changes in respiratory or cardiovascular parameters during the 1 h infusion and for 4 h after. Temperature, heart rate, mean arterial pressure and respiratory rate assessed at 15 min, 30 min, 1 h, 2 h, 3 h and 4 h | No patient developed significant infusion reaction. SAEs in 7 patients, of which 2 likely to be related to MSC administration: appendicitis (with appendectomy 9 d after infusion and complete colectomy for medically refractory CD after 120 d) and *C. difficile* colitis (30 d after infusion) |
| Panés *et al*[63] (2016) | Phase 3, randomized, placebo-controlled trial | 107 patients, mean age = 39 | Actively draining perianal fistulizing CD refractory to conventional therapies | 24 wk after local injection | Allogeneic ASC | 12 × $10^6$ | TEAEs (MedDRA version 17.0) | 18 patients of the ASC treated group *vs* 30 of 107 in the placebo group developed treatment-related adverse events, (anal abscess and proctalgia) |

SAEs: Serious adverse events; TEAEs: Treatments emergent adverse events; ASC: Adipose-derived stem cells; CD: Crohn's disease; MSC: Mesenchymal stromal cells; BM-MSC: Bone Marrow derived mesenchymal stromal cells. CTCAE: Common Terminology Criteria for Adverse Events; MedDRA: Medical Dictionary for Regulatory Activities.

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REVIEW

# Genetic alterations in hepatocellular carcinoma: An update

Zhao-Shan Niu, Xiao-Jun Niu, Wen-Hong Wang

Zhao-Shan Niu, Laboratory of Micromorphology, School of Basic Medicine, Medical Department of Qingdao University, Qingdao 266071, Shandong Province, China

Xiao-Jun Niu, Oncology Specialty, Medical Department of Qingdao University, Qingdao 266071, Shandong Province, China

Wen-Hong Wang, Department of Pathology, School of Basic Medicine, Medical Department of Qingdao University, Qingdao 266071, Shandong Province, China

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Correspondence to: Zhao-Shan Niu, MD, Laboratory of Micromorphology, School of Basic Medicine, Medical Department of Qingdao University, Room 201, Building Boya, 308 Ningxia Road, Qingdao 266071, Shandong Province, China. niumiao1993@hotmail.com
Telephone: +86-532-83780012
Fax: +86-532-83780012

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## Abstract

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide. Although recent advances in therapeutic approaches for treating HCC have improved the prognoses of patients with HCC, this cancer is still associated with a poor survival rate mainly due to late diagnosis. Therefore, a diagnosis must be made sufficiently early to perform curative and effective treatments. There is a need for a deeper understanding of the molecular mechanisms underlying the initiation and progression of HCC because these mechanisms are critical for making early diagnoses and developing novel therapeutic strategies. Over the past decade, much progress has been made in elucidating the molecular mechanisms underlying hepatocarcinogenesis. In particular, recent advances in next-generation sequencing technologies have revealed numerous genetic alterations, including recurrently mutated genes and dysregulated signaling pathways in HCC. A better understanding of the genetic alterations in HCC could contribute to identifying potential driver mutations and discovering novel therapeutic targets in the future. In this article, we summarize the current advances in research on the genetic alterations, including genomic instability, single-nucleotide polymorphisms, somatic mutations and deregulated signaling pathways, implicated in the initiation and progression of HCC. We also attempt to elucidate some of the genetic mechanisms that contribute to making early diagnoses of and developing molecularly targeted therapies for HCC.

**Key words:** Genetic alterations; Chromosomal instability; Somatic mutations; Signaling pathways; Hepatocellular carcinoma



**Core tip:** Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide. The poor survival rate is mainly due to late diagnosis of

HCC. Elucidating the molecular mechanisms underlying hepatocarcinogenesis is critical for making early diagnoses of and developing targeted therapies for HCC. Recent studies on HCC using deep sequencing have provided increasing lines of evidence indicating that genetic alterations play important roles in the initiation and progression of HCC, which are summarized in this article. We also attempt to elucidate some of the genetic mechanisms underlying HCC, which may help in making early diagnoses of and developing molecularly targeted therapies for this disease.

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third leading cause of cancer-related deaths[1]. HCC has a high incidence rate, and patients with this disease have a poor prognosis. Rising incidence and mortality rates for HCC have been observed in most countries, particularly in eastern/south-eastern Asia and in Africa[2]. Currently, it is generally accepted that persistent hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are the primary causes of chronic liver disease leading to liver cirrhosis and HCC. Aflatoxin B1 (AFB1) exposure and chronic alcohol abuse are also important risk factors for developing HCC[2]. Despite improved overall survival (OS) rates among patients with HCC due to advancements in surgical techniques, 5-year OS remains low at 18%[3]. The survival rate of HCC patients is poor because most patients cannot be treated by surgical resections or liver transplantation (LT), mainly due to late diagnosis. In addition, HCC is associated with a high recurrence rate, which exceeds 50% at 5 years after surgery[4]. Therefore, the early detection of HCC is urgently needed to perform curative and effective treatments and to improve long-term survival rates. There is a need for a deeper understanding of the molecular mechanisms underlying the initiation and progression of HCC because this understanding is critical to making early diagnoses and developing novel therapeutic strategies.

It is widely accepted that carcinogenesis is a multistep process triggered by the accumulation of genetic alterations that activate different signal transduction pathways and drive the progressive transformation of normal cells into malignant cells[5,6]. The precise molecular mechanisms underlying the initiation and progression of HCC remain obscure. The phenotypic (morphological and microscopic) and genetic heterogeneity of HCCs also adds a new level of complexity to our understanding of hepatocarcinogenesis. However, despite many remaining challenges, substantial progress has been made in this field. As in other solid cancers, numerous genetic alterations accumulate during the process of hepatocarcinogenesis. Genetic alterations accumulate slowly in a limited number of genes and chromosomal loci during the early preneoplastic stage and accelerate throughout dysplasia and into the development of HCC[7]. Previous studies have shown that the incidence of genetic alterations in HCC is relatively rare and limited to a subset of a few cancer-specific genes[8]. Encouragingly, functional genomic approaches that have been applied in recent years, such as array-based comparative genomic hybridization, genome-wide association studies (GWAS) and next-generation sequencing (NGS), have advanced our understanding of the genetic basis of HCC. Specifically, recent advances in NGS technologies have identified major cancer-driving genes and associated oncogenic signaling pathways that play important roles in the initiation and progression of HCC.

It is known that HCC cells are extremely resistant to almost all conventional chemotherapeutic drugs, and until now, there have been only a limited number of chemotherapeutic agents available for the treatment of patients with HCC, especially those with advanced, unresectable cancer. Currently, oncologists are testing novel, molecularly targeted agents for treating HCC. Therefore, in an era of precision cancer medicine, monitoring clinically relevant genetic alterations is important for stratifying patients for targeted therapies[9].

The molecular mechanisms leading to the development of HCC are extremely complicated and consist of prominent genetic and epigenetic alterations[10]. Although it has been widely accepted that epigenetic alterations also play a significant role in hepatocarcinogenesis, this topic is beyond the scope of this article. Instead, in this article, we focus on the current advances in understanding the genetic alterations, including genomic instability, single-nucleotide polymorphisms (SNPs), somatic mutations, and the deregulated signaling pathways implicated in the initiation and progression of HCC. We also attempt to elucidate some of the underlying genetic mechanisms, which could contribute to making early diagnoses of and developing molecularly targeted therapies for HCC. The impact of genetic alterations on hepatocarcinogenesis is presented in Figure 1.

## GENOMIC INSTABILITY

Genomic instability (also known as "genetic instability" or "genome instability") is defined as a high frequency of mutations within the genome, including changes in nucleic acid sequences, chromosomal rearrangements, or aneuploidy[11]. However, it remains unclear whether genomic instability is a cause or a consequence of

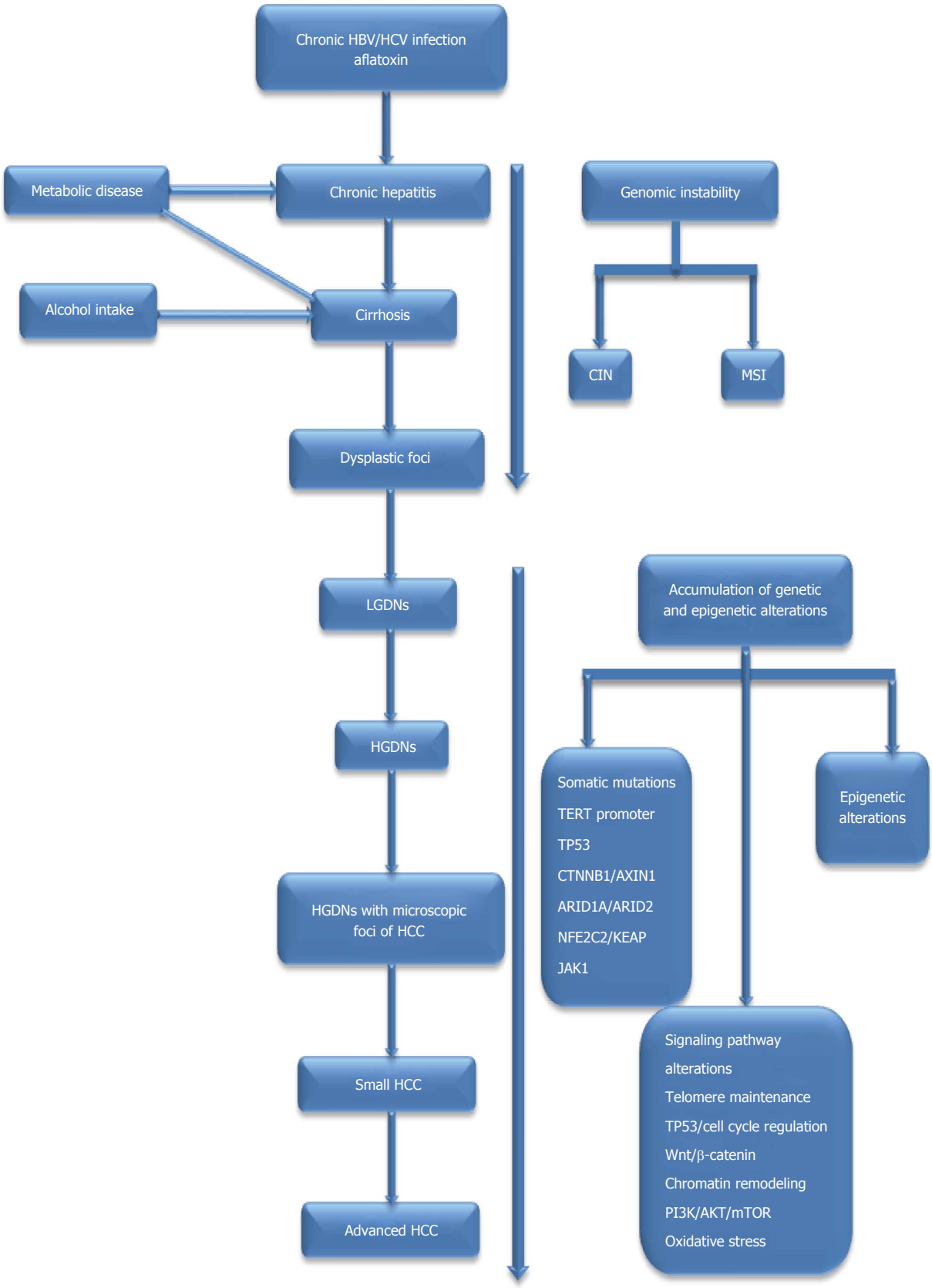


**Figure 1 The impact of genetic alterations on hepatocarcinogenesis.** Genetic alterations in hepatocarcinogenesis are connected to underlying etiologies, such as HBV, HCV, dietary AFB1 exposure and alcohol intake. Genomic instability accumulates slowly in a limited number of genes during the early preneoplastic stage, such as the development of cirrhosis, and the accumulation of genetic and epigenetic alterations accelerates throughout the formation of preneoplastic lesions, such as LGDNs and HGDNs, and into the development HCC; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFB1: Aflatoxin B1; LGDN: Low grade dysplastic nodule; HGDN: High grade dysplastic nodule; HCC: Hepatocellular carcinoma; CIN: Chromosomal instability; MSI: Microsatellite instability; TERT: Telomerase reverse-transcriptase; ARID1A: AT-rich interactive domain-containing protein 1A; ARID2: AT-rich interactive domain-containing protein 2; NFE2L2 or NRF2: Nuclear factor erythroid-derived 2-like 2; KEAP1: Kelch-like ECH-associated protein 1; JAK1: Janus kinase 1; RPS6KA3: Ribosomal protein S6 kinase polypeptide 3.

**Table 1 The characteristics of chromosomal instability and possible correlations with clinical and pathological parameters in hepatocellular carcinoma discussed in this review**

| Chromosome | Type of aberration | Targeted genes | Correlations with clinical and pathological parameters | Ref. |
|---|---|---|---|---|
| 1q21 | Gain | *CHD1L, CKS1B, JTB, SHC1* | Progression of HCC | Hyeon *et al*[43] |
| 1q21-23 | Gain | - | Early development | Yim *et al*[40] |
| 1q21-q22 | Gain | - | Metastasis | Wang *et al*[41] |
| 1q21.1-q23.2 | Gain | *BCL9, ARNT, TPM3, MUC1, NTRK1* | Poorly differentiated HCV-associated HCC | Liu *et al*[42] |
| 1q22-23.1 | Gain | *CD1d* | Diagnosis and prognosis | Zhang *et al*[44] |
| 1q24.1-24.2 | Gain | *MPZL1* | Intrahepatic metastasis | Jia *et al*[45] |
| 8q24.21-24.22 | Gain | *MYC, DDEF1, MLZE* | Prognosis (DFS and OS) | Pedica *et al*[47] |
| 8q21.13 | Gain | *HEY1* | Proliferation | Jia *et al*[37] |
| 8q22.3 | Gain | *CTHRC1* | Aggressive HCC | Tameda *et al*[48] |
| 8q24.3 | Gain | *BOP1* | Advanced-stage HCC, microvascular invasion and shorter DFS | Chung *et al*[50] |
| 7q21.3 | Gain | *SGCE, DYNC1I1, PEG10* | Hepatocarcinogenesis | Tsuji *et al*[51] |
| 4q34.3-35 | LOH | *ING2* | Progression | Zhang *et al*[56] |
| 4q13.3-q35.2 | LOH | *ADH4, ADH1C, ADH1A, ADH6* | HBV- and AFB1-related HCC carcinogenesis | Qi *et al*[58] |
| 8p | LOH | *DLC1, CCDC25, ELP3, PROSC, SH2D4A, SORBS3* | Early stage of hepatocarcinogenesis, poor outcomes | Tornillo *et al*[59]; Roessler *et al*[30] |
| 8p22-p23 | LOH | *MCPH1, KIAA1456, TUSC3, ZDHHC2* | Metastasis and prognosis | Peng *et al*[62] |
| D4S2964 | LOH | *ARD1B, SEPT11* | Prognosis (OS) | Huang *et al*[63] |
| 6q26-q27 | LOH | *M6P/IGF2R* | Poor outcomes | Jang *et al*[64] |

HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AFB1: Aflatoxin B1; DFS: Disease-free survival; OS: Overall survival; CHD1L: Chromodomain helicase/ATPase DNA binding protein 1-like; CKS1B: Cyclin-dependent kinases regulatory subunit 1; JTB: Jumping translocation breakpoint; SHC1: SHC-transforming protein 1; BCL9: B-cell CLL/lymphoma 9 protein; ARNT: Aryl hydrocarbon receptor nuclear translocator; TPM3: Tropomyosin alpha-3 chain; MUC1: Mucin 1; NTRK1: Neurotrophic tyrosine kinase receptor type 1; CD1d: Antigen-presenting glycoprotein; MPZL1: Myelin protein zero-like protein 1; MYC: Myelocytomatosis viral oncogene; DDEF1: Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 1; MLZE: Human melanomaderived leucine zipper extra-nuclear factor; HEY1: YRPW motif protein 1; CTHRC1: Collagen triple helix repeat containing 1; BOP1: Block of proliferation 1; SGCE: Epsilon-sarcoglycan; DYNC1I1: Cytoplasmic dynein 1 intermediate chain 1; PEG10: Parternal express gene 10; ING2: Interferon regulatory factor 2; ADH4: Alcohol dehydrogenase 4; ADH1C: Alcohol dehydrogenase 1C; ADH1A: Alcohol dehydrogenase 1A; ADH6: Alcohol dehydrogenase 6; DLC1: Deleted in liver cancer 1; CCDC25: Coiled-coil domain-containing protein 25; ELP3: Longator complex protein 3P; ROSC: Proline synthetase co-transcribed bacterial homolog; SH2D4A: SH2 domain-containing protein 4A; SORBS3: Sorbin and SH3 domain containing 3; MCPH1: Microcephalin 1; KIAA1456: tRNA methyltransferase 9-like; TUSC3: Tumor suppressor candidate 3; ZDHHC2: DHHC-type containing 2; ARD1B: ARD1 homolog B (*S. cerevisiae*); SEPT11: Mus musculus septin 11; M6P/IGF2R: Mannose 6-phosphate/insulin-like growth factor 2 receptor.

tumorigenesis. In recent years, accumulating evidence has strongly indicated that genomic instability could be a major driving force in tumorigenesis and the development of cancer[12-18]. In neoplasms, genomic instability can be broadly classified based on its origin as chromosomal instability (CIN) or, less commonly, microsatellite instability (MSI)[19]. Currently, there are many technologies that can be used to detect genomic instability, including karyotyping, flow cytometry, fluorescent *in situ* hybridization (FISH), array comparative genome hybridization (aCGH), high-density single-nucleotide polymorphism (SNP) arrays, the random amplified polymorphic DNA (RAPD) technique, and NGS technology.

### Chromosomal instability

In cancer, aneuploidy is a consequence of an increased rate of whole-chromosome missegregation during mitosis, a process known as chromosomal instability (CIN)[20]. CIN usually involves both numerical and structural chromosomal changes. Numerical CIN is characterized by gross chromosomal abnormalities, such as the gain or loss of whole chromosomes, leading to an altered DNA copy number (aneuploidy)[21]. Structural CIN might involve only fractions of chromosomes, resulting in the gain or loss of chromosome fragments, translocations, inversions, amplifications, deletions and allelic loss [loss of heterozygosity (LOH)][22]. CIN is a hallmark of human cancer and is believed to contribute to tumorigenesis, tumor progression, and the development of therapy resistance[20]. In addition, it has been widely accepted that CIN is associated with clinical and pathological parameters in solid tumors, and CIN is one of the most frequent abnormalities in HCC. The characteristics of CIN and its possible correlations with clinical and pathological parameters in HCC patients are summarized in Table 1. In addition, we also review the role of micronuclei, which are indicators of CIN, and chromothripsis, which is a new class of complex catastrophic chromosomal rearrangement.

DNA copy number alterations (CNAs) are important subclasses of somatic mutations, with aberrant chromosomal regions of amplifications or deletions commonly associated with overexpressed oncogenes or the loss of tumor suppressor genes (TSGs)[23].

Thus, CNAs can be used as an effective method for identifying driver genes with causal roles in carcinogenesis[24]. Such alterations are related to certain types of cancer, including HCC, and it is possible that the identification of driver genes by means of cancer-specific CNAs could provide new insights for understanding the molecular mechanisms underlying the initiation and progression of HCC. In particular, the elucidation of the molecular roles of CNAs could contribute to developing clinically relevant prognostic and predictive markers and novel therapeutic targets for treating HCC, which might ultimately be used in personalized therapeutics. Currently, CNAs in HCC cells are usually detected *via* conventional methods, such as FISH, comparative genomic hybridization, aCGH and SNP arrays. Lately, NGS technology has been used to detect CNAs in several types of tumors[25-27]. These studies have suggested that NGS has obvious advantages in sensitivity, reliability and accuracy in detecting CNAs relative to the use of aCGH and SNP arrays. However, there is currently only one study that has reported NGS-based CNAs detected in HCC[28].

Although the distribution of aberrant chromosomal arms differs among HCCs, numerous studies have shown, using aCGH data and SNP arrays, that certain regions are frequently affected in HCC, including gains in chromosomes 1q, 5p, 6p, 7q, 8q, 17q, and 20q and losses in 1p, 4q, 6q, 8p, 9p, 13q, 14q, 16p-q, 17p, 21p-q, and 22q[28-33]. These findings reflect a high degree of CIN in HCC[34], contributing to hepatocarcinogenesis. In addition, some of these regions contain CNA-associated oncogenes or TSGs, such asc-myelocytomatosis viral oncogene (*c-myc*) (8q), *cyclin A2* (4q), *cyclin D1* (11q), retinoblastoma 1 (*Rb1*) (13q), axis inhibition protein 1 (*AXIN1*) (16p), *p53* (17p), mannose-6-phosphate receptor (*IGFRII/M6PR*) (6q), *p16* (9p), epithelial cadherin (*E-cadherin*) (16q), suppressor of cytokine signaling (*SOCS*) (16p), and phosphatase and tensin homolog (*PTEN*) (10q), which have been identified to be associated with HCC[35,36]. These findings could provide us with information critical for understanding the genetic events involved in the pathogenesis and progression of HCC. However, studies employing unbiased genome-wide searches for HCC driver genes have been limited, particularly for genes related to cancer prognosis[30]. Hence, an integrated approach, such as a combined analysis of CNAs and gene expression, might be necessary to identify driver mutations.

A copy number gain at 1q is one of the most frequently detected alterations in HCC (58%-86%), and it has been suggested to be an early genomic event in the development of HCC[37]. Notably, the region 1q21 is the most frequent minimal amplifying region (MAR)[38]. A research group showed that 1q21 was the most frequently amplified region in chromosome 1q; its amplification was detected in 36 of 60 (60%) HCC specimens[39]. In addition, a gain in 1q21-23 was identified as a genomic event associated with the early development of HCC[40], and regional 1q21-q22 gains were found in 40% of advanced metastatic HCC cases[41]. In particular, a gain of 1q21.1-q23.2 was significantly associated with grades Ⅱ-Ⅳ HCC and moderately or poorly differentiated HCV-associated HCCs. 1q21.1-q23.2 target genes encode five cancer genes: B-cell CLL/lymphoma 9 protein (*BCL9*), aryl hydrocarbon receptor nuclear translocator (*ARNT*), tropomyosin alpha-3 chain (*TPM3*), mucin 1 (*MUC1*), and neurotrophic tyrosine kinase receptor type 1 (*NTRK1*)[42]. These findings indicate that 1q21 might harbor many potential oncogenes, and the overexpression of these genes *via* amplification plays an important role in the pathogenesis of HCC[38]. In recent years, several research groups have focused on the identification and characterization of 1q21 target genes, such as chromodomain helicase/ATPase DNA binding protein 1-like *(CHD1L),* cyclin-dependent kinase regulatory subunit 1 (*CKS1B),* jumping translocation breakpoint (*JTB*) and SHC-transforming protein 1 (*SHC1),* in the progression of HCC. Of these, *CHD1L* was shown to be amplified and overexpressed in HCC cases[39]. A recent study found no nuclear immunoreactivity for *CHD1L* in normal livers or dysplastic nodules (DNs). In contrast, *CHD1L* expression in cases of HCC was significantly associated with microvascular invasion, major portal vein invasion, and higher American Joint Committee on Cancer (AJCC) T stage values[43], suggesting that *CHD1L* expression might not be an early event in hepatocarcinogenesis, whereas it is an independent predictor of lower disease-free survival (DFS) rates in HCC patients after surgical resection. Given these findings, it is vital to elucidate the roles of candidate target genes within 1q21 amplicons in the initiation and progression of HCC, which could contribute to our understanding of HCC carcinogenesis.

In addition to chromosome 1q21, a novel potential oncogene antigen-presenting glycoprotein (*CD1d*) amplicon at 1q22-23.1 could be a potential target for this amplicon in HCC[44]. In addition, using an integrated analysis of copy number and expression profiling data, one recent study found that the recurrent region of the 1q24.1-24.2 amplicon specifically targets the myelin protein zero-like protein 1 (*MPZL1*) gene in HCC; the expression levels of *MPZL1* were positively correlated with the intrahepatic metastasis of the HCC specimens[45].

Chromosome 8q is the second most frequently amplified region in HCC. More specifically, 8q24.21-24.22 is the most frequently amplified region in chromosome 8q, with amplification occurring in 53.4% of samples and targeting the known oncogenes myelocytomatosis viral oncogene (*MYC*), Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein 1 (*DDEF1*), and human melanoma-derived leucine zipper extra-nuclear factor (*MLZE*)[45]. *MYC* has been identified as a central regulator of malignant transformations in early hepatocarcinogenesis[46], and

*c-myc* gene amplification has also been found to be significantly correlated with DFS and OS in patients with HCC after surgical resection[47]. These findings suggest that *c-myc* gene amplification plays an important role in the pathogenesis and progression of HCC. Additionally, three other recurrent amplified regions at chromosome 8q have been found: 8q21.13, 8q22.3, and 8q24.3. The 8q21.13 region targets the hairy/enhancer-of-split related with YRPW motif protein 1 (*HEY1*), and functional experiments have shown that the enhanced expression of *HEY1* significantly promotes the *in vitro* and *in vivo* proliferation of HCC cells[37]. The 8q22.3 region targets two genes: collagen triple helix repeat containing 1 (*CTHRC1*) and grainy head-like transcription factor 2 (*GRHL2*). *CTHRC1* has the potential to be a new biomarker of aggressive HCC[48], while a gain in *GRHL2* was found to be associated with an early recurrence of HCC[49]. The 8q24.3 region contains several genes that could be functionally related to HCC, including scribble (*SCRIB*) and block of proliferation 1 (*BOP1*). It has been reported that increased expression of *BOP1* is associated with advanced-stage HCC, microvascular invasion and lower DFS[50].

Other amplifications include the 7q21.3 locus, which might contribute to the development or progression of HCC. Epsilon-sarcoglycan (*SGCE*), cytoplasmic dynein 1 intermediate chain 1 (*DYNC1I1*) and paternal express gene 10 (*PEG10*) have been identified as putative oncogenes located within the amplified 7q21.3 locus in HCC[51,52]. These results indicate that the amplification of 7q21.3 might be implicated in hepatocarcinogenesis.

The LOH is a marker of CIN that involves the loss of one of the two alleles at one or more loci in a heterozygote[53]. The LOH is one of the main mechanisms for the inactivation of TSGs, and the identification and characterization of LOHs could provide potential means for finding HCC-related TSGs. The LOH is frequently observed on chromosomes 1p, 4q, 6q, 8p, 9p, 10q, 11p, 13q, 14q, 16q, and 17p and is commonly observed in HCC patients[54,55]. Of these, losses on 4q and 8p are the most frequent chromosomal alterations in HCC.

The LOH at 4q has been reported to be strongly correlated with increased in alpha-fetoprotein (AFP) levels in HCC[56], and it has been found to be significantly more frequently in poorly differentiated HCCs[57]. These results suggest that the inactivation of TSGs on chromosome 4q might be a late progression event that occurs after malignant transformation. Using a high-throughput SNP array, 4q24-26 and 4q34.3-35 were found to be hot regions of chromosome 4q in HCC[56]. Three TSGs, including nei endonuclease VIII-like 3 (*NEIL3*), interferon regulatory factor 2 (*IRF2*) and inhibitor of growth family member 2 (*ING2*), are located on chromosome 4q34.3-35, but only *ING2* is a potential TSG associated with HCC[48]. In addition, the loss of 4q13.3-q35.2 is related to both HBV- and AFB1-related HCC[58], suggesting that genetic abnormalities in 4q13.3-q35.2 might play a role in both HBV- and AFB1-related HCC carcinogenesis. Four TSGs, including alcohol dehydrogenase 4 (*ADH4*), alcohol dehydrogenase 1C (*ADH1C*), alcohol dehydrogenase 1A (*ADH1A*), and alcohol dehydrogenase 6 (*ADH6*), are located in this region[58].

The LOH on chromosome 8p is one of the most common alterations in HCC. A group of researchers found that allelic losses on 8p were observed in high-grade dysplastic nodules (HGDNs)[59], indicating that these losses might occur in the early stage of hepatocarcinogenesis. Chromosome 8p is rich in candidate and validated TSGs, with a cluster of six genes, including deleted in liver cancer 1 (*DLC1*), coiled-coil domain-containing protein 25 (*CCDC25*), elongator complex protein 3 (*ELP3*), proline synthetase co-transcribed bacterial homolog (*PROSC*), SH2 domain-containing protein 4A (*SH2D4A*), and sorbin and SH3 domain containing 3 (*SORBS3*), located on chromosome 8p that have been deleted in HCC samples from patients with poor outcomes[30]. Notably, numerous studies have revealed a high frequency for LOH on 8p22-p23 in HCC[60,61], and deletions of alleles on 8p22-p23 have been found to be associated with metastasis and poor prognoses for HCC patients[56]. Four specific genes - microcephalin 1 (*MCPH1*), tRNA methyltransferase 9-like (*KIAA1456*), tumor suppressor candidate 3 (*TUSC3*), and zinc finger, DHHC-type containing 2 (*ZDHHC2*) - are located in this region. Of these genes, a LOH for *ZDHHC2* might contribute to the early metastatic recurrence of HCC after LT[62]. These findings suggest that 8p22-p23 harbors numerous TSGs that play important roles in the progression of HCC, which could contribute to assessing the risk of metastasis and recurrence in HCC patients.

In addition, a few recent studies have investigated the associations between LOH for new TSGs and the clinicopathological features of HCC. For example, LOH in the genes ARD1 homolog B (*S. cerevisiae*) (*ARD1B*) and *Mus musculus* septin 11 (*SEPT11*) were found to be significant prognostic factors for poor OS[63], and LOH in mannose 6-phosphate/insulin-like growth factor 2 receptor (*M6P/IGF2R*) was found to be predictive of poor clinical outcomes in surgically resected primary HCC patients[64].

In summary, the aforementioned findings provide valuable information that could contribute to our understanding of HCC carcinogenesis. However, there are still many important LOH regions that must be explored with regard to the genes that are involved in carcinogenesis and their biological and clinical implications[63].

MN are extra-nuclear bodies that contain damaged chromosome fragments and/or whole chromosomes that are not incorporated into the nucleus after cell division[65]. The frequency of MN is higher in tumor cells and cells with defective DNA damage repair systems or

disrupted cell cycle checkpoint machinery; hence, MN could serve as indicators of CIN[66,67]. In one study, the micronucleus index was found to gradually increase along with the progression of hepatocarcinogenesis. HCCs showed the highest micronucleus index values, which were significantly greater than those of HGDNs and DNs with HCC foci[68]. In another study, a progressively increasing number of MN were also documented in the transition from cirrhotic nodules (CNs) to large regenerative nodules (LRNs), DNs and HCC; MN were significantly more frequent in DNs than in CNs or LRNs[69]. These results suggest that CIN might occur in the early stage of hepatocarcinogenesis, and HCC cells generally have acquired chromosomal abnormalities; therefore, the degree of CIN could increase during the progression of HCC.

Recently, chromothripsis has been identified using whole-genome sequencing(WGS) as a new class of complex catastrophic chromosomal rearrangement. Chromothripsis is a single cellular crisis in which a chromosome is broken and reassembled by a DNA repair mechanism, resulting in a large number of rearrangements clustered in a chromosomal region[70]. Although chromothripsis appears to be relatively rare, it can be an extreme outcome of a mutagenic mechanism that could be widespread in human cancers[71]. Furthermore, chromothripsis could affect cancer gene function and thereby have a major impact on the progression, prognosis, and therapeutic response of cancer[72]. To date, we are aware of only one study that investigated the role of chromothripsis in the incidence of HCC. In this study, chromothripsis and CIN were found to recurrently affect chromosomal arms 1q and 8q to create gene amplifications, suggesting that chromothripsis might contribute to hepatocarcinogenesis[33]. It seems that more attention should be paid to this concept.

### *MSI*

MSI is the result of defects in mismatch repair genes that leads to the expansion and contraction of short nucleotide repeats called microsatellites[18]. Microsatellites are simple tandem repeats that are present at millions of loci in the human genome. MSI can result in the inactivation of TSGs or can disrupt other noncoding regulatory sequences, thereby playing a role in carcinogenesis[73]. MSI has been described in cirrhosis, mainly when cirrhosis is associated with an HBV infection[74,75]. Recent limited data are available on the incidence of MSI in HCCs. Several studies have suggested that MSI might play a minor role in hepatocarcinogenesis[76,77]. Furthermore, MSI is not implicated in the pathogenesis of a subset of HCCs affecting elderly patients without chronic liver disease[78]. Nevertheless, two studies have shown that high levels of MSI (MSI-H > 30%) were significantly associated with more aggressive histological tumor features and shorter median delays before recurrence[79], and the degree of MSI was significantly correlated with the poor differentiation and portal vein involvement of HCC[80]. These findings suggest that MSI could play a minor role in hepatocarcinogenesis and might be associated with the progression of HCC in patients with a background of chronic hepatitis and/or cirrhosis.

## SNPS

SNPs are the most common form of human genetic polymorphisms that can contribute to an individual's susceptibility and progression to cancer. Accumulating evidence suggests an association between SNPs in certain genes and HCC susceptibility[81]. GWAS have emerged as a new approach for identifying less penetrant cancer susceptibility alleles that might be associated with the initiation and progression of cancer.

Recent GWAS have identified numerous SNPs associated with the risk of HCC (Table 2); however, most findings have been both conflicting and inconsistent. For example, three researchers investigated whether an SNP (rs17401966) of kinesin-like factor 1 B (*KIF1B)* might be associated with the risk of HBV-related HCC in Chinese individuals. One study found that it was[82], but another study found that it was not[83]. A third study found that *KIF1B* alone was not associated with the risk but that the gene-environment interaction between the *KIF1B* variant and alcohol consumption was associated with the risk of HCC[84]. These inconsistent findings could be attributed to a lack of controlling for confounding variables, such as epidemiological and environmental risk factors in the first two studies. Therefore, it is important to evaluate the role of *KIF1B* rs17401966 in the genetic susceptibility to HCC and gene-environment interactions. Interestingly, three studies found that *KIF1B* rs17401966 was not associated with the development of HBV-related HCC in Thai, Japanese, and Saudi Arabian patients[85-87], and two other studies identified that *KIF1B* rs17401966 exerted protective effects against the susceptibility to HBV-related HCC in Chinese patients[88,89]. These inconsistencies might partly be because different ethnicities or study populations have distinct genetic architectures. In another example, three GWAS identified that MHC class Ⅰ polypeptide-relatedsequence A (*MICA*) and DEP domain containing 5 (*DEPDC5*) SNPs were strongly associated with HCC in Japanese populations with chronic HCV infections[90-92]. However, two other studies found that neither *DEPDC5* rs1012068 nor *MICA* rs2596542 was associated with HCC in Europeans with chronic HCV infections[93] or in Chinese populations with chronic HBV infections[94]. The discrepancies among these studies might be due to different study designs[93] or to differences in the different racial/ethnic groups. The inconsistent findings for HBV- and HCV-related HCC suggest that whether SNPs in

**Table 2 Summary of single-nucleotide polymorphisms associated with the risk of hepatocellular carcinoma identified from genome-wide association studies**

| Related gene | SNP | Etiology of HCC | Odds ratio (95%CI) | *P* value | Ref. |
|---|---|---|---|---|---|
| *TPTE2* | rs2880301 | HBV/HCV, Republic of Korea | 0.27 (0.19-0.39) | $1.74 \times 10^{-12}$ | Clifford *et al*[96] |
| *KIF1B* | rs17401966 | HBV, China | 0.61 (0.55-0.67) | $1.70 \times 10^{-18}$ | Zhang *et al*[82] |
| *KIF1B* | rs17401966 | HBV interacting with alcohol consumption, China | 2.36 (1.49-3.74) | | Chen *et al*[84] |
| *GRIK1* | rs455804 | HBV, China | 0.84 (0.80-0.89) | $5.24 \times 10^{-10}$ | Li *et al*[94] |
| *HLA-DQA1/DRB1* | rs9272015 | HBV, China | 1.28 (1.22-1.35) | $1.13 \times 10^{-19}$ | Li *et al*[94] |
| *MICA* | rs2596542 | HCV, Japan | 1.39 (1.27-1.52) | $4.21 \times 10^{-13}$ | Kumar *et al*[91] |
| *HLA-DQ* | rs9275319 | HBV, China | 1.51 (1.38-1.66) | $8.65 \times 10^{-19}$ | Jiang *et al*[97] |
| *DEPDC5* | rs1012068 | HCV, Japan | 1.75 (1.51-2.03) | $1.27 \times 10^{-13}$ | Miki *et al*[90] |
| *DDX18* | rs2551677 | HBV/HCV, Republic of Korea | 3.38 (2.07-5.53) | $1.41 \times 10^{-10}$ | Clifford *et al*[96] |
| *FasL* | rs763110 | HBV/HCV, Egypt | 1.970 (1.250-3.105) | 0.003 | Khalifa *et al*[98] |
| *DLC1* | rs3816747 | HBV, China | 0.486 (0.245-0.962)/ 0.51 (0.267-0.974) | 0.037/0.039 | Xie *et al*[99] |
| *STAT4* | rs7574865 | HBV, China | 1.22 (1.15-1.29) | $1.66 \times 10^{-11}$ | Jiang *et al*[97] |
| *FOXP3* | rs3761549 | HBV, China | 1.32 (1.03-1.70) | 0.030 | Chen *et al*[100] |

SNP: Single-nucleotide polymorphism; HCC: Hepatocellular carcinoma; GWAS: Genome-wide association studies; HBV: Hepatitis B virus; HCV: Hepatitis C virus; TPTE2: Transmembrane phosphoinositide 3-phosphatase and tensin homolog 2; KIF1B: Kinesin-like factor 1 B; GRIK1: Glutamate receptor, ionotropic, kainate 1; HLA-DQA1/DRB1: Major histocompatibility complex, class II, DQ alpha 1, DR beta 1; MICA: MHC class I polypeptide-relatedsequence A; HLA-DQ: Major histocompatibility complex class II antigen; DEPDC5: DEP domain containing 5; DDX18: DEAD (Asp-Glu-Ala-Asp) box polypeptide 18; FasL: Fas ligand; DLC1: Deleted in liver cancer 1; STAT4: Signal transducer and activator of transcription 4; FOXP3: Forkhead box P3.

the *MICA* and *DEPDC5* loci affect the susceptibility to HCC is subject to race/ethnicity-specific differences. Undoubtedly, the same variability also applies to all the other HCC-related SNPs, which could be explained by gene-gene and gene-environment interactions contributing to the inconsistent findings in different racial or ethnic groups that have been studied[95].

Taken together, the available results show that most findings related to the SNPs detected in GWAS on HCC can be problematic to replicate due to differences among different racial/ethnic groups, different study designs, and genetic heterogeneity. GWAS have so far identified numerous SNPs associated with HCC susceptibility[90-100]; however, most of these investigations were limited by relatively small sample sizes or the inclusion of only one racial/ethnic group. The inconsistency of these findings could be attributed to many factors, such as a lack of control for confounding variables, different study designs or the different racial/ethnic groups in the studies. Given the high variability/inconsistency in findings related to SNPs found in GWAS, at least to date, we cannot recommend the continued study of SNPs in relation to HCC as a means for identifying reliable markers of the initiation and progression of HCC. Therefore, further well-designed investigations with larger sample sizes and multiple races/ethnicities are warranted to elucidate the impact of SNPs on susceptibility to HCC.

## SOMATIC MUTATIONS IN HCC

Similar to any other cancer, HCCs consist of highly heterogeneous tumors with multiple genetic alterations, particularly somatic mutations. Recent advances in NGS technologies, such as WGS or whole-exome sequencing (WES), have enabled us to identify global driver genes related to the development of HCC. In addition to confirming the high frequency of somatic mutations in tumor protein p53 (*TP53*), catenin beta 1 (*CTNNB1*) and *AXIN1*, recent studies applying deep-sequencing analyses have identified numerous novel mutations in genes, such as mutations in genes related to chromatin remodeling (*ARID1A* and *ARID2*), oxidative stress (*NFE2L2* and *KEAP1*), RAS/MAPK signaling (*RPS6KA3*), and the janus kinase/signal transducers and activators of the transcription (JAK/STAT) pathway (*JAK1*)[28,101-103]. With the exception of *ARID1A* (10%-16%), most of these newly identified driver genes are mutated in less than 10% of HCC cases. It is encouraging that recurrent telomerase reverse transcriptase (*TERT*)-promoter mutations have been recently identified as the most frequent molecular alterations in HCC and as the first gene that is recurrently mutated in cirrhotic preneoplastic lesions[104,105]. There is abundant evidence to support the notion that *TERT, TP53, CTNNB1, ARID1A* and *AXIN1* are recurrently mutated genes involved in HCC[28,102,103,106-111]. Specifically, driver mutations in *TERT, TP53,* and *CTNNB1* are among the most frequent genetic alterations that have been defined as additive events in the development of HCC, irrespective of etiological background[28,106-108,112-115].

In this section, we briefly summarize previously well-known driver mutations and some novel gene mutations discovered in NGS studies. *CTNNB1* and *AXIN1* are subsequently reviewed in relation to the Wnt/β-catenin signaling pathway. The role and characteristics of frequent recurrent somatic mutations in HCC and their associations with clinical pathological

**Table 3 The characteristics of frequent recurrent somatic mutations and their correlations with clinical and pathological parameters in hepatocellular carcinoma based on deep-sequencing analyses**

| Gene | Altered pathway | Correlations with clinical and pathological parameters | Ref. |
|---|---|---|---|
| *TERT* promoter | Telomere stability | Hepatocarcinogenesis | Nault *et al*[104]; Yang *et al*[165] |
| *TP53* | Cell cycle control | Under debate: an early event in the context of aflatoxin exposure and chronic HBV infection, or it might not play a role in carcinogenesis | Qi *et al*[136] |
| | | Poor prognosis | El-Din *et al*[117] |
| | | | Cleary SP *et al*[138] |
| *CTNNB1* | Wnt/β-catenin signaling | Under debate: a late event for malignant progression or earlier during hepatocarcinogenesis | Park *et al*[253]; Vilarinho *et al*[256] |
| | | Under debate: worse outcomes or better outcomes | Tornesello *et al*[263]; Wang *et al*[269] |
| *AXIN1* | Wnt/β-catenin signaling | Hepatocarcinogenesis and progression | Guan *et al*[242] |
| *ARID1A* | Chromatin remodeling | Initiation and progression of HCC | Schulze *et al*[106] |
| *ARID2* | Chromatin remodeling | Initiation and progression of HCC | Totoki *et al*[107] |
| *NFE2L2* | Oxidative stress | Hepatocarcinogenesis and progression | Nault JC *et al*[105] |
| *KEAP1* | Oxidative stress | Hepatocarcinogenesis and progression | Schulze *et al*[106] |
| *JAK1* | JAK/STAT pathway | Hepatocarcinogenesis | Kan *et al*[28] |
| *RPS6KA3* | RAS/MAPK signaling | Hepatocarcinogenesis | Guichard *et al*[102] |

TERT: Telomerase reverse-transcriptase; ARID1A: AT-rich interactive domain-containing protein 1A; ARID2: AT-rich interactive domain-containing protein 2; NFE2L2/NRF2: Nuclear factor erythroid-derived 2-like 2; KEAP1: Kelch-like ECH-associated protein 1; JAK1: Janus kinase 1; RPS6KA3: Ribosomal protein S6 kinase polypeptide 3.

parameters are summarized in Table 3.

### *TP53*

*TP53* is a key molecule in the *TP53*/cell cycle signaling pathway. The mutation or deletion of the *p53* gene, which plays an important role in cell growth, division and apoptosis by acting as a transcription factor or by forming complexes with other proteins, is one of the most frequent genetic changes detected in HCC[116,117]. Strikingly, *TP53* mutation rates in HCC vary in different geographic areas, reflecting differences in etiological agents and susceptibility factors[118]. The *TP53* mutation in HCC occurs most commonly in sub-Saharan Africa and Southeast Asia, where the combination of widespread dietary AFB1 exposure and endemic hepatitis B fosters a high rate of mutagenesis in the liver[119]. In these areas, AFB1 is a particularly common mutagen of *TP53*, causing G:C to T:A transversions at the third base of codon 249 in TP53 (R249S), and the rate of *TP53* R249S mutations can be accelerated in the presence of a viral infection[120,121]. This mutation was not detected in HCC cases from non-aflatoxin-contaminated areas[119].

Accumulating evidence shows that the HBV X (HBx) protein is a multifunctional regulator that plays a crucial role in HBV-associated hepatocarcinogenesis[122]. However, the potential synergistic effects between the HBx protein and *TP53* mutations during hepatocarcinogenesis remain unclear. Several studies have suggested that the HBx protein affects the function of the P53 protein and contributes to the development of HCC. For example, complete HBx sequences were often associated with the presence of *TP53* R249S mutations[123], and HBx was found to be associated with *TP53* R249S mutations in HCC patients with no documented history of cirrhosis[124]. In addition, HBx mutations were found to interact with *TP53* R249S mutations in altering cell proliferation and chromosome stability in hepatocytes[125]. HBx has also been shown to bind to *p53* and to block *p53*-sequence-specific DNA-binding and *p53*-dependent transcription, ultimately blocking *p53*-mediated apoptosis[126]. HBx and *TP53* mutations have been suggested to synergistically contribute to the formation of HCC in animal models[127]. These findings suggest that HBx is involved in the etiology of *TP53* mutations during the molecular pathogenesis of HCC.

Persistent HCV infections could play a role in hepatocarcinogenesis; however, the mechanisms underlying this process remain unclear. A possible mechanism of HCV-induced oncogenesis seems to result from the interference of HCV proteins in the intracellular signal transduction processes *via* a mechanism including the dysregulation of cell cycle control[128]. In the presence of DNA damage, the P53 protein can be activated, promoting the expression of several important genes involved in cell cycle arrest, DNA repair, and apoptosis[129]. Accordingly, whether HCV infections occur concurrently with other genomic alterations, such as *TP53* mutations, in hepatocarcinogenesis is of interest. Currently, several studies have provided some evidence for the direct action of HCV-related proteins on *TP53*. For example, HCV infection impairs the function of P53 through the overexpression of 3β-hydroxysterol delta 24-reductase (*DHCR24*), which up-regulates the interaction between P53 and MDM2 (mouse double minute 2 homolog, also known as HDM2, a P53-specific E3 ubiquitin ligase) in the cytoplasm and suppresses P53 acetylation in the nucleus[130]. Additionally, a novel *TP53* mutation, 616ins14del1 (14-1 microindel), has been detected in a case of HCC associated with an HCV infection,

providing evidence that HCCs characterized by HCV infections are typically associated with the mutational inactivation of the *TP53* gene[131]. In addition, genetic changes in *TP53* have been detected in non-neoplastic lesions linked to chronic HCV infections[132]. Collectively, the aforementioned findings suggest that HCV is implicated in the etiology of *TP53* mutations during hepatocarcinogenesis. However, these results were obtained *in vitro* using cell culture models or animal models, and the synergistic effects of *TP53* mutations and HCV infections in human hepatocarcinogenesis must be further investigated.

A *TP53* mutation has been identified as one of the most frequent molecular alterations in HCC; however, the role of *TP53* mutations in hepatocarcinogenesis remains debatable. Strikingly, a missense mutation in exon 7 (R249S) of *p53* has been found specifically in HCC patients from regions with high levels of AFB1 exposure[133]. Several studies have suggested that *TP53* R249S mutations are likely to occur as early events in association with aflatoxin exposure and chronic HBV infection[134-136]. A recent study showed that *TP53* R249S mutations are an important factor in HCC carcinogenesis in Brazil, where aflatoxin exposure levels are high[137]. In contrast, *TP53* mutations can occur as a late event in carcinogenesis without a typical mutational pattern in areas with low levels of AFB1 intake[135]. Furthermore, another study showed that *TP53* R249S mutations might not play a role in the carcinogenesis of HCC in Egypt, where HCV infections are highly prevalent and are a major risk factor for the development of HCC[117]. Taken together, these findings show that *TP53* mutations could play an important role in hepatocarcinogenesis in populations with chronic HBV infections, especially in those exposed to excessive levels of AFB1. It follows that these inconsistent and even conflicting results regarding the role of *TP53* mutations in hepatocarcinogenesis might primarily be due to heterogeneity in the geographic and etiological backgrounds of the cases studied.

Recent reports have shown that *TP53* mutations can be used to predict HCC. For example, mutations in *TP53* were found to be associated with a significantly higher rate of recurrence and a lower DFS[138]. In addition, two systematic reviews concluded that *TP53* mutations were associated with poor OS, relapse-free survival rates (RFS), and DFS in HCC patients, with similar results found between patients with HBV infections and HCV infections[139,140]. However, a recent study showed that *TP53* mutations were associated with shorter survival time only in cases of HBV-related HCC, although R249S hot spot mutations were not associated with survival rates in patients of European origin with HBV-related HCC[141]. In contrast, another study found that *TP53* mutations, particularly the hot spot mutations R249S and V157F, regardless of sample origin, were associated with poor prognoses in patients with HCC[142]. This finding was echoed by another recent study on the relationship between *TP53* mutations and the recurrence of HCC in patients with HCC of various etiologies[143]. Taken together, these inconsistent and even conflicting findings might be largely due to the use of different racial and regional groups as well as other possible contributing factors, including the small sample sizes of the studies. Therefore, these confounding factors should be considered when evaluating the prognostic value of *TP53* mutations in HCC.

Increasing evidence suggests that the stabilization of mutant *p53* in tumors is crucial for its oncogenic activities, while the depletion of mutant *p53* attenuates the malignant properties of cancer cells. Thus, mutant *p53* is an attractive drug target for cancer therapies[144].

### *Telomerase reverse-transcriptase*

The human telomerase reverse transcriptase (*hTERT*) gene encodes a rate-limiting catalytic subunit of telomerase, which maintains the length of telomeric DNA and chromosomal stability[145]. *hTERT* is the major determinant of telomerase activity, and it plays a key role in cellular immortalization and the development and progression of human cancers. The reactivation of telomerase activity is observed in approximately 90% of human cancers, enabling cells to overcome replicative senescence and to escape apoptosis, which are fundamental steps in the initiation of malignant transformation[146,147]. The precise mechanism behind the reactivation of telomerase activity in cancer remains elusive, but it likely involves multiple changes that occur during the progression of cancer, including mutations and chromosomal rearrangements[148].

In two recent studies, researchers identified mutations that created new binding sites in the *TERT* promoter for particular transcriptional regulators, such as E-twenty-six (*ETS*)/ternary complex factors (*TCFs*) factors, and resulted in increased transcriptional activity at the *TERT* promoter, which could in turn lead to the increased expression of the gene and the endless cell division characteristic of cancer cells[149,150]. These findings suggest that *TERT* promoter mutations could be potential mechanisms for *TERT* reactivation in cancer cells. In more recent studies, investigators found that two highly recurrent point mutations (G228T and G250T) in the *TERT* promoter might be among the fundamental mechanisms underlying telomerase reactivation/expression in several types of human cancers[149,151-154].

The molecular mechanisms involved in telomerase reactivation in HCC have been only partially elucidated, with the most important being *TERT* promoter mutations[104]. *TERT* amplification and the recurrent integration of HBx into the *TERT* gene promoter are alternative explanations for telomerase reactivation[107,155-157]. In particular, *TERT* promoter mutations were found to be associated with *CTNNB1* mutations in HCC[104,106,107,158], suggesting that

*TERT* promoter mutations and the deregulation of the Wnt/β-catenin pathway could interact in the malignant transformation of hepatocytes. Overall, the identification of *TERT* promoter mutations in association with HCC has provided new insights into telomerase reactivation and telomere maintenance in hepatocarcinogenesis[148]. Despite these compelling findings, the functional role of *TERT* promoter mutations in HCC remains unclear and must be further explored.

To date, recurrent somatic mutations in the *TERT* promoter have been identified as the most frequent non-coding mutations in multiple cancer types, suggesting that *TERT* promoter mutations are driver mutations in these cancers[154,159,160]. The frequency of *TERT* promoter mutations in HCC varies substantially across the different geographical regions studied. For example, cases of HCC with *TERT* promoter mutations have been reported from the United States[161], Europe[104,158,162], Africa[163], and East Asia (except for Japan)[103,104,164-166], with mutation frequencies of 44%, 47%-59%, 53%, and 20.7%-38.8%, respectively. These data indicate that *TERT* promoter mutations are less frequent among Asian patients with HBV-related HCC than among those with HCV-related HCC. The lower rate of *TERT* promoter mutations in patients with HBV-related HCC might be partially explained by the frequent insertion of HBV DNA in the *TERT* promoter, which is known to induce telomerase transcription[103,155]. These findings suggest that various etiological factors could be involved in different mechanisms that preserve telomeres during the carcinogenesis of HCC[164]. Despite these differences, *TERT* promoter mutations are currently considered the most frequent somatic genetic alterations in HCC regardless of patients' geographical origin[163,167]. In the past few years, many investigators have explored the role of *TERT* mutations in HCC. In a recent study, *TERT* promoter mutations were found in 6% of low-grade dysplastic nodules (LGDNs), 19% of HGDNs, 61% of early HCCs and 42% of small and progressed HCCs. However, mutations in other classic HCC driver genes (*i.e*., *CTNNB1, TP53, ARID1A*, or *ARID2*) were not identified in LGDNs, HGDNs, or early HCC[105]. In another recent study, *TERT* mutations were found to occur at an early stage of tumorigenesis. Specifically, they were observed in 57% of preneoplastic lesions and in 30% of stage I HCCs[165], indicating that *TERT* promoter mutations occur early during malignant transformation and persist throughout tumor progression. These findings have been further confirmed by two recent studies using exome or DNA sequencing of liver tumor samples in which *TERT* promoter mutations occurred early during hepatocarcinogenesis[106,164]. In addition, when *hTERT mRNA* was measured *via* real-time quantitative RT-PCR, the *hTERT mRNA* levels were found to be increased in association with the progression of hepatocarcinogenesis, and most HGDNs strongly expressed *hTERT mRNA* at levels similar to those in HCC samples[168]. In a recent study, the authors found that the activation and expression of *hTERT* played extremely critical roles in the incidence and progression of HCC[169]. Previous studies also showed that telomere shortening and telomerase reactivation occurred in DNs during the early stages of hepatocarcinogenesis[163]. Indeed, alterations in telomerase restriction fragment (TRF) length, telomerase activity (TA), and *hTERT* and *hTR* expression were identified in both the early and late stages of hepatocarcinogenesis[170]. These findings demonstrate that telomere status is a factor in hepatocarcinogenesis.

*hTERT mRNA* has been reported to be detectable in the serum of patients with HCC, and it has been reported that the sensitivity and specificity for serum *hTERT mRNA* in detecting HCC were 77.14% and 100%, respectively, which are higher than the sensitivity and specificity for AFP in the early detection of HCC[171]. In another report, the sensitivity/specificity for serum *hTERT mRNA* in diagnosing HCC was found to be 90.2%/85.4%, which is superior to using alpha-fetoprotein (*AFP*), *AFP-L3*, and des-gamma-carboxy prothrombin (*DCP*) in the diagnosis of HCC at an early stage[172]. Therefore, measuring serum *hTERT mRNA* levels might serve as a potential diagnostic tool for HCC.

Taken together, these findings suggest that *TERT* promoter mutations are among the earliest genetic alterations in hepatocarcinogenesis, occurring at preneoplastic stages and behaving as a "gatekeeper" during the malignant transformation sequence[173,174].

Considering that *TERT* promoter mutations are among the earliest recurrent genetic events in tumorigenesis and are also the most frequent somatic genetic alterations in HCC, telomerase inhibition shows potential as an ideal therapeutic target in treating HCC. Currently, different strategies for telomerase inhibition, such as the use of nucleoside analogs, oligonucleotides, small molecule inhibitors, G-quadruplex stabilizers, immunotherapy, and gene therapy in different cancers, are currently in development, preclinical studies or clinical trials[175].

### *ARID1A and ARID2*

Increasing evidence has demonstrated that the misregulation of ATP-dependent chromatin remodeling complexes (chromatin remodelers) contributes to tumorigenesis[176], tumor heterogeneity[177], and the cellular response to anticancer drugs[178-182]. Among the different ATP-dependent chromatin remodelers, genes encoding SWitch/sucrose nonfermentable (SWI/SNF) complex subunits are now recognized as among the most commonly mutated targets affecting chromatin remodeling, as they are present in 20% of human cancers[183-185]. SWI/SNF chromatin remodeling has been linked to a variety of epigenetic processes,

including roles in maintaining nucleosome positioning and interacting with other chromatin modifiers[186]. The SWI/SNF complexes can be divided into two broad categories based on the presence of the AT-rich interactive domain containing protein 1A-B (*ARID1A/B*) subunits (BAF complex) or *ARID2* and polybromo 1 (*PBMR1*) subunits (PBAF complex)[187].

Recent exome and WGS studies of HCC have shown that recurrent inactivating mutations in SWI/SNF subunits are involved in the molecular basis of hepatocarcinogenesis[101,102,188-190]. However, the functional role and molecular mechanisms underlying these mutations in the initiation and progression of HCC are not yet completely understood. Genes involved in coding for chromatin-modifying proteins are commonly mutated in HCC. In particular, two inactivating mutations in genes encoding subunits of the SWI/SNF complex, and *ARID1A* and *ARID2* have been identified in approximately 10% of HCC cases[101,106,107,189,191]. Therefore, it is not surprising that chromatin remodeling complex alterations might play important roles in the initiation and progression of HCC. Interestingly, the frequency of *ARID1A* and *ARID2* mutations occurring in HCC varies considerably across HCC cases, depending on the different etiologies of the disease. For example, *ARID1A* mutations are significantly more frequent in HCC related to alcohol intake than in tumors of other etiologies[102], and *ARID2* mutations commonly occur in HCV-associated HCC[188,192]. However, several studies did not observe an association between *ARID1A* and *ARID2* mutations and the etiology of HCC. *ARID1A* was mutated in 13% of HBV-associated cases of HCC[189], and *ARID2* mutations were not significantly associated with HCV infections[102]. A recent study also demonstrated that *ARID1A* alterations were not correlated with HBV infection, HCV infection or the heavy use of alcohol[193]. These findings suggest that *ARID1A* and *ARID2* mutations are universally present in association with HCC related to hepatitis virus infection and alcohol intake.

The mechanisms by which mutations in SWI/SNF subunits drive tumorigenesis are unclear. Most *ARID1A* and *ARID2* mutations detected in cancer cells to date are inactivating mutations, suggesting that both proteins function as tumor suppressors[194]. Several possible mechanisms for this effect have been suggested. *ARID1A* has been indicated in preventing DNA entanglements during mitosis. Hence, its mutational inactivation could lead to genomic instability and alter gene expression, which could contribute to tumorigenesis[195]. In addition, it has been found that *ARID1A* mutations tend to interact with the activation of the PI3K/AKT pathway in promoting tumorigenesis in many human cancers of diverse origins[196-203]. Furthermore, a recent study found that *ARID1A* mutations alone did not cause the development or progression of cancer but that a combination of *ARID1A* inactivation and a PI3K/AKT pathway aberration was sufficient to initiate tumorigenesis[204]. Theoretically, the two mechanisms mentioned above in other solid tumors might also apply to HCC. The functional significance of *ARID1A* and *ARID2* mutations remains to be elucidated in relation to the initiation and progression of HCC.

In a recent study, HCC cases with altered *ARID1A* expression showed inverse correlations with the nuclear localization of P53 and beta-catenin, suggesting that the *ARID1A* pathway might represent an alternative pathway to the *p53* and beta-catenin pathways in HCC. Thus, *ARID1A* might constitute a promising therapeutic target for treating a subset of HCCs[193].

### *NFE2L2/NRF2 and KEAP1*

Oxidative stress involves elevated intracellular levels of reactive oxygen species (ROS) that cause damage to lipids, proteins and DNA[205]. Recent studies have shown that persistent oxidative stress due to elevated ROS levels is associated with carcinogenesis and the progression of cancer[206-210]. The NRF2-KEAP1 pathway is the major regulator of cytoprotective responses to endogenous and exogenous stresses caused by ROS and electrophiles[211,212]. The key proteins within the NRF2-KEAP1 pathway are the transcription factor *NRF2*, which mediates oxidative stress responses, and *KEAP1*, which is a negative regulator of *NRF2* activity.

*NRF2* has been traditionally considered a tumor suppressor because of its cytoprotective functions[213]. In fact, accumulating evidence from genetic analyses of human tumors suggests that the deregulation of *NRF2* is a critical determinant in oncogenesis, and somatic mutations of either *NRF2* or *KEAP1* have frequently been detected in a variety of cancer types[107,214-216]. These findings indicate that mutations in *NRF2* and *KEAP1* frequently play important roles in carcinogenesis.

Recent exome sequencing of HCC samples has revealed that the oxidative stress pathway is activated in 12% of HCC patients, primarily as a result of mutations of *NRF2* or *KEAP1*[106]. Numerous genomic studies on cancer have reported somatic mutations of *NRF2* and inactivating mutations of *KEAP1*(6%-10% and 3%-8% of HCC patients, respectively)[102,106,107,138,217,218]. A recent functional experiment found that *NRF2/KEAP1* mutations were present in 71% of early preneoplastic lesions and in 78.6% and 59.3% of early and advanced HCCs[219], respectively, suggesting that the onset of NRF2/KEAP1 mutations is a very early event in rat hepatocarcinogenesis. In contrast, mutations of *NRF2* and *KEAP1* in humans were observed only in advanced HCC and not in premalignant nodules or early HCC, suggesting that these mutations are late events in hepatocarcinogenesis in humans[105,106]. Despite some differences in the role of mutations of *NRF2*

and *KEAP1* between rats and humans, it is evident that the dysregulation of the NRF2/KEAP1 pathway and mutations of these genes play important roles in hepatocarcinogenesis in both species. The NRF2/KEAP1 pathway might contribute to hepatocarcinogenesis through the following mechanisms. First, the NRF2/KEAP1 pathway might cause epigenetic instability, leading to HCC[220]. Second, either *NRF2* acts by itself as a proto-oncogene or *NRF2* or *KEAP1* mutations support the accumulation of additional mutations of proto-oncogenes[215,221]. Third, the NRF2/KEAP1 pathway could alter the chromatin status, leading to abnormal methylation of TSGs, which might contribute to hepatocarcinogenesis[222]. Interestingly, recent analyses of somatic mutations in HCC have revealed that mutations in *NRF2* or *KEAP1* are significantly correlated with the deregulation of the Wnt/β-catenin pathway *via CTNNB1* or *AXIN1* mutations[102,217]. These results suggest that the NRF2/KEAP1 pathway might interact with Wnt/β-catenin signaling to promote hepatocarcinogenesis. Nevertheless, the exact molecular mechanism underlying the role of *NRF2* in the pathogenesis of HCC must still be investigated.

The finding of recurrent mutations in HCC revealed that *NRF2* activation was a driver event in the progression of tumors[102,138]. Collectively, *NRF2/KEAP1* mutations might be involved in the pathogenesis and progression of HCC. The genetic or pharmacologic inhibition of *NRF2* expression/activity in HCC cells increased the anticancer activity of erastin and sorafenib *in vitro* and in tumor xenograft models[223]. Intriguingly, the accumulation of phosphorylated P62, a selective autophagy substrate, was found to cause the persistent activation of *NRF2*, contributing to the development of HCC[223-225]. In addition, in Japanese HCC patients, *NRF2* activation was associated with the phosphorylation of P62 but not with the *KEAP1* status[226].These results suggest that there might be crosstalk between the NRF2/KEAP1 pathway and P62-mediated selective autophagy, and selective *NRF2* inhibitors or inhibitors of the interaction between phosphorylated P62 and *KEAP1* should be developed as potential therapeutic agents against human HCC.

### *Janus kinase 1*

The JAK/STAT signaling pathways have been identified as promoters of carcinogenesis in a subset of HCCs *via* cytokine-induced JAK/STAT pathway activation[28,227,228]. A previous study using single-strand conformational polymorphisms (SSCPs) and direct sequencing reported a low frequency (1/84, 1.2%) of Janus kinase 1 (*JAK1*) mutations in HCC[229]. Recently, a comprehensive whole genome analysis revealed that *JAK1* mutations appeared in 9.1% of HCCs, and the JAK/STAT pathway was altered in 45.5% of HCCs[28]. These findings indicate that the JAK/STAT pathway might act as one of the major oncogenic drivers in HCC and suggest the possibility of its use as a promising therapeutic approach for HCC treatment.

### *Ribosomal protein S6 kinase polypeptide 3*

Ribosomal protein S6 kinase polypeptide 3 (*RPS6KA3*) encodes a component of the RAS/MAPK signaling pathway, *i.e.*, a gene located on chromosome X that encodes ribosomal S6 protein kinase 2 (RSK2). Recurrent mutations in *RPS6KA3* have been found in 2%-9% of HCCs[102,106,189], suggesting that *RPS6KA3* could act as a newly identified potential driver of the pathogenesis of HCC. Specifically, *RPS6KA3* tended to be mutated in poorly differentiated HCCs[230] and was found in HCCs that developed without cirrhosis[102]. In addition, *RPS6KA3* mutations were frequently associated with *AXIN1* mutations[102], suggesting that *RPS6KA3* inactivation might cooperate with Wnt/β-catenin signaling to promote hepatocarcinogenesis.

## SIGNALING PATHWAYS IMPLICATED IN HCC

The recurrent mutated genes reviewed above were found to be highly enriched in multiple key driver signaling processes, including telomere maintenance, *TP53*, cell cycle regulation, the Wnt/β-catenin pathway (*CTNNB1* and *AXIN1*), chromatin remodeling (*ARID1A* and *ARID2*), the phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway, and oxidative/endoplasmic reticulum stress (*NFE2L2* and *KEAP1*). In the following section, we briefly summarize two of the most common molecular cellular pathways, Wnt/β-catenin and PI3K/AKT/mTOR, in human HCC[28,35,107,231]. Other pathways are summarized in the section above.

### *Wnt/β-catenin signaling pathway*

The WNT/β-catenin pathway can be classified into canonical (β-catenin dependent) and noncanonical (β-catenin independent) pathways[232]. In the absence of Wnt proteins, β-catenin is phosphorylated at amino-terminal serine and threonine residues by casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK-3β)[233]. β-catenin phosphorylation is facilitated by the axis inhibition protein (AXIN) and adenomatous polyposis coli (APC). Wnt signaling is activated upon Wnt-ligand binding to frizzled receptors (FZD), followed by the cytosolic accumulation of β-catenin through the prevention of *GSK-3β*-mediated phosphorylation of the β-catenin Ser/Thr domain[234]. The absence of β-catenin phosphorylation releases it from the degradation complex composed of APC, AXIN, GSK-3β and CK1, resulting in an accumulation of β-catenin in the cytoplasm[234]. Subsequently, cytosolic β-catenin can translocate to the nucleus to initiate the transcription of target genes through interactions with T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factors[234]. Hepatocytes with the nuclear translocation

of β-catenin displayed abnormal cellular proliferation and expressed membrane proteins associated with HCC, metastatic behavior, and cancer stem cells[235].

The deregulation of WNT/β-catenin signaling has been found in 40%-70% of HCC patients[236]. Increasing evidence suggests that the Wnt/β-catenin signaling cascade plays a major role in the pathogenesis of HCC[234,237]. Some studies have suggested possible mechanisms for this role. For example, research has found that the occurrence of HCC may be closely related to allelic loss, chromosomal changes and mutations in Wnt/β-catenin signaling pathway genes[238]. In addition, the Wnt/β-catenin signaling pathway contributes to angiogenesis, infiltration and metastasis in HCC by regulating the expression of angiogenic factors, such as matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor-A (VEGF-A), vascular endothelial growth factor-C (VEGF-C) and basic fibroblast growth factor (bFGF)[239]. However, the precise molecular mechanism remains uncertain.

Mutations in exon 3 of the *CTNNB1* gene, which encodes β-catenin, constitute a crucial molecular mechanism leading to the aberrant activation of the Wnt/β-catenin pathway, which is strongly associated with hepatocarcinogenesis[240]. In addition to gain-of-function mutations in positive modulators of Wnt signaling, such as β-catenin, the Wnt pathway can be activated by loss-of-function mutations in negative modulators, such as *AXIN* and *APC*[241]. It has been suggested that *AXIN* might play an important role in the pathogenesis and progression of HCC *via* the Wnt signaling pathway[242]. Moreover, the overexpression of the Frizzled-7 (FZD-7) receptor and glycogen synthase kinase-3 (GSK-3) inactivation may also lead to aberrant β-catenin pathway activation[243] as the FZD-7 receptor has been found to be up-regulated in 90% of human HCCs[244,245], suggesting that the consequent activation of Wnt/Frizzled-mediated signaling plays a key role in hepatic carcinogenesis. Specifically, one study analyzed the spectrum of mutations in a series of 125 cases of HCC, and the authors identified significant associations between mutations in *ARID1A, RPSK6KA3* or *NFE2L2* and mutations in *CTNNB1* or *AXIN1*, suggesting that Wnt/β-catenin signaling might interact with oxidative stress responses, chromatin remodeling or the RAS/MAPK pathway to promote hepatocarcinogenesis[217].

Mutations in the Wnt/β-catenin pathway have been described in 20%-40% of HCCs[246]. In HBV-related HCC, *β-catenin* mutations have been found at a lower frequency[103,246,247], whereas higher incidences of *β-catenin* mutations have been shown to occur mainly in alcohol- and HCV-related HCCs[101,102,188,248]. These findings suggest that *β-catenin* mutations are associated with the etiology of the HCC, which might be explained in part by actions of the HCV core protein synergizing Wnt-induced stabilization and the accumulation of β-catenin, perhaps playing an important role in the pathogenesis of HCV[249]. In HCC occurring in association with HBV, patients display β-catenin activation, which is induced in a mutation-dependent manner by the expression of the HBx protein[250]. Furthermore, one explanation for why *β-catenin* mutations tend to occur in non-HBV-associated casesis that *AXIN* mutations (and rarely *β-catenin* mutations) are mainly found in chromosome-unstable tumors associated with HBV infections, and *β-catenin* mutations are mainly found in non-HBV, well-differentiated, chromosome-stable tumors[251]. Thus, these two components of the Wnt pathway, β-catenin and *AXIN1*, could operate in distinct ways in human HCC[252].

The verdict on the role of *β-catenin* mutations in the initiation and progression of HCC is currently uncertain. A few studies have demonstrated that *β-catenin* mutations are found only in association with HCC and not in DNs[104,253,254].These results suggest that *β-catenin* mutations might be a late event in malignant progression rather than *β-catenin* being an early event gene or a gatekeeper gene in the multistep process of hepatocarcinogenesis. Nevertheless, another study concluded that β-catenin accumulates in the cytoplasm and the nuclei in precancerous lesions of the liver and might contribute, at least in part, to hepatic carcinogenesis[255]. Moreover, a clonality analysis predicted that the *CTNNB1* mutation was clonal and occurred earlier during hepatocarcinogenesis[256]. To date, numerous studies have investigated the possible mechanisms underlying the role of *β-catenin* mutations in the initiation and progression of HCC. For example, *CTNNB1* mutations are likely to occur as late events in the context of aflatoxin exposure and chronic HBV infection, whereas *CTNNB1* mutations might represent early events in carcinogenesis without a typical mutational pattern in areas with low AFB1 intake[135]. Transcription complexes, formed by a combination of intranuclear β-catenin and transcription factors, activate downstream target genes and regulate the expression of corresponding genes, leading to HCC tumorigenesis[257]. Although a *β-catenin* mutation might represent an important event leading to tumorigenic changes in hepatocytes, several studies using transgenic animal models have shown that the overexpression of mutant or stable forms of *β-catenin* on its own is not sufficient to induce HCC[258,259]. A recent study found that the up-regulated genes v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog G (*MAFG*) and synovial sarcoma, X breakpoint 1 (*SSX1*) significantly synergized with the transcriptional activity of β-catenin, and the overexpression of the downregulated genes one cut homeobox 1 (*Onecut1*) and forkhead box protein A3 (*FOXA3*) potently inhibited the growth of a *CTNNB1*-mutation-positive (HepG2) cell line and negative (Huh-7 and Hep3B) cell lines[260]. In another study, the over-expression of cysteine-rich protein 61 (Cyr61/CCN1) was positively correlated with increased

levels of β-catenin in human HCC samples, indicating that Cyr61 is a direct target of β-catenin signaling in HCC[261]. Therefore, the findings of these studies indicate that *β-catenin* mutations can interact with other oncogenic alterations or pathways to result in hepatocarcinogenesis more often than previously recognized.

Similarly, there have been conflicting data in the literature on the question of whether *β-catenin* mutations in HCC are associated with favorable or unfavorable prognoses[262]. Some studies have found associations of *β-catenin* mutation or activation with worse outcomes, such as moderately/poorly differentiated HCV-related HCC, larger tumor sizes, multiple nodules and increased vascular invasion[263,264]. In contrast, other studies have reported that HCCs harboring *β-catenin* mutations had better outcomes, such as less invasive and less frequent portal vein involvement[138,260,265-268]. A recent meta-analysis also revealed that *β-catenin* mutations could predict a favorable prognosis in patients with HCC[269]. In addition, one study reported that *β-catenin* mutations were not associated with prognoses in patients with advanced HCC[238]. Interestingly, the expression of the noncanonical *Wnt5a*, which is known to inhibit canonical Wnt signaling, was increased in poorly differentiated HCC cell lines[270]. Based on this result, the authors proposed that canonical and noncanonical Wnt pathways play complementary roles in HCC, with canonical signaling contributing to tumor initiation and noncanonical signaling contributing to tumor progression[270]. Accordingly, the noncanonical activation of *Wnt* in HCC deserves further research. Furthermore, a possible mechanism underlying *β-catenin* mutations with favorable outcomes was proposed in another study. In this study, the presence of cytokeratin 19 (CK19) expression or the absence of *β-catenin* mutations was found to be predictive of early tumor recurrence (ETR), and CK19 expression abolished the suppressive effects of *β-catenin* mutations on the progression of HCC. CK19 expression and *β-catenin* mutations were found to play dramatically opposite roles in vascular invasion, ETR and the prognosis of HCC patients[271].

Considering these findings, future prospective studies to determine the initiation, progression and outcome of HCC as a function of the WNT/β-catenin pathway will be essential. Specifically, such studies should consider the geographical origin, etiology and heterogeneity of the patients as well as the modes of WNT/β-catenin pathway activation[272].

### *PI3K-AKT-mTOR pathway*

The phosphoinositide 3-kinase-AKT-mammalian target of rapamycin (PI3K-AKT-mTOR) pathway is one of the most frequently deregulated pathways in human cancers, and it is a master regulator of processes that contribute to tumorigenesis and tumor maintenance[273]. The membrane lipid phosphatidylinositol 4, 5-bisphosphate (PIP2) is phosphorylated by *PI3K* into phosphatidylinositol 3, 4, 5-triphosphate (PIP3), which binds to and activates the serine/threonine kinase *AKT*[274]. The tumor suppressor gene product *PTEN* deleted on the chromosome is antagonistic to *PI3K* activity; the inactivation of *PTEN* through gene deletion increases $PIP_3$ levels and activates *AKT*, which inhibits apoptosis, leading to the development of tumors[275]. Activated *AKT* initiates a cascade of downstream signaling events, including the mTOR pathway. Once activated by *AKT, mTOR* promotes cell growth and proliferation by stimulating protein synthesis through the phosphorylation of 4E-BPs and the S6 kinases[275].

The PI3K/AKT/mTOR pathway is frequently deregulated in human hepatocarcinogenesis[276]. Furthermore, the deregulation of key genes of the PI3K/AKT/mTOR pathway has clinical importance in HCC[277,278]. As a negative regulator of the PI3K/AKT/mTOR pathway, *PTEN* is considered a tumor suppressor gene. *PTEN* mutations rarely occur in HCC, whereas *PTEN* heterozygosity, resulting in reduced *PTEN* expression, has been observed in 32%-44% of HCC patients[279]. Recent studies have demonstrated that the underexpression of *PTEN* is associated with poorly differentiated HCC, advanced TNM (tumor-node-metastasis) stage and intrahepatic metastasis, and poor patient survival[278,280-282]. *PI3KCA* is an upstream regulator of *AKT*, although there is some controversy regarding the role of *PI3KCA* mutations in HCC. A recent study identified *PIK3CA* mutations in 14% of patients.These mutations were strongly correlated with tumor size, suggesting that *PIK3CA* mutations could be used as prognostic markers in HCC[283]. However, other more recent studies have shown that hot spot mutations in *PIK3CA* are completely absent or rare in HCC[263,284-287]; *PI3K* mutations were not associated with either hepatic carcinogenesis or the postoperative prognosis of HCC patients[284,285,288].

*AKT*, also known as protein kinase B, is a central effector in the PI3K pathway. Many HCCs have demonstrated the activation of *AKT*, and it has been reported that both hepatitis B and hepatitis C could activate PI3K/AKT signaling[289]. It is well established that *AKT* plays a key role in tumorigenesis by stimulating cell proliferation and inhibiting apoptosis. The phosphorylation of *AKT* at S473 was detected in up to 71% of HCC samples and was associated with the invasion, metastasis, and vascularization of HCC[278]. As an *AKT* effector, S6 ribosomal protein (pS6) could be used as a prognostic indicator of HCC[290]. In addition, phospho-AKT (*pAKT*) expression showed a significant correlation with decreased OS[291], suggesting a worse prognosis for HCC patients with activated *AKT*[292].

*mTOR* is a key component of the *PI3K* and *AKT* pathways that activate downstream kinases required for G1 to S phase transition[293]. *mTOR* deregulation

has been reported to play a significant role in the pathogenesis and progression of HCC. A recent study showed that high *mTOR* expression levels were correlated with Edmondson tumor grades and cirrhosis[294]. Additionally, data from preclinical studies have indicated that the deregulated expression of mTOR pathway effectors occurred in 40%-50% of HCCs, and the activation of the mTOR pathway was associated with less differentiated tumors, earlier tumor recurrence, and lower survival rates[290,295]. *mTOR* acts by directly activating p70S6 kinase (p70S6K/S6K1) and inhibiting 4E binding protein 1 (4E-BP1)[296]. *mTOR* forms two multiprotein complexes, called *mTORC1* (*mTOR* complexed with raptor) and *mTORC2* (*mTOR* complexed with rictor)[297]. Both *mTORC1* and *mTORC2* participate in regulating the migration and invasion of HCC cells[298]. A recent study showed that a high ratio of the levels of rictor and raptor mRNAs in tumors was an independent prognostic indicator of DFS[297]. This finding suggests that an analysis of *mTOR* expression in cancer tissues could serve as a predictive marker of HCC recurrence after curative treatment.

Currently, many inhibitors targeting the PI3K/AKT/mTOR pathway are being evaluated for treating HCC in preclinical and clinical studies[299,300]. It is hoped that the efficacy of inhibitors of the PI3K/AKT/mTOR pathway, in combination with other anticancer agents, might represent a promising new strategy for treating HCC patients.

## PROBLEMS AND PERSPECTIVES

Although numerous genes are altered in association with HCC, only a small number of them are considered alterations that drive clonal expansion and invasion. Most of the somatic alterations appear to be passengers that are neutral for tumor cell selection[301]. So far, most of the genetic events that initiate HCC remain unknown. Therefore, the identification of key driver genes in HCC is crucial to elucidating the genetic mechanism of hepatocarcinogenesis and providing new molecularly targeted therapies for HCC patients.

Recent advancements in NGS technology have allowed for the identification of recurrently mutated genes in the pathogenesis of HCC. For example, a recent study of NGS analyses was performed to identify mutations in the *TERT* promoter, *TP53*, and *CTNNB1* genes that are major drivers of the development of HCC[103]. To date, however, no potential drivers of specific oncogenes (oncogene addiction, which is a term used when a cancer cell is found to be dependent on a single gene to survive) corresponding to targeted therapies have emerged, likely due to the genomic heterogeneity of HCC. In addition, the most prevalent of the critical driver mutations that have been identified in HCC are not yet drug-accessible targets[302]. Although several molecularly targeted agents have been evaluated in clinical trials in advanced HCC, no novel, fully effective molecularly targeted agents for the treatment of patients with advanced HCC have been produced, except for sorafenib. There are two factors, *i.e*., the lack of a clearly identified driver oncogene and the presence of underlying cirrhosis, that are primarily responsible for the frequently unsuccessful results in studies on the use of novel drugs in treating HCC[303].

It is anticipated that studies including large sample sizes combined with the integration of multiple levels of data, such as data on genomic instability, SNPs, and somatic mutations, in conjunction with integrative functional genomic approaches, will contribute to identifying driver genes in the pathogenesis of HCC. The identification of these driver genes will lead to the development of effective molecularly targeted therapies and personalized medicine.

Currently, it has been widely realized that signaling pathways, rather than individual genes, govern the course of carcinogenesis[304]. In fact, HCC is considered a multigenic disease with a multifactorial etiology, and hepatocarcinogenesis is an extremely complex multistep process, in which multiple signaling pathways are altered to some extent. In brief, due to the high complexity and heterogeneity of HCC genomes, it is important to emphasize that identifying the altered signaling pathways implicated in HCC, rather than individual mutated genes, may be the key in elucidating the genetic mechanisms underlying hepatocarcinogenesis. Furthermore, insights into the key signaling pathways will likely aid in defining previously unrecognized oncogenic addiction loops in HCC and in developing more effective targeted therapies[305]. Recent extensive research has identified multiple signaling pathways implicated in the pathogenesis of HCC; however, unfortunately, no single dominant signaling pathway is specifically altered in HCC. Future investigations into associated signaling pathways should elucidate the crosstalk between different signaling pathways, *i.e*., how different signaling pathways interact and how they are coordinately regulated in HCC. It is hoped that targeting these crosstalk pathways will result in superior clinical efficacy in treating HCC patients.

Taken together, current evidence suggests that there are no major mutated genes and signaling pathways corresponding to the development of tumors in the majority of cases of HCC, which might primarily be due to the heterogeneity in their geographic and etiologic backgrounds. Due to the intertumor and intratumor heterogeneity of HCCs, future studies must evaluate in detail genetic alterations in relation to the geographic origin of the disease, both across and within individual patients, and chronologically during tumor progression[306]. At the same time, the geographic and etiologic backgrounds of cases of HCC should also be considered in the design of future clinical trials testing molecularly targeted therapies. Such consideration will aid in identifying personalized

therapies for treating HCC patients.

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MINIREVIEWS

# Th17 involvement in nonalcoholic fatty liver disease progression to non-alcoholic steatohepatitis

Carla Melisa Chackelevicius, Sabrina Eliana Gambaro, Claudio Tiribelli, Natalia Rosso

Carla Melisa Chackelevicius, Sabrina Eliana Gambaro, Claudio Tiribelli, Natalia Rosso, Italian Liver Foundation, Area Science Park, Trieste, 34149 TS, Italy

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Correspondence to: Natalia Rosso, PhD, Senior Scientist, Italian Liver Foundation, Area Science Park, Ed Q, SS14 Km 163.5 Basovizza, Trieste, 34149 TS, Italy. natalia.rosso@csf.units.it
Telephone: +39-40-3757922
Fax: +39-40-3757832

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## Abstract

The nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome. NAFLD encompasses a wide histological spectrum ranging from benign simple steatosis to non-alcoholic steatohepatitis (NASH). Sustained inflammation in the liver is critical in this process. Hepatic macrophages, including liver resident macropaghes (Kupffer cells), monocytes infiltrating the injured liver, as well as specific lymphocytes subsets play a pivotal role in the initiation and perpetuation of the inflammatory response, with a major deleterious impact on the progression of fatty liver to fibrosis. During the last years, Th17 cells have been involved in the development of inflammation not only in liver but also in other organs, such as adipose tissue or lung. Differentiation of a naïve T cell into a Th17 cell leads to pro-inflammatory cytokine and chemokine production with subsequent myeloid cell recruitment to the inflamed tissue. Th17 response can be mitigated by T regulatory cells that secrete anti-inflammatory cytokines. Both T cell subsets need TGF-β for their differentiation and a characteristic plasticity in their phenotype may render them new therapeutic targets. In this review, we discuss the role of the Th17 pathway in NAFLD progression to NASH and to liver fibrosis analyzing different animal models of liver injury and human studies.

**Key words:** Th17; Interleukin-17; Nonalcoholic fatty liver disease; Non-alcoholic steatohepatitis; Inflammation



**Core tip:** Interleukin-17 producing cells are important in maintaining inflammation since they are a source of pro-inflammatory cytokines and chemokines with a

critical role in fighting extracellular bacteria. In the last years, this lymphocyte subset has been linked to the pathogenesis of multiple immune mediated diseases and in some cases to the progression to fibrosis. In this review, we discuss the role of the Th17 pathway in nonalcoholic fatty liver disease progression to non-alcoholic steatohepatitis and to liver fibrosis analyzing previously published data obtained from different animal models and human studies of liver injury.

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is defined as an abnormal accumulation of fat in the liver, evidenced by either imaging or histology without any known cause of secondary hepatic fat accumulation such as alcohol consumption, steatogenic medication or hereditary disorders[1]. The histological spectrum of NAFLD comprises benign simple steatosis and a more severe form with inflammation, hepatocyte injury with or without fibrosis called Non-alcoholic steatohepatitis (NASH), this last entity can progress to cirrhosis, liver failure and hepatocellular carcinoma. The incidence of NAFLD and NASH is growing worldwide associated with obesity and diabetes, becoming a common cause of chronic liver disease and need of liver transplantation. The prevalence in the European general population is between 20%-30%, reaching 90% among obese patients[2]. Sustained inflammation in the liver is critical in the progression from benign simple steatosis to NASH. Hepatic macrophages, comprising liver resident macropaghes (Kupffer cells), monocytes infiltrating the injured liver, as well as specific lymphocytes subsets play a pivotal role in the initiation and perpetuation of the inflammatory response, with a major deleterious impact on key steps of fatty liver progression to fibrosis[3]. During the last years, a specific subset of CD4 T effector cells, Th17 subpopulation has been suggested to be involved in this process[4,5]. In this review, we discuss the role of the Th17 pathway in NAFLD progression to NASH and to liver fibrosis analyzing previously published data obtained from different animal models and human studies of liver injury

## LITERATURE SEARCH

For this review, we used PubMed and Google Scholar databases to search for relevant articles using the following mesh terms: "Th17 cells"; "NASH"; "NAFLD" "liver inflammation"; "liver fibrosis"; "induced liver injury" "IL17"; "Tregs"; "CD4 T cells" and "regulatory T cells". Only the articles published between 2006 and 2016 were included.

**Table 1 Interleukin-17 family ligands and receptors**

| IL-17 family ligands | Binding receptor | Produced mainly by |
|---|---|---|
| IL-17 A | IL-17 RA, IL-17 RC | T cells |
| IL-17 A/F | IL-17 RA, IL-17 RC | T cells |
| IL-17 B | IL-17 RB | Numerous cells |
| IL-17 C | Unknown | Prostate, kidney cells |
| IL-17 D | Unknown | Numerous cells |
| IL-17 E (IL-25) | IL-17 RB (IL-25 R) | Numerous cells |
| IL-17 F | IL-17 RA, IL-17 RC | T cells |

IL-17: Interleukin-17.

## Th17 CELLS

### *Th17 differentiation*

CD4 T helper cells that recognize antigens in the context of Mayor Histocompatibility Complex type II (MHC II) can be polarized into different types of effector T cells to coordinate different immunopathological responses[6]. Th17 cells play a role in pathogen clearance and tissue inflammation but are also implicated in the pathogenesis of autoimmune diseases[7,8]. The differentiation of naïve CD4 T cells into Th17 cells in humans is triggered by the combined action of transforming growth factor (TGF)-β, interleukin (IL)-6 and IL-1β, these cytokines induce the expression of the key lineage defining transcription factor orphan nuclear receptor (RORc). RORc is necessary and sufficient for the differentiation of Th17 cells whereas IL-23 is required only for the pathogenicity and expansion of this lineage[9,10]. Th17 pathway is suppressed by IFN-γ and IL-4 that promote Th1 or Th2 respectively[11]. The major target genes for IL-17 include pro-inflammatory chemokines, hematopoietic cytokines, acute phase response genes and antimicrobial substances[12].

### *IL-17 family cytokine and IL-17 family receptor*

Though six IL-17 ligands have been described, IL-17A is the best characterized. IL-17F has 60% homology with IL-17A but it has 10 times less affinity for their receptors[13] (Table 1). They can form homo or heterodimers. Once they bind their cognate heterodimeric receptor IL-17RA, propagates a cascade of events that lead to neutrophil recruitment, inflammation and host defense[14]. Secretion of IL-17 is triggered and perpetuated by IL-6 and IL-23 through at least two transcription factors. The first one is Janus kinase - signal transducer and activator of transcription (JAK-STAT) and the second one is phosphoinositide-3-kinase (PI3K) through the nuclear factor-κB (NF-κB)[15,16]. STAT3 and/or NF-κB, respectively, translocate to the nucleus to promote IL-17 production (Figure 1).

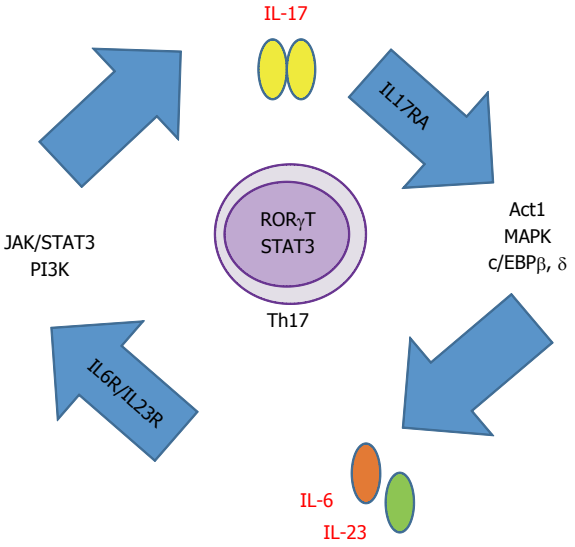


**Figure 1 Interleukin-17 signaling cascade and amplification loop.** IL-17 upregulates the production of pro inflammatory cytokines IL-6 and IL-23 through a complex intracellular signal involving IL-17 RA downstream Act1, MAPK and C/EBP transcription factors and kinases. IL-6 and IL-23 after binding their receptors, stimulate IL-17 production by PI3K and JAK/STAT3 that release NF-κB to translocate to the nucleus. IL-17: Interleukin-17; Act1: Activator 1; JAK/STAT3: Janus kinase/signal transducer and activator of transcription 3; PI3K: Phosphoinositide-3-kinase.

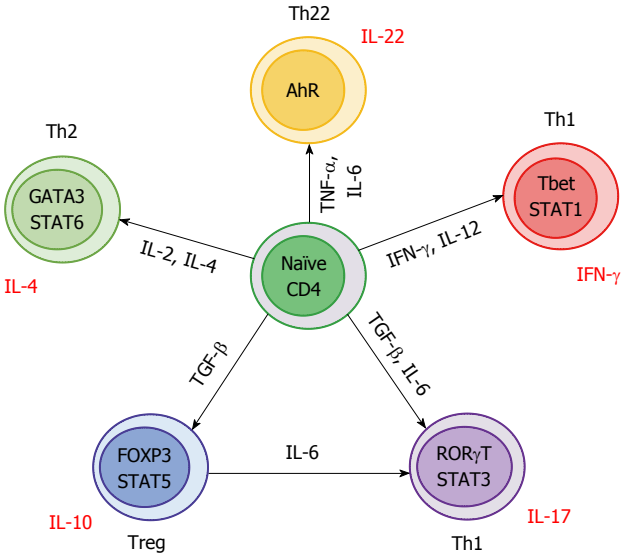


**Figure 2 T cell differentiation and plasticity.** A naïve CD4 T cell differentiates into different T effector cell subsets depending on the cytokines present in the enviroment. Effector T cells secrete their characteristic cytokines represented in red. In the presence of pro-inflammatory IL-6, already differentiated Tregs can switch their phenotype to Th17 and secrete IL-17. IL-17: Interleukin-17; Treg: Regulatory T cells; TGF-β: Transforming growth factor β; IFN-γ: Interferon-γ.

Regarding IL-17 receptors, there are five different heterodimeric receptors for the IL-17 family ligands. IL-17 RA is ubiquitously expressed on a wide range of tissues (liver, intestine, lung, adipose tissue) and cell types (endothelial and immune cells). IL-17RA downstream signaling involves activation of NF-κB activator 1 (Act1), CCAAT/enhancer binding protein beta (C/EBPβ), CCAAT/enhancer binding protein delta (C/EBPδ) and mitogen-activated protein kinase (MAPK) activation, followed by NF-κB and JNK nuclear translocation. Thus, leading to the production of pro-inflammatory cytokines and chemokines and subsequent myeloid cell recruitment to the inflamed tissue[15,17].

### *Th17 cells diversity and plasticity*

Even though Th17 and T regulatory cells (Tregs) have different functions, they do share some similarities. Depending on the stimulus, both T cells populations are capable to change their regulation and function[18]. TGF-β for example, is essential for differentiation of both cell types, but in the absence of pro-inflammatory signals promotes the expansion of inducible Tregs (iTregs)[19]. On the other hand, Th17 development requires the presence of both TGF-β and IL-6[16,17].

This effect could be explained by a TGF-β concentration-dependent function. TGF-β at low concentrations acts synergistically with IL-6 and IL-21 to promote IL-23 receptor (IL-23R) expression, favoring Th17 differentiation[20,21]. On the contrary, at high concentrations, TGF-β suppresses IL-23R and Tregs development is favored by Foxp3+ expression (which in turn inhibits RORγt function)[22,23].

Several studies have established that differentiation of Foxp3+ Tregs is not static and that they can transdifferentiate into Th17 cells[24,25]. In mice, IL-6 showed to convert Foxp3+ cells into Th17 cells in the absence of TGF-β[25] (Figure 2).

IL-17 has been linked to the pathogenesis of many immune mediated diseases like psoriasis, pulmonary fibrosis, systemic sclerosis, myocardial fibrosis, systemic lupus erythematosus, inflammatory bowel disease, rhino sinusitis, encephalomyelitis, multiple sclerosis, asthma, and uveitis[7,8,26-37]. Still, the role of the Th17 pathway in human liver disease is not fully understood.

## ROLE OF Th17 CELLS IN THE PROGRESSION FROM NAFLD TO NASH

The association between obesity and NAFLD/NASH implicates the crosstalk of many cells types and organs. Due to the limitation of using human samples, the best approach is to study deeply the different cell interactions in murine models.

There is evidence regarding IL-17 axis playing a broad role in multiple models of NAFLD via modulation of hepatic inflammation. Among resident hepatic cells, hepatic stellate cells (HSC), Kupffer cells, hepatocytes and endothelial cells express the IL-17RA and are known to activate inflammatory pathways which exacerbate the disease[38,39]. On the other hand, other studies showed that hepatocytes and endothelial cells do not transmit IL-17 signals despite IL-17RA expression and that they do not produce IL-17[39-41]. As regard the production of IL-17 in liver, is not only limited to CD4+

**Table 2 Th17 in mouse models of liver injury**

| Model | Th17 cells | Th17/Tregs | IL-17 expression | Ref. |
|---|---|---|---|---|
| CCL4 | ↑ | ↑ | ↑ | Meng *et al*[39]<br>Sun *et al*[45] |
| BDL | ↑ | | ↑ | Meng *et al*[39]<br>Zhang *et al*[49] |
| MCDD | ↑↑ | | ↑ | Rolla *et al*[52]<br>Giles *et al*[53]<br>Liu *et al*[51] |
| HFD | ↑↑ | | ↑ | Tang *et al*[55] |

IL-17: Interleukin-17; Th17: IL17 secreting T helper; Treg: Regulatory T cells; CCL4: Carbon tetrachloride; BDL: Bile duct ligation; MCDD: Methionine choline deficient diet; HFD: High fat diet.

and CD8+ T cells. Natural Killer T cells, macrophages, neutrophils, γδ T cells and Innate Lymphoid Cells are also capable of producing IL-17[39,42,43]. At least for now, only Th17 CD4 T cells, macrophages and neutrophils are known to be involved in the development of steato-hepatitis inflammation process.

### *Th17 studies in different animal models of NAFLD*

As mentioned before, the progression from NAFLD to NASH involves a wide spectrum of events such as lipid deposition, inflammation, oxidative stress, fibrosis[44]. To study the mediators involved in this process, were characterized and described several animal models.

One of the oldest model for liver fibrosis is the $CCl_4$ toxin-based damage. During the development of liver fibrosis by this approach, CD4+ and CD8+ T cells both exhibited increased IL-17A expression. However the major source of this interleukin was represented by neutrophils. Moreover, HSC were activated and responded by increasing IL-6, α-SMA, TNF-α and TGF-β mRNA expression[39,45,46]. Therefore, when studied the balance of Th17/Treg in the liver, it was favored toward Th17, thus promoting inflammation[45].

*In vivo* and *in vitro* analysis of this model demonstrated that in HSC, IL-17 increases the expression of Collagen-α1 through STAT3 signaling. Stimulation of HSCs with IL-17 results in Collagen-α1 up-regulation via IL-17RA. Moreover, in a STAT3-deficient mice, HSCs do not up-regulate Collagen-α1 in response to IL-17A, confirming that this mediator is a required target of IL-17 signaling[39,47].

Another model of liver injury is the bile duct ligation (BDL) where the bile flow is disrupted, resulting in severe inflammatory cholestatic liver injury that induces a strong fibrotic response after 21 to 28 d[48]. During the inflammatory process CD4+ T cells exhibited an increase in IL-17 expression in the liver. For the CD8+ T cells controversial results were observed, in some studies was reported that IL-17 was produced whereas others indicated the opposite[39,49]. However, neutrophils keep on representing the major source of IL-17 among the infiltrating cells in liver after BDL[49].

Inflammatory cytokines, TGF-β, IL-6, IL-1β , and TNF-α were increased after BDL, but when anti-IL-17mAb treatment or knock out (KO) IL-17RA mice was performed, a marked improvement in liver function was observed. Suppressed Kupffer cells and HSC activation (collagen-α1 production through STAT3), macrophages infiltration and decreased proinflammatory mediators level in serum and injured liver in mice were shown[39,49].

Diet induced models of liver damage have been characterized. One of the most used is the Methionine Choline deficient diet (MCDD) where steatohepatitis occurs at day 10 and fibrosis is observed by 8-10 wk in mice[50]. The main disadvantage of this model is that obesity and insulin resistance are not present. MCDD-driven NAFLD was related to increased hepatic IL-17RA expression and IL-17A/IL-17F production. Moreover, was observed an increase of Tregs (peak at 4 wk of diet) and Th17 (peak 8 wk of diet or further)[51]. When MCDD animals were treated *in-vivo* with neutralizing antibodies against CD25 or IL-17, the liver injury (measured by ALT and AST levels) was alleviated or worsen respectively. However, no evident histological changes were found[51]. On the other hand, when KO mice of IL-17RA, IL-17A or IL-17F were challenged with the diet, a reduction in proinflammatory cytokine and chemokine production, immune cell infiltration and hepatocellular damage was observed[52,53]. The anti-inflammatory and/or immune-regulatory mediators normally inhibited by the IL-17 axis were restored, for instance when IL-17A or IL-17F were missing Treg cell expansion and activation returned to normal. Rolla *et al*[52] described no changes in Treg cells but observed the presence of Th22 cells. Interestingly, was shown in IL-17 KO mice that Th22 cells seemed to be protective in NASH preventing from lipotoxicity[52].

Another widely used diet induced model of liver injury in mice is the high fat diet (HFD). Even if it is a good model for glucose intolerance and obesity, fibrosis is rarely observed and usually additional events such as LPS challenge are required to develop it. The increased oxidative stress produced in the fatty liver causes the apoptosis of Tregs, and increase the Th17 cells[54,55]. When IL-17 is neutralized in HFD mice the challenge with LPS promotes a decrease in serum transaminases levels and a reduced hepatic inflammatory cell infiltrate[55]. In *in vitro* high fat models (HepG2 and primary mice hepatocytes) the exposure to IL-17 induced a higher IL-6 release in the culture medium, higher triglyceride intracellular content and interfered insulin-signaling pathway[55] (Table 2).

### *Th17 studies in humans*

NAFLD prevalence is higher in morbid obese (MO) patients than in the lean population, and these patients present a higher risk for developing NASH and its complications. In a prospective study that included 112 obese patients with NAFLD, the Th17/

**Table 3 Th17 in human tissues**

| | Th17 cells | Th17/ Tregs | IL-17 expression | Disease | Ref. |
|---|---|---|---|---|---|
| Liver | ↑ | ↑ | ↑ | NAFLD - MO | Rau *et al*[4] |
| | | | | PBC | Shi *et al*[62] |
| | | | | CH - CIRR | Tan *et al*[46] |
| VAT | | | ↑↑ | MO | McLaughlin *et al*[59] |
| | | | | MO | Zapata-Gonzalez *et al*[58] |
| SAT | | | ↑ | MAO | Fabbrini *et al*[57] |
| | | | | MO | McLaughlin *et al*[59] |
| PBMC | ↑↑↑ | ↑↑ | | NAFLD - MO | Rau *et al*[4] |
| | | | | T2D | Zeng *et al*[60] |
| | | | | Obesity | Łuczyński *et al*[64] |
| | | | | PBC | Shi *et al*[62] |

IL-17: Interleukin-17; Th17: IL-17 secreting T helper; Treg: Regulatory T cells; VAT: Visceral adipose tissue; SAT: Subcutaneous adipose tissue; PBMC: Peripheral blood mononuclear cells; NAFLD: Nonalcoholic fatty liver disease; MO: Morbid obesity; PBC: Primary biliary cirrhosis; CH: Chronic hepatitis; CIRR: Cirrhosis; T2D: Type II diabetes mellitus.

Tregs ratio correlated positively with NASH progression (by histology) and CK-18 expression (one of the proposed biomarkers of NAFLD progression) analyzed in peripheral blood and in intra hepatic lymphocytes. One year after bariatric surgery, there was a decrease in the Th17/Tregs ratio that became similar to healthy lean controls[4]. In Vonghia *et al*[56] prospective study, a decrease in the IL-10/IL-17A ratio marked an accentuated pro-inflammatory state in obese patients with NASH in comparison to those without NASH.

Studies with MO patients evaluated subcutaneous adipose tissue CD4 T cells content from lean, metabolically normal obese and metabolically abnormal obese subjects. They found that CD4+ gene expression was increased progressively and skewed towards Th17 phenotype. JNK activation was proposed as the mechanism responsible for IL-17 induced insulin resistance[57].

IL-17 mRNA expression from visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) of MO patients was increased in comparison to normal weight women being higher in VAT than in SAT[58]. Moreover, SAT, VAT and peripheral blood mononuclear cells (PBMC) from overweight/moderately obese and MO subjects presented a marked increase in the Th17 population (VAT higher than SAT and peripheral blood)[59]. Positive correlations between IL-17 *vs* IL-6 or Resistin at mRNA levels were found but not correlations for the percentages of Th17 cell with insulin resistance values have been established[58,59].

Contrarily to what is reported in mice[52], to our knowledge the study published by Zapata-Gonzalez *et al*[58] is the only one that reported higher plasmatic IL-17 concentration in the normal weight group than in MO patients.

Diabetes mellitus type II (T2D) is a common disorder among NAFLD patients. In the work of Zeng *et al*[60], CD4 T cells from PBMC were analyzed by flow cytometry. A reduction in the absolute number and in the percentage of Tregs was shown favoring the Th17/Tregs ratio toward Th17 cells[60]. Even though functionality of Tregs cells was conserved, their number was decreased because of impaired survival ability. Interestingly, Th17 cells were higher in patients that presented more T2D complications[57]. Conversely, no differences were found in IL-17 plasma of T2D compared to age-matched healthy controls[61].

In liver fibrosis secondary to primary biliary cirrhosis (PBC), patients presented higher peripheral Th17 cells when compared to healthy controls. In the liver, IL-17+ cells gathered around the portal areas[62]. Furthermore, in cirrhotic liver tissue IL-17+ cell infiltration was higher than controls[46].

*In vitro* studies of human hepatic stellate cells (HSC) exposed to IL-17 showed a dose dependent activation and proliferation response that was neutralized by an IL-17 antagonist[62]. Fabre *et al*[63] evaluated HSC activation (LX2 cell line and primary human hepatic stellate cells) by IL-17. They observed that IL-17 by itself was insufficient to activate the cells, but when combined with a suboptimal TGF-β dose generated a strong activation enhancing TGF-β response by increasing cell surface expression of its receptor and the profibrotic signaling[63].

Regarding the pediatric population, much less is known; we found only a study conducted by Łuczyński *et al*[64] in children with central obesity. They showed higher percentages of Th17 cells in the peripheral blood in comparison with healthy lean children[61]. In other pediatric diseases these T cells were involved, principally in inflammation, such as autoimmune thyroid disease or Mycoplasma pneumoniae infection[65,66] (Table 3).

## CONCLUSION

A pro-inflammatory state is crucial for the initiation and maintenance of inflammation in the onset and progression of NAFLD/NASH. T cells resident in non-lymphoid tissues are able to regulate local inflammation by modulating immunological and non-immunological responses. Many studies in different animal models have proved the important role of the Th17 pathway in inflammation and HSC activation. Much less is known about human physiopathology of NAFLD due to the limitations and difficulty to obtain samples. Studies with obese or diabetic patients obtained higher Th17 cells in blood with no changes or decrease in Tregs. If IL-17 is elevated or not in plasma is still controversial. Adipose tissue and intrahepatic Th17 lymphocyte subsets have been assessed in NAFLD/obese/PBC patients, being higher compared to control individuals.

It has been widely argued if inflammation occurs first in liver than in adipose tissue or the other way around. Until now, this is still unraveled but it is

known that the adipose tissue inflammation and their adipokines, free fatty acids, and gut derived microbial products could promote Th17 differentiation in the liver, with the consequent imbalance towards inflammation. Obesity may maintain a positive feedback loop that promotes Th17 survival in the inflamed liver. This would explain how weight loss after bariatric surgery can reverse clinical and histopathological features of NASH. On the other hand, it seems that the T cell imbalance occurs in situ, but to date there is not enough evidence to explain the connection between adipose tissue inflammation and hepatic injury progression.

Studies that analyze the crosstalk between the different organs during the NAFLD/NASH progression should be promoted in order to evaluate and establish the main players in this disease.

Although there is evidence that implicates the Th17 pathway as a key player in the progression of NALFD, it seems that there is a lot more to be elucidated. Plasticity of this cell subtype may render it a therapeutic target.

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ORIGINAL ARTICLE

Basic Study

# Dysregulation of innate immunity in ulcerative colitis patients who fail anti-tumor necrosis factor therapy

Angela C Baird, Dominic Mallon, Graham Radford-Smith, Julien Boyer, Thierry Piche, Susan L Prescott, Ian C Lawrance, Meri K Tulic

Angela C Baird, Ian C Lawrance, Faculty of Medicine and Dentistry, School of Medicine and Pharmacology, University of Western Australia, Perth, WA 6009, Australia

Dominic Mallon, Department of Immunology, Fremantle Hospital, Perth, WA 6160, Australia

Graham Radford-Smith, Royal Brisbane and Women's Hospital, Brisbane, Queensland, 4006, Australia

Julien Boyer, Thierry Piche, Meri K Tulic, University of Nice Sophia-Antipolis, EA6302, 06202 Nice, France

Julien Boyer, Thierry Piche, Department of Gastroenterology and Nutrition, l'Archet Hospital 2, 06202 Nice, France

Susan L Prescott, School of Paediatrics and Child Health, University of Western Australia, Subiaco, WA 6008, Australia

Susan L Prescott, Meri K Tulic, International Inflammation network (in-FLAME) of the World Universities Network, University of Western Australia, Subiaco, WA 6008, Australia

Ian C Lawrance, Centre for Inflammatory Bowel Diseases, St John of God Subiaco Hospital, Perth, WA 6009, Australia

Meri K Tulic, Mediterranean Center for Molecular Medicine (C3M)-INSERM U1065, Team 12, 06204 Nice, France

**Author contributions:** Baird AC and Tulic MK were involved in data acquisition, analysis, interpretation of the data and writing of the manuscript; Baird AC, Mallon D, Radford-Smith G, Lawrance IC and Tulic MK were involved in study design; Prescott SL provided protocols and provision of laboratory space for some experiments; Boyer J, Piche T and Lawrance IC gave invaluable intellectual input into the study and clinical direction; Lawrance IC and Tulic MK proposed the original hypothesis of this study and approved the final version of this manuscript.

**Institutional review board statement:** The study was reviewed and approved by the Southern Metropolitan Health Service Human Ethics Committee and Institutional Review Board.

**Informed consent statement:** All biological samples from the patients were taken after informed consent.

**Conflict-of-interest statement:** To the best of our knowledge, there is no conflict of interest to declare.

**Data sharing statement:** Two senior co-authors (Lawrance IC and Tulic MK) have access to all data and dataset available from the corresponding author at meri.tulic@unice.fr.



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**Correspondence to:** Meri K Tulic, PhD, INSERM, CR1, Professor, Mediterranean Center for Molecular Medicine (C3M)-INSERM U1065, Team 12, Batiment Archimed, 151 route Saint-Antoine de Ginestiere, 06204 Nice, France. meri.tulic@unice.fr
**Telephone:** +33-4-89064326
**Fax:** +33-4-89064221

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## Abstract

***AIM***

To study the innate immune function in ulcerative

colitis (UC) patients who fail to respond to anti-tumor necrosis factor (TNF) therapy.

***METHODS***

Effects of anti-TNF therapy, inflammation and medications on innate immune function were assessed by measuring peripheral blood mononuclear cell (PBMC) cytokine expression from 18 inflammatory bowel disease patients pre- and 3 mo post-anti-TNF therapy. Toll-like receptor (TLR) expression and cytokine production post TLR stimulation was assessed in UC "responders" ($n$ = 12) and "non-responders" ($n$ = 12) and compared to healthy controls ($n$ = 12). Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels were measured in blood to assess disease severity/activity and inflammation. Pro-inflammatory (TNF, IL-1β, IL-6), immuno-regulatory (IL-10), Th1 (IL-12, IFNγ) and Th2 (IL-9, IL-13, IL-17A) cytokine expression was measured with enzyme-linked immunosorbent assay while TLR cellular composition and intracellular signalling was assessed with FACS.

***RESULTS***

Prior to anti-TNF therapy, responders and non-responders had similar level of disease severity and activity. PBMC's ability to respond to TLR stimulation was not affected by TNF therapy, patient's severity of the disease and inflammation or their medication use. At baseline, non-responders had elevated innate but not adaptive immune responses compared to responders ($P$ < 0.05). Following TLR stimulation, non-responders had consistently reduced innate cytokine responses to all TLRs compared to healthy controls ($P$ < 0.01) and diminished TNF ($P$ < 0.001) and IL-1β ($P$ < 0.01) production compared to responders. This innate immune dysfunction was associated with reduced number of circulating plasmacytoid dendritic cells (pDCs) ($P$ < 0.01) but increased number of CD4+ regulatory T cells (Tregs) ($P$ = 0.03) as well as intracellular accumulation of IRAK4 in non-responders following TLR-2, -4 and -7 activation ($P$ < 0.001).

***CONCLUSION***

Reduced innate immunity in non-responders may explain reduced efficacy to anti-TNF therapy. These serological markers may prove useful in predicting the outcome of costly anti-TNF therapy.

**Key words:** Ulcerative colitis; Innate immunity; Anti-tumor necrosis factor therapy; Toll-like receptor; IRAK4; Inflammatory bowel disease



**Core tip:** Anti-tumor necrosis factor (TNF) therapy is effective in approximately 60% of ulcerative colitis (UC) patients. Currently we do not know which patients are likely to benefit from this costly treatment. Here we show that differences in innate immune function [measured by patients response to toll-like-receptor (TLR), TLR agonists] exist between UC responders and non-responders. Differences exist in (1) content of immune and regulatory cells in their blood; (2) capacity of their cells to produce cytokines; and (3) in their signalling following TLR activation. Serological measure of TLR function may prove to be a useful tool in clinic to predict patient's response to anti-TNF treatment.

Baird AC, Mallon D, Radford-Smith G, Boyer J, Piche T, Prescott SL, Lawrance IC, Tulic MK. Dysregulation of innate immunity in ulcerative colitis patients who fail anti-tumor necrosis factor therapy. *World J Gastroenterol* 2016; 22(41): 9104-9116 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i41/9104.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i41.9104

## INTRODUCTION

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC) are life-long, immunologically-mediated disorders that are increasing in frequency[1,2]. One of the main pro-inflammatory cytokines involved in ongoing and uncontrolled inflammation in IBD is tumor necrosis factor alpha (TNFα). Although the use of anti-TNF therapy (Infliximab® and Adalimumab®) has revolutionized the treatment of the disease[3-6], one third of patients fail to respond and significant proportion loose sensitivity or become steroid dependent. In the past, serologic and faecal[7] as well as genetic[8,9] markers have been used to predict response to anti-TNF therapy, however these are often not effective or extremely expensive. Our lack of understanding why certain patients respond to anti-TNF therapy and others don't hinders our progress in predicting which patients are likely to benefit from this costly treatment.

Inflammation in IBD is thought to result from inappropriate activation of the innate immune system by intestinal luminal antigens or a defect in its signaling regulation in genetically susceptible individuals[1]. Toll-like receptors (TLR 1-10) are crucial activators of innate immunity. All TLRs signal through MyD88-dependent pathway except TLR3. TLR4 can signal through both MyD88-dependent and MyD88-independent pathways but requires CD14 (Figure 1). IRAK4 plays a critical role in initiating nuclear factor kappa B (NFκB) intracellular signalling pathway and therefore production of pro-inflammatory cytokines. The role of TLRs in IBD is mounting[10-12]; polymorphisms in TLR genes are associated with increased risk of IBD[13-15] and genes regulating TNF signalling and TNF production have been shown to be important predictors of anti-TNF therapy[13]. Together these finding suggest a strong pathogenic association between the TLRs and IBD.

Previously we have demonstrated that measurement of early innate immune function in peripheral blood of children (TLR responses during their first 5 years of

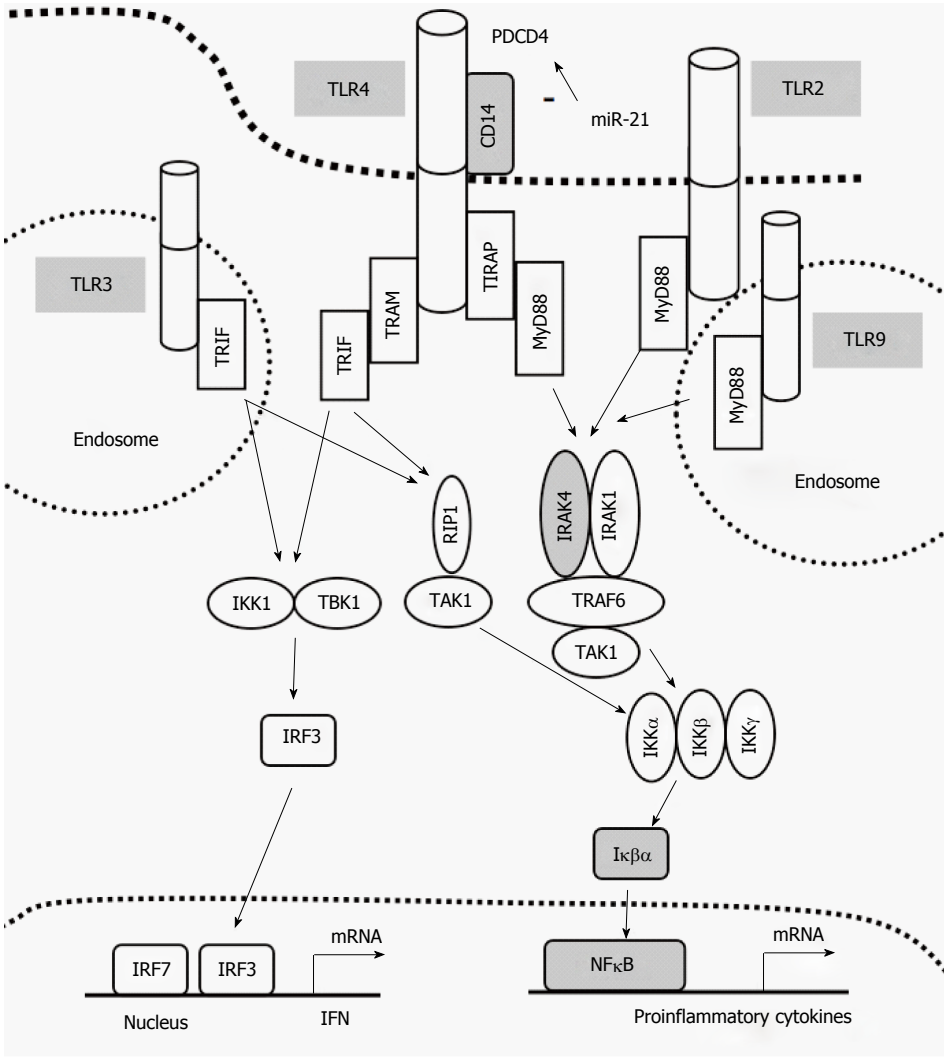


**Figure 1 Toll-like receptor signalling pathway.** TLR: Toll-like receptor.

life) identified striking differences in the developmental of their innate immune responses and profile of these responses were a good indicator of their subsequent risk of development of allergic disease[16]. As the inflammatory extra-intestinal manifestations in UC suggest an immunological component not isolated to the intestine, the same methodology was used to investigate the global innate immune function in UC patients to determine if differences can explain the heterogeneity in their clinical responses to anti-TNF therapy.

This study investigated the interaction between the TLR activity in UC patients responsive or non-responsive to anti-TNF therapy to determine if TLR levels, activity and/or TLR signalling pathways correlate with patient's response to anti-TNF therapy. It was hypothesised that there are inherent differences in innate immune function between responders and non-responders which may explain differences in their clinical effectiveness of treatment. These novel results extend our understanding of intestinal inflammation pathogenesis and implications of innate immunity in UC patients' response to anti-TNF therapy.

## MATERIALS AND METHODS

### *Study design*

This study was conducted as a prospective and retrospective observational study. The former, to determine whether inflammation levels, medication use, patient demographics, surgery and anti-TNF therapy itself influenced patient outcome (response or non-response to TNF therapy), and the latter to determine if there were differences in the underlying mechanisms responsible for TLR recognition and innate immune response. To address these aims, pro-inflammatory cytokine levels, TLR expression, TLR signalling and cell populations were analysed from isolated PBMCs pre- and post-anti-TNF therapy and; (1) correlated back to inflammation levels, medication use, patient demographics, surgery and anti-TNF therapy itself, and (2) were then compared between responders and non-responders.

### *Participants*

IBD patients ($n$ = 42) and healthy controls ($n$ = 12) were recruited from Centre for Inflammatory

Bowel Diseases, Fremantle Hospital, Perth, Australia. The diagnosis of CD and UC was made based upon clinical, endoscopic, histopathological and radiological findings, and classified by the "Montreal classification". Patient demographic data included data of birth, age at diagnosis, age at time of study, timing of anti-TNF therapy, concurrent immunosuppressive medications, surgeries, family and smoking history. To examine the effects of disease, anti-TNF therapy, inflammation and medication use on innate immunity, blood was taken from 18 IBD patients (13 with CD and 5 with UC) prior (pre-anti-TNF) and 3 mo after anti-TNF therapy commenced (post-anti-TNF). To study immune responses in *responder* and *non-responder* UC patients, blood was collected from separate 24 UC patients and compared to 12 healthy controls. UC patients achieving clinical remission, defined by a Colitis Activity Index (CAI) $\leqslant$ 4, and normal C reactive protein (CRP) $\leqslant$ 10 mg/L, were considered responders (*Rs*, $n$ = 12), whilst those who failed to respond with a reduction in CAI of < 4 points and a consistently elevated CRP as non-responders (NRs, $n$ = 12).

### Blood collections and processing

Sixty millilitres of peripheral blood was collected and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation and cryopreserved (7.5% DMSO) at -80 ℃ for future use.

### PBMC stimulation

PBMCs were cultured alone or with various TLR agonists including lipoteichoic acid (LTA 1 μg/mL, TLR2 ligand), Poly I:C (50 μg/mL, TLR3 ligand), *E. coli* lipopolysaccharide (LPS 10 ng/mL, TLR4 ligand), Flagellin (1 μg/mL, TLR5 ligand), Imiquimod (10 μg/mL, TLR7 ligand), Gardiquimod (10 μg/mL, TLR8 ligand) or CpG oligonucleotide (CpG 3 μg/mL, TLR9 ligand); all purchased from InvivoGen, CA, United States. All cultures were plated in duplicate in 96-well round-bottom plates in 250 μL RPMI (Gibco, Life Technology, Grand Island, NY, United States) supplemented with 10% foetal calf serum (Australia Biosearch, Australia) and incubated at 37 ℃ with 5% $CO_2$ for 24 h (LTA, Poly I:C, LPS and Flagellin) or 48 h (Imiquimod, Gardiquimod or CpG). The supernatants were then removed and stored at -20 ℃ until cytokine analysis.

### Multiplex bead assay

Cytokines [TNFα, interferon γ (IFNγ), interleukin (IL)-1β, IL-6, IL-9, IL-10, IL-12, IL-13 and IL-17A] were measured from culture supernatants. Multiplex beads for the Bio-Plex® multiplex system (Life Sciences, Bio-Rad Laboratories Pty, Ltd., Vic, Australia) were diluted 1:2 in bead diluents and the 9plex bead assay according to the manufacturer's protocol using a Luminex®200 Bead array with Xmap® multiplexing technology located at the Centre of Microscopy, characterisation and Analysis (CMCA), UWA, Australia. The limit of detection was 3 pg/mL for all cytokines. Data was analysed using the xPONENT 4.2 for MAGPIX software (Luminex Corporation, Austin, TX, United States).

### Flow cytometric analysis

PBMC cells were stained with monoclonal antibodies to identify macrophages/monocytes [Mφ] ($HLADR^+CD14^+$), natural killer cells ($CD16^+CD56^+$), myeloid ($Lin1^-HLADR^+CD123^-CD11c^+$) and plasmacytoid ($Lin1^-HLADR^+CD123^+CD11c^-$) dendritic cells (DC), effector T cells ($CD4^+$ or $CD8^+$), T regulatory cells ($CD4^+CD25^+CD127^-$ or $CD8^+CD25^+CD127^-$), memory T cells ($CD45RO^+$) and naïve T cells ($CD45RA^+CD4^+$) (Supplement Table 1). Isotype-matched antibodies were used as controls (Supplement Table 1) and assessed by FACS analysis. For the analysis of TLR and CD14 receptor levels, unstimulated and stimulated PBMCs were stained with TLR2 (1:20 dilution, PE; eBiosciences, San Diego, CA, United States), TLR4 (1:50 dilution, APC; eBiosciences), TLR9 (1:20 dilution, APC; BD Pharmingen, San Diego, CA, United States) and CD14 (1:20 dilution, FITC; eBiosciences) prior to fixing according to manufacturer's instructions. Isotype-matched antibodies were used as controls.

To address differences in MyD88-dependent signalling in unstimulated and stimulated PBMCs, PBMCs were cultured alone or with TLR2, TLR3, TLR4, TLR7 TLR9 agonists as previously described for 15 min at 37 ℃ with 5% $CO_2$. Cells were fixed and permeabilized according to manufacturer's instructions (BD Biosciences, San Diego, CA, United States) and stained for phosphorylated NFκB (pNFκB) (1:5 dilution, AF488; BD Biosciences), total IRAK4 (1:5 dilution, PE; BD Biosciences) and total Iκβα (1:5 dilution, AF647; BD Biosciences). Stained cells were captured using the FACScanto II bench top flow cytometer (BD Biosciences) at the CMCA, UWA, Australia and analysed using FlowJO v7.6.3 research software (Tree Star Inc. Oregon, United States).

### Statistical analysis

Significance between groups at 95% confidence level was determined by paired and Mann-Whitney non-parametric unpaired $t$ tests, using Graphpad Prism 4.0 software package (Graphpad, San Diego, CA, United States). Results were expressed as median geometric mean with 95% confidence interval, fold-change from basal ± SD, mean percentage of total cell population ± SD or mean fluorescence intensity (MFI) ± SD. Correlation between medications and patient's response to anti-TNF therapy was determined by multiple regression analyses using SPSS version 14.0 software package for Windows PC (IBM, Armonk, NY, United States). Statistical significance was considered as $P < 0.05$.

**Table 1 Inflammatory bowel disease patient demographics and characteristic**

| Characteristics | *n* or mean ± SD (range) |
|---|---|
| Male:female | 7:11 |
| Age at diagnosis(yr) | 26.6 ± 11.3 (12-56) |
| Disease duration | 8.4 ± 7.9 (0-30) |
| Montreal classification | |
| CD | 13 |
| UC | 5 |
| Age at diagnosis (yr) | |
| A1 ( ⩽ 16) | 1 |
| A2 (17-40) | 14 |
| A3 (> 40) | 3 |
| Smoking status | |
| Never smoked | 7 |
| Ex-smoker | 5 |
| Current smoker | 6 |
| Anti-TNF response | |
| Responder | 13 |
| Non-responder | 5 |

CD: Crohn's disease; UC: Ulcerative colitis; TNF: Tumor necrosis factor.

**Table 2 Clinical data of responders and non-responders**

| | Responders (*n* = 13) (*n* or mean ± SEM) | Non-responders (*n* = 5) (*n* or mean ± SEM) | *P* value |
|---|---|---|---|
| Immuno-suppressants | | | |
| Thiopurine | 10 | 4 | NS |
| Tacrolimus | 0 | 0 | |
| Prednisone | 6 | 4 | NS |
| Prednisone/thiopurine | 5 | 2 | NS |
| Methotrexate | 0 | 0 | |
| 5-ASA | 1 | 2 | |
| Corticosteroids | 9 | 2 | NS |
| ESR | 23.9 ± 4.8 (*n* = 11) | 9.6 ± 2.2 (*n* = 4) | NS |
| CRP | 25.8 ± 6.8 (*n* = 13) | 9.2 ± 3.7 (*n* = 4) | NS |
| Partial Mayo[1] (out of 9 - UC patients only) | 8 ± 1.3 (*n* = 3) | 8 (*n* = 3) | NS |
| CDAI (CD patients only) | 321.9 ± 35.4 (*n* = 9) | 385 ± 55 (*n* = 3) | NS |

[1]Partial Mayo scores presented, as not all patients had endoscopic examination at time of blood draw. CDAI: Crohn's disease activity index; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; UC: Ulcerative colitis.

**Table 3 Crohn's disease vs ulcerative colitis pre-anti-tumour necrosis factor therapy**

| | CD (*n* = 13) (*n* or mean ± SEM) | UC (*n* = 5) (*n* or mean ± SEM) | *P* value |
|---|---|---|---|
| Immunosuppressant | | | |
| Thiopurine | 10 | 4 | NS |
| Tacrolimus | 0 | 0 | |
| Prednisone | 6 | 4 | NS |
| Prednisone/ thiopurine | 3 | 3 | |
| Methotrexate | 0 | 0 | |
| 5-ASA | 2 | 3 | NS |
| ESR | 17.4 ± 3.3 (*n* = 11) | 27.6 ± 11.8 (*n* = 4) | NS |
| CRP | 19.5 ± 6.3 (*n* = 13) | 29.8 ± 12 (*n* = 4) | NS |
| Partial Mayo[1] (out of 9 - UC patients only) | - | 8.5 ± 0.9 (*n* = 4) | |
| CDAI (CD patients only) | 337 ± 29.8 (*n* = 12) | - | |

[1]Partial Mayo scores were presented, as not all patients had undergone endoscopic examination at time of blood draw. CD: Crohn's disease; CDAI: Crohn's disease activity index; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; UC: Ulcerative colitis.

## RESULTS

### *Baseline inflammation, medication use and disease type in UC patients prior to anti-TNF therapy*

**Clinical data comparison of patient population:** To determine if innate immune response were altered by the use of immuno-suppressants and anti-inflammatory medications, disease type or the level of inflammation measured by erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels, CD activity index (CDAI) and partial Mayo score pre-anti-TNF therapy, the clinical data of 18 IBD patients were compared (Tables 1 and 2).

Prior to anti-TNF therapy, clinical data of responders and non-responders demonstrated no significant differences in the number of patients on individual or combined immune-suppressants, methotrexate, 5-ASA or corticosteroids (Table 2). Although the ESR and CRP levels appeared higher in responders, they were not significantly different to non-responders suggesting similar level of disease severity/activity between the two groups. This suggests that whether a patient responds or not to anti-TNF therapy is not predicted by their ESR, CRP, CDAI or partial Mayo score, nor their medication use. As expected, the partial Mayo scores were significantly higher in non-responders (7.3 ± 0.6, $n = 3$) than in responders (0.5 ± 0.5, $n = 4$) post-anti-TNF therapy ($P = 0.004$), as were the CDAI scores (198 ± 42, $n = 2$ *vs* 80.1 ± 21.7, $n = 9$ respectively, $P = 0.04$). This was also true of the CRP levels (23 ± 1.9, $n = 5$ *vs* 7.4 ± 3.4, $n = 13$ respectively, $P = 0.01$) (data not shown). Considering CD and UC patients separately, all patients suffered from moderately-severe inflammation and there were no significant differences in medications, ESR or CRP levels pre anti-TNF therapy (Table 3). At the time of the second blood draw (3 mo post-anti-TNF induction therapy), two thirds of the patients had gone into remission with anti-TNF therapy (CDAI < 150, CAI ⩽ 4 and CRP ⩽ 10 mg/L) and had ceased steroid therapy. Five ($n = 5$) did not respond to anti-TNF therapy, three ($n = 3$) continued on steroid therapy (1 responder and 2 non-responders $P > 0.05$) and two ($n = 2$) underwent surgery with cessation of immunomodulation.

**Basal and stimulated PBMC cytokine production pre- and post-anti-TNF therapy:** To investigate whether medication use, anti-TNF therapy, disease type or inflammation affected baseline PBMC function, isolated PBMCs were cultured *in vitro* and basal and stimulated supernatant cytokine levels measured.

**Table 4 Ulcerative colitis cohort demographics and characteristics**

| | UC non-responders (NR) ($n$ = 12) | UC responders (R) ($n$ = 12) | $P$ value NR *vs* R | Controls ($n$ = 12) |
|---|---|---|---|---|
| Male:female | 6:6 | 4:8 | NS | 4:8 |
| Mean age at diagnosis (yr) (SD/range) | 27.5 (12.2/15-59) | 24.5 (8.8/10-36) | NS | NA |
| Mean age at assessment (yr) (SD/range) | 33.6 (14/20-67) | 38.6 (14.2/21-64) | NS | 33.4 (13/18-52) |
| Mean disease duration (yr) (SD/range) | 6.9 (2.7/13.1/20-67) | 17.5 (10.6/4-37) | 0.01 | NA |
| Median time of blood draw post-therapy (wk) (range) | 156 (12-364) | 208 (12-468) | NS | NA |
| Montreal classification, *n* | | | | NA |
| Age at diagnosis (yr) | | | | |
| A1 - ⩽ 16 | 1 | 1 | NS | |
| A2 - 17-40 | 9 | 10 | NS | |
| A3 - > 40 | 2 | 1 | NS | |
| Disease location | | | | |
| E1 - Proctitis | 2 | 0 | NS | |
| E2 - Left sided | 1 | 4 | NS | |
| E3 - Extensive | 9 | 8 | NS | |
| Smoking status (*n*) | | | | |
| Never smoked | 8 | 8 | NS | 8 |
| Ex-smoker | 3 | 4 | NS | 2 |
| Current smoker | 1 | 0 | NS | 2 |

UC: Ulcerative colitis.

Basal innate (TNF, IL-1β, IL-6), immunoregulatory (IL-10), Th1 (IL-12 and IFNγ) and Th2 (IL-9, IL-13 and IL-17A) cytokine expression was similar pre- and post-anti-TNF therapy suggesting it was not affected by treatment (Supplementary Figure 1).

To determine if anti-TNF therapy affected the PBMC's ability to recognise and respond to stimulation, TNF production pre- and post-anti-TNF therapy was measured post TLR activation. TNF production was unaffected or even higher ($P$ = 0.03 for TLR4 stimulation) post-TNF therapy compared to production pre-therapy (Supplementary Figure 2) suggesting that anti-TNF therapy does not reduce the PBMC's ability to recognise, or responds to TLR activation. None of the other cytokine levels were affected post therapy (data not shown). TNF production post TLR3 and TLR4 activation was approximately 10-fold higher than stimulation of other TLRs.

### *The immune response of UC responders and non-responders to anti-TNF therapy*

As the use of anti-TNF therapy for the treatment of UC is associated with higher rates of primary and secondary non-responses than in CD patients, we next set out to study innate immune responses in UC patients who respond or do not respond to treatment.

**Demographic and clinical data comparison of UC patient cohort:** PBMCs from 24 UC patients ($n$ = 12 responders and $n$ = 12 non-responders to anti-TNF therapy), and 12 healthy controls were isolated. Bloods were taken post-anti-TNF therapy, with the median time being 208 (12-468) wk for responders and 156 (12-364) wk for non-responders ($P$ = 0.52). No significant demographic differences were detected between the UC populations except that responders had longer disease duration ($P$ = 0.01) (Table 4). None of the controls were on any medications or suffering from any infections or inflammatory conditions. There was no correlation between any of the medications (alone or in combination) and patient's response to anti-TNF therapy. At time of blood draw (post-anti-TNF therapy) all responders were in clinical remission (CAI ⩽ 4 and CRP ⩽ 10 mg/L) with 5 patients on maintenance anti-TNF therapy, 8 on thiopurine and 4 on 5-ASA. Of the non-responders, 3 had blood drawn following recovery from colectomy from uncontrolled UC, 1 was in remission on tacrolimus and 4 had ongoing inflammation with 6 taking oral corticosteroids and all receiving thiopurines (Table 5).

**Basal cytokine production in UC responders and non-responders:** PBMCs from UC patients (responders and non-responders) had significantly greater basal IL-1β, IL-6 and IL-10 levels compared to healthy controls (Figure 2). Non-responders had significantly increased TNF, IL-1β and IL-10 compared to responders. There were no differences in basal IL-12 production between UC groups compared to controls or production of any of the Th2 cytokines measured (IL-5, IL-9, IL-13 or IL-17A) (Figure 2). The Th1 cytokine IFNγ was significantly elevated in non-responders compared to responders and controls (Figure 2).

**Differences in stimulated cytokine production by UC responders and non-responders:** In general, responders had similar TNF, IL-1β, IL-6 and IL-10 responses to healthy controls following TLR stimulation; exceptions being increased TLR9-induced TNF in responders (Figure 3A), and reduced TLR7-induced IL-1β (Figure 3B) as well as reduced TLR-3, -5 and -7 induced IL-6 responses ($P$ = 0.04) (Figure 3C). In contrast, TNF, IL-1β, IL-6 and IL-10 responses

**Table 5 Clinical data pre- and post-anti-tumour necrosis factor therapy**

| | Non-responder ($n$ = 12) ($n$ or mean ± SEM) | Responder ($n$ = 12) ($n$ or mean ± SEM) | $P$ value |
|---|---|---|---|
| Pre-anti-TNF therapy | | | |
| Immunosuppressant | | | |
| Thiopurine | 7 | 6 | NS |
| Tacrolimus | 2 | 0 | NS |
| Prednisone | 9 | 7 | NS |
| Prednisone/thiopurine | 6 | 2 | NS |
| Methotrexate | 1 | 0 | |
| 5-ASA | 6 | 5 | NS |
| ESR | 42.5 ± 12.9 ($n$ = 7) | 33.1 ± 10.4 ($n$ = 7) | NS |
| CRP | 36.3 ± 10.1 ($n$ = 11) | 27.4 ± 7.4 ($n$ = 12) | NS |
| Partial Mayo[1] | 7.3 ± 0.5 ($n$ = 7) | 7.5 ± 0.2 ($n$ = 11) | NS |
| Post-anti-TNF therapy | | | |
| Immunosuppressant | | | |
| Thiopurine | 12 | 8 | NS |
| Tacrolimus | 4 | 0 | 0.045 |
| Prednisone | 6 | 0 | 0.007 |
| Prednisone/thiopurine | 0 | 0 | |
| Methotrexate | 0 | 0 | |
| 5-ASA | 6 | 4 | NS |
| ESR | 22.9 ± 7.9 ($n$ = 4) | 13.5 ± 4.7 ($n$ = 9) | NS |
| CRP | 51.2 ± 14.6 ($n$ = 12) | 4.6 ± 1.2 ($n$ = 12) | 0.0043 |
| Partial Mayo[1] | 7.6 ± 0.4 ($n$ = 12) | 0.3 ± 0.2 ($n$ = 12) | < 0.0001 |

[1]Partial Mayo scores presented as not all patients had endoscopic examination at time of blood draw. CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate.

to all TLR agonists were significantly lower in non-responders compared to healthy controls ($P$ < 0.01) (Figure 3A-D). Non-responders had significantly lower TNF and IL-1β production to all TLRs compared to responders (Figure 3A and B) as well as reduced TLR9-induced IL-6 (Figure 3C) and TLR-3, -4, -8 and -9-induced IL-10 (Figure 3D).

### *PBMC characterisation*

Isolated PBMCs are a mixed cell population and differences in cytokine production are likely to be attributed to differences in distribution of cellular populations. Characterization of cellular populations in the three study groups have shown both UC subgroups to have higher percentage of monocytes in circulating blood compared to controls (Figure 4A). Non-responders had a significantly lower plasmacytoid DC (pDC) frequency compared to responders and controls ($P$ < 0.01). This decrease in pDCs was associated with increased percentage of $CD4^+$ regulatory T cell (Tregs) compared to controls ($P$ = 0.03) (Figure 4A). Increased Tregs was of borderline significance in responders compared to controls ($P$ = 0.09; Figure 4A). We found no difference in memory, naïve, $CD4^+$, $CD8^+$ effector T cells, $CD8^+$ Treg cells, NK cells or myeloid DC (mDC) between the groups (data not shown).

### *PBMC expression of TLR2, -4, -7, -9 and CD14*

As pDC frequency varied between responders and non-responders, the level of basal and stimulated TLR-2, TLR-4, TLR-7, TLR-9 and CD14 expression levels were assessed. The baseline expression of TLRs and CD14 were similar between the three groups and was increased to similar levels in each of the groups following stimulation (Figure 4B). No differences were observed in basal or stimulated TLR/CD14 expression levels between the groups suggesting that the percentage pDC did not impact expression of TLRs or CD14.

### *TLR signalling*

MyD88-dependent signalling was assessed following TLR-2, -3, -4, -7 and -9 stimulation of PBMCs by measuring total IRAK4, total Iκβα and phosphorylated NFκB (pNFκB) activity. TLR-3 was used as a control as TLR3-mediated signalling is independent of MyD88, IRAK4 and IRAK1[17]. Total IRAK4 levels did not change upon TLR-3 activation and no differences in protein levels between the 3 groups were identified (data not shown). When comparing basal to stimulated total IRAK4 levels; responders and controls had similar response profiles, that is, significantly lower total IRAK4 levels upon TLR stimulation (Figure 5), whilst non-responders failed to reduce total IRAK4 following TLR-2, -4, and -7 stimulation (Figure 5). Total IRAK4 was significantly increased in non-responders following TLR-9 activation compared to basal levels ($P$ = 0.03, Figure 5). This suggests that in the non-responders, the degradation/inhibition of IRAK4 may be dysregulated resulting in its aberrant accumulation. Whilst total Iκβα were significantly decreased in all groups following stimulation ($P$ < 0.02 for all), pNFκB were significantly increased in all groups ($P$ < 0.02 for all; Figure 5).

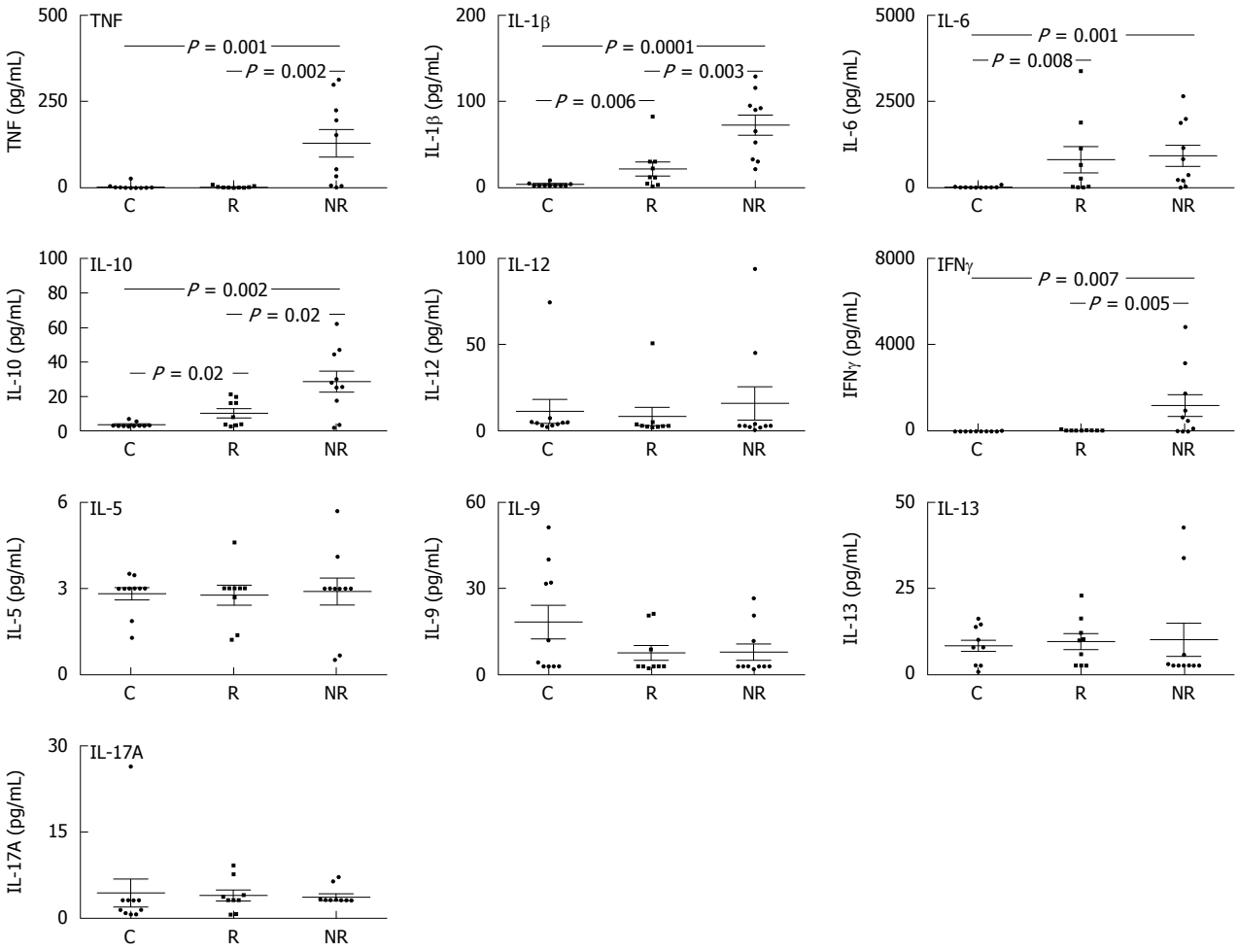


**Figure 2 Basal cytokine production in responders and non-responders compared to healthy controls.** The basal expression of pro-inflammatory (TNF, IL-1β, IL-6), regulatory (IL-10), Th1 (IL-12, IFNγ) and Th2 (IL-5, -9, -13, 17A) cytokines were assessed and compared in peripheral blood mononuclear cells isolated from healthy controls (C) ($n$ = 12) and UC patients who are in remission following anti-TNF therapy, responders (R) ($n$ = 12) and those who failed to respond, non-responders (NR) ($n$ = 12). Results were expressed as mean with 95%CI. The $P$ values represent statistical significance of < 0.05 between the groups denoted. TNF: Tumor necrosis factor; IL: Interleukin.

## DISCUSSION

Non-communicable disease including cardiovascular, metabolic, IBD and allergic diseases are now surpassing infectious disease accounting for more than 60% of all global deaths[18]. The IBDs are incurable, disabling life-long conditions. Albeit expensive, anti-TNF therapy is an effective treatment for approximately 60% UC patients; however, the mechanisms responsible for lack of responses to treatment are unknown. Here we have shown, for the first time, clear differences in innate immune function in peripheral blood of responders and non-responders UC patients given anti-TNF therapy. We have demonstrated that whilst, in general, responders have similar innate cytokine responses to healthy controls, non-responders have diminished innate responses to all TLR agonists compared to controls and reduced TNF and IL-1β responses compared to responders. These results suggest dysregulation of innate immunity in non-responders and may explain heterogeneity in clinical effectiveness of anti-TNF treatment in UC patients. Individuals innate immune function may prove be a useful tool to predict cost effective application of this treatment.

As there were no significant differences in medication use, baseline ESR, CRP and inflammation levels (as indicated by partial Mayo scores and CDAIs) between responders and non-responders prior to anti-TNF induction (part 1 of study), this strengthens the hypothesis that the differences are intrinsic and that there is an inherent difference within the innate immune response of these two cohorts, *i.e.*, their PBMCs function differently in response to TLR stimulation. This was clearly demonstrated in *non*-responders having significantly higher basal Th1 cytokine production compared to responders. Having higher Th1 but not Th2 cytokine production was also an indicator that the problem lays within the innate and not the adaptive immune response. By using both UC and CD patients in the initial experiments, it demonstrated that these intrinsic differences may be inherent in both UC and CD patients, and the ability to be able to target and treat patients based on their

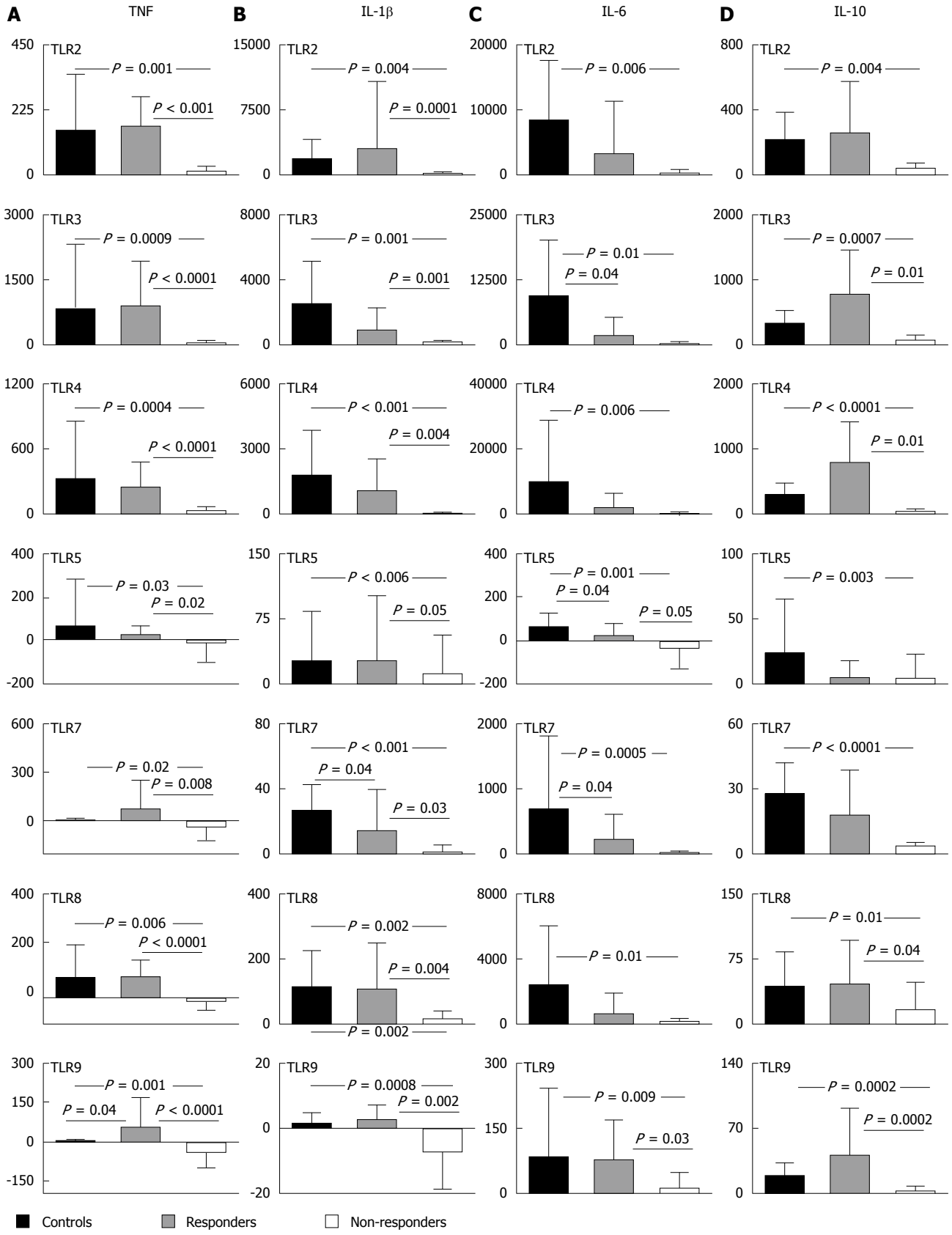


**Figure 3 Toll-like receptor-induced tumour necrosis factor, interleukin-1β, -6 and -10 in responders and non-responders compared to healthy controls.** The differences in basal and stimulated (A) TNF, (B) IL-1β, (C) IL-6 and (D) IL-10 production in PBMCs post TLR stimulation. Results were calculated and expressed as fold-change from baseline (± SD). The *P* values represent statistical significance of < 0.05 between the groups as denoted. PBMC: Peripheral blood mononuclear cells; TLR: Toll-like receptor; TNF: Tumor necrosis factor; IL: Interleukin.

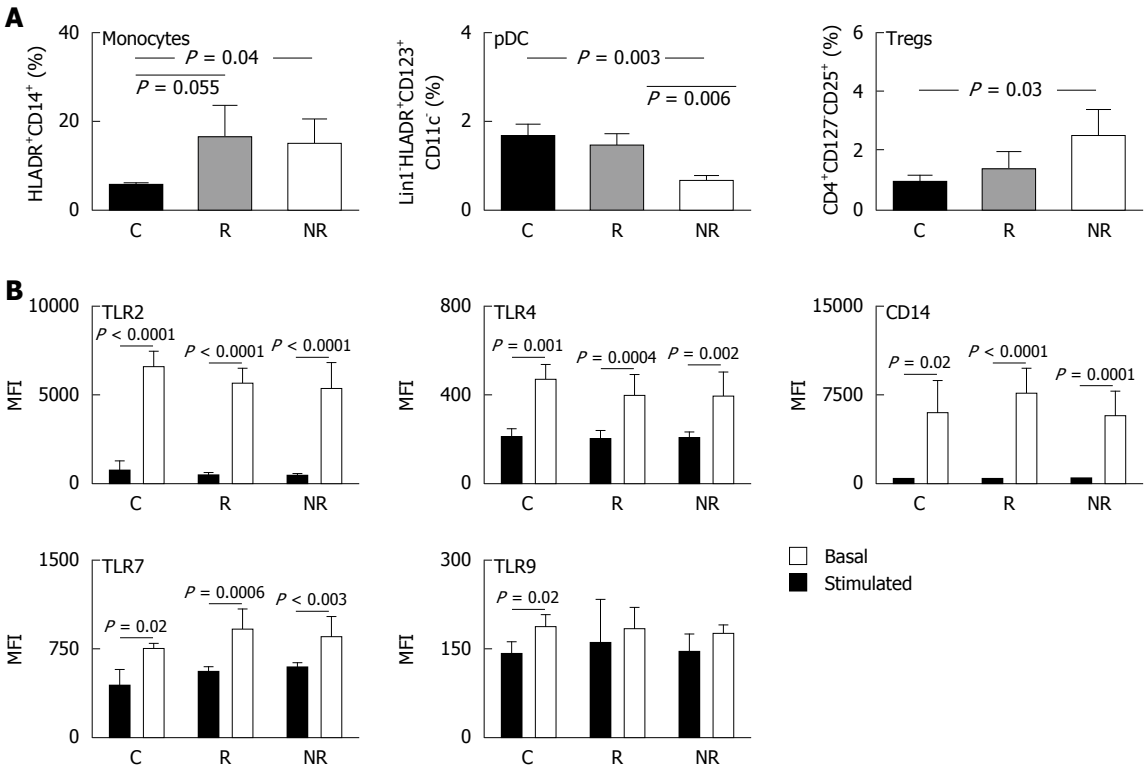


**Figure 4 Peripheral blood mononuclear cells phenotype and toll-like receptor /CD14 protein levels in responders and non-responders compared to healthy controls.** A: Percentage of monocytes, pDC and CD4+ regulatory T cells were determined from total population of PBMCs isolated from responders (R, *n* = 12), non-responders (NR, *n* = 12) and healthy controls (C, *n* = 12) by FACS analysis. Data are expressed as mean percentage (± SD) of total cell population; B: Basal (black columns) and stimulated (white columns) TLR2, TLR4, TLR7, TLR9 and CD14 protein levels in PBMCs isolated from C (*n* =12), R (*n* =12) and NR (*n* = 12) were assessed by surface and intracellular staining followed by FACS analysis. Data are expressed as mean fluorescence intensity (MFI ± SD). The *P* values represent statistical significance of < 0.05 between the groups as denoted. PBMC: Peripheral blood mononuclear cells; TLR: Toll-like receptor; pDC: Plasmacytoid dendritic cells.

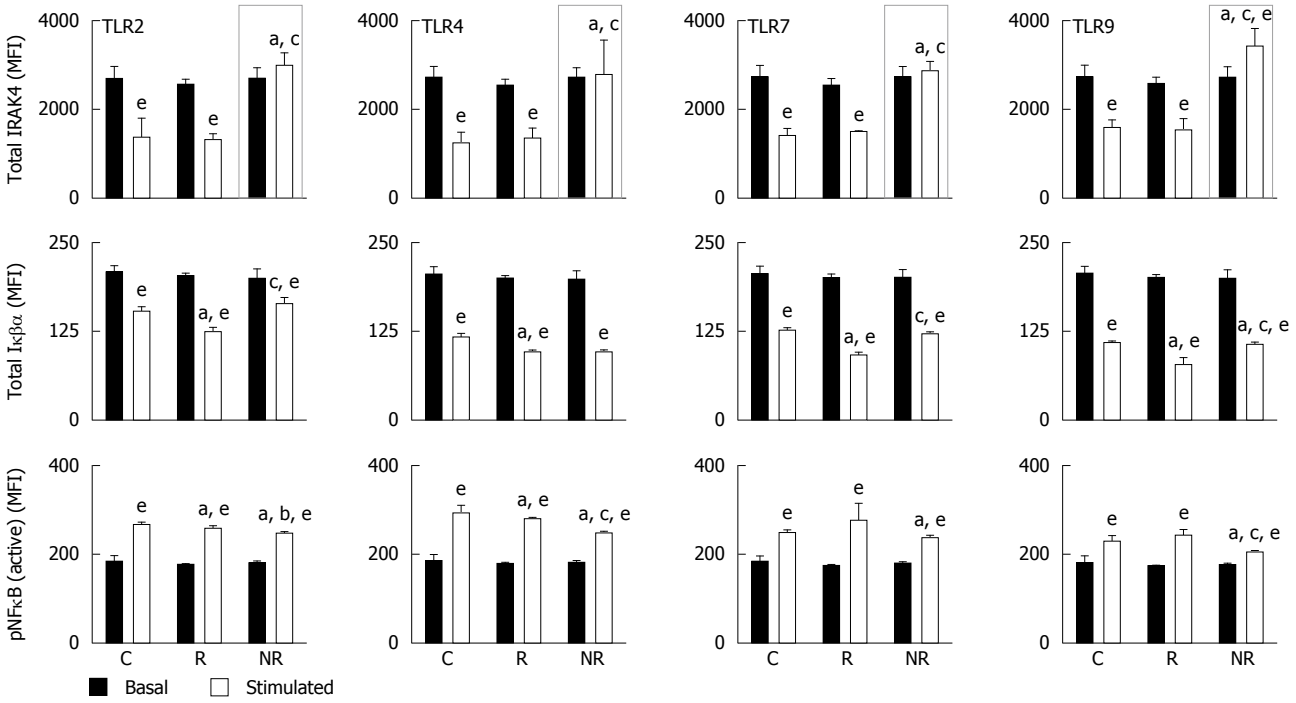


**Figure 5 Basal and stimulated toll-like receptor signalling pathways in responders and non-responders compared to healthy controls.** PBMCs isolated from responders (R, *n* = 12), non-responders (NR, *n* = 12) and healthy controls (C, *n* = 12) were stimulated with TLR2, TLR4, TLR7 or TLR9 agonists for 24-48 h prior to intracellular staining for total IRAK4 (top), total IκBα (middle) and phosphorylated (activated) NFκB (pNFκβ) (bottom) followed by FACS analysis. Data are expressed as MFI ± SD. [a]*P* < 0.05 *vs* stimulated healthy control C (white open bars); [c]*P* < 0.05 compared to stimulated responders (white open bars); [e]*P* < 0.05 compared to basal (black solid bars). Grey open boxes represent differences in total IRAK4 expression compared to responders and controls. PBMC: Peripheral blood mononuclear cells; TLR: Toll-like receptor; NFκB: Nuclear factor kappa B; IκBα: Inhibitor of NFκB.

innate immune response may be applied to both of the IBD cohorts.

When specifically looking at differences between the UC responders and non-responders, it was noted that the non-responders had a significantly higher constitutive or basal cytokine production than responders and controls, and smaller fold change in cytokine production upon TLR stimulation. High basal cytokine levels and the lack of fluctuation in cytokine production upon TLR stimulation suggests that the mechanisms involved in the negative regulation of TLR signalling may be impaired. Indeed, low levels of immunoregulatory cytokine IL-10 seen in non-responders compared to responders following TLR activation (Figure 3) may explain why inflammation cannot be controlled with anti-TNF therapy in these patients.

We demonstrated that non-responders had a significantly lower number of pDCs in their peripheral blood compared to responders and to healthy controls. Others have shown that UC and CD patients experience a significant drop in their peripheral pDC populations during acute inflammation and significant increase in numbers within the intestinal mucosa[19]. The decreased pDC frequency we see in non-responders may result from continuous migration of peripheral pDCs into the intestinal mucosa whereupon they mature, activate and contribute to gut inflammation, thus resulting in an elevated basal Th1 cytokine profile which is characteristic of this population. The maturational status of the peripheral pDCs in non-responders may also be of importance, as healthy individuals display an immature pDC phenotype which normally induces T cell unresponsiveness[20], whilst IBD patients have a lack of immature peripheral pDCs which would perpetuate inflammation[21]. Further investigation into the distribution of pDCs in the peripheral blood and the intestinal mucosa, and their maturational status in the UC subgroups is required. The increased frequency of $CD4^+$ Treg cells in non-responders could suggest a problem with Treg homing to the mesenteric lymph nodes and lamina propria to inhibit pathogenic T effector cells during inflammation *via* direct contact with $cD11c^+$ dendritic cells[22], thus leading to ongoing inflammation. Consistent with the increase in pro-inflammatory cytokines, monocyte frequency was significantly greater in both UC subgroups compared to controls, and no differences were observed in naïve, memory or $CD8^+$ effector or $CD8^+$ Treg cell frequency, which again supports the concept that the differences in immunologic responses between the UC subgroups lie within the innate immune system.

Downstream of the TLRs, we saw accumulation of total IRAK4 in non-responders upon stimulation, particularly following TLR-9 activation. IRAK4 is a key signalling component in the innate immune response[23] and IRAK4 deficiencies have been implicated in IBD[24]. We know that IBD patients who do not respond to anti-TNF therapy maintain an increased expression of pro-inflammatory cytokines[25]. In our *non-responder* population, this is associated with IRAK4 accumulation and we may speculate such accumulation may lead to prolonged activation of the signalling pathway resulting in sustained and excessive pro-inflammatory cytokine production seen in UC patients. Our signalling data shows non-responders to have normal IRAK4 kinase activity however, other mechanisms which may contribute to its accumulation such as defects in IRAK4 degradation or inhibition remains to be tested. It's been previously shown that IL-10 can induce IRAK4 ubiquitination and proteasomal degradation[26,27]. Our results support reduced ability of non-responders to induce IRAK4 ubiquitination due to their reduced capacity to produce IL-10 following TLR stimulation. Alternatively, IRAK4 activity is inhibited by cleavage into its inactive form[26]. Cleavage occurs by an NFκB-induced protease resulting in a smaller molecular weight protein (32 kDa) that can also be recognised by anti-IRAK4 antibodies[26]. As IRAK1 phosphorylation and NFκB activation precedes IRAK4 cleavage, this suggests that this may be part of a negative feedback inhibition loop[26].

There is no doubt that anti-TNF therapy can be effective in UC but only in some patients. The ability to predict patient's response to anti-TNF therapy would allow for more targeted therapy with better cost-effectiveness. Here we provide evidence which suggests that heterogeneity in the innate immune function between UC patients may give us an important insight into their subsequent responses to future anti-TNF therapy. This would be particularly beneficial for the patient with acute severe colitis requiring rescue therapy when a choice must be made between cyclosporine or anti-TNF therapy. It is important to acknowledge that one of the potential limitations of this study is the relatively small and a mixed population of patients (CD and UC) used. Moreover, it is known that pathogenesis of CD and UC are different and disease can be more severe in the elderly[28]. In our cohort, results could not be explained by differences in age between groups and our functional innate differences between responders and non-responders have been performed in UC patients only. Our data offers promise for serological measure of innate immune function in UC patients as a potential application in clinic to predict response to costly anti-TNF therapy. These data remain to be confirmed in a larger cohort of not only UC but also CD patients. With better prediction of the response to therapy, targeted patient treatment may be possible in the future, resulting in improved efficacy and cost-effectiveness of treatment for all IBD patients.

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## COMMENTS

### Background

Monoclonal antibodies against tumour necrosis factor [anti-tumour necrosis factor (TNF) therapy] can be used to treat patients with ulcerative colitis (UC) who are no longer responding to corticosteroids. Anti-TNF treatment is expensive and 30%-40% of patients do not respond.

### Research frontiers

Unravelling the mechanisms involved in lack of response to anti-TNF is paramount for prediction of response to treatment.

### Innovations and breakthroughs

Here the authors show, for the first time, that differences in innate immune function exist between UC patients who respond to anti-TNF therapy and those that don't. Both quantitative (difference in presence of inflammatory cells in their peripheral blood) and qualitative (production of cytokines and signalling capacity following activation of innate immune pathways) differences exist between responders and non-responders.

### Applications

Measurement of innate immune function in the blood of UC patients (their response to TLR agonists) may be a useful tool in predicting patient's response to anti-TNF treatment. With improved prediction of the response to therapy, targeted and individualised patient treatment may be possible in future, resulting in improved efficacy and cost-effectiveness.

### Terminology

Innate immune function is measured by cellular response to toll-like receptor (TLR) stimulation. To understand which TLRs are implicated in lack of response to anti-TNF treatment, we examined peripheral blood mononuclear cells responses to a wide range of TLR agonists and closely examined the TLR signalling pathway molecules.

### Peer-review

In the presented article the authors aimed to predict anti-TNF response in IBD patients by means of alterations in immune functions. There are two parts of the study. The effects of disease, treatment and inflammation on innate immunity were evaluated in 18 patients. In the second part, the differences between responders and non-responders were evaluated in 24 patients. The study adds new knowledge to the current literature.

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ORIGINAL ARTICLE

Basic Study

# Altered pattern of tumor necrosis factor-alpha production in peripheral blood monocytes from Crohn's disease

Claudia Loganes, Alessia Pin, Samuele Naviglio, Martina Girardelli, Anna Monica Bianco, Stefano Martelossi, Alberto Tommasini, Elisa Piscianz

Claudia Loganes, Samuele Naviglio, Elisa Piscianz, Department of Medicine, Surgery and Health Sciences, University of Trieste, 34128 Trieste, Italy

Alessia Pin, Martina Girardelli, Anna Monica Bianco, Stefano Martelossi, Alberto Tommasini, Department of Pediatrics, Institute for Maternal and Child Health, IRCCS "Burlo Garofolo", 34137 Trieste, Italy

Author contributions: Loganes C, Martelossi S and Tommasini A conceived and designed the research; Loganes C, Pin A and Girardelli M performed experiments for data acquisition and performed the statistical analysis; Bianco AM and Piscianz E analyzed the data; Naviglio S and Martelossi S enrolled the patients and provided patient samples; Loganes C, Tommasini A and Piscianz E wrote the paper; Bianco AM and Martelossi S revised the manuscript critically for important intellectual content; all authors read and approved the final version of this manuscript.

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Data sharing statement: Dataset available from the corresponding author at claudia.loganes@gmail.com.



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Correspondence to: Claudia Loganes, BSc, Department of Medicine, Surgery and Health Sciences, University of Trieste, Strada di Fiume 447, 34128 Trieste, Italy. claudia.loganes@gmail.com
Telephone: +39-040-3785422

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## Abstract

### *AIM*

To evaluate the inflammatory state in Crohn's disease (CD) patients and correlate it with genetic background and microbial spreading.

### *METHODS*

By means of flow cytometry, production of tumor necrosis factor-alpha (TNF-α) was measured in peripheral blood monocytes from patients suffering from CD, ulcerative colitis (UC) and in healthy subjects after stimulation of the NOD2 and TLR pathways. CD patients were genotyped for the three most common *NOD2* variants (R702W, G908R and L1007Pfs*2) and basal production of TNF-α was correlated to *NOD2* genotype. Also, production of TNF-α was correlated to plasmatic levels of LPS Binding Protein (LBP), soluble (s) CD14 and to the activity state of the disease.

### *RESULTS*

The patients with CD were characterized by a significantly higher monocyte basal expression of TNF-α

compared with healthy subjects and UC patients, and after stimulation with $Pam_3CSK_4$ (ligand of TLR2/1) and MDP-L18 (ligand of NOD2) this difference was maintained, while other microbial stimuli (LPS, ligand of TLR4 and PolyI:C, ligand of TLR3) induced massive activation in CD monocytes as well as in UC and in healthy control cells. There was no significant difference in the production of TNF-α between patients who carried CD-associated heterozygous or homozygous variants in *NOD2* and patients with wild type *NOD2* genotype. Although serum LBP levels have been shown to correlate positively with the state of activity of the disease, TNF-α production did not show a clear correlation with either LBP or sCD14 levels in plasma. Moreover, no clear correlation was seen between TNF-α production and activity indices in either CD or UC.

***CONCLUSION***

Peripheral monocytes from CD express higher basal and stimulated TNF-α than controls, regardless of *NOD2* genotype and without a clear correlation with disease activity.

**Key words:** Crohn's disease; Ulcerative colitis; Tumor necrosis factor-α; *NOD2* variants; Toll like receptors; Dysbiosis; Activity index; LPS-binding protein



**Core tip:** Crohn's disease (CD) is characterized by an aberrant activation of the mucosal immune system in genetically susceptible subjects, who often harbor variants in genes involved in the innate immunity. To study the integrity of innate immune response, the activity of the TLR and NOD2 pathways was investigated, measuring TNF-α expression in peripheral blood monocytes. CD monocytes showed a higher production of TNF-α, which was not clearly related to disease activity, to *NOD2* genotype or to the presence of translocated bacteria (indirectly measured by serum LPS-binding protein), indicating that this TNF-α hyper-production may rely on a NOD2-independent pathway and is not due to systemic exposure to LPS.

Loganes C, Pin A, Naviglio S, Girardelli M, Bianco AM, Martelossi S, Tommasini A, Piscianz E. Altered pattern of tumor necrosis factor-alpha production in peripheral blood monocytes from Crohn's disease. *World J Gastroenterol* 2016; 22(41): 9117-9126 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i41/9117.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i41.9117

## INTRODUCTION

Inflammatory bowel diseases (IBD) are complex inflammatory conditions that include different chronic and relapsing intestinal diseases, such as Crohn's disease (CD) and ulcerative colitis (UC). Both genetic and environmental factors are thought to play a role in determining the disease phenotype[1,2].

On the one hand, CD and UC both involve intestinal mucosa and share some clinical symptoms, *i.e.*, bloody diarrhoea, abdominal pain and weight loss. On the other hand, they present peculiar clinical and pathogenic features. Indeed, major differences between the two pathologies are found in the localization of lesions: while CD interests the small intestine (ileum) in particular, UC manifestations are localized mainly in the colon and rectum. Moreover, CD intestinal lesions, differently from UC, cause transmural inflammation and are characterized by skip lesions, alternating inflamed and not inflamed regions[3].

Diagnosis is based on clinical symptoms and medical history of the patients and then confirmed by endoscopic evaluations and histology. The classification of the disease, which is useful for prognosis and management of patients, is based on index of activity. In particular, the reference scores for pediatric patients is the Pediatric Crohn's disease Activity Index (PCDAI)[4] and the Pediatric Ulcerative Colitis Activity Index (PUCAI)[5,6].

The immunopathogenesis underlying CD involves immune cells, such as monocytes, macrophages, neutrophils, recruited and activated *via* inflammatory cytokines, *i.e.*, interleukin 6, interleukin 10, Interferon-gamma and Tumor Necrosis Factor-alpha (TNF-α) as well as commensal microbiota[7]. In contrast, lymphocytic immune dysregulation seems to play an important role in UC, as shown by the increase of T cells activation markers and cytokines. Several gene variants are associated with the activity of the T cells and with the control of mucosal inflammation[8].

Although the etiopathology of CD is still unclear, several studies have linked the onset of the disease with an imbalance between mucosal immune system and microbiota[9,10]. For this reason, it has been suggested that CD inflammation may represent the compensatory response to a variety of minor defects in mucosal innate immunity. These deficiencies may account for changes in the intestinal microbiota (dysbiosis), which, together with multifactorial damages of the intestinal barrier, may facilitate bacterial translocation to the *lamina propria* and engagement of inflammatory cells. Microbial components from translocated bacteria can reach mesenteric lymph nodes and the bloodstream, making it possible to measure lipopolysaccharide (LPS), LPS-binding Protein (LBP) and soluble (s)CD14, as markers of bacterial spread across the intestinal mucosa[11]. Moreover, the circulating microbial components can induce excessive stimulation of toll like receptors (TLRs) followed by exacerbated activation of the immune system. It has been proposed that genetic variants in nucleotide oligomerization domain 2 (*NOD2*), the

first gene associated with IBD[12-14], may contribute to increasing the risk of disease by interfering with the response of monocytes to bacterial compounds. NOD2 is a cytoplasmic receptor that specifically recognizes muramyl dipeptide (MDP) present on the bacterial wall. It is involved in the regulation of acute inflammation, secreting pro-inflammatory cytokines such as TNF-α[15]. However, despite the huge amount of researches on this topic, it is still not clear how CD-associated *NOD2* variants may contribute to the pathogenesis of the disease. On the one hand, dysfunctional pathogen associated molecular patterns (PAMPs) sensing in CD, involving NOD2 and other TLR pathways, could lead to altered shaping of gut microbial species, or dysbiosis[16-18]. On the other hand, NOD2 deficiency could lead to impaired autophagy and inflammation, with excessive response to TLR stimulation[19].

All this considered, a first aim of this study was to evaluate NOD2 and TLR signaling pathway integrity in CD. Immune activation was evaluated in terms of production of TNF-α by peripheral monocytes, since the NOD2 pathway is particularly important in these cells. Moreover peripheral blood monocytes are good representatives of the innate immune system and can easily be obtained from patients during blood sampling performed for clinical purposes.

CD monocytes were activated with different purified microbial compounds [MDP-L18 (NOD2 ligand), LPS (TLR4 ligand), $Pam_3CSK_4$ (TLR2/1 ligand), Poly I:C (TLR3 ligand)]. Production of TNF-α was then evaluated by flow cytometry in comparison with samples from UC and healthy donors. Intracellular production of TNF-α was correlated with the activity state of the disease, with the genotype of the patients and with the levels of plasmatic LBP and sCD14.

As expected, CD patients displayed higher monocyte TNF-α production in basal condition and after stimulation of NOD2 and TLR2/1. Conversely, stimulation of TLR3 and TLR4 induced similar TNF-α production among CD, UC and healthy donors, suggesting that in CD, monocytes might be primed by mycobacterial components that can induce reduced tolerance to PAMPs. Neither the variants in *NOD2* susceptibility gene nor the state of activity of the disease (PCDAI score) correlated with intracellular TNF-α. Moreover, the levels of plasmatic LPB, which have been shown to correlate positively with the activity of the disease, did not exhibit a clear correlation with monocyte activation.

## MATERIALS AND METHODS

### *Patient recruitment*

Monocytes were obtained from heparinized blood of 38 CD patients, 31 UC patients (diseased controls) and 50 healthy donors (HD; negative controls) subsequent to receiving written consent. All subjects were recruited from the Gastroenterology and Clinical Nutrition Unit of the Institute for Maternal and Child Health IRCCS "Burlo Garofolo", Trieste, Italy. The IBD patients were classified according to the disease activity evaluated by physician global assessment (based on PCDAI and PUCAI scores).

The present study was approved by the Institutional Review Board of the Institute for Maternal and Child Health "Burlo Garofolo" (RC 03/2009).

### *Intracellular cytokine staining*

This cytometric technique allows for the detection of intracellular TNF-α in monocytes stimulated with synthetic microbial stimuli, to investigate the functionality of the innate immune pathway. The protocol was adapted from Takada *et al*[20].

Briefly, Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole heparinized blood by centrifugation on Ficoll separating solution (Lympholite, Cederlane, Burlington, NC, United States) at 500 × *g* for 30 min at room temperature. 2 × $10^5$ PBMCs were resuspended in 200 μL of culture medium (RPMI, EuroClone, Milano, Italy) supplemented with 10% human AB serum (Sigma Aldrich, Milano, Italy), 2 mmol/L L-glutamine (EuroClone), 100 U/mL penicillin (EuroClone) and 0.1 mg/mL streptomycin (EuroClone) and stimulated with TLR and NOD2 ligands: 100 ng/ml Lipopolysaccharide (LPS; TLR4 ligand, Sigma Aldrich), 10 μg/mL Polyinosinic-polycytidylic acid sodium salt (PolyI:C; TLR3 ligand, Sigma Aldrich), 500 ng/mL L-18 Muramyl DiPeptide (MDP-L18; NOD2 ligand, InvivoGen, San Diego, CA, United States), 500 ng/mL $Pam_3CSK_4$ (TLR2/1 ligand, InvivoGen) for an initial period of 30 min at 37 ℃, in a $CO_2$ incubator. Subsequently, 10 μg/mL of Brefeldin A (BFA, Sigma Aldrich) were added to inhibit the secretion of newly synthesized cytokines and cells were incubated for additional 3.5 h. BFA, without any stimulus, was added to the unstimulated control tube. After incubation, FITC conjugated anti-CD14 antibody (eBiosciences, San Diego, CA, United States) was added for surface staining to identify monocytes, followed by a fixation step with FACS Lysing Solution (BD Biosciences, San Jose, CA, United States). After centrifugation at 300 × *g* for 8 min, cells were permeabilized using FACS Permeabilizing Solution 2 (BD Biosciences). PBMCs were washed with Wash Buffer (PBS + 1% BSA + 0.1% $NaN_3$), and anti-TNF-α PE antibody (BD Biosciences) was added to perform the intracellular staining. A fluorescent-conjugated isotype control antibody was used to detect non-specific bindings (PE IgG Isotype control, BD Biosciences). Finally, after an additional wash, the cells were fixed with PBS + 1% paraformaldehyde for analysis on a flow cytometer. Data were acquired on a CyAn ADP flow cytometer (Beckman Coulter, Fort Collins, CO, United States) and analyzed using FlowJo software v 7.6 (TreeStar, Ashland, OR, United States). Results are expressed as percent of TNF-α positive monocytes, after gating on CD14 positive cells.

**Table 1 Characteristics of Inflammatory bowel diseases patients included in the study**

| Patients ($n$ = 69) | Age (yr) (mean ± SD) | Male/female | Active/remission |
|---|---|---|---|
| CD ($n$ = 38) | 13 ± 4.47 | 25/13 | 18/20 |
| UC ($n$ = 31) | 13 ± 5.13 | 15/16 | 19/12 |

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

### *NOD2 variant analysis*

CD patients were screened using targeted gene sequencing to identify the three principal variants in *NOD2* susceptibility gene [c.2104C>T;p.R702W (rs2066844), c.2722G>C;p.G908R (rs2066845) and c.3016_3017insC; p.L1007Pfs*2 (rs2066847)].

DNA samples were collected from patients suffering from CD ($n$ = 36). Genomic DNA of each patient was extracted from 1-2 ml EDTA-anticoagulated blood using the EZ1 DNA Blood Kit (QIAGEN, Valencia, CA, United States) according to the manufacturer's instructions. To analyze the three IBD associated variants in the *NOD2* gene (NM_022162), Polymerase chain reaction (PCR) amplification was performed using the KAPA 2G Fast Hot Start Readymix (RESNOVA, Rome, Italy). Purified PCR products were directly sequenced in both directions using the amplification primers by the ABI PRISM 3130XL automated DNA Sequencer (Applied Biosystems, United States). Sequences were analyzed using Seqman II Software (DNASTAR I Lasergene 7.0, Madison, WI, United States). Primers, used for PCR and sequencing were designed using Primer Blast, based on the relative sequence deposited in GeneBank. The specific primers for each variant are listed below:

R702W_For 5'-CTTCAACCTTCTGCAGGGC-3'
R702W_Rev 5'- GGTGGCAGAGGCGAAGCT-3'
R702W_sequencing_For 5'-TGCTGATGTGCCACCAG-3'
G908R_For 5'-CTGCCCCTCTGGCTGGGACT-3'
G908R_Rev 5'-CCCAGCTCCTCCCTCTTC-3'
L1007Pfs*2_For 5'-GTAGACTGGCTAACTCCTGC-3'
L1007Pfs*2_Rev 5'-AGGAGGGCGGGAGCTGACTT-3

### *LPS-Binding Protein and soluble CD14 quantification*

Samples were collected from patients suffering from CD ($n$ = 27), UC ($n$ = 22) and from healthy individuals ($n$ = 36). Plasma samples were obtained by centrifuging heparinized blood at 1300 × $g$ for 10 min and were stored at -80 ℃ until LBP and sCD14 levels were measured.

Quantifications were performed using the LPS-binding Protein (Human) ELISA kit (Abnova, Taipei City, Taiwan) and the human CD14 ELISA kit (RayBio, Norcross GA, United States), following the manufacturer's instructions.

Absorptions were measured at 450 nm with a GloMax®-Multi+ Microplate Multimode Reader (Promega Corporation, Madison, United States). The concentration of each sample was calculated from the standard curve, obtained by plotting absorbance *vs* known standard concentrations.

### *Statistical analysis*

All statistical analyses were performed using GraphPad Prism software version 5 (GraphPad, SanDiego, United States).

Data on intracellular TNF-α production are expressed as mean ± standard error of mean (SEM), while values of plasmatic proteins are described as medians and interquartile ranges and are represented with box plots using Tukey's whiskers.

Statistical significance was assessed by one-way analysis of variance (ANOVA) with Kruskal-Wallis non-parametric test and denoted by letters ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$).

## RESULTS

The clinical characteristics of all the patients included in the study are presented in Supplementary Table 1 and summarized in Table 1.

### *Monocyte intracellular TNF-α expression*

To study the production of TNF-α by monocytes, cells were isolated from peripheral blood of patients with CD and UC and of healthy donors (HD). Monocytes were stimulated with TLR and NOD2 purified stimuli: LPS (TLR4 ligand), $Pam_3CSK_4$ (TLR2/1 ligand), Poly I:C (TLR3 ligand) and MDP-L18 (NOD2 ligand). TNF-α expression in monocytes was analyzed by flow cytometry after gating on CD14 positive cells.

Both CD and UC showed increased basal expression of TNF-α (unstimulated) compared to healthy donors (HD 4.3 ± 0.4; CD 14.5 ± 2.5, $P < 0.01$; UC 10.7 ± 1.9, $P < 0.05$) (Figure 1A).

After stimulation with $Pam_3CSK_4$ (TLR2/1 ligand), a higher percentage of activated monocytes producing TNF-α was recorded in CD compared with HD (CD *vs* HD: 68.4 ± 2.7 *vs* 55.6 ± 2.1, $P < 0.01$) (Figure 1B), while stimulation with MDP-L18 (NOD2 ligand) induced a significant higher production of TNF-α in CD compared to both HD and UC (CD 44.2 ± 3.0; UC 30.2 ± 3.0, $P < 0.01$; HD 24.9 ± 1.4, $P < 0.001$) (Figure 1C).

Stimulation with LPS (TLR4 ligand) or Poly I:C (TLR3 ligand) did not lead to significant differences among the three groups (respectively Figure 1D and Figure 1E).

### *TNF-α expression and NOD2 genotype*

A genetic survey was carried out on 36 available patients with CD (out of 38). PCR and DNA sequencing analyses revealed that 13 patients were homozygous or heterozygous for one or more of the three CD-associated genetic variants in *NOD2* (Table 2). The remaining 23 patients were found to be wild type for the three polymorphisms (Supplementary Table 1).

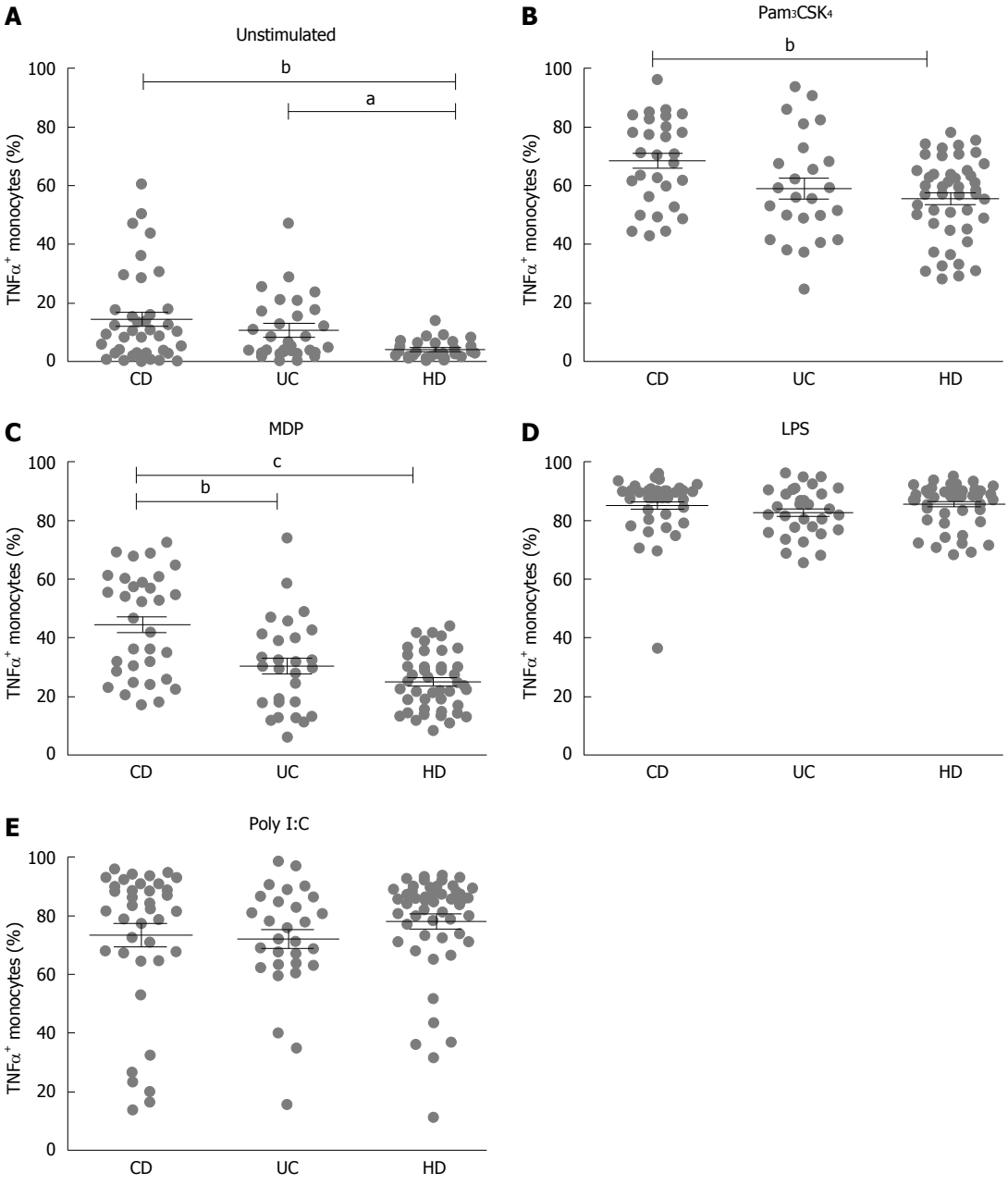


**Figure 1 Intracellular tumor necrosis factor-alpha expression in peripheral monocytes.** Intracellular TNF-α expression in peripheral monocytes derived from patients affected by Crohn's disease (CD), ulcerative colitis (UC) and healthy donors (HD), in basal condition (A), or after stimulation with $Pam_3CSK_4$ (B), MDP-L18 (C), LPS (D) or Poly I:C (E). Data are presented as mean ± SEM. Statistical significance is denoted by letters ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ *vs* HD).

After grouping CD patients based on *NOD2* genotype, no significant differences in TNF-α positive monocytes were detected between patients with wild type or mutated genotype (CD WT: 14.87 ± 3.1 *vs* CD Mut 13.82 ± 4.5) (Figure 2). Data regarding the single *NOD2* variants are shown in Supplementary Figure 1.

### *TNF-α expression and disease activity index*

Intracellular production of TNF-α by monocytes from CD and UC patients was correlated to the disease activity score, expressed as PCDAI (Pediatric Crohn's Disease Activity Index) or PUCAI (Pediatric Ulcerative Colitis Activity Index). No correlation between the two parameters was found, either in CD ($R^2 = 0.004$) or in UC ($R^2 = 0.01$) subjects (Figure 3).

### *TNF-α expression and LBP and sCD14 levels*

Plasmatic levels of LBP and sCD14 were measured to verify if the basal inflammatory state in CD patients could be due to exposition of monocytes to microbial components in peripheral blood.

Levels of LBP were higher in plasma from patients with CD compared to HD [(median CD 19.11 (16.35-21.89), HD 15.01 (11.85-18.84), $P < 0.05$)] (Figure 4A) and this condition was noticed also when patients were grouped by disease activity [(median activeCD: 20.44 (16.81-26.31) *vs* HD: 15.01 (11.85-18.84); $P < 0.05$)] (Figure 4B); furthermore a significant difference was also found

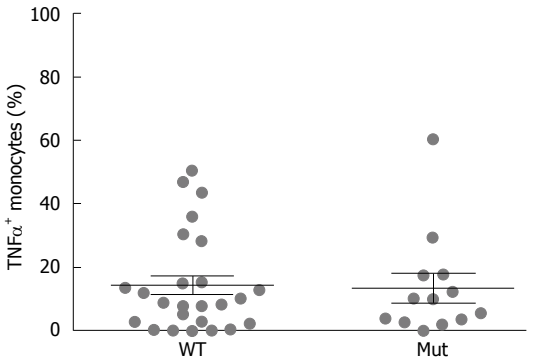


**Figure 2 Intracellular tumor necrosis factor-alpha expression in wild type or mutation-carrier patients.** Intracellular TNF-α expression in basal conditions measured in peripheral monocytes derived from patients affected by Crohn's disease (CD), grouped into Wild Type (WT) or mutation carriers (Mut) for the three *NOD2* polymorphisms (R702W, G908R and L1007Pfs*2). Data are presented as mean ± SEM. No statistical significances was found.

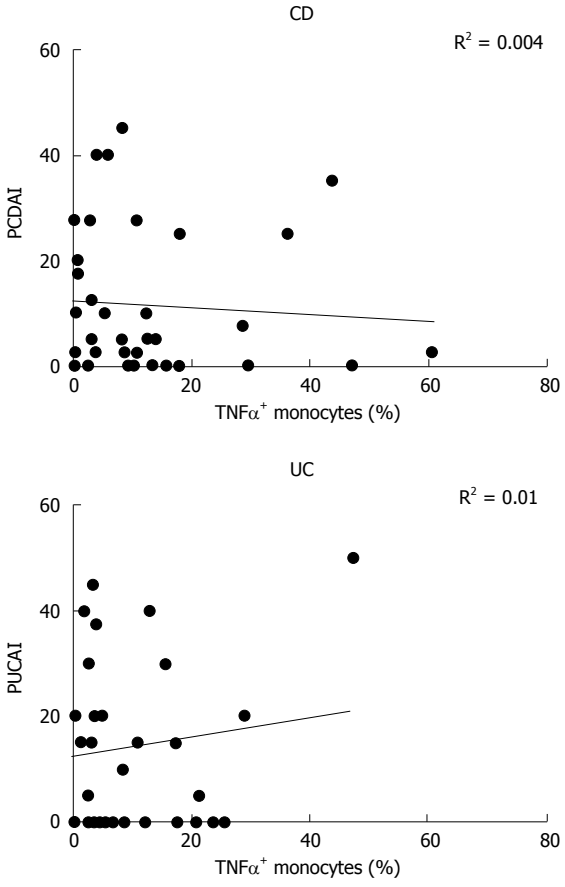


**Figure 3 Correlation of disease activity index with monocyte expression of tumor necrosis factor-alpha in inflammatory bowel disease patients.** Correlation of activity index, expressed by PCDAI (Pediatric Crohn's disease Activity Index) or PUCAI (Pediatric Ulcerative Colitis Activity Index) with monocyte expression of TNF-α in patients suffering from Crohn's disease (CD) and ulcerative colitis (UC). The squared Pearson correlation coefficients ($R^2$) are shown.

between UC in active or remissive phase [(median UC: 20.74 (14.55- 21.57) *vs* rUC: 12.35 (8.35-13.5); $P < 0.05$)] (Figure 4B).

LBP values displayed a significant correlation with the disease activity index, especially for CD ($R^2 = 0.40$, $P < 0.001$) (Figure 4C and D). However, there was no correlation between monocyte TNF-α expression in basal condition and plasmatic levels of LBP in any of the analyzed groups (CD: $R^2 = 0.04$, UC: $R^2 = 0.0004$, HD: $R^2 = 0.005$) (Figure 5).

The quantification of sCD14 was not significantly different among the three groups and did not correlate with the degree of monocyte activation in CD (data not shown).

**Table 2 *NOD2* genotype analysis in Crohn's disease patients**

| CD patient | *NOD2* genotypes | | |
|---|---|---|---|
| | Ex. 4 C>T R702W | Ex. 8 G>C G908R | Ex. 11 InsC L1007Pfs*2 |
| 3 | C/C | G/G | InsC/- |
| 4 | T/T | G/G | -/- |
| 7 | C/T | G/G | InsC/- |
| 14 | C/C | G/C | -/- |
| 18 | C/C | G/C | InsC/- |
| 24 | C/T | G/G | -/- |
| 25 | C/C | G/C | -/- |
| 44 | C/C | G/G | InsC/- |
| 45 | C/C | G/G | InsC/- |
| 47 | C/C | G/C | InsC/- |
| 53 | C/C | G/G | InsC/- |
| 54 | C/C | G/C | -/- |
| 63 | C/C | G/G | InsC/- |

Details of the three *NOD2* polymorphisms (R702W, G908R, L1007Pfs*2) that were found in heterozygous or in homozygous form in 13 of the CD patients. The 23 patients who were found to be wild type are not listed in the table.

## DISCUSSION

The activation of the innate immune system is thought to play a pivotal role in the pathogenesis of CD. Most genetic variants associated with the disease involve genes that are expressed in monocytes and that contribute to functional innate response to microbes. For example, *NOD2* and *ATG16L1* are involved in the response to molecular patterns associated with pathogens (PAMPs) and in autophagy, a process that can amplify the inflammatory response to phagocytized materials. Notably, there is a number of genes and processes involved in phagocytic function and innate immunity that have been shown to be responsible for rare monogenic forms of Crohn's-like intestinal inflammation. For instance, early onset CD-like inflammatory bowel disease has been described in neutropenias and in functional deficiencies of phagocytes, such as chronic granulomatous disease and leukocyte adhesion deficiency, as well as in defects of other genes interacting with the NOD2 pathway of response to PAMPs, such as XIAP[8,21]. These findings have raised the question of whether CD should be considered more like an immunodeficiency than an autoinflammatory condition[22-24].

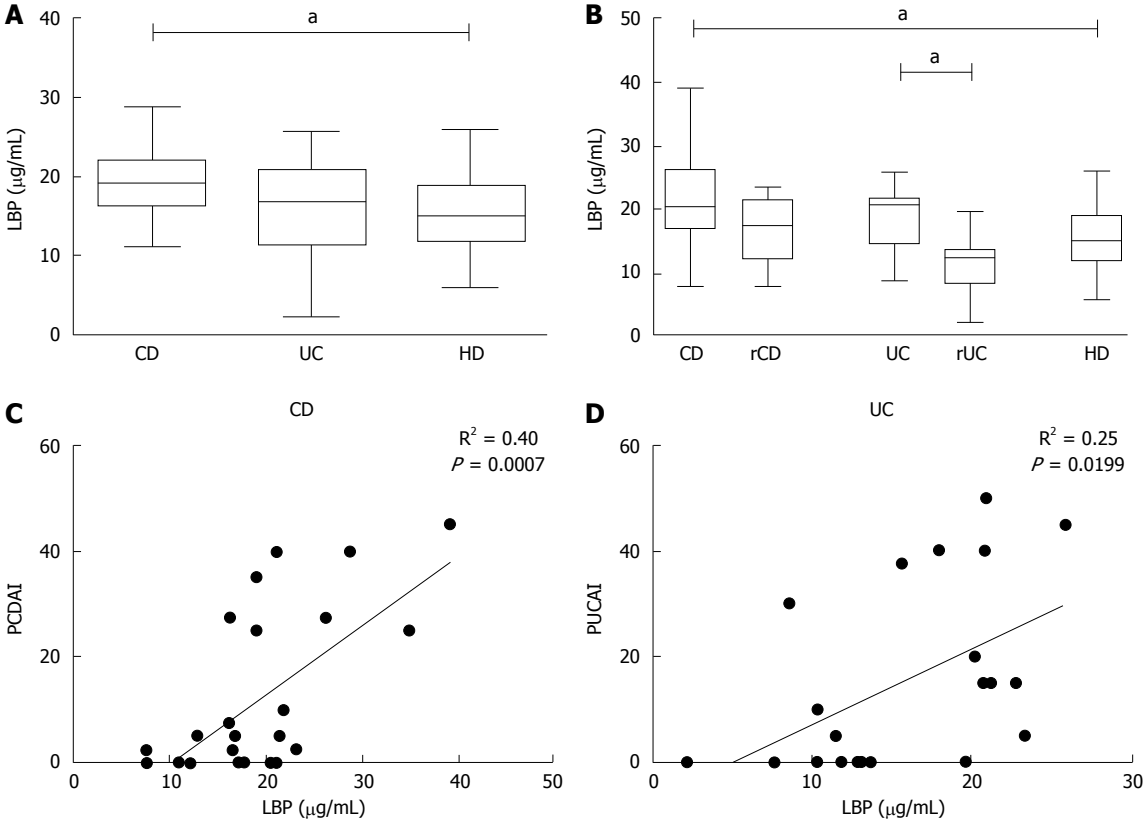


**Figure 4 Dosage of LPS-binding protein and correlation with disease activity index.** A, B: Quantification of plasmatic LPS-Binding Protein (LBP) from patients affected by active Crohn's disease (CD), remissive Crohn's disease (rCD), active Ulcerative Colitis (UC), remissive Ulcerative Colitis (rUC) and healthy donors (HD). Data are represented with box plots using Tukey's whiskers. Statistical significance is denoted by letters ($^aP < 0.05$); C, D: Correlation between activity Index, expressed by PCDAI (Pediatric Crohn's Disease Activity Index) or PUCAI (Pediatric Ulcerative Colitis Activity Index) with plasmatic level of LBP in patients with Crohn's disease (CD) (C, $R^2 = 0.40$) or Ulcerative Colitis (UC) (D, $R^2 = 0.25$). The squared Pearson correlation coefficients ($R^2$) and the *P* value are shown.

For this reason, several studies have investigated the function of peripheral blood monocytes, an easily available sample cell that can be, at least in part, representative of the innate immune profile of CD. The observation of impaired monocyte/macrophage function against yeast particles dates back decades[25]. However, it is still not clear whether these defects are a cause or a consequence of CD inflammation. For example, it has been hypothesized that CD-associated monocytes may display hyporesponsiveness because of massive mucosal stimulation[26]. Indeed, we and other groups have demonstrated that peripheral blood monocytes from CD show an increased expression of activation markers[27-29], whilst *ex-vivo* maturation of dendritic cells may be somehow impaired[30]. Overall, these results have highlighted the paradox of defective and excessive immunities in CD[31]: CD monocytes display increased activation and production of inflammatory cytokines, but they may present functional defects in terms of capacity to mature effectively and to clear bacteria efficiently[32]. Indeed, the depletion of monocytes could improve the response to anti-inflammatory treatments in CD[33]. Thus, the contribution of monocytes to the pathogenesis of CD seems to be quite complex, involving hyper-activation and defective functionality at the same time.

Beynon *et al*[34] demonstrated that in spite of increased basal activation, CD-associated monocytes display reduced response to MDP stimulation, in a manner that greatly depends on *NOD2* genotype. The effect of *NOD2* variants on the activation of monocytes has been confirmed by other studies showing that homozygous mutations can almost abrogate TNF-α production after MDP stimulation[35]. However, NOD2 defects can also impair MDP-induced tolerance, resulting in sustained monocyte activation and TNF-α production[36].

The aim of our research was the analysis of the integrity of the different pathways of the innate immune network. By means of a cytometric protocol, adapted from a clinically oriented technique already used for the screening of some primary immunodeficiency[20], we analysed the production of TNF-α triggered in monocytes by different stimuli, to depict possible alterations in different pathways of innate immunity to PAMPs.

We demonstrated that peripheral monocytes from CD show significant basal production of TNF-α and that this is not clearly related to either the disease activity or the *NOD2* genotype, supporting the idea that, in these cases, hyper-production of TNF-α relies on NOD2-independent pathways. Indeed, stimulation with

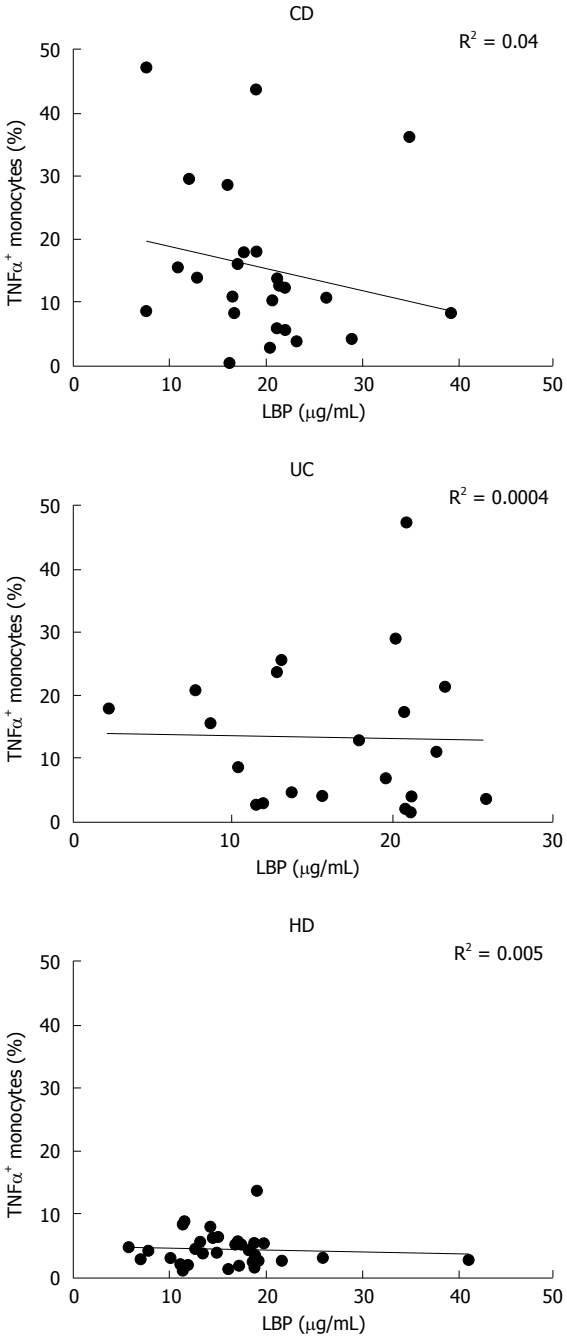


**Figure 5 Correlation of monocyte tumor necrosis factor-alpha expression and LPS-binding protein levels in inflammatory bowel disease patients.** Correlation between monocyte expression of tumor necrosis factor-alpha (TNF-α) and plasmatic level of LBP in patients affected by Crohn's disease (CD) or ulcerative colitis (UC) and in healthy subjects (HD). The squared Pearson correlation coefficients ($R^2$) are shown. No statistical significances were found.

MDP or with $Pam_3CSK_4$ led to higher TNF-α production in CD patients than in controls. The stronger response in CD after the stimulation with the NOD2 ligand did not find a clear explanation, but it highlights the importance of NOD2 signalling in CD and not in UC. This result is coherent with the knowledge that NOD2 is the main genetic factor associated with CD, even though the mechanisms underlying the pathogenesis of the disease remain controversial.

Thus, the activation of monocytes resulting from the mucosal immunopathology of CD could play a greater role in inducing TNF-α than the *NOD2* genotype, not only in the intestinal mucosa but also in peripheral blood. A possible hypothesis is that excessive activation of monocytes occurs in response to bacterial translocation across the epithelium and to minor defects in bacterial clearance or in MDP-induced tolerance. Notably, the paradoxical efficacy of GM-CSF in some cases of CD may be due to the correction of such defects in epithelial monocytes, for example by rescuing their epithelial repair function[37]. Another critical function for mucosal monocytes may concern the induction of antimicrobial peptide production by Paneth cells[38]; indeed, a defective production of defensins after bacterial translocation may result in altered shaping of gut microbiota, leading to inflammatory amplification in a vicious circle.

We investigated whether increased activation of peripheral monocytes correlated with plasma levels of LPS-binding protein (LBP). We showed that plasma concentrations of LBP correlate positively with disease activity, both in CD and in UC, in agreement with previous studies[11]. However, there was no significant correlation between LBP levels and activation of peripheral monocytes, suggesting that monocytes are not activated by systemic exposure to LPS. We cannot, however, rule out the existence of other bacterial molecular patterns.

An added value of our research is that we studied monocyte function after stimulation of different TLR pathways. Several studies have already reported that altered patterns of TLR expression can be differentially found in CD, rather than in UC[39], even though no definitive association between the pathway dysregulations and the disease phenotypes has been ever identified. We interestingly found that peripheral blood monocytes from CD patients displayed increased response to $Pam_3CSK_4$, which is a mycobacterial-like ligand for TLR2/1. The increased response to $Pam_3CSK_4$ was quite specific to CD monocytes and was not found in UC. Thus, it is unlikely that the hyper-response to TLR2/1 signaling depended on monocyte activation alone. A possible explanation is that peripheral monocytes from patients with CD are primed by mycobacterial components released in the intestinal mucosa, maybe as a result of colonization with bacteria such as *mycobacterium avium paratuberculosis* (MAP). The presence of MAP in CD intestinal mucosa and bloodstream has been proved by several works and accomplished by different techniques; it has been repetitively associated with the pathogenesis of CD but its role in the etiology remains to be defined[40]. Our results should prompt further investigation on the immune response to MAP in healthy population and in CD.

In conclusion, we showed that peripheral monocytes from patients with CD have higher basal

production of TNF-α than controls, regardless of *NOD2* genotype and without a clear correlation with disease activity. Moreover, peripheral monocyte activation does not seem to be due to plasma exposition to LPS. There is also an increased response of monocytes to MDP or $Pam_3CSK_4$ stimulation, which could reflect a reduced tolerance to bacterial PAMPs. Based on these data, it is worth investigating the possible priming of monocytes by mycobacterial colonization, for example with MAP.

## ACKNOWLEDGMENTS

The authors would like to thank Alessandra Knowles for revising carefully the manuscript.

## COMMENTS

### Background

Crohn's disease (CD) is characterized by an aberrant activation of the mucosal immune system in genetically susceptible subjects, due to an altered crosstalk between innate immune system and environmental factors, with particular reference to gut microbiota.

### Research frontiers

CD could be considered as a paradoxical condition in which defective immune functions coexist with excessive inflammation. Indeed, peripheral blood monocytes from CD patients display increased activation and production of pro-inflammatory tumor necrosis factor-alpha (TNF-α), but they seem to present functional defects in their capacity to respond to muramyl dipeptide (MDP) and to bacteria. Several studies have investigated the function of peripheral blood monocytes as representative cells of the innate immune profile of CD.

### Innovations and breakthroughs

The analysis of intracellular TNF-α allowed the evaluation of the integrity and functionality of NOD2 and TLR signaling pathways in peripheral monocytes. Unexpectedly, CD monocytes showed increased basal and MDP induced TNF-α production, irrespective of *NOD2* genotype and disease activity.

### Applications

Hyper-production of TNF-α by CD monocytes probably relies on a NOD2-indipendent pathway and is not due to systemic exposure to LPS, but to other bacterial molecular patterns. The fact that CD monocytes display higher production of TNF-α compared to controls, both in basal and in stimulated conditions, may be due to reduced tolerance to bacterial PAMPs. The finding of increased response to TLR2/1 stimulation may indicate a possible priming of monocytes by mycobacterial colonization, for example by MAP.

### Peer-review

The manuscript described that peripheral monocytes from CD express higher basal and stimulated TNF-α than controls, regardless of *NOD2* genotype and without a clear correlation with disease activity. In general, the results are very interesting.

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ORIGINAL ARTICLE

Basic Study

# Esophagogastric anastomosis in rats: Improved healing by BPC 157 and L-arginine, aggravated by L-NAME

Zeljko Djakovic, Ivka Djakovic, Vedran Cesarec, Goran Madzarac, Tomislav Becejac, Goran Zukanovic, Domagoj Drmic, Lovorka Batelja, Anita Zenko Sever, Danijela Kolenc, Alen Pajtak, Nikica Knez, Mladen Japjec, Kresimir Luetic, Dinko Stancic-Rokotov, Sven Seiwerth, Predrag Sikiric

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Author contributions: Djakovic Z, Djakovic I, Drmic D, Batelja L, Luetic K, Stancic-Rokotov D, Seiwerth S and Sikiric P designed the research; Djakovic Z, Djakovic I, Cesarec V, Madzarac G, Becejac T, Zukanovic G, Drmic D, Batelja L, Zenko Sever A, Kolenc D, Pajtak A, Knez N, Japjec M and Luetic K performed the research; Stancic-Rokotov D, Seiwerth S and Sikiric P contributed reagents and analytic tools; Djakovic Z, Djakovic I, Becejac T, Drmic D, Batelja L, Zenko Sever A, Luetic K, Stancic-Rokotov D, Seiwerth S and Sikiric P analyzed the data; Djakovic Z, Djakovic I, Drmic D, Batelja L, Stancic-Rokotov D, Seiwerth S and Sikiric P wrote the paper.

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Correspondence to: Predrag Sikiric, MD, PhD, Professor, Departments of Pharmacology and Pathology Medical Faculty University of Zagreb, Salata 11, POB 916, 10000 Zagreb, Croatia. sikiric@mef.hr
Telephone: +385-1-4566833
Fax: +385-1-4920050

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## Abstract

***AIM***

To cure typically life-threatening esophagogastric anastomosis in rats, lacking anastomosis healing and sphincter function rescue, in particular.

***METHODS***

Because we assume esophagogastric fistulas represent a particular NO-system disability, we attempt to identify the benefits of anti-ulcer stable gastric pentadecapeptide BPC 157, which was in trials for ulcerative colitis and currently for multiple sclerosis, in rats with esophagocutaneous fistulas. Previously, BPC 157 therapies have promoted the healing of intestinal anastomosis and fistulas, and esophagitis and gastric lesions, along with rescued sphincter function. Additionally, BPC 157 particularly interacts with the NO-system. In the 4 d after esophagogastric anastomosis creation, rats received medication (/kg intraperitoneally

once daily: BPC 157 (10 μg, 10 ng), L-NAME (5 mg), or L-arginine (100 mg) alone and/or combined or BPC 157 (10 μg, 10 ng) in drinking water). For rats underwent esophagogastric anastomosis, daily assessment included progressive stomach damage (sum of the longest diameters, mm), esophagitis (scored 0-5), weak anastomosis (mL $H_2O$ before leak), low pressure in esophagus at anastomosis and in the pyloric sphincter (cm $H_2O$), progressive weight loss (g) and mortality. Immediate effect assessed blood vessels disappearance (scored 0-5) at the stomach surface immediately after anastomosis creation.

***RESULTS***

BPC 157 (all regimens) fully counteracted the perilous disease course from the very beginning (*i.e.*, with the BPC 157 bath, blood vessels remained present at the gastric surface after anastomosis creation) and eliminated mortality. Additionally, BPC 157 treatment in combination with L-NAME nullified any effect of L-NAME that otherwise intensified the regular course. Consistently, with worsening (with L-NAME administration) and amelioration (with L-arginine), either L-arginine amelioration prevails (attenuated esophageal and gastric lesions) or they counteract each other (L-NAME + L-arginine); with the addition of BPC 157 (L-NAME + L-arginine + BPC 157), there was a marked beneficial effect. BPC 157 treatment for esophagogastric anastomosis, along with NOS-blocker L-NAME and/or NOS substrate L-arginine, demonstrated an innate NO-system disability (as observed with L-arginine effectiveness). BPC 157 distinctively affected corresponding events: worsening (obtained with L-NAME administration that was counteracted); or amelioration (L-arginine + BPC 157-rats correspond to BPC 157-rats).

***CONCLUSION***

Innate NO-system disability for esophagogastric anastomoses, including L-NAME-worsening, suggests that these effects could be corrected by L-arginine and almost completely eliminated by BPC 157 therapy.

**Key words:** Esophagogastric anastomosis; L-NAME; Aggravation; BPC 157; L-arginine; Curative treatment; Rats



**Core tip:** In rats underwent esophagogastric anastomosis, BPC 157 (given intraperitoneally or in drinking water) fully counteracted an otherwise serious disease course since very beginning (*i.e.*, with BPC 157 bath blood vessels remained present at the gastric surface after anastomosis creation) and eliminated mortality. Additionally, BPC 157 treatment, along with L-NAME, nullified any effect of L-NAME that otherwise intensified the regular course. Consistently, with worsening (with L-NAME administration) and amelioration (with L-arginine), either L-arginine-amelioration prevails (*i.e.*, esophageal and gastric lesions are attenuated) or they counteract each other (L-NAME + L-arginine), an effect which is further reversed toward a marked beneficial effect with the addition of BPC 157 (L-NAME + L-arginine + BPC 157).

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## INTRODUCTION

We acknowledged curative treatment of esophagogastric anastomosis in rats with stable gastric pentadecapeptide BPC 157 (an anti-ulcer peptide stable in human gastric juice), as a novel mediator of Robert's cytoprotection that was effective in the entire gastrointestinal tract, which was originally tested in clinical trials for ulcerative colitis and multiple sclerosis[1-7]. Additionally, BPC 157 treatment of esophagogastric anastomosis along with a NO-synthase (NOS) blocker, L-NAME, and/or NOS substrate L-arginine would evidence an innate NO-system disability, and investigate the effect on the corresponding worsening (obtained with L-NAME administration) or amelioration (due to L-arginine).

In general, in the curative treatment of esophageal cancer, the most feared complication is the highest rate of anastomotic leakage[8] compared with anastomoses involving other parts of the gastrointestinal tract[9].

Likely, BPC 157 exhibits some favorable effects for esophagogastric anastomosis healing. Together, intestinal anastomosis[10-14] and fistulas[15-20] healing, esophagitis and gastric lesion healing, alongside with rescued sphincter function[10,11,17,18,20-25] could certainly improve the possible curative peptides therapy for rat esophagogastric anastomosis. Until now, only to improve anastomosis healing, tested were keratinocyte growth factor-2 (KGF-2) (shown to be ineffective given intraperitoneally)[26] (regardless to therapeutic efficacy of a mutant of KGF-2 on trinitrobenzene sulfonic acid-induced rat model of Crohn's disease[27]) and FGF-beta (effective given topically[28]).

Additionally, unlike other anastomoses, the esophagogastric anastomosis should not only resist leakage, it should maintain some "sphincter" function at the anastomosis site, a point that thus far been unappreciated for standard peptide growth factors[26,28]. As a result, these standard peptide growth factors[26,28] only support the particular perilous course of the esophagogastric anastomosis and show little improvement in rats[29,30]. On the other hand, these combined BPC 157 effects

may be more useful for both anastomosis healing and sphincter function rescue[10-14,17,18,20-25]. As a result, the esophagogastric anastomosis healing should both resist leakage and maintain some "sphincter" function at the anastomosis site. These effects may be the maintained esophageal and stomach integrity, and they counteracted the development of both esophagitis and gastric lesions.

Another point may be the perilous effect of ischemia[31-33]. To accelerate anastomosis healing, several studies implicate the positive effect of the induced angiogenesis that follows partial devascularization of the stomach after a certain period (*i.e.,* two-week period)[34-37]. As a very active cytoprotective agent, BPC 157[6], confronted with an injurious course, rapidly induces strong endothelium protection[38] as with standard cytoprotective agents[39], but it has a more prominent angiogenic effect[40] that may significantly contribute to healing in esophagogastric anastomosis. Finally, with BPC 157 designated as a "wound healing therapy"[1-7], these were attributed to the stimulation of the early growth response-1 (EGR1) gene and its co-repressor nerve growth factor 1-A binding protein-2 (NAB2), which affected cytokine and growth factor generation and, thereby, early extracellular matrix (collagen) and blood vessel formation[41]. As a result, a particular feedback-process for the simultaneous healing of different tissues was suggested, leading to both internal and external wound healing, anastomosis and fistulas[1-7]. Others correlated the BPC 157 beneficial effects with the activation of a cellular FAK-paxillin signaling pathway and, subsequently, demonstrated that BPC 157 dose- and time-dependently increased the expression of growth hormone receptor, Janus kinase 2, which belongs to the downstream signal pathway of growth hormone receptor and may interact with other molecular pathways[42-44].

Additionally, BPC 157, based on the beneficial activities noted[1,5,7,17,18,19,45-51], would have particular effects on the NO-system (for review[1-7]), as observed in different models and species[1,5,7,17,18,19,45-51], but it has not previously been tested in anastomosis healing. Likewise, the NO-system plays a particular role in the gastrointestinal lesion healing[1]. It has been more frequently investigated in gastric lesions[1] than in esophagitis lesions[18,52]; despite inconsistencies, L-arginine has a beneficial effect, while L-NAME has an ulcerogenic effect[1], and they have not been investigated in esophagogastric anastomosis.

For practical purposes, the stable gastric pentadecapeptide BPC 157, was given daily, intraperitoneally or orally, in drinking water, using the previous efficacious regimens[7,15-25]. In addition to these effects, the possible simultaneous healing (stable gastric pentadecapeptide BPC 157, along with both NOS-blockade, L-NAME, and NOS-substrate L-arginine application[1]) would define the esophagogastric anastomosis healing, define esophagitis and gastric defects healing, rescue "sphincter" pressure at the site of anastomosis and preserve pyloric sphincter pressure, as a new NO-system related phenomenon.

## MATERIALS AND METHODS

### Animals

Wistar Albino male rats (200 g b.w.) were randomly assigned to the experiments (at least 10 animals per experimental group). All experiments were approved by the Local Ethics Committee. Furthermore, all experiments were performed under a blind protocol, and the effect was assessed by examiners who were blinded to the given protocol.

### Drugs

Pentadecapeptide BPC 157 (GEPPPGKPADDAGLV, M.W. 1419), (Diagen, Ljubljana, Slovenia) dissolved in saline, was used in all experiments. BPC 157, a peptide, is part of the sequence of human gastric juice protein BPC, and it is freely soluble in water at pH 7.0 and saline. The peptide was prepared, as described previously[15-25], with 99% high pressure liquid chromatography (HPLC) purity, expressing 1-des-Gly peptide as an impurity. L-NAME (Sigma, United States) and L-arginine (Sigma, United States) were used accordingly[1,5,7,17-19,45-51].

### Surgical procedure

In deeply anaesthetized rats, an esophagogastric anastomosis (PDS 6.0 suture, Johnson & Johnson, USA) was created at the apical part of the forestomach and distal part of the cut and transferred esophagus.

### Experimental protocol

BPC 157 was given perorally, in drinking water (10 μg/kg, 10 ng/kg, 0.16 μg/mL, 0.16 ng/mL, and 12 mL/rat per day) until sacrifice, or it was administered intraperitoneally (10 μg/kg and 10 ng/kg) with the first application at 30 min after surgery, once daily, and the last at 24 h before sacrifice.

Combination studies: L-NAME (5 mg/kg intraperitoneally) and/or L-arginine (100 mg/kg intraperitoneally) were given alone or together with the first application at 30 min after surgery, once daily, and the last at 24 h before sacrifice. BPC 157 (10 μg/kg and 10 ng/kg intraperitoneally) was given with L-NAME (5 mg/kg intraperitoneally) and/or L-arginine (100 mg/kg intraperitoneally). Controls simultaneously received an equal volume of saline (5.0 mL/kg ip) or water alone. The full assessment was performed at days 1, 2, 3, and 4, as follows (due to subsequent mortality).

To demonstrate the direct effect of BPC 157 administration on the blood vessel presentation immediately after the creation of esophagogastric anastomosis, a bath containing 2 μg/mL of BPC 157 or a corresponding volume of saline was applied to the ventral surface of the stomach.

### *Assessment of esophageal and gastric lesions and anastomosis*

A precise caliper was used to verify the final size of the stomach lesions and largest diameter of the gastric lesions (mm)[53-55]. The esophagitis scoring[20-23] was modified to a 0-5 scoring system, normal, glistening mucosa (score 0); edematous mucosa with focal hemorrhagic spots (score 1); multiple erosions with hematins attached (score 2); tiny esophagus with hemorrhagic and linear yellowish lesions (score 3); tiny esophagus with coalesced hemorrhagic and yellowish lesions (score 4); and tiny esophagus with coalesced hemorrhagic, yellowish lesions and dehiscent anastomosis (score 5), which was also photographed and further verified using the program ISSA (VAMSTEC Software Company, Zagreb, Croatia), as described previously[1-7]. The tissue was placed in 10% formalin and used for histopathological examination, and processed for further microscopic analysis[1-7].

To assess anastomosis leakage, a separate group of animals received a volume of water intragastrically to induce leakage[17].

### *Pressure in the esophagus at the site of anastomosis assessment and pyloric sphincter pressure assessment*

As described previously[17,18,20-23], manometrical evaluation (cm $H_2O$) was performed in all rats, with a water manometer connected to the drainage port of the Foley catheter, as previously described (values of 68-76 cm $H_2O$ for the lower esophageal sphincter, and 68-74 cm $H_2O$ for the pyloric sphincter, were considered normal)[17,18,20-23]. The proximal side of the esophageal incision, or distal side of the duodenal incision, was ligated to prevent regurgitation[17,18,20-23].

### *Weight assessment*

As described in prior works[13,18], animals were weighed before surgery, once daily thereafter, and before sacrifice. Weight loss (g) was presented as the $\Delta$ between the initial and final weight[13,18].

### *Mortality*

In separate group of animals, mortality was assessed daily until post-operative day 7, as described previously[13,18].

### *Very early effects: disappearance of blood vessels at the stomach surface in rats that underwent esophagogastric anastomosis*

For disappearance and presentation of blood vessels at the stomach surface in rats that underwent esophagogastric anastomosis, we described blood vessel presentation at the ventral stomach surface (scored 0-5) throughout stomach distension and/or alcohol instillation into the stomach[53], as follows: presentation completely reduced, only the main tree of the left gastric artery present (score 0); thin vessels present in the fore stomach only (score 1); thin vessels present in the entire stomach (score 2); moderate vessels present in the whole stomach (score 3); prominent vessels present in the fore stomach (score 4); and prominent vessels present in the entire stomach (score 5). Continuous camera (Veho discovery VMS-004 deluxe) recordings (5 cm above the tissue; magnification 30-100 x) were used in deeply anesthetized rats for the next 15 min.

### *Statistical analysis*

Statistical analysis was performed by a non-parametric Kruskal-Wallis ANOVA test and, later, a Mann-Whitney *U*-test, to compare groups. The Fisher exact probability test was used for mortality assessment. Values of $P < 0.05$ were considered statistically significant.

## RESULTS

### *Esophagogastric anastomosis course*

In general, since the beginning, the rats that underwent esophagogastric anastomosis without medication suffered a very severe course (as assessed until post-operative day 4) that would eventually be lethal (at post-operative day 5). These rats had relatively small gastric lesions (Figure 1) compared with severe esophagitis lesions (Table 1) and poor anastomosis (constantly small water volume that could be sustained before leakage) (Figure 2). Considering the esophagus at the site of the anastomosis (Figure 3) and pyloric sphincter (Figure 4), the pyloric pressure seems to be more affected (constantly low pyloric sphincter pressure) than the esophageal pressure at the anastomotic site. The esophageal pressure was initially considerably lower that the lower esophageal pressure in normal rats; however, on the fourth day, the esophageal pressure approached to that values. These changes, however, shortly preceded the lethal outcome on post-operative day 5. Meanwhile, these rats suffered considerable weight loss.

**BPC 157 therapy:** On the other hand, the effect of BPC 157 (both μg- and ng regimens, intraperitoneal and drinking water applications) seems to be important considering the severe and perilous course without it, after rats underwent esophagogastric anastomosis. Gastric lesions (Figure 1) and esophagitis lesions (Table 1) were attenuated, and anastomoses were strengthened (water volume before anastomosis leakage was more than two times that in the controls) (Figure 2); the pressure in the esophagus at the site of the anastomosis (Figure 3) and pyloric sphincter (Figure 4) markedly increased. Lethal outcomes were completely avoided (Fisher exact probability test *vs* control $P < 0.05$). Weight loss was attenuated (Figure 5).

**L-arginine therapy:** Rats that underwent esophagogastric lesions and were treated with L-arginine had an attenuated course. Gastric (Figure 1) and esophagitis lesions (Table 1) were attenuated and anastomosis was

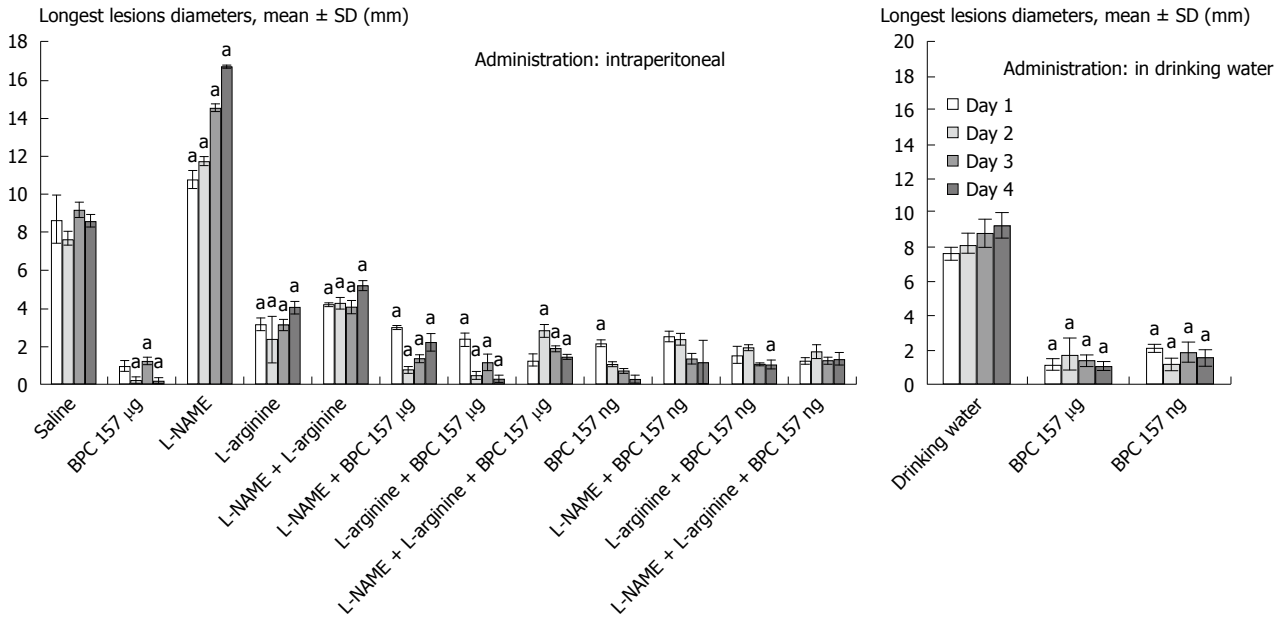


**Figure 1 Gastric lesions, sum of the longest lesions diameters, mean ± SD, mm, in rats that underwent esophagogastric anastomosis.** Medication (/kg) given intraperitoneally (ip) (once time daily) or continuously in drinking water (po) after the creation of an esophagogastric anastomosis in rats. BPC 157 (10 μg, 10 ng), L-NAME (5 mg), and L-arginine (100 mg) given alone and/or combined intraperitoneally with the first application at 30 min after anastomosis creation and the last at 24 h before sacrifice. Drinking water alone (12 mL/d per rat) or BPC 157 in drinking water (10 μg, 10 ng/kg; 0.16 μg, 0.16 ng/mL) was provided continuously until sacrifice. [a]*P* < 0.05, at least, *vs* control.

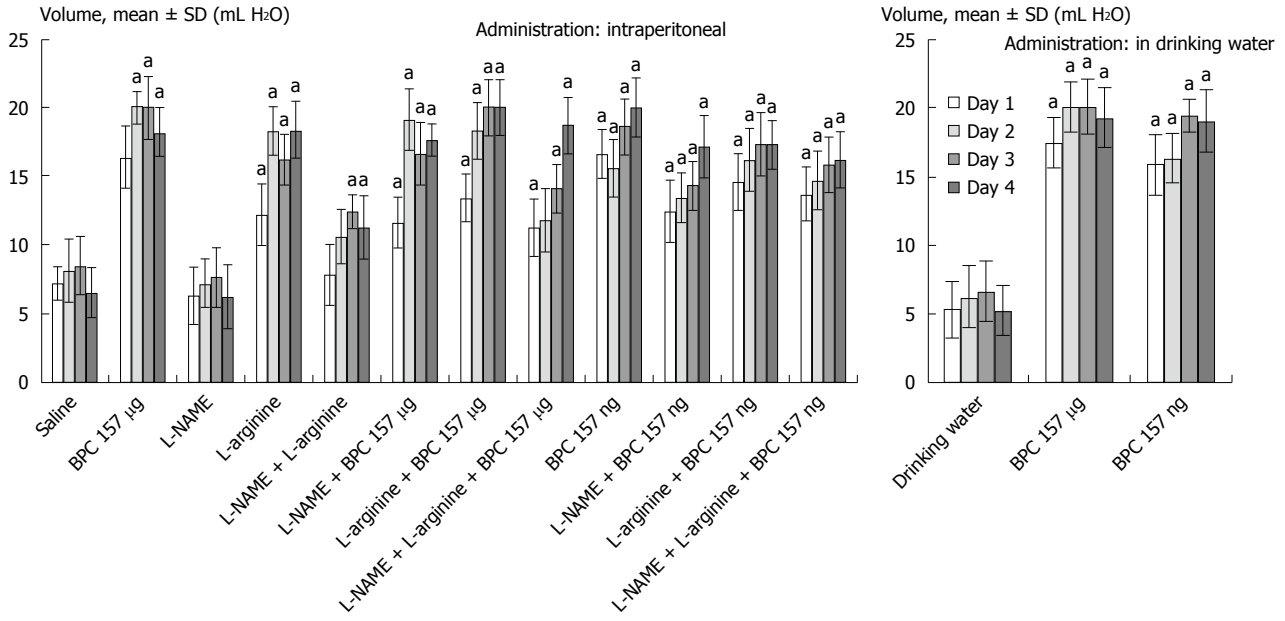


**Figure 2 Anastomosis strength.** Water volume that could be sustained before leakage, mean ± SD, mL, in rats that underwent esophagogastric anastomosis. Medication (/kg) given intraperitoneally (ip) (once time daily) or continuously in drinking water (po) after the creation of esophagogastric anastomosis in rats. BPC 157 10 μg and 10 ng, L-NAME 5 mg, and L-arginine 100 mg given alone and/or combined intraperitoneally with the first application at 30 min after anastomosis creation and last at 24 h before sacrifice. Drinking water alone (12 mL/d per rat) or BPC 157 in drinking water (10 μg, 10 ng/kg; 0.16 μg, 0.16 ng/mL) was provided continuously until sacrifice. [a]*P* < 0.05, at least, *vs* control.

strengthened (more water volume before anastomosis leakage than in controls) (Figure 2). The pressures in the esophagus at the site of the anastomosis (Figure 3) and pyloric sphincter (Figure 4) markedly increased. Lethal outcomes were completely avoided (Fisher exact probability test *vs* control, $P < 0.05$). Weight loss was continuously attenuated before the last day (Figure 5).

**L-NAME therapy:** Rats that underwent esophagogastric lesions and treated with L-NAME had an aggravated course. Gastric (Figure 1) and esophagitis lesions (Table 1) constantly worsened; other disturbances were less expressed. For instance, weakened anastomosis constantly sustained a small volume water before anastomosis leakage such as in controls

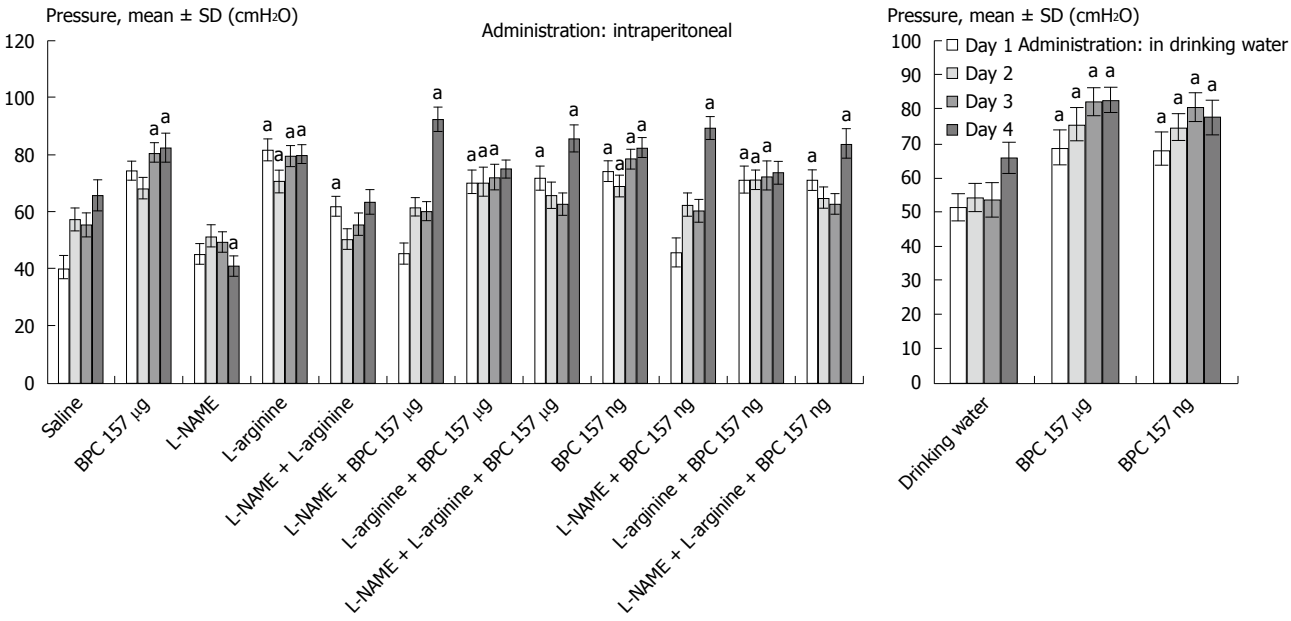


**Figure 3 Pressure in the esophagus at the anastomosis site.** Mean ± SD, cmH2O, in rats that underwent esophagogastric anastomosis. Medication (/kg) given intraperitoneally (ip) (once time daily) or continuously in drinking water (po) after the creation of esophagogastric anastomosis in rats. BPC 157 10 μg and 10 ng, L-NAME 5 mg, and L-arginine 100 mg given alone and/or combined intraperitoneally with the first application at 30 min after anastomosis creation and last at 24 h before sacrifice. Drinking water alone (12 mL/d per rat) or BPC 157 in drinking water (10 μg, 10 ng/kg; 0.16 μg, 0.16 ng/mL) was provided continuously until sacrifice. [a]*P* < 0.05 at least *vs* control. The values of 68-76 cm H2O for the lower esophageal sphincter were considered to be normal, as determined previously[17,18,20-23].

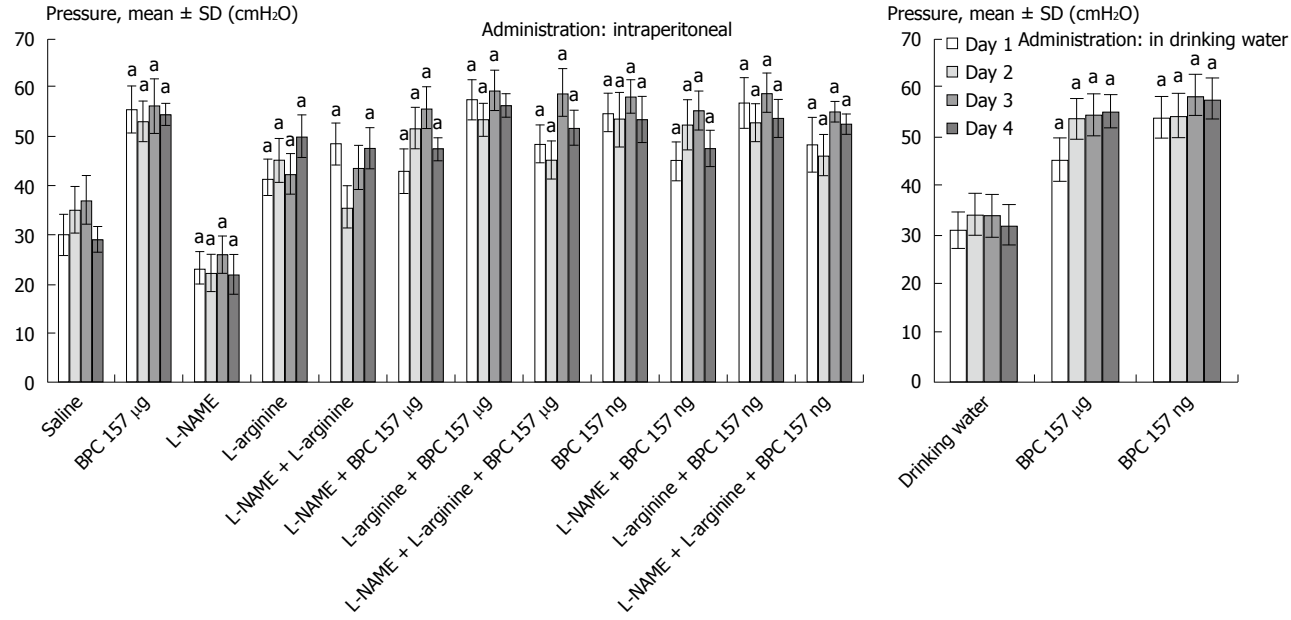


**Figure 4 Pressure in the pyloric sphincter.** Mean ± SD, cmH2O, in rats that underwent esophagogastric anastomosis. Medication (/kg) given intraperitoneally (ip) (once time daily) or continuously in drinking water (po) after the creation of esophagogastric anastomosis in rats. BPC 157 10 μg and 10 ng, L-NAME 5 mg, and L-arginine 100 mg given alone and/or in combination intraperitoneally with the first application at 30 min after anastomosis creation and last at 24 h before sacrifice. Drinking water alone (12 mL/d per rat) or BPC 157 in drinking water (10 μg, 10 ng/kg; 0.16 μg, 0.16 ng/mL) was provided continuously until sacrifice. [a]*P* < 0.05, at least *vs* control. The values of 68-74 cm H2O for pyloric sphincter were considered normal, as previously determined[17,18,20-23].

(Figure 2); there was small pressure in the esophagus at the site of the anastomosis (Figure 3) and in the pyloric sphincter (Figure 4); and weight loss increased on post-operative days 1 and 4 (Figure 5). However, they could not survive post-operative day 4 (Fisher exact probability test *vs* control $P < 0.05$, at least).

**Combined therapies:** L-NAME-induced worsening was commonly reversed with medication combinations (L-NAME + L-arginine; L-NAME + BPC 157; and L-NAME + L-arginine + BPC 157). Gastric (Figure 1) and esophagitis lesions (Table 1) were constantly attenuated. However, in particular, L-NAME + L-arginine rats do not immediately have increased anastomosis

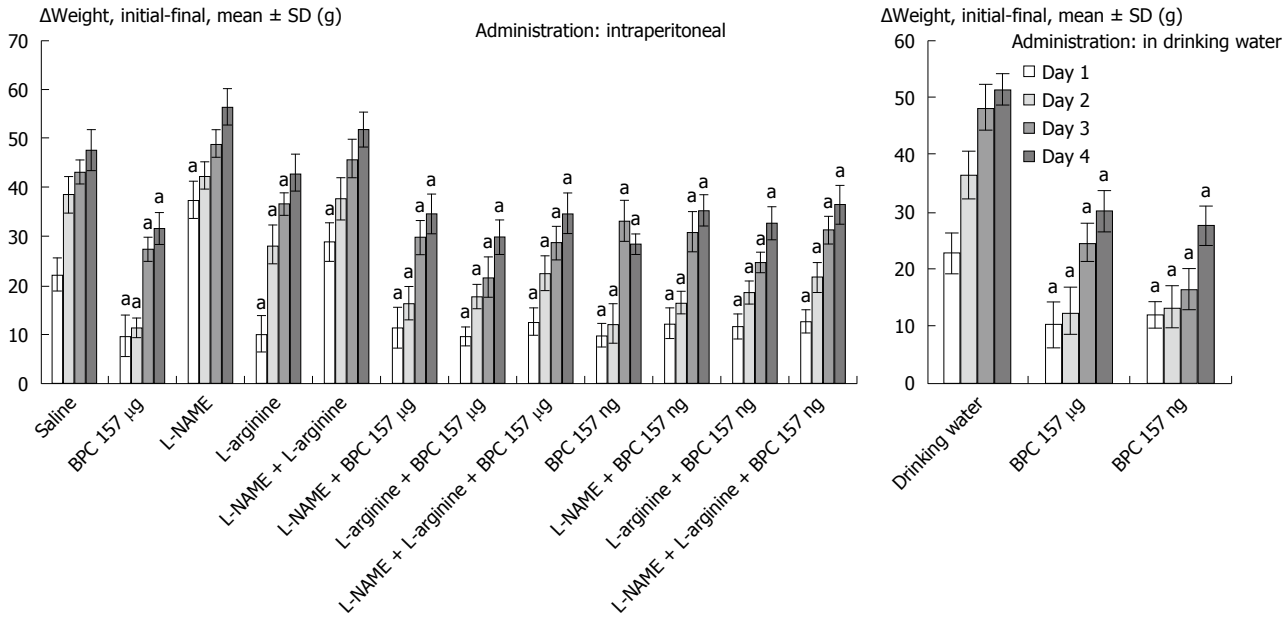


**Figure 5 Weight loss (g) presented as the Δ between the initial and final weights[13,18], mean ± SD, in rats that underwent esophagogastric anastomosis.** Medication (/kg) given intraperitoneally (once time daily) or continuously in drinking water after the creation of an esophagogastric anastomosis in rats. BPC 157 10 μg and 10 ng, L-NAME 5 mg, and L-arginine 100 mg given alone and/or combined intraperitoneally with the first application at 30 min after anastomosis creation and the last at 24 h before sacrifice. Drinking water alone (12 mL/d per rat) or BPC 157 in drinking water (10 μg and 10 ng/kg; 0.16 μg and 0.16 ng/mL) was provided continuously until sacrifice. [a]$P < 0.05$, at least, *vs* control.

**Table 1 Esophagitis score (0-5) Min/Med/Max in rats that underwent esophagogastric anastomosis**

| Medication (/kg) given ip (once time daily) or continuously in drinking water (po) after the creation of esophagogastric anastomosis in rats | Esophagitis score (0-5) Min/Med/Max in rats that underwent esophagogastric anastomosis | | | |
|---|---|---|---|---|
| | 1 d Min/Med/Max | 2 d Min/Med/Max | 3 d Min/Med/Max | 4 d Min/Med/Max |
| Saline 5 mL | 4/4/4 | 4/4/4 | 4/4/4 | 4/4/4 |
| BPC 157 10 μg | 0/1/2[a] | 0/1/2[a] | 0/1/2[a] | 0/1/2[a] |
| L-NAME 5 mg | 5/5/5[a] | 5/5/5[a] | 5/5/5[a] | 5/5/5[a] |
| L-arginine 100 mg | 0/1/2[a] | 1/2/3[a] | 1/2/3[a] | 1/2/3[a] |
| L-NAME 5 mg + L-arginine 100 mg | 2/3/4[a] | 0/1/2[a] | 1/2/3[a] | 1/2/3[a] |
| L-NAME 5 mg + BPC 157 10 μg | 1/2/3[a] | 1/2/3[a] | 0/1/2[a] | 0/1/2[a] |
| L-arginine 100 mg + BPC 157 10 μg | 0/1/2[a] | 0/1/2[a] | 0/1/2[a] | 0/1/2[a] |
| L-NAME 5 mg + L-arginine 100 mg +BPC 157 10 μg | 0/1/2[a] | 0/1/2[a] | 1/2/3[a] | 0/1/2[a] |
| BPC 157 10 ng | 0/1/2[a] | 0/1/2[a] | 0/1/2[a] | 0/1/2[a] |
| L-NAME 5 mg + BPC 157 10 ng | 1/2/3[a] | 2/3/4[a] | 1/2/3[a] | 0/1/2[a] |
| L-arginine 100 mg + BPC 157 10 ng | 0/1/2[a] | 0/1/2[a] | 0/1/2[a] | 0/1/2[a] |
| L-NAME 5 mg + L-arginine 100 mg +BPC 157 10 ng | 1/2/3[a] | 0/1/2[a] | 1/2/3[a] | 0/1/2[a] |
| Drinking water 12 mL/d per rat | 4/4/4 | 4/4/4 | 4/4/4 | 4/4/4 |
| BPC 157 10 μg | 0/1/2[a] | 0/1/2[a] | 0/1/2[a] | 0/1/2[a] |
| BPC 157 10 ng | 0/1/2[a] | 0/1/2[a] | 0/1/2[a] | 0/1/2[a] |

Medication (/kg) given intraperitoneally (ip) (once daily) or continuously in drinking water (po) after the creation of an esophagogastric anastomosis in rats. BPC 157 10 μg and 10 ng, L-NAME 5 mg, and L-arginine 100 mg given alone and/or combined intraperitoneally with the first application at 30 min after anastomosis creation and the last at 24 h before sacrifice. Drinking water alone (12 mL/d per rat) or BPC 157 in drinking water (10 μg, 10 ng/kg; 0.16 μg, 0.16 ng/mL) was provided continuously until sacrifice. [a]$P < 0.05$, at least *vs* control.

strength (they develop it later), and pressure in the esophagus at anastomosis site and in pyloric sphincter is only occasionally increased (Figures 2, 3 and 4). Weight loss initially remained increased; then, it was reversed to control values (Figure 5). In rats that additionally received BPC 157 (L-NAME + BPC 157 and L-NAME + L-arginine + BPC 157), as well as rats treated with L-arginine + BPC 157), these parameters were constantly improved. Lethal outcomes were commonly avoided (Fisher exact probability test *vs* control $P < 0.05$, at least).

Generally, the described macroscopical healing (Figure 6) is along with microscopic presentation followed and thereby counteracted as described above (Figures 7 and 8). In the period after esophagogastric anastomosis creation, at the site of anastomosis, the control animals showed severe necrosis along the anastomosis line, including a large necrotic

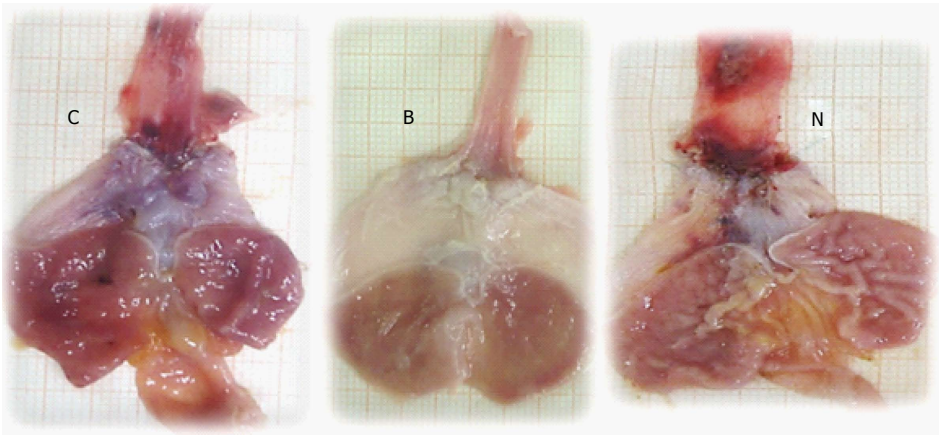


**Figure 6 Illustrative gross presentation of rats that underwent esophagogastric anastomosis at post-operative day 4 and then received medication saline (control, C), BPC 157 (B), or L-NAME (N).**

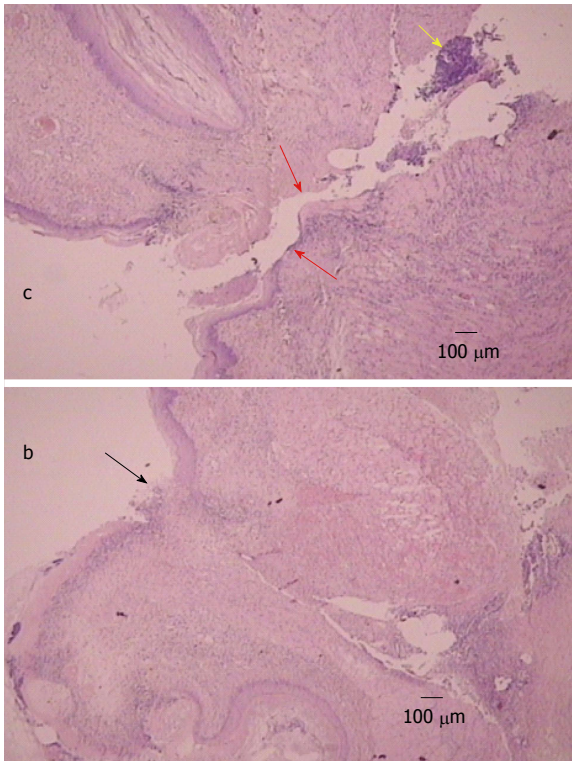


**Figure 7 Illustrative microscopic presentation in rats that underwent esophagogastric anastomosis at post-operative day 2, hematoxylin eosin staining.** Separated anastomosis edges (red arrows) with neutrophils within the edges (yellow arrow) were consistently noted in controls (c). Separated wound edges (red arrows). Connected edges in rats that underwent esophagogastric anastomosis and treated with BPC 157 regimens (black arrow) (b).

area of the superficial epithelium and broad band of necrotic subcutaneous tissue and muscle. Abundant, predominantly polymorphonuclear infiltration was present along the anastomosis. The inflammation extended to the adipose tissue. Grossly, regular confluent hemorrhagic and yellowish lesions appear in advanced esophagitis; microscopically, ulcerations with pronounced subepithelial and muscular edema,

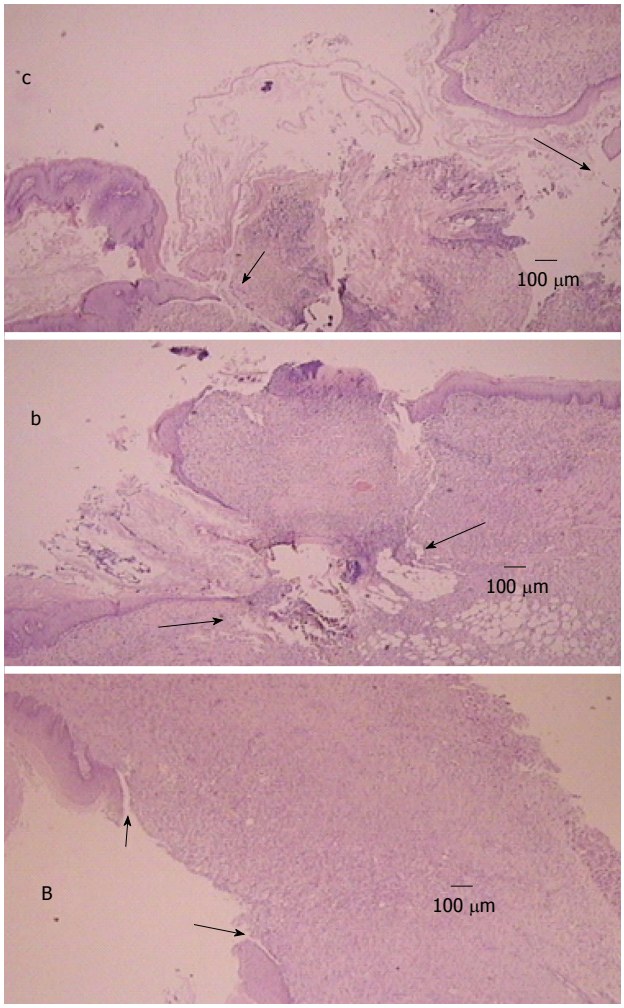


**Figure 8 Illustrative microscopic presentation in rats that underwent esophagogastric anastomosis at post-operative day 4 [controls (c), BPC 157 (b)] and at post-operative day 7 [BPC 157 (B)], hematoxylin eosin staining.** Tissue destruction at the anastomosis edges (arrows) were consistently noted in controls (c), which is a presentation that precedes lethal outcome. Initial organization of the exudate (b) (arrows) and wound completely closed with granulation tissue (B) (arrows), which is consistently noted in rats that underwent esophagogastric anastomosis and were treated with BPC 157.

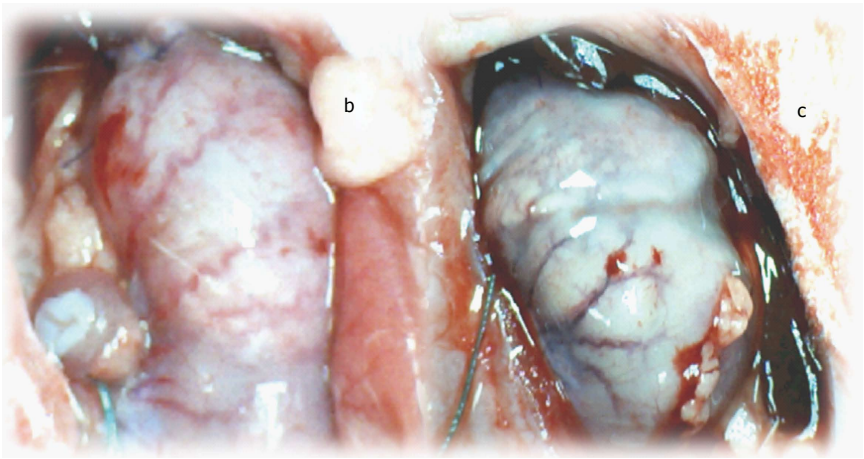


**Figure 9 Blood vessels presentation at the ventral site of the stomach surface in rats that just underwent esophagogastric anastomosis.** In general, immediately after anastomosis creation and saline bath application, blood vessels disappeared from the rat gastric surface, and this effect lasts at least the next 15 min (scored Min/Med/Max 0/0/0), a period that was carefully monitored (control, c). With a BPC 157 bath immediately after anastomosis creation, the blood vessels did not disappear from the rat gastric surface; instead, these vessels remained present during at least the next 15 min of monitoring (BPC 157, b).

mononuclear infiltration, thinner epithelium and superficial corneal layers are present. Gastric mucosal lesions mostly presented with hemorrhagic lesions that were surrounded by edema of the lamina propria and submucosa with a mixed inflammatory reaction. However, some presented with extensive necrosis to all parts of the mucosa, and they had sharp edges with infiltrated granulocytes at the bases.

**Very early findings:** The following very early findings could likely be illustrative of all post-operative courses. In control rats that underwent esophagogastric anastomosis immediately after anastomosis creation and saline bath application at the ventral side of the stomach, blood vessels disappeared from the rat gastric surface, lasting for at least 15 min (scored Min/Med/Max 0/0/0), a period that was carefully monitored (Figure 9). Conversely, immediately after anastomosis creation and BPC 157 bath application, blood vessels did not disappear from the rat gastric surface; these vessels remained present, at least during the next 15 min of monitoring (scored Min/Med/Max 5/5/5, *vs* control $P < 0.05$) (Figure 9).

## DISCUSSION

We report on the curative treatment of esophagogastric anastomosis in rats with stable gastric pentadecapeptide BPC 157[1-7]. It was highly successful against a perilous and mortal course even when it had to be markedly aggravated by L-NAME application. Namely, as observed before, rats undergoing esophagogastric anastomosis are severely affected[29,30]. They exhibited failed anastomosis healing[30,31], but they also presented with progressive esophagitis and gastric lesions, leakage, failed pressure within the anastomosis site that was markedly below values noted in the rat's lower esophageal sphincter, a dysfunctional pyloric sphincter, weight loss, a short-life, and inescapable lethal outcomes.

As mentioned, BPC 157 treatment along with an NO-synthase (NOS) blocker, L-NAME, nullified any effect of L-NAME that would otherwise markedly intensify the regular course. Consistently, with worsening (obtained with L-NAME administration) and amelioration (with L-arginine), either L-arginine-amelioration prevails (*i.e.,* esophageal and gastric lesions attenuated) or they counteract each other (L-NAME + L-arginine) with an effect that was further reversed toward a marked beneficial effect by the addition of BPC 157 (L-NAME + L-arginine + BPC 157). Together, these provide evidence for an innate NO-system disability (L-NAME-worsening) that could be corrected by the administration of a NOS substrate, such as L-arginine, and almost completely eliminated by BPC 157 therapy. Accordingly, in various models and species[1,5,7,17,18,20,45-51], BPC 157 counteracted the L-NAME effect better than L-arginine[1,5,7,17,18,20,45-51] as well as induced NO-release in the gastric mucosa from rat stomach tissue homogenates, even in conditions in which L-arginine is not working[50,56]. No further beneficial effect was observed when BPC 157 and L-arginine were co-administered[1,5,7,17,18,20,45-51].

In the rats that underwent esophagogastric anastomosis, the particular point of BPC 157 effectiveness involving both anastomosis healing and sphincter rescue was the realized anastomosis creation already in controls that at least partly rescued the sphincter function at the site of anastomosis, while pressure in the pyloric sphincter remains constantly low. Of note, pylorus sphincter failure was thought to reflect lower esophageal sphincter failure[17,18,20-23]. This was further additionally improved in rats that underwent BPC 157 therapy, and pressure in the pyloric sphincter is also rescued, which is an important point now reported.

Previously, we demonstrated that BPC 157 maintains sphincter function (lower esophageal, pyloric[17,18,20-23],

urethral[24], and pupil[25]). Specifically, simultaneous lower esophageal and pyloric sphincter function assessment, as a hallmark of reinstated function and tissue integrity[17,18,20-23], demonstrates that when there are more lesions present, the sphincter pressure is lower[17,18,20-23]. Of note, BPC 157 therapy regularly counteracted the disability of the lower esophageal and pyloric sphincter[17,18,20-23], induced in various ways (*i.e.,* stretching sphincters with temporal tube insertion[21-23], potassium chloride overdose application[20], bile duct ligation-induced pancreatitis[21], esophagocutaneous[18] or duodenocutaneous fistula creation[17], and lower esophageal sphincter dysfunction instantly induced pyloric sphincter failure, and vice versa[21-23]). In fistula conditions, this was shown to be a NO-system related phenomenon[7,17,18]. With respect to the outcome of esophagogastric anastomosis, an interesting anastomosis analogy could be made, providing that these surgically created fistulas are actually anastomosis between two different tissues (*i.e.,* esophagus and skin[17]; duodenum and skin[18]; colon and skin[7]) and, thereby, sphincter function rescue could be observed along with anastomoses healing.

Of note, indicatively, anastomosis creation that better rescued the sphincter function at the site of anastomosis (as well as the pyloric sphincter function) could be also obtained in L-arginine-treated rats. Additionally, sphincter failure is proposed as a hallmark of ongoing injury[17,18,20-23] along with an injurious effect of L-NAME itself[1,5,7,17,18,20,45-51] that overrides previous considerations about NO-sphincter relationships[57] while being unrelated to injurious conditions (*i.e.,* in dogs, ferrets and muscle strips[58-60]). In rats that underwent esophagogastric anastomosis and L-NAME therapy, the final drop of pressure within the esophagus at the site of anastomosis on day 4 occurs just prior to death. Here, moreover, we have to assume dysfunction of the nitrergic pathway; for instance, excision-immediate heavy loss of endothelium cells from the vascular wall results in a lower NO-production ability[61], which has different action for the damaged tissue integrity.

Thereby, in rats with esophagogastric anastomosis that were treated with L-NAME, the degree of sphincter failure was higher, in accordance with the worst esophageal and gastric lesions, and accelerated lethal outcomes.

Finally, it is reasonable to assume also in the esophagogastric anastomosis studies that constant vessel presentation could predict the beneficial effect of the applied agent[53]. Thereby, it is interesting to note the perilous effect of ischemia[31-33] and, conversely, angiogenesis in improving esophagogastric anastomosis healing triggered in the conditioned stomach (partial stomach devascularization)[34-37], as evidenced in a period of one week[34-37]. These observations have to be further corroborated with the noted beneficial effect of BPC 157 in rats with esophagogastric anastomosis. Namely, BPC 157 exhibits a rapid, beneficial effect (since the first day), and BPC 157 is a cytoprotective agent[1-7,38,53] that rapidly induces strong endothelium protection[38] and prominent angiogenic effects (seen when placed in the classic sponge inserted into the rat's back or through various tissues healing[2,40,62] with VGEF expression[2,40,62]). As a result, BPC 157 obviously has an additional, more direct beneficial effect on blood vessel presentation[1-7,38,40,53,62].

The constant vessel presentation synergizes the beneficial effect of BPC 157[53], and it is worth noting that after pentadecapeptide BPC 157 instillation into the stomach following distension or alcohol instillation into the stomach, the vessel presentation remains constant, while left gastric artery blood vessels clearly disappear at the serosal site, indicative of loss in the integrity and function within a minute[53]. These findings[53] correlate with the findings noted immediately after the creation of esophagogastric anastomosis in rats, wherein left gastric artery blood vessels clearly disappear at the serosal site, unlike the constant vessel presentation in rats that underwent BPC 157 therapy. This may be an early, essential point for achieving the further full healing effect.

The esophagogastric anastomosis point provides the anastomosis strength (*i.e.*, with various anastomosis leakage, the highest rates belong to this anastomotic leakage alone[8,9]). Since the very beginning, stable gastric pentadecapeptide BPC 157 significantly improved all parameters of anastomotic wound healing in rats with esophagogastric anastomosis, as has previously been shown with various intestinal anastomoses[10-14] (note BPC 157 also improves the blood vessel and peripheral nerve anastomoses[63,64]), and with both external and internal fistulas[7,15-19], which were originally created as the surgical anastomoses between various tissues[7,15-19]. As a result, BPC 157 especially improves the anastomotic strength. Furthermore, we noted comparable, complex functional and biomechanical improvement of various tissues[65-68], as well as their suitable healing and functional restoration (*i.e.,* increased tensile breaking force, relative elongation of the burned skin[65,66], failure of the load of the transected tendon[67] or muscle[68], improved walking[67,68], and absent post-injury contracture[67,68]). Therefore, since these results were obtained with the same dosage regimen, increased tensile strength of the anastomosis is a direct reflection of the successful repair process[69], and an essential healing point[1-7,53] could be achieved along with the stimulation of the early growth response-1 (EGR-1) gene and its co-repressor nerve growth factor 1-A binding protein-2 (NAB2), cytokine and growth factor generation and thereby, early extracellular matrix (collagen) and blood vessel formation[41], and other molecular pathways[41-44].

These processes may be involved in a particular feedback-process for the simultaneous healing of different tissues, which can improve esophagogastric anastomosis healing and counteract all consequences

of an otherwise fatal injury course.

In addition, for a new NO-system phenomenon, stable gastric pentadecapeptide BPC 157, along with NOS-blockade, L-NAME, and NOS-substrate L-arginine application[1], would favorably define esophagogastric anastomosis healing, esophagitis and gastric defect healing, as well as rescue the "sphincter" pressure at the site of anastomosis while preserving the pyloric sphincter pressure. These approaches should be used to counteract the frequently dangerous course after esophagogastric anastomosis creation.

## COMMENTS

### Background

BPC 157 treatment of esophagogastric anastomosis along with a NO-synthase (NOS) blocker, L-NAME, and/or NOS substrate L-arginine would evidence an innate NO-system disability, and investigate the effect on the corresponding worsening (obtained with L-NAME administration) or amelioration (due to L-arginine).

### Research frontiers

BPC 157 treatment of esophagogastric anastomosis along with a NO-synthase (NOS) blocker, L-NAME, and/or NOS substrate L-arginine would evidence an innate NO-system disability, and investigate the effect on the corresponding worsening (obtained with L-NAME administration) or amelioration (due to L-arginine).

### Innovations and breakthroughs

The stable gastric pentadecapeptide BPC 157, was given daily, intraperitoneally or orally, in drinking water, using the previous efficacious regimens. In addition to these effects, the possible simultaneous healing (stable gastric pentadecapeptide BPC 157, along with both NOS-blockade, L-NAME, and NOS-substrate L-arginine application) would define the esophagogastric anastomosis healing, define esophagitis and gastric defects healing, rescue "sphincter" pressure at the site of anastomosis and preserve pyloric sphincter pressure, as a new NO-system related phenomenon.

### Applications

A new NO-system phenomenon, stable gastric pentadecapeptide BPC 157, along with NOS-blockade, L-NAME, and NOS-substrate L-arginine application[1], would favorably define esophagogastric anastomosis healing, esophagitis and gastric defect healing, as well as rescue the "sphincter" pressure at the site of anastomosis while preserving the pyloric sphincter pressure. These approaches should be used to counteract the frequently dangerous course after esophagogastric anastomosis creation.

### Peer-review

This manuscript presents stable gastric pentadecapeptide BCP157, along with NOS-blockade, L-NAME, and NOS-substrate L-argnine application would favorably define the esophagogastric anastomosis healing, esophagitis and gastric defects healing and rescued sphincter pressure.

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ORIGINAL ARTICLE

Basic Study

# HER2-induced metastasis is mediated by AKT/JNK/EMT signaling pathway in gastric cancer

Yiseul Choi, Young San Ko, Jinju Park, Youngsun Choi, Younghoon Kim, Jung-Soo Pyo, Bo Gun Jang, Douk Ho Hwang, Woo Ho Kim, Byung Lan Lee

Yiseul Choi, Jinju Park, Youngsun Choi, Woo Ho Kim, Byung Lan Lee, Department of Tumor Biology, Cancer Research Institute, Seoul National Universit, College of Medicine, Seoul 110-799, South Korea

Young San Ko, Douk Ho Hwang, Byung Lan Lee, Department of Anatomy, Seoul National University College of Medicine, Seoul 110-799, South Korea

Younghoon Kim, Woo Ho Kim, Department of Pathology, Seoul National University College of Medicine, Seoul 110-799, South Korea

Jung-Soo Pyo, Department of Pathology, Eulji University Hospital, Eulji University College of Medicine, Daejon 35233, South Korea

Bo Gun Jang, Department of Pathology, Jeju National University Hospital, Jeju 690-767, South Korea

Byung Lan Lee, Ischemic/Hypoxic Disease Institute Medical Research Center, Seoul National University College of Medicine, Seoul 110-799, South Korea

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Correspondence to: Byung Lan Lee, MD, PhD, Department of Anatomy, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 110-799, South Korea. dslanat@snu.ac.kr
Telephone: +82-2-7408218
Fax: +82-2-7459528

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## Abstract

***AIM***

To investigated the relationships between HER2, c-Jun N-terminal kinase (JNK) and protein kinase B (AKT) with respect to metastatic potential of HER2-positive

gastric cancer (GC) cells.

***METHODS***

Immunohistochemistry was performed on tissue array slides containing 423 human GC specimens. Using HER2-positve GC cell lines SNU-216 and NCI-N87, HER2 expression was silenced by RNA interference, and the activations of JNK and AKT were suppressed by SP600125 and LY294002, respectively. Transwell assay, Western blot, semi-quantitative reverse transcription-polymerase chain reaction and immunofluorescence staining were used in cell culture experiments.

***RESULTS***

In GC specimens, HER2, JNK, and AKT activations were positively correlated with each other. In vitro analysis revealed a positive regulatory feedback loop between HER2 and JNK in GC cell lines and the role of JNK as a downstream effector of AKT in the HER2/AKT signaling pathway. JNK inhibition suppressed migratory capacity through reversing EMT and dual inhibition of JNK and AKT induced a more profound effect on cancer cell motility.

***CONCLUSION***

HER2, JNK and AKT in human GC specimens are positively associated with each other. JNK and AKT, downstream effectors of HER2, co-operatively contribute to the metastatic potential of HER2-positive GC cells. Thus, targeting of these two molecules in combination with HER2 downregulation may be a good approach to combat HER2-positive GC.

**Key words:** Gastric cancer; HER2; c-Jun N-terminal kinase; Protein kinase B; Metastatic potential



**Core tip:** We investigated the significance of c-Jun N-terminal kinase (JNK) and its interaction with protein kinase B (AKT) in the HER2 signaling with respect to metastatic potential of HER2-positive gastric cancer (GC). In clinical GC samples, we found positive relationships between HER2, JNK and AKT. Inhibition studies using HER2-positive SNU-216 and NCI-N87 GC cell lines demonstrated that positive crosstalk exists between HER2 and JNK, and that JNK is a downstream signaling molecule of AKT. In addition, JNK and AKT increased EMT and co-operatively contributed to the metastatic potential of HER2-positive GC cell lines. Thus, HER2 signaling contributes to GC metastasis through activation of AKT/JNK/EMT pathway.

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## INTRODUCTION

Gastric cancer (GC) is the fourth most common malignancy and the second leading cause of cancer death worldwide[1]. Although metastasis is the major obstacle in the treatment of malignant cancer, the underlying molecular mechanism responsible for GC metastasis needs to be further elucidated.

Human epidermal growth factor receptor 2 (HER2/ERBB2/neu), a member of the epidermal growth factor receptor family of receptor tyrosine kinases[2], is overexpressed in 7%-34% of GC cases[3]. Since recent studies have reported a high concordance between HER2 protein overexpression in immunohistochemistry and gene amplification by fluorescence *in situ* hybridization or chromogenic *in situ* hybridization[4], HER2 overexpression seems to be directly correlated with HER2 amplification in most cases[5]. Although our previous study[6] showed that HER2 downregulation decreased cell migration, invasion and metastasis of GC, the efficacy of anti-HER2 treatment of GC patients was limited due to intrinsic and acquired drug resistance. However, the underlying molecular mechanism of HER2-induced GC metastasis remains largely unknown.

Major downstream signaling pathways of HER2 include the mitogen-activated protein kinase (MAPK) pathway and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway[7]. MAPKs are serine (Ser)/threonine (Thr)-specific protein kinases and include extracellular signal-regulated kinases (ERKs), p38 MAPK and c-Jun N-terminal kinases (JNKs). After phosphorylation, MAPKs are activated and can translocate to the nucleus followed by regulation of various transcription factors[8], which control the proliferation, differentiation, survival and migration of specific cell types. The specific role of individual MAPKs is dependent on cell-context and cell-type[9].

Aberrant expression and activation of JNK is found in many cancer cell lines and tissue samples of cancer patients[9]. In general, JNK has been established as a key kinase in cancer cell apoptosis[10]. Recently, the role of JNK in HER2 signaling pathway has gained much attention, because JNK activation plays a critical role in the lapatinib-resistance in HER2-positve breast cancer cells[11,12]. However, regarding GC, the biological significance of JNK in relation to HER2 signaling has not been reported. Thus, the role of JNK and its interaction with other signaling molecules in HER2-positive GC need to be investigated.

It has been shown that AKT promotes cell migration and invasion of GC cells *in vitro*[13] and that Akt activation significantly correlates with HER2 expression in GC specimens[14]. Although the association between AKT and JNK in various cancer cells has been reported previously[10,15-17], the results have been inconsistent. In breast cancer cells, AKT induced JNK activation[15]. In lung cancer cells, JNK induced AKT activation[16]. In glioblastoma cells, there was crosstalk between

JNK and AKT[10]. In addition, in osteosarcoma cells, JNK inhibited AKT activation[17]. Thus, these findings indicate that the association between JNK and AKT could be different according to the cancer cell type investigated. Thus, interaction of these two molecules in terms of cancer cell metastasis of each cancer type needs to be elucidated. However, the relationship between JNK and AKT in GC has not been described previously.

The present study performed a large scale immunohistochemical analysis and examined the associations between the activation of HER2, JNK and AKT in 423 GC specimens. In addition, we evaluated the *in vitro* effect of these molecules alone or in combination on the metastatic potential of HER2-positive GC cell lines SUN-216 and NCI-N87. Furthermore, the effect of JNK/AKT inhibition on epithelial-mesenchymal transition (EMT) of these cell lines were investigated since previous studies have demonstrated that EMT plays a critical role in not only tumor metastasis but also drug resistance[18].

## MATERIALS AND METHODS

### *Patients and tissue array methods*

A total of 423 surgically resected gastric carcinoma cases were obtained from the Department of Pathology, Seoul National University College of Medicine from 2 January to 29 December, 2006. Eight paraffin tissue array blocks were prepared as previously described[19]. Briefly, core tissue biopsies (2 mm in diameter) were taken from individual paraffin-embedded gastric tumors (donor blocks) and arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus. Each tissue block was able to contain up to 60 cases, allowing eight array blocks to contain 423 cases. The staining results of the different intratumoral areas of gastric carcinomas in these tissue array blocks showed an excellent agreement[20]. A core was chosen from each case for analysis. We defined an adequate case as a tumor occupying more than 10% of the core area. Sections of 4 μm thicknesses were cut from each tissue array block, deparaffinized, and rehydrated. This protocol was reviewed and approved by the Institutional Review Board of Seoul National University.

### *Immunohistochemistry*

Immunohistochemistry was performed after antigen retrieval using a Bond-max automated immunostainer (Leica Microsystems, Newcastle, United Kingdom). The primary antibodies used were against HER2 (1:100, DAKO, Glostrup, Denmark), active form of JNK phosphorylated at Thr183 and Tyr185 (pJNK) (1:50, Cell Signaling Technology, Beverly, MA, United States) and active form of AKT phosphorylated at Ser473 (pAKT) (1:100, New England Biolabs, Beverly, MA, United States). Antibody binding was detected with the Bond Polymer Refine Detection kit (Leica Microsystems). All immunostained sections were then lightly counterstained with Mayer's haematoxylin. Throughout the above analysis, negative controls were prepared by omitting the primary antibody.

For statistical analysis, the results of immunostaining were considered positive if immunoreactivity (nuclear pJNK, and nuclear and cytoplasmic pAKT) was seen in $\geqslant$ 10% of the tumor cells, as described in previous studies[20,21]. Regarding HER2 immunostaining, immunoreactivity was scored in accordance with the HER2 scoring system for GC as described in a previous study[22]. Briefly, cases showing weak to strong staining of the entire or basolateral membrane in $\geqslant$ 10% of the tumour cells were considered HER2 immuno-positive.

### *Cell cultures*

Human GC cell lines SNU-216 and NCI-N87 were purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI1640 (Life Technologies, Grand Island, NY, United States) supplemented with 10% fetal bovine serum (FBS), 2 mg/mL sodium bicarbonate, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technoloiges) at 37 ℃ in a humidified 95% air and 5% $CO_2$ atmosphere.

### *Lentivirus-mediated short hairpin RNA (shRNA) silencing of HER2*

Lentiviral particles containing non-targeting shRNA or HER2 shRNA were purchased (Sigma, St. Louis, MO, United States). The sequence of HER2 shRNA was 5′-CCGGTGTCAGTATCCAGGCTTTGTACTCGAG TACAAAGCCTGGATACTGACATTTTTG-3′. The control shRNA particles contain four base pair mismatches within the short hairpin sequence to any known human or mouse gene. Viral infection was performed by incubating GC cells in the culture medium containing lentiviral particles for 12 h in the presence of 5 μg/mL Polybrene (Santa Cruz Biotechnology, Santa Cruz, CA, United States). Pooled puromycin (2 μg/mL)-resistant cells were used for further analysis.

### *Western blot*

Cell lysates were prepared in 100-200 μL of 1 × sodium dodecyl sulfate (SDS) lysis buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 0.004% bromophenol blue, and 20% glycerol]. Protein contents were measured using BCA Protein Assay Reagent (Pierce, Rockford, IL, United States). Equal amounts of proteins were separated on an 8% discontinuous SDS-polyacrylamide gel and electrophoretically transferred to PVDF membranes (Millipore Corporation, Billerica, MA, United States) blocked with 5% nonfat dry milk in phosphate-buffered saline-Tween 20 (0.1%, v/v) for 1 h. The membranes were then incubated at 4 ℃ overnight. The primary antibodies used were against phospho-HER2$^{Tyr1221/1222}$ (1:1000, Cell Signaling Technology), HER2 (1:1000, Cell Signaling

Technology), pJNK (1:1000, Cell Signaling Technology), JNK (1:1000, Cell Signaling Technology), E-cadherin (1:1000, BD Biosciences, San Jose, United States), Snail (1:1000, Santa Cruz Biotechnology), Vimentin (1:1000, Neomarkers), pAKT (1:1000, Cell signaling Technology), matrix metalloproteinase (MMP9) (1:1000; Neomarkers, Fremont, CA, United States) and β-actin (1:1000, Santa Cruz Biotechnology). Horse-radish peroxidase-conjugated anti-rabbit IgG (1:4000, Santa Cruz Biotechnology) or anti-mouse IgG (1:4000, Santa Cruz Biotechnology) was used as a secondary antibody. Enhanced chemiluminescence (Pierce) was used to detect the immunoreactive proteins. Equal protein loading was confirmed by β-actin.

### *Immunofluorescence staining*

SNU-216 cells (1 × $10^4$ cells/well) were cultured on 4-well chamber slide (Thermo Scientific, Rockford, IL, United States). After 24 h, cells were fixed with 4% paraformaldehyde for 10 min, and blocked with 5% normal donkey serum containing 0.5% Triton X-100 for 5 min. Cells were incubated overnight at 4 ℃ with mixture of the following primary antibodies: rabbit anti-HER2 (1:200; Cell Signaling Technology) and mouse anti-pJNK (1:200, Santa Cruz Biotechnology). Alexa fluor-555-conjugated anti-rabbit IgG (1:200, Life Technologies) and Alexa fluo-488-conjugated anti-mouse IgG (1:200, Life Technologies) were used as secondary antibodies. To examine whether JNK inhibition reorganizes cytoskeleton, filamentous actin (F-actin) was visualized. Cells were incubated with 165 nmol/L Alexa Fluor-633-conjugated phalloidin (Invitrogen, Carlsbad, CA, United States) for 10 min, followed by 4'6'-diamidio-2-phenoylindole (DAPI) staining. Immunofluorescence was observed under a fluorescence microscope.

### *Pharmacological inhibition of JNK and AKT*

Cells were seeded and allowed to attach for 24 h. To inhibit endogenous JNK activity, cancer cells were treated with a specific JNK inhibitor SP600125 (20 μmol/L for SNU-216 and 30 μmol/L for NCI-N87) (Cell Signaling Technology) dissolved in dimethylsulfoxide (DMSO). For AKT inhibition, cells were treated with 20 μmol/L of a PI3K/AKT inhibitor LY294002 (Cell Signaling Technology) dissolved in DMSO, as described in previous study[23].

### *Semi-quantitative reverse transcription-polymerase chain reaction*

Semiquantitative reverse transcription-polymerase chain reaction (SQ RT-PCR) was performed to determine the transcript level of HER2 in human gastric cancer cells, and the amplification of β-actin transcripts was used as the control to normalize the transcript levels of HER2. Total RNAs were isolated using TRIZOL reagent (Invitrogen), and reverse transcription was performed to synthesize cDNAs in a 20 μg reaction mixture containing each gene-specific primer, 1 μg RNA, 2 × reaction buffer, 0.4 μg Taq polymerase, and 1.2 mM MgCl2. The cDNAs of HER2 transcripts were all amplified for 20 cycles (denaturation at 94 ℃ for 30 s, annealing at 52 ℃ for 30 s, and extension at 70 ℃ for 30 s), and the cDNAs of b-actin transcripts were amplified for 18 cycles (94 ℃ for 30 s, 52 ℃ for 30 s, and 70 ℃ for 30 s). The PCR cycling numbers had been optimized to avoid the amplification saturation. Then, 5 μL of RT-PCR product was separated on 1% agarose gels, which were subsequently stained with ethidium bromide. Primer sequences were 5'-GGGAGAGAGTTCTGAGGATT-3' and 5'-CGTCCGTAGAAAGGTAGTTG-3' for HER2, and 5'-ACACCTTCTACAATGAGCTG-3' and 5'-CATGATGGAGTTGAAGG TAG-3' for β-actin.

### *Cell invasion and migration assay*

A 24-well Insert System with an 8 μm pore size polyethylene terephthalate membrane was purchased from BD Biosciences. Transwell inserts were coated with Matrigel, followed by rehydration with medium for 2 h. Ten percent FBS-containing medium was placed in the lower chambers to be used as a chemoattractant. SNU-216 cells (1 × $10^4$ cells/insert) or NCI-N87 cells (5 × $10^4$ cells/insert) in 300 μL volume of 1% FBS-containing medium. After incubation for 48 h at 37 ℃, non-invasive cells were removed with a cotton swab. Invasive cells on the bottom surface of the insert were stained with 0.2% crystal violet in 20% methanol for 30 min and were photographed with an inverted microscope. Stained cells were lysed with 10% SDS for 30 min, and absorbance was measured at 570 nm using an ELISA reader (Bio-Rad) as described previously[6]. Migration assays were performed the same way as the invasion assays, using Transwell compartment except that Matrigel was not included[6].

### *Assessment of cell growth*

SNU-216 (2 × $10^4$ cells/each well) and NCI-N87 cells (5 × $10^4$ cells/each well) were seeded into 24-well plates and were allowed to grow for 3 d. Cell numbers were measured indirectly by using the crystal violet assay as reported by Kim *et al*[24]. Briefly, cells were stained with 0.2% crystal violet aqueous solution in 20% methanol for 10 min, dissolved in 10% SDS, transferred into 96-well plates, and the absorbance was measured at 570 nm using an ELISA reader (Bio-Rad, Hercules, CA, United States).

### *Statistical analysis*

For tissue array analysis, statistical analyses were conducted using SPSS version 11.0 statistical software program (SPSS, Chicago, IL, United States), and the $\chi^2$ test was used to determine the correlations between the expressions of HER2, pJNK and pAKT.

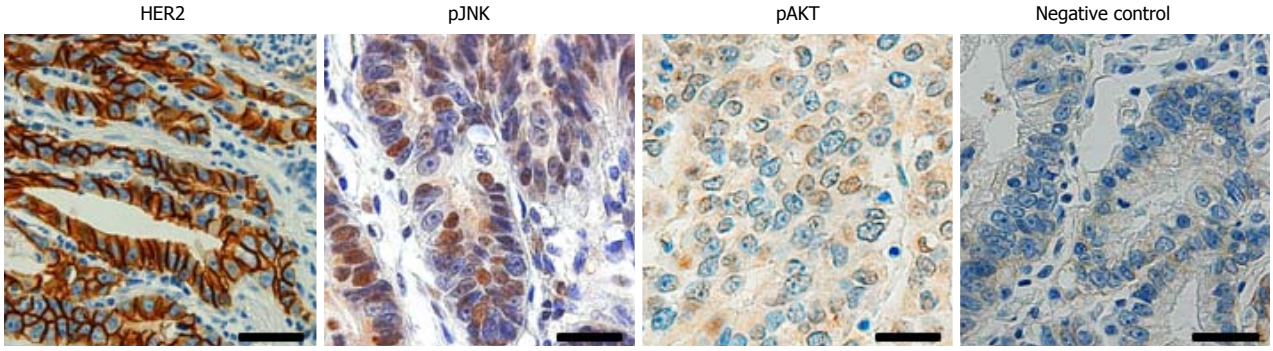



**Figure 1 Representative immunohistochemical features of HER2, pJNK and pAKT in human gastric cancer specimens.** HER2-positive, pJNK-positive, pAKT-positive and negative control treated without primary antibodies. Original magnification, × 400. Bars: 100 μm.

**Table 1 Correlation between expressions of human epidermal growth factor receptor 2, phospho-Thr183 and Tyr185-c-Jun N-terminal kinase and phosphor-Ser473-protein kinase B in human gastric cancer specimens**

| | HER2 (*n*) | | Total |
|---|---|---|---|
| | Positive | Negative | |
| Total | 56 | 367 | 423 |
| pJNK | | | |
| Positive | 24 | 106 | 130 |
| Negative | 32 | 261 | 293 |
| pAKT | | | |
| Positive | 9 | 27 | 36 |
| Negative | 7 | 340 | 347 |
| | pJNK (*n*) | | Total |
| | Positive | Negative | |
| Total | 130 | 293 | 423 |
| pAKT | | | |
| Positive | 17 | 19 | 36 |
| Negative | 113 | 274 | 387 |

[a]*P* value was statistically significant. HER2: Human epidermal growth factor receptor 2; pJNK: Phospho-Thr183 and Tyr185-c-Jun N-terminal kinase; pAKT: Phosphor-Ser473-protein kinase B.

For cell culture experiments, data were analyzed using GraphPad Prism software for Windows 7 (version 4; GraphPad Software, San Diego, CA, United States), and the significances of the results were determined by the two-tailed Student's *t*-test. *P* values of $< 0.05$ were considered statistically significant for all statistical analyses.

## RESULTS

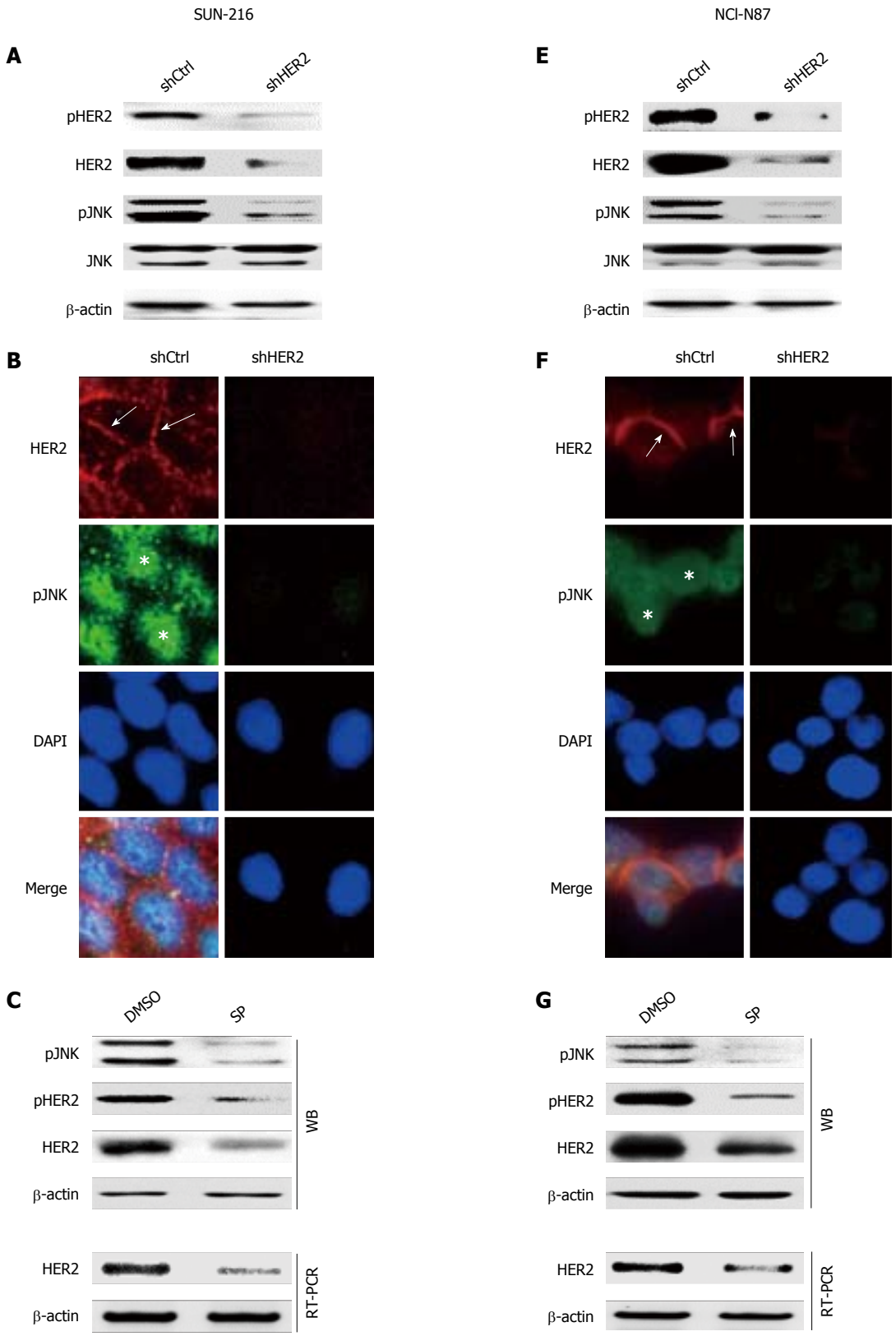
### *HER2, pJNK and pAKT are positively correlated with each other in GC specimens*

To investigate the association between HER2, JNK and AKT in human GC, immunohistochemical tissue array analysis of 423 human GC specimens was performed. Figure 1 shows the representative findings of the immunohistochemical stainings. Cancer cells with membranous HER2 expression were considered to exhibit HER2 activation, and those with nuclear staining of pJNK, regardless of cytoplasmic staining, were considered to exhibit JNK activation. For pAKT staining, immunoreactivity in both nucleus and cytoplasm was interpreted to show AKT activation. We found positive immunoreactivity for membranous HER2 in 56 (14%), pJNK in 130 (31%) and pAKT in 36 (8%) of 423 GC cases, respectively. Data concerning the correlations between the expressions of membranous HER2, nuclear pJNK and pAKT are summarized in Table 1. HER2 activation was found to be positively correlated with JNK activation ($P = 0.035$) and AKT activation ($P = 0.029$). In addition, JNK activation was also correlated with AKT activation ($P = 0.025$).

### *Positive crosstalk exists between HER2 and JNK in GC cells*

Although a current study[12] reported that HER2 inhibition suppressed JNK activation in HER2-positive breast cancer cells, the relationship between these molecules could be different according to cellular context and cell type. To investigate the direct effect of HER2 on JNK activation, we performed *in vitro* experiments. Since HER2 protein expression in GC cell lines varied, we selected GC cell lines SNU-216 (Figure 2A-D) and NCI-N87 (Figure 2E-H) showing a high level of HER2 expression[6]. To investigate the relationship between HER2 and JNK in GC cells, we first produced stable cell lines infected with lentiviral particles containing non-targeting (control) or HER2-targeting shRNA. Western blot (Figure 2A and E) confirmed that HER2 shRNA overexpression downregulated HER2 activation (manifested by pHER2 expression) and expression in both cell lines. HER2 silencing also decreased JNK activation (manifested by pJNK expression), but not expression. Additionally, double immunofluorescence staining for HER2 and pJNK were performed (Figure 2B and F). In both cell lines, control shRNA cells showed immunofluorescence for HER2 at the plasma membrane (red) and pJNK staining in the nucleus (green). In contrast, cells with HER2 silencing showed reduced immunofluorescence for both HER2 and pJNK compared to the control cells.

Next, we examined whether JNK has a role in HER2 activation and expression. Western blot and RT-PCR (Figure 2C and G) showed that protein expressions of


SUN-216
NCI-N87
A
shCtrl
shHER2
pHER2
HER2
pJNK
JNK
β-actin
E
shCtrl
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HER2
pJNK
JNK
β-actin
B
shCtrl
shHER2
HER2
pJNK
DAPI
Merge
F
shCtrl
shHER2
HER2
pJNK
DAPI
Merge
C
DMSO
SP
pJNK
pHER2
HER2
β-actin
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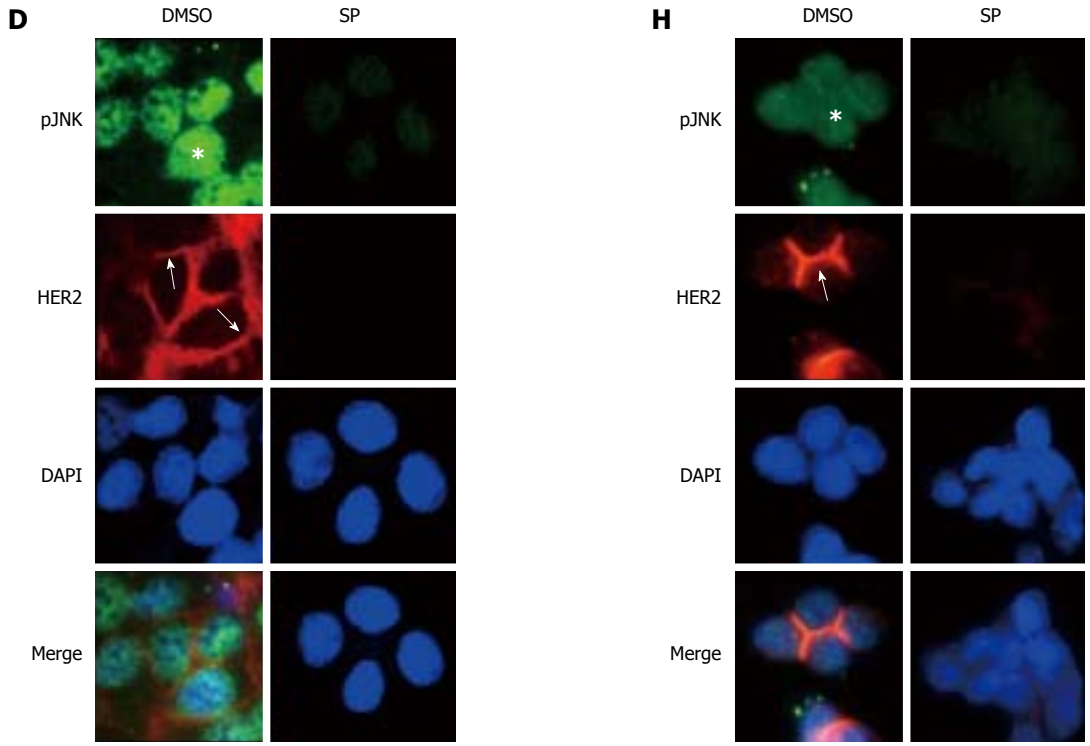


**Figure 2 Relationship between HER2 and JNK in SNU-216 and NCI-N87 cells.** A, B, E and F: Cancer cells were infected with a lentivirus containing either control shRNA (shCtrl) or HER2 shRNA (shHER2); A, E: Protein expressions of pHER2, HER2, pJNK and JNK were determined by Western blot; B, F: Double immunofluorescence staining for HER2 (red, arrows) and pJNK (green, asterisks) was performed. Cell nuclei were visualized by DAPI staining (blue). Original magnification, × 400; C, D, G, H: Cells were treated with either DMSO (vehicle control) or SP600125 (SP); C, G: Protein expressions of pJNK, pHER2 and HER2 were determined by Western blot (WB) and HER2 mRNA expression was determined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR); D, H: Double immunofluorescence staining for pJNK (green, asterisks) and HER2 (red, arrows) was performed. Cell nuclei were visualized by DAPI staining (blue). Original magnification, × 400.

pHER2 and HER2 as well as HER2 mRNA expression were substantially decreased by SP600125 treatment. Consistently, immunofluorescence staining (Figure 2D and H) revealed that SP600125-treated cells showed faint stainability for both pJNK and HER2 compared to DMSO control cells. Taken together, these results indicate that JNK controls and is controlled by HER2 with a positive relationship.

### *Pharmacological JNK inhibition decreases migration, invasion and EMT of HER2-positive GC cells*

To evaluate the effect of JNK on the metastatic potential of HER2-positive GC cells, Transwell assay was performed. Figure 3A shows that SP600125 treatment for 48 h significantly suppressed the cancer cell migration (by 43%, $P$ = 0.0288) and invasion (by 39%, $P$ = 0.005) of SNU-216 cells compared to DMSO control cells. Consistent results were shown in NCI-N87 cells (Figure 3D).

In the initial steps of metastasis of carcinoma cells, epithelial cancer cells change their phenotype to mesenchymal phenotype and become motile and invasive by a process called EMT[25]. To examine whether JNK activation is related to EMT phenotype of cancer cells, Western blot (Figure 3B and E) was performed. After SP600125 treatment for 24 h, the expression of the representative epithelial marker E-cadherin enhanced, whereas the expressions of mesenchymal markers Snail, Vimentin and MMP9 reduced. To further confirm these results, immunofluorescence staining was performed. Since actin-dependent membrane protrusions are regarded as a critical determinant of EMT[26], we examined actin organization. Staining of F-actin with fluorescein isothiocynate-conjugated phalloidin revealed that SP600125 treatment induced apparent changes in actin organization, leading to the loss of many filopodia-like cellular projections shown in DMSO control cells (Figure 3C and F).

### *Pharmacological JNK inhibition combined with HER2 shRNA transfection exerts an additive effect on the metastatic potential*

The above results indicate that JNK enhances metastatic potential and that positive crosstalk exists between HER2 and JNK in GC cells. To investigate whether the combination of JNK and HER2 has an additive effect on GC cell metastasis, we performed dual inhibition of these molecules. Western blot showed that combination of HER2 downregulation and SP600125 treatment induced lower protein expressions of pHER2 and pJNK than individual inhibitions (Figure 4A and D). Consistently, cell migration assay using SNU-216 cells (Figure 4B) showed that SP600125

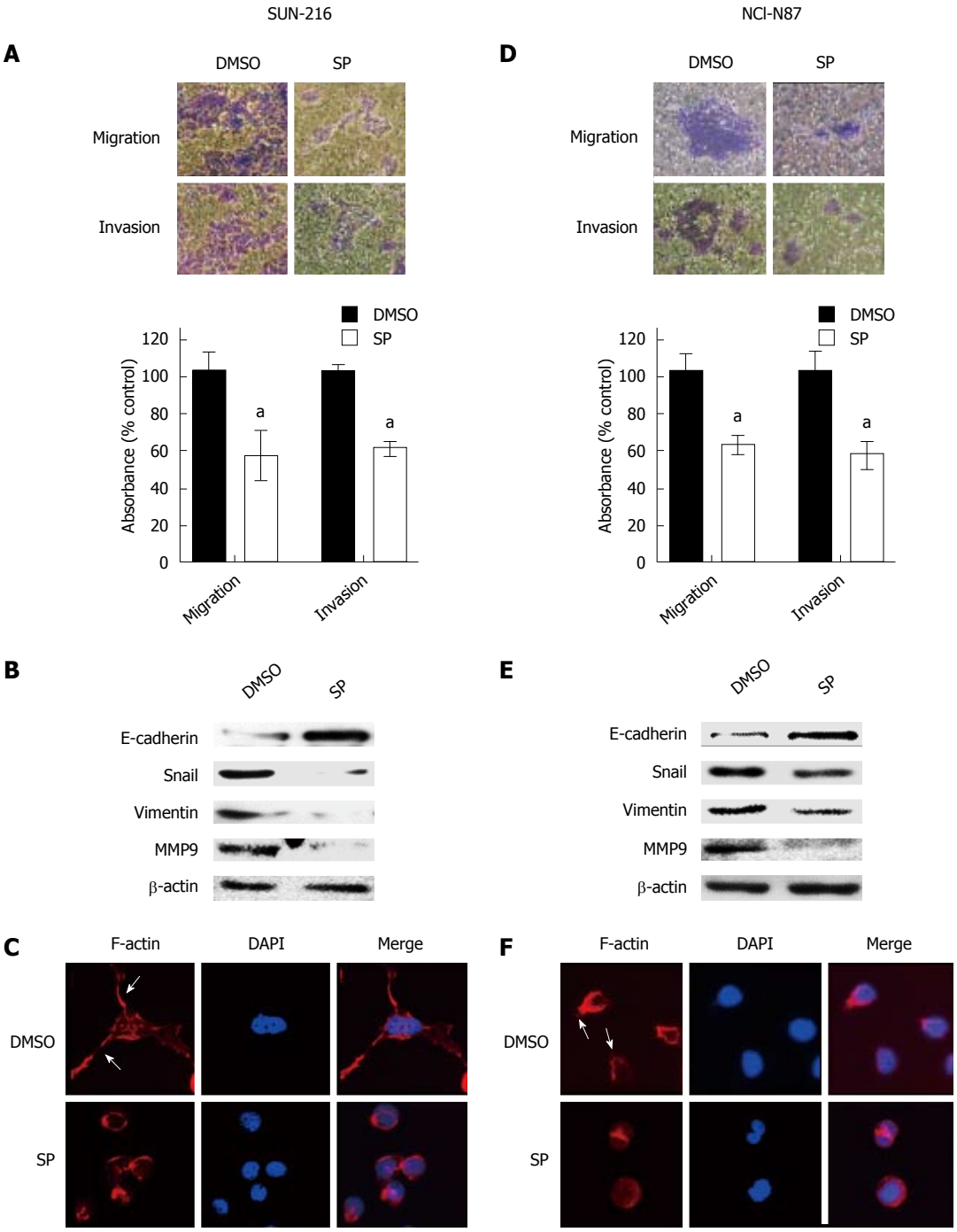


**Figure 3 Effect of pharmacological inhibition of JNK on cell migration, invasion, EMT marker expressions and actin cytoskeleton organization.** SNU-216 and NCI-N87 cells were treated with either DMSO or SP600125 (SP). A, D: Cell migration and invasion were analyzed by Transwell assay followed by cell viability assessment using the crystal violet assay. Representative images of migrated/invasive cells taken 48 h after plating into a transwell insert are on the upper, and the quantification of migrated/invasive cells is on the lower. Results were calculated as percentages relative to DMSO vehicle control. Data are expressed as mean ± SD ($n$ = 4 per each group). $^{a}P < 0.05$ *vs* DMSO vehicle control; B, E: EMT marker expressions were determined by Western blot; C, F: The organization of the actin cytoskeleton was determined by immunofluorescence staining. Alexa Fluor 633-conjugated phalloidin was used to visualize F-actin (red), and DAPI staining (blue) was used for visualization of cell nuclei. Arrows indicate the FITC-labelled filopodia-like projections. Photographs were taken with a fluorescence microscope. Original magnification, × 400.

treatment decreased cell motility by 49%, and HER2 downregulation by 47% compared to control cells. Dual inhibition of HER2 and JNK further decreased cell motility by 61% compared to control cells. Similar results were shown in the invasion assay (by 63% in cells with dual inhibition compared to control cells) (Figure 4C). Consistent results were shown in the experiments using NCI-N87 cells (Figure 4E and F).

### *JNK activation is regulated by AKT activation in HER2-positive GC cells*

In GC, AKT has also been previously described as a

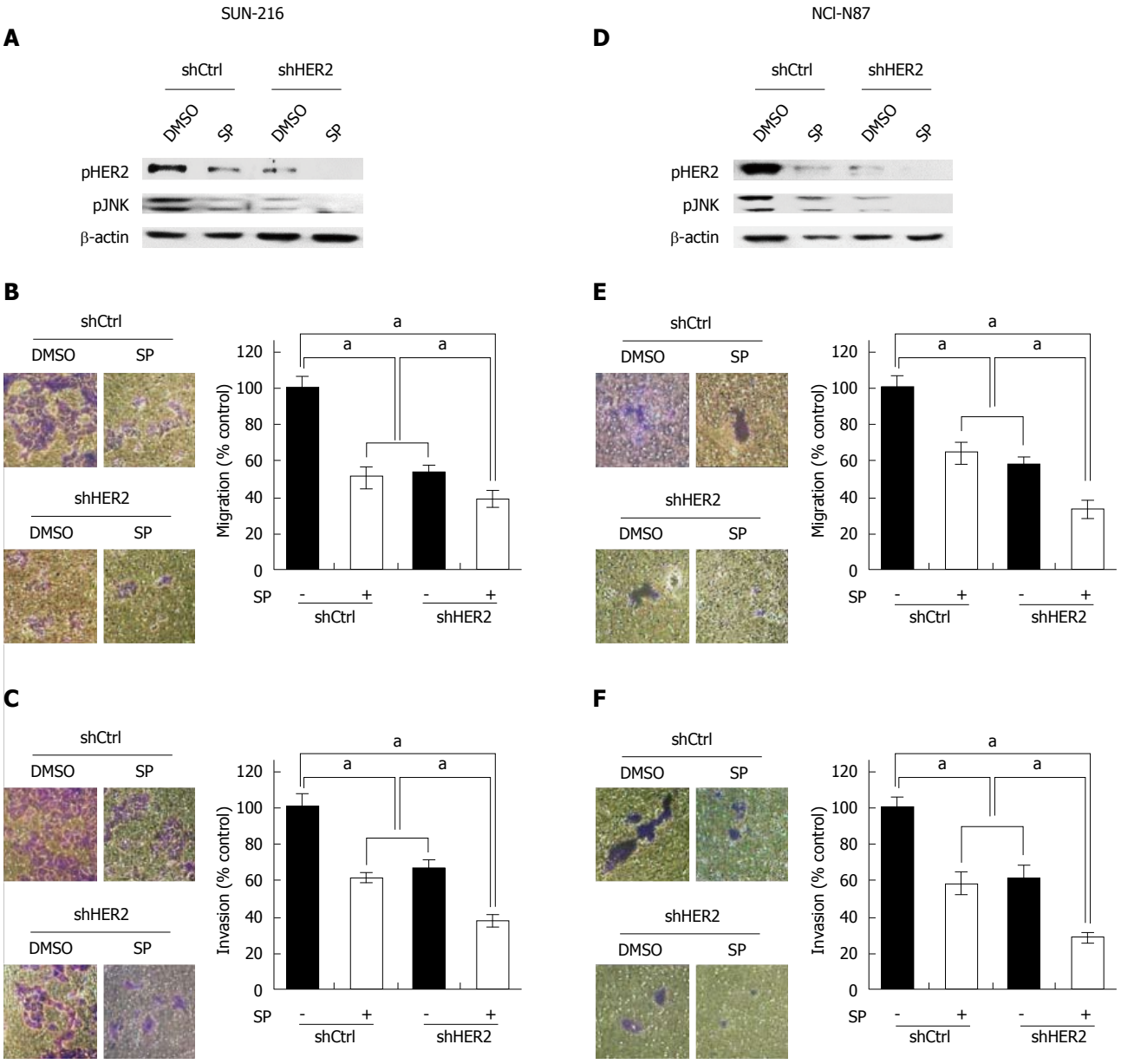


**Figure 4 Combined effects of HER2 silencing and JNK inhibition on metastatic potential.** SNU-216 and NCI-N87 cells were infected with a lentivirus containing either control shRNA (shCtrl) or HER2 shRNA (shHER2) followed by treatment with either DMSO or SP600125 (SP). A, D: After treatment with SP for 24 h, protein expressions of pHER2 and pJNK were determined by Western blot; B, E: After treatment with SP for 48 h, cell migration was evaluated by Transwell migration assay; C, F: After treatment with SP for 48 h, invasion was evaluated by cell invasion assay. Results were calculated as percentages relative to control cells. Data are expressed as mean ± SD ($n$ = 4 per each group). $^{a}P < 0.05$ *vs* control cells.

downstream effector of HER2 and becomes upregulated in response to HER2 oncogene activation[27,28]. Therefore, we investigated whether AKT is involved in HER2/JNK pathway in GC cell lines SNU-216 and NCI-N87. Our data showed that AKT activation decreased in HER2 shRNA transfectants compared to control shRNA transfectants (Figure 5A and E).

Next, we observed the correlation between JNK and AKT in these cells. Western blot showed that SP600125 treatment did not change AKT activation manifested by pAKT expression (Figure 5B and F). In contrast, AKT inhibition by treatment with a PI3K/AKT inhibitor LY294002 decreased JNK activation in both cell lines (Figure 5C and G). These results were confirmed by dual inhibition of AKT and JNK (Figure 5C and G). These data indicate that AKT is an upstream effector of JNK in HER2/JNK pathway in relation to metastatic potential of HER2-positive GC cells. Additionally, we examined the effect of AKT inhibition on the EMT marker expressions (Figure 5D and H). Western blot showed that LY294002 treatment increased E-cadherin expression but decreased Snail expression, which demonstrated that AKT increases mesenchymal phenotype.

### *JNK and AKT co-operatively induce migration of HER2-positive GC cells*

To determine the combined effect of JNK and AKT on

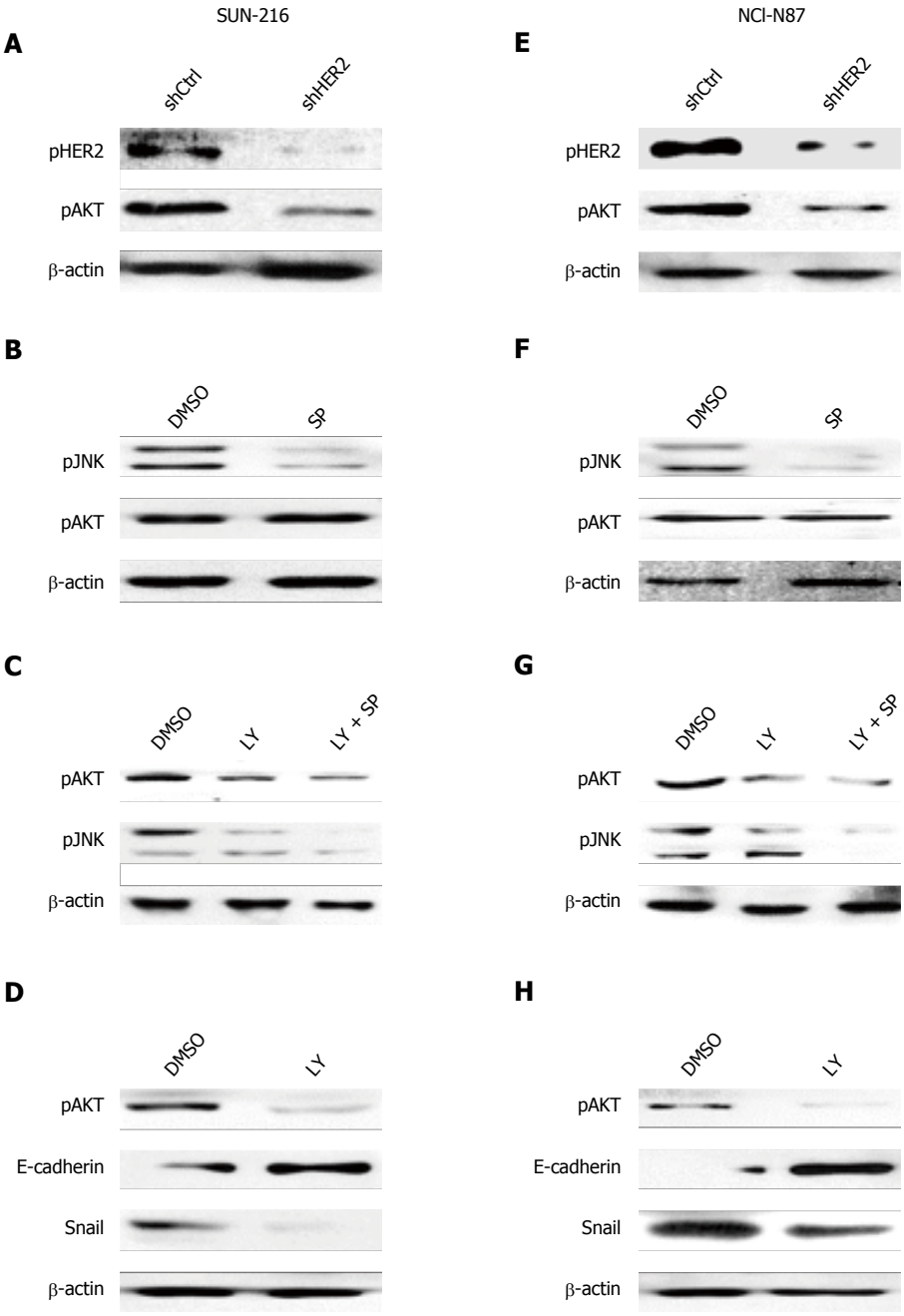


**Figure 5 Association between AKT and JNK as well as EMT marker expressions in SNU-216 and NCI-N87 cells.** Western blot was performed to determine protein expressions of pHER2, pAKT, E-cadherin, Snail and pJNK. A, E: Cells were infected with a lentivirus containing either control shRNA (shCtrl) or HER2 shRNA (shHER2); B, F: Cells were treated with either DMSO or SP600125 (SP); C, G: Cells were treated with LY with or without SP; D, H: Cells were treated with either DMSO or LY294002 (LY).

the metastatic potential of HER2-positive GC cells, Transwell migration assay was performed (Figure 6A and B). We found that cell migration capacity was significantly suppressed by treatment with either LY294002 (by 46% in SNU-216 cells and by 50% in NCI-N87 cells) or SP600125 (by 45% in SNU-216 and NCI-N87 cells). Dual inhibition of both JNK and AKT led to greater inhibition of migration (by 80% in SNU-216 cells and by 79% in NCI-N87 cells) than single molecule inhibition.

### *Both JNK and AKT increased cell growth of HER2-positive GC cell lines*

Since cell proliferation and survival affects the results of cell migration and invasion, we examined the effect of treatment with either SP600125 (Figure 7A and C) or LY294002 (Figure 7B and D) on the growth of SNU-216 and NCI-N87 cells. We found that both

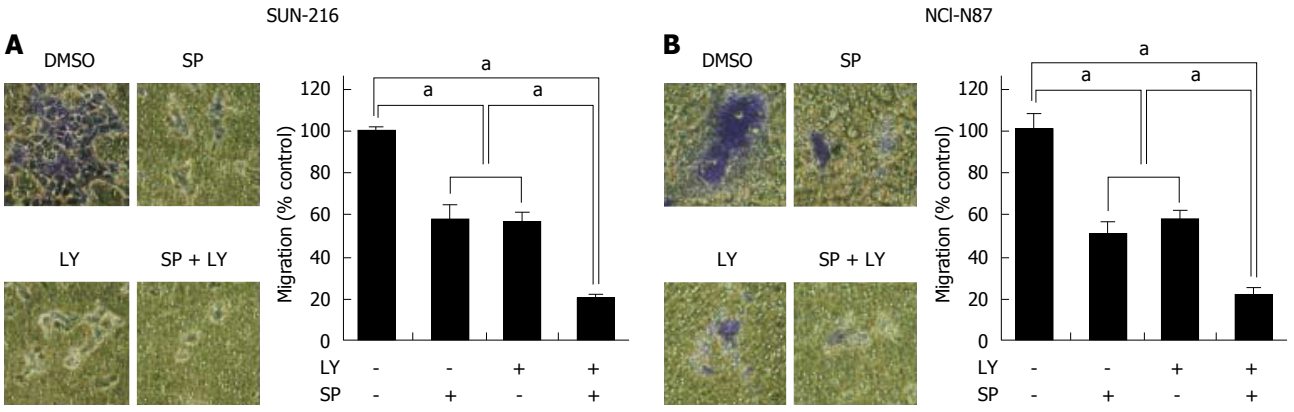


**Figure 6 Synergistic effect of JNK and AKT on metastatic potential of HER2-positive gastric cancer cells.** Combined effects of AKT and JNK on the metastatic potential of SNU-216 and NCI-N87 were determined by Transwell migration assay. A, B: Cells were treated with DMSO or SP600125 (SP) in the presence or absence of LY294002 (LY). Results were calculated as percentages relative to DMSO vehicle control. Data are expressed as mean ± SD ($n$ = 4 per each group). $^{a}P < 0.05$ *vs* DMSO vehicle control.

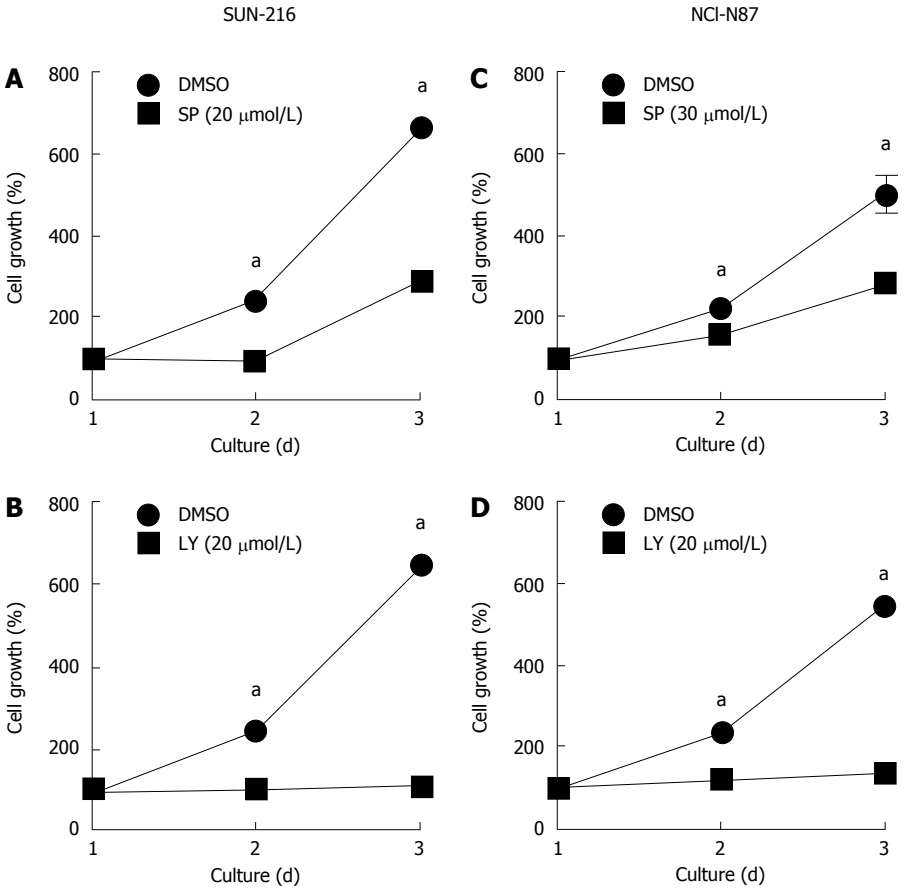


**Figure 7 Effect of JNK or AKT on cell growth of human epidermal growth factor receptor 2-positive gastric cancer cell lines.** A, C: GC cell lines were treated with either DMSO or SP600125 (SP); B, D: Cells were treated with either DMSO or LY294002 (LY). Cell growth rates were analyzed using crystal violet assay on the indicated times, and absorbance was measured. Results were calculated as percentages relative to DMSO vehicle control. Data are expressed as mean ± SD ($n$ = 4 per each group). $^{a}P < 0.05$ *vs* DMSO vehicle control.

inhibitors suppressed cell growth of these cell lines compared to DMSO (vehicle) control.

## DISCUSSION

Understanding the oncogenic signaling pathways may lead to the development of therapeutic strategies for cancer treatment. In GC, the pivotal role of HER2 metastasis has been shown[6,29], but not much is known about the downstream effectors in this process. Although most studies have implicated AKT and ERK as promising therapeutic targets for HER2-positive tumors[27,28,30], JNK, especially in breast cancer, is now emerging as an important molecule in HER2 signaling

pathways[11,12]. In the present study, we found positive associations between HER2, JNK and AKT in terms of GC metastasis. This is the first study, to the best of our knowledge, to show the associations between JNK and HER2/AKT pathway in GC.

In the present study, immunohistochemical tissue array analysis of 423 GC specimens demonstrated constitutive activation of HER2 (14%), JNK (31%) and AKT (8%), which were positively related with each other. In addition, cell culture experiments using HER2-positive GC cell lines SNU-216 and NCI-N87 showed that HER2 silencing by RNA interference reduced JNK activation (manifested by pJNK expression), but not JNK expression. On the other hand, pharmacological inhibition of JNK reduced not only HER2 activation (manifested by pHER2 expression) but also HER2 protein and mRNA expressions. These results indicate that there is a positive reciprocal regulatory loop between HER2 and JNK, and that JNK increases HER2 expression at the transcriptional level, possibly through the regulation of the transcription factor. To the best of our knowledge, this is the first report on crosstalk between HER2 and JNK in the regulation of human cancer cells, including GC cells.

The inter-relationship between the PI3K and MAPK pathways is complex and incompletely understood[30]. Although we previously found that both JNK and AKT are overexpressed in GC tissue specimens[20], the relationship between these two molecules in GC has not been reported. In the present study, we found a positive association between the activations of JNK and AKT in human GC tissue specimens. In addition, cell culture experiments showed that treatment of HER2-positive GC cells with a PI3K/AKT inhibitor LY294002 decreased JNK activation, whereas JNK did not modulate AKT activation. Since HER2 downregulation suppressed AKT activation, it seems that HER2 inhibited JNK activation in GC cells through PI3K/AKT signaling.

In the present study, we found that pharmacological inhibition of JNK decreased the expressions of Snail, Vimentin and MMP9, but increased the expression of an epithelial marker E-cadherin. Moreover, SP600125 treatment decreased cancer cell migration and invasion. Since similar effects on EMT were induced by both HER2[6] and AKT, our results indicate that both JNK and AKT might contribute to malignant progression, including metastasis and drug resistance, of HER2-positive GC cells.

Although targeted therapies may increase patient selectivity and treatment efficacy, mostly their effects are not durable when they are used alone. For this reason, combination therapies are often needed for effective treatment of malignant tumors. In the present study, we found that treatment with either SP600125 or LY294002 significantly reduced metastatic potential of HER2-positive GC cells to the similar level, and that co-treatment with both of these inhibitors induced a further decrease compared to treatment with either alone. Thus, our findings suggest that combined targeting of JNK and AKT significantly impairs GC cell migration in concert with HER2 downregulation. However, additional animal experiments to evaluate these inhibitions are needed.

In the present study, we found that either inhibition of JNK/AKT suppresses cell growth in both HER2-positive GC cell lines. Thus, JNK/AKT-induced cell growth of these cell lines might affect the results of cell migration and invasion assays observed in the present study. However, our results also indicate that inhibition of JNK/AKT decreases mesenchymal phenotype in individual GC cells based on the EMT marker expressions. Thus, we speculate that JNK/AKT contributes to metastatic potential as well as cell growth of HER2-positive gastric cancer cells.

In conclusion, our results showed that JNK and AKT are co-expressed in a subset of HER2-positive GC cases, and that HER2, JNK and AKT are positively associated with each other. In addition, inhibition of either JNK or AKT decreased cancer cell motility of HER2-positive GC cells through reversing EMT. Since dual inhibition of JNK and AKT induced more profound effect on cancer cell motility, combined targeting of these molecules might be used to regulate the GC metastasis in a subgroup of GC patients.

## COMMENTS

### Background

Human epidermal growth factor receptor 2 (HER2/ERBB2/neu), a member of the epidermal growth factor receptor family of receptor tyrosine kinases, is overexpressed in 7%-34% of gastric cancer (GC) cases.

### Research frontiers

The present study performed a large scale immunohistochemical analysis and examined the associations between the activation of HER2, JNK and AKT in 423 GC specimens. In addition, the authors evaluated the *in vitro* effect of these molecules alone or in combination on the metastatic potential of HER2-positive GC cell lines SUN-216 and NCI-N87.

### Innovations and breakthroughs

The effect of JNK/AKT inhibition on epithelial-mesenchymal transition (EMT) of these cell lines were investigated since previous studies have demonstrated that EMT plays a critical role in not only tumor metastasis but also drug resistance.

### Peer-review

This manuscript has clearly shown that HER2, JNK and AKT play important roles on invasion of gastric cancer cells. It provided important contribution to the role of anti-HER2 treatment of gastric cancer patients. They performed a large scale analysis and the manuscript was well written. However, certain revisions are required.

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ORIGINAL ARTICLE

Retrospective Cohort Study

# Effect of airplane transport of donor livers on post-liver transplantation survival

Yi Huang, Gerry MacQuillan, Leon A Adams, George Garas, Megan Collins, Albert Nwaba, Linjun Mou, Max K Bulsara, Luc Delriviere, Gary P Jeffrey

Yi Huang, Gerry MacQuillan, Leon A Adams, George Garas, Gary P Jeffrey, School of Medicine and Pharmacology, University of Western Australia, Nedlands, WA 6009, Australia

Gerry MacQuillan, Leon A Adams, George Garas, Megan Collins, Albert Nwaba, Linjun Mou, Luc Delriviere, Gary P Jeffrey, Western Australian Liver Transplantation Service, Sir Charles Gairdner Hospital, Nedlands, WA 6009, Australia

Max K Bulsara, Institute of Health Research, University of Notre Dame, Fremantle, WA 6160, Australia

Author contributions: Huang Y and Jeffrey GP were responsible for study design, data collection and data analysis; Huang Y and Bulsara MK were responsible for statistical analysis of the date; all authors took part in data interpretation, review of the draft report, and content development.

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Informed consent statement: Due to the nature of the study design, no direct contact of participants was required. The informed consent was waived by Sir Charles Gairdner Hospital Human Research Ethics Committee.

Data sharing statement: Additional data are available from the corresponding author at gary.jeffrey@uwa.edu.au.



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Correspondence to: Gary P Jeffrey, MB, BS, MD, FRACP, FRCP, Professor, School of Medicine and Pharmacology, University of Western Australia, 5th Floor, Harry Perkins Institute of Medical Research, 6 Verdun Street, Nedlands, WA 6009, Australia. gary.jeffrey@uwa.edu.au
Telephone: +61-8-93462098
Fax: +61-8-93463098

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## Abstract

***AIM***

To evaluate the effect of long haul airplane transport of donor livers on post-transplant outcomes.

***METHODS***

A retrospective cohort study of patients who received a liver transplantation was performed in Perth, Australia from 1992 to 2012. Donor and recipient characteristics information were extracted from Western Australian liver transplantation service database. Patients were followed up for a mean of six years. Patient and graft survival were evaluated and compared between patients who received a local donor liver and those who received an airplane transported donor liver. Predictors of survival were determined by univariate and multivariate analysis using cox regression.

***RESULTS***

One hundred and ninety-three patients received a

local donor liver and 93 patients received an airplane transported donor liver. Airplane transported livers had a significantly lower alanine transaminase (mean: 45 U/L *vs* 84 U/L, $P = 0.035$), higher donor risk index (mean: 1.88 *vs* 1.42, $P < 0.001$) and longer cold ischemic time (CIT) (mean: 10.1 h *vs* 6.4 h, $P < 0.001$). There was a weak correlation between CIT and transport distance ($r^2 = 0.29$, $P < 0.001$). Mean follow up was six years and 93 patients had graft failure. Multivariate analysis found only airplane transport retained significance for graft loss (HR = 1.92, 95%CI: 1.16-3.17). One year graft survival was 0.88 for those with a local liver and was 0.71 for those with an airplane transported liver. One year graft loss was due to primary graft non-function or associated with preservation injury in 20.8% of recipients of an airplane transported liver compared with 4.6% in those with a local liver ($P = 0.027$).

***CONCLUSION***

Airplane transport of donor livers was independently associated with reduced graft survival following liver transplantation.

**Key words:** Airplane transportation; Cold ischemic time; Graft survival; Donor location; Organ damage



**Core tip:** This study demonstrated a significantly decreased graft and patient survival for patients who received an airplane transported donor liver compared to a local donor liver not requiring airplane transport. The hazard ratio for airplane transported donor livers compared to local donor livers was 1.98 for graft survival and 1.86 for patient survival. The effect of airplane transportation was independent of cold ischemic time.

Huang Y, MacQuillan G, Adams LA, Garas G, Collins M, Nwaba A, Mou L, Bulsara MK, Delriviere L, Jeffrey GP. Effect of airplane transport of donor livers on post-liver transplantation survival. *World J Gastroenterol* 2016; 22(41): 9154-9161 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i41/9154.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i41.9154

## INTRODUCTION

The combination of the large geographic area of Western Australia and relatively small and centralised population has resulted in the need for donor livers to be transported by airplane over long distances. Australian donor allocation policy is based on a regional (state) and national model. Sharing between regions is mandatory for urgent patients and for non-urgent patients sharing occurs when there is no suitable local recipient. As a result a significant number of patients in Perth have received a long distance airplane transported donor liver from other states in Australia and New Zealand. The shortest transport distance was from Adelaide (2140 km) and the longest was from Auckland (5364 km) and this is similar to that between Dallas and Los Angeles and Nuuk (Greenland) and Los Angeles respectively.

It is well established that the cold ischemic time (CIT) has a major effect on donor organ quality and graft survival following liver transplantation and most transplant centres attempt to maintain the CIT less than12 h[1]. One study found that air transport of donor livers for more than 322 km increased CIT and decreased graft survival and it was recommended that long distance transport be avoided if other adverse donor risk factors were present[2]. The donor risk index and other donor risk models that have been developed to predict short term graft survival have used a variety of donor factors that include donor age, body mass index (BMI), time in Intensive Care Unit, use of inotropes, hypernatremia, cause of death, liver function tests, pre-existing donor liver disease, warm ischaemic time, CIT, MELD score and location of donor[3-7]. Interestingly, none have analysed if the type of transport used to transfer the organ could add to the utility of the model. Airplane transport is commonly used for long distance donor liver transportation, but its unique conditions such as low cabin pressure (0.7 Atm), reduced partial pressure of oxygen, acceleration and deceleration forces and engine vibrations have the potential to cause damage to donor organs.

The geographic isolation of Perth allows a unique opportunity to evaluate the effect of long distance airplane transport of donor livers on graft and patient survival. The aim of this study was firstly to evaluate the association between airplane donor liver transport distance and CIT and secondarily determine the effects of liver transport type on graft and patient survival.

## MATERIALS AND METHODS

286 patients who had a liver transplant (LT) performed by the Western Australian liver transplant service, Sir Charles Gairdner Hospital from 1992 to 2012 were included. All patients received a donation after brain death donor liver. Exclusion criteria included living donor liver transplantation.

### *Donor organ retrieval*

Donor livers were preserved in cold (4 ℃) UW solution, sealed in two plastic bags and placed in an insulated cooler that contained a slurry of iced water (Figure 1). All Western Australian donor liver retrievals were performed in Perth. Ventilated patients in regional areas of Western Australia are transferred by the Royal Flying Doctor Service to Perth and only Perth based intensive care units will declare brain death. Interstate donor liver retrieval is performed by the regional donor team. The cold stored donor livers are transported by commercial flights (passenger or freight) in the cabin. Charter jets are rarely used due to the expense.

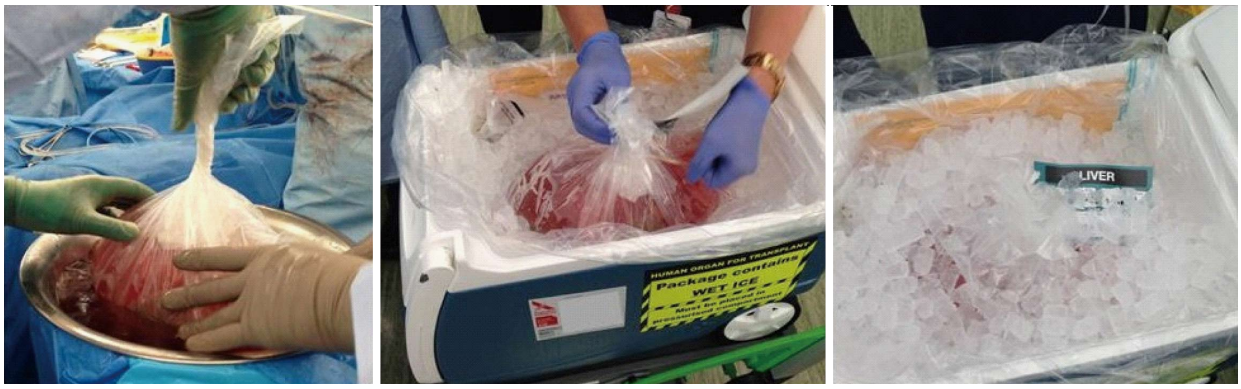


**Figure 1 Donor liver preservation for airplane transport.**

### *Data source*

Clinical data were prospectively recorded and retrospectively extracted from the Western Australian liver transplantation service database. Donor factors collected were regional area of donation, history of airplane travel, age, gender, weight, height, liver function test [alanine transaminase (ALT), aspartate transaminase (AST), bilirubin and alkaline phosphatase(ALP)], blood type, CIT, cause of death, past cytomegalovirus (CMV) infection, smoking/drinking history. The donor risk index (age, cause of death, race, partial/split liver, height, CIT, regional/national share and donation after cardiac death) was also calculated[3]. Recipient factors collected were age, gender, race, weight, height, blood type, MELD score, LT indication, past CMV infection. Follow-up was performed at Sir Charles Gairdner Hospital on all patients till death, re-transplantation or December 2012. The study was approved by the Sir Charles Gairdner Hospital Human Research Ethics Committee.

### *Endpoints and statistical analysis*

The primary endpoints were graft and patient survival. Primary graft non-function was defined as severe and immediate liver dysfunction that lead to death of the patient or re-transplantation during the first seven postoperative days[8]. Primary graft dysfunction was defined as transaminases > 2000 U/L immediately post-LT[9]. Early graft failure was associated with primary graft dysfunction or progressive deterioration of liver function tests from the time of transplantation. Continuous variables were expressed as mean and standard deviation. Mean values between groups were compared using the *t* test. Categorical variables were expressed as count and percentages. Percentages were compared using the $\chi^2$ test. The correlation between transport distance and CIT was assessed using linear regression analysis. Survival was assessed using Kaplan Meier curves and significance determined by the log rank test. Predictors of survival were determined by univariate and multivariate analysis using cox regression. Two sided *P* values of < 0.05 were considered significant.

## RESULTS

Two hundred and eighty-six patients were included: 193 (67%) patients received a local donor liver and 93 (33%) patients received a donor liver airplane transported from other states in Australia or New Zealand. Donor and recipients characteristics are shown in Table 1. Local and airplane transported donor livers were well matched for factors that are known to affect graft and patient survival following liver transplantation. Airplane transported donor livers had a lower mean ALT level (45 U/L *vs* 84 U/L, *P* = 0.035) and a higher mean donor risk index (1.88 *vs* 1.42, *P* < 0.001). There was a trend for less alcohol use in airplane transported donor livers but this was non-significant. Recipients who received an airplane transported donor liver were significantly younger than those who received a local donor liver (50 years *vs* 47 years, *P* = 0.019), had a higher mean MELD score (18.2 *vs* 14.5, *P* = 0.0007) and more often had acute liver failure (16.1% *vs* 2.6%, *P* < 0.001).

Local donor livers had a significantly shorter mean CIT of 6.4 h *vs* 10.1 h for airplane transported livers (*P* < 0.001). Only 4% of local donor livers had a CIT $\geqslant$ 12 h compared to 24% of airplane transported livers. Livers transported from the central states (South Australia, Northern Territory) had a mean CIT of 9.0 h and those from the eastern states (Queensland, New South Wales, Victoria, Tasmania) had a significantly longer mean CIT of 10.7 h (*P* = 0.01). Linear regression analysis found that CIT significantly increased with transport distance with a coefficient of 1.3 (95%CI: 1.1-1.6) per 1000 km, *P* < 0.001 (Figure 2). However the correlation was poor with a model fit (R square value) of 0.295, indicating that other factors apart from transport distance affected CIT. Some of these included availability of commercial flights, flight delays and flight diversions.

Recipients were followed after LT for a mean of 6 years (range: 0.1-19 years). 93 (33%) developed graft failure, 15 (5%) had a repeat LT and 78 (27%) died. The one and 5-year graft survival was 83% and 73% and patient survival was 86% and 76% respectively.

**Table 1 Patient characteristics**

| Characteristics | Local liver transport ($n$ = 193) | Airplane liver transport ($n$ = 93) | $P$ value |
|---|---|---|---|
| Donor characteristics | | | |
| Age (yr) | 41 (16.8) | 44 (14.8) | 0.23 |
| Gender-male/female | 55%/45% | 56%/44% | 0.87 |
| Height (cm) | 171 (11.5) | 172 (9.3) | 0.74 |
| Weight (kg) | 77 (17.4) | 77 (13.4) | 0.96 |
| BMI | 26 (7.5) | 26 (4.2) | 0.66 |
| Bilirubin (μmol/L) | 15 (8.1) | 14 (9.0) | 0.38 |
| ALP (U/L) | 78 (35.4) | 80 (35.8) | 0.73 |
| AST (U/L) | 70 (84.9) | 62 (69.2) | 0.52 |
| ALT (U/L) | 84 (167.6) | 45 (45.8) | 0.035 |
| Smoker | 63% | 64% | 0.94 |
| Etoh drinker | 82% | 69% | 0.07 |
| CMV positive | 57% | 63% | 0.421 |
| Cause of Death - trauma/ cerebrovascular accident/anoxia/other | 37%/52% /9%/2% | 25%/67% /7%/1% | 0.129 |
| Donor risk index | 1.42 (0.35) | 1.88 (0.43) | < 0.0001 |
| Recipient characteristics | | | |
| Age (yr) | 50 (10.8) | 47 (13.4) | 0.019 |
| Gender-male/female | 72%/28% | 63%/37% | 0.175 |
| Non-Caucasian | 19% | 22% | 0.53 |
| MELD score | 14.5 (7.7) | 18.2 (10.2) | 0.0007 |
| Height (cm) | 171 (9.7) | 171 (8.2) | 0.65 |
| Weight (kg) | 78 (15.7) | 75 (16.2) | 0.08 |
| BMI | 27 (4.5) | 26 (4.7) | 0.21 |
| CMV positive | 61% | 65% | 0.58 |
| Transplant indication Acute liver failure/ chronic liver disease | 2.6%/97.4% | 16.1%/83.9% | < 0.001 |
| Transplant factors | | | |
| CIT (h) | 6.4 (2.8) | 10.1 (2.9) | < 0.001 |
| ABO incompatible | 3.4% | 3.8% | 1.00 |

Age, height, weight, BMI, bilirubin, ALP, AST, ALT, MELD score, cold ischemia time and donor risk index were expressed as mean (standard deviation). Other variables were expressed as percentage. Donor allocation, gender, recipient age, gender, transplant indication was available in all patients, Missing data count: donor factors: age: 1; BMI: 50; Bilirubin: 51; ALP: 58; AST: 110; ALT: 54; Smoking history: 113; Drinking history: 107; CMV infection: 19; Cause of death: 24; Donor risk index: 54; Recipient factors: race: 6; MELD score: 5; BMI: 28; CMV infection: 20; Transplantation factors: CIT: 3; Blood type: 27.

Univariate analysis found that airplane donor transport and long CIT were significantly associated with worse graft survival and patient survival (Table 2). After adjusting for potential confounders (donor and recipient age, donor and recipient gender, CIT, transplant indication), multivariate analysis found that only airplane donor transport was significantly associated with decreased graft and patient survival (Table 2). The hazard ratio for airplane transported donor livers compared to local donor livers was 1.98 (95%CI: 1.20-3.27) for graft survival and 1.86 (95%CI: 1.07-3.22) for patient survival. Recipients with airplane transported livers had significantly worse graft survival ($P$ = 0.0005) and patient survival ($P$ = 0.003) than those who received a local liver (Figure 3). One year and five year graft survival was 0.88 and 0.79 for those with a local liver and was 0.71 and 0.61 for those with an airplane transported liver. One year and

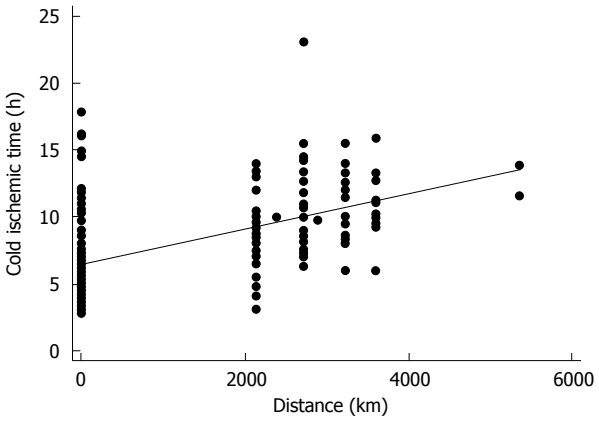


**Figure 2 Correlation between cold ischemic time and liver transport distance.**

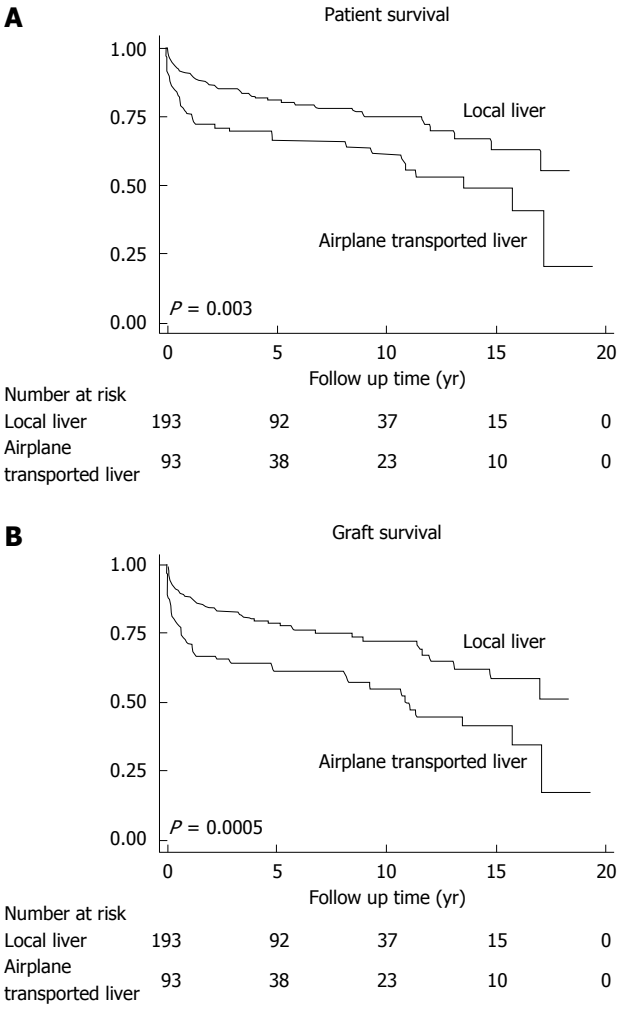


**Figure 3 Post-transplantation outcome for recipient with local liver and those with airplane transported liver.** A: Patient survival; B: Graft survival.

five year patient survival was 0.91 and 0.81 for those with a local donor liver and was 0.76 and 0.66 for those with an airplane transported liver. The significant reduction in graft survival for recipients with an airplane transported liver was observed immediately after liver transplantation with graft loss within seven days of 8.6% (8/93) compared to 1% (2/193) for

**Table 2 Predictors for patient survival and graft survival**

| Factors | HR, 95%CI, *P* value | | | |
|---|---|---|---|---|
| | Patient death | | Graft loss | |
| | Univariate | Multivariate | Univariate | Multivariate |
| Cold ischemic time | 1.07 (1.002-1.14)<br>*P* = 0.041 | 1.04 (0.96-1.13)<br>*P* = 0.300 | 1.07 (1.01-1.14)<br>*P* = 0.018 | 1.04 (0.96-1.11)<br>*P* = 0.348 |
| Airplane transport liver *vs* local liver | 1.95 (1.25-3.04)<br>*P* = 0.003 | 1.86 (1.07-3.22)<br>*P* = 0.027 | 2.03 (1.35-3.05)<br>*P* = 0.001 | 1.98 (1.20-3.27)<br>*P* = 0.008 |

Donor age, donor gender, recipient age, recipient gender, cold ischemic time, transplant indication and donor liver transport were included in multivariate analysis.

**Table 3 Cause of graft loss within one year and primary graft dysfunction rate *n* (%)**

| | Local liver transport (*n* = 22) | Airplane liver transport (*n* = 26) | *P* value |
|---|---|---|---|
| Cause of graft loss | | | 0.027 |
| Primary graft non-function | 1 (4.6) | 3 (11.5) | |
| Early graft failure | 0 (0) | 5 (19.3) | |
| MOF due to sepsis | 5 (22.7) | 9 (34.6) | |
| others | 16 (72.7) | 9 (34.6) | |
| Primary graft dysfunction | 1 (4.6) | 10 (38.5) | 0.006 |

MOF: Multi-organ failure.

those with local livers ($P$ = 0.02). This difference in graft survival increased until one year post-transplant (28% *vs* 11.4% respectively, $P$ = 0.001) and then was maintained until the end of follow up. Primary graft non-function and early graft failure associated with preservation injury accounted for 20.8% of graft loss within the first year in those with an airplane transported liver and only for 4.6% for those with a local liver ($P$ = 0.027) (Table 3). The primary graft dysfunction rate was also significantly higher in recipients with an airplane transported liver than those with a local liver (38.5% *vs* 4.6%, $P$ = 0.006) (Table 3).

Analysis of survival stratified by CIT (CIT $\geqslant$ 12 h, CIT < 12 h) found that airplane donor liver transport was significantly associated with decreased graft survival in both groups ($P$ = 0.032 and $P$ = 0.004 respectively) (Figure 4). Stratification by cause of liver failure found a significant reduction of graft survival for airplane transported livers in recipients with chronic liver disease ($P$ = 0.002) but not for recipients with acute liver failure ($P$ = 0.243) (Figure 4). The non-significant difference for acute liver failure was possibly due to small numbers ($n$ = 20) and lack of statistical power. For those patients transplanted for chronic liver disease, further stratification analysis by MELD score (MELD $\geqslant$ 20, MELD < 20) found a significant correlation between airplane transported liver and graft survival in both groups ($P$ = 0.013 and $P$ = 0.019 respectively). Finally there was no significant difference in graft or patient survival when comparing recipients who received an airplane transported liver from the central states compared with the eastern states, $P$ = 0.88 and 0.93 respectively.

## DISCUSSION

In this longitudinal study, we found that airplane transport of donor liver organs was associated with significantly reduced patient and graft survival independently of CIT and donor and recipient characteristics. Donor characteristics were well matched in local and airplane transported liver groups apart from a lower mean ALT level and the expected longer CIT in the airplane transported liver group. The lower ALT level in this group is likely due to a better quality donor liver being accepted because of the added risk of interstate airplane transport. Recipient characteristics differed in that there were an increased proportion of acute liver failure recipients and a higher MELD score in those that received an airplane transported liver. National mandatory donor sharing accounted for 75% of all donor livers being transported by airplane for this urgent indication.

There was a weak but significant correlation between donor transport distance and CIT. CIT increased by 1.3 h for each additional 1000 km of flight distance. Clearly other transport related factors apart from transport distance influenced CIT and these included delays in ground transport to and from airports, delayed airplane departures and increased flight times. On one occasion Perth airports closed due to adverse weather conditions and caused a flight diversion. These delays become more significant in that surgery may be commenced prior to arrival of the donor organ in an attempt to reduce CIT.

Overall graft and patient survival were excellent and not different from those reported by the Transplantation Society of Australia and New Zealand for transplantation during this period[10]. There was however a significantly reduced graft and patient survival in recipients that received an airplane transported donor liver. For those who received a local donor the one year graft and patient survival was 88% and 91% respectively compared to 71% and 76% respectively for those with an airplane transported liver. The increased graft loss in airplane transported livers was evident early within seven days after LT

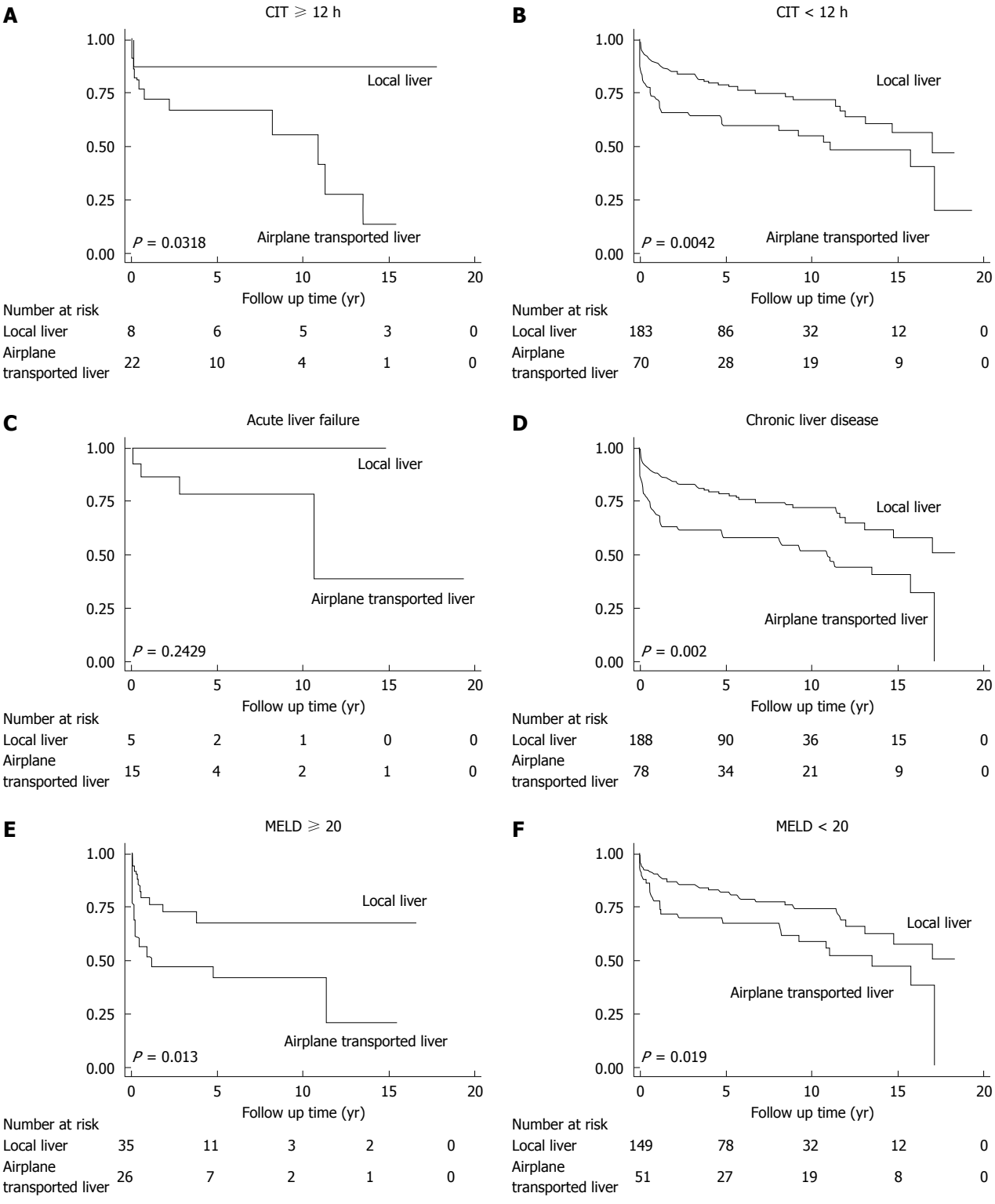


**Figure 4 Graft survival curves after stratification.** A: Recipients with cold ischemic time (CIT) ⩾ 12 h; B: Recipients with CIT < 12 h; C: Recipients transplanted for acute liver failure; D: Recipients transplanted for chronic liver disease; E: Recipients transplanted for chronic liver disease and with MELD score ⩾ 20; F: Recipients transplanted for chronic liver disease and with MELD score < 20.

with the maximum difference observed after one year. Moreover, primary graft non-function and early graft failure accounted for 28% of all graft loss in the first year for recipients with an airplane transported liver compared with 4.6% in those with a local liver. This suggested a role of graft damage during transportation for recipients with an airplane transported liver.

Airplane transport was the only factor that was independently associated with either graft survival or patient survival. Univariate analysis found CIT was associated with both end-points but this did not maintain significance in multivariate analysis. After

stratifying graft survival results for livers that had CIT ⩾ 12 h or < 12 h; for recipients with a MELD ⩾ 20 or < 20 and for recipients transplanted for chronic liver disease, there remained a significant difference between airplane transported and local donor livers. This further confirmed the independent effect of airplane transportation on graft survival. Other donor risk factors such as pre-existing liver disease, the use of inotropes, hypernatremia and warm ischemia time were not available for analysis in this study. It is unlikely that these factors varied between groups. It is also unlikely that donor organ retrieval by the other states contributed to the worse graft survival as all donor procurement surgery was performed by experienced surgical teams that also perform the service for each of the home states. Others have shown that non-local organ procurement had no effect on graft survival[11]. Future studies that include these clinical factors are of great interest.

Up to date, this is the only study that has evaluated the effect of airplane transport on post-transplantation survival. Two large studies from the US and European found that distant donor location (local *vs* reginal *vs* national) was independently associated with decreased survival after adjusting for CIT[3,7]. This decreased graft survival was potentially due to damage of the donor liver caused by airplane transport. In the current environment where donor sharing across a large geographical area is increasing, further clinical and laboratory investigation is needed to determine the potential mechanism of the damage caused by airplane transport and search for possible solutions. Airplane transport has a number of well documented environmental effects that have the potential to cause damage to cold stored donor livers. Cabin pressure is routinely maintained at approximately 8000 ft which is equivalent to approximately 0.7 Atm. The direct and indirect effects of this pressure change on the isolated organ with tissue swelling and bubble expansion in preservation fluid both have the potential to adversely affect graft quality[12]. The decreased partial pressure of oxygen to 108 mmHg is less likely to affect the donor liver because cellular metabolism at 4 ℃ is negligible. Direct trauma from the walls of the container and acceleration and de-acceleration forces could also damage the isolated liver. Finally airplane engine and other vibrations are well known to cause tissue damage particularly in the resonance frequency range for organs of 4-5 Hz.

In summary, this "proof of concept" study demonstrated the significant effect of airplane transportation of donor livers on post-liver transplantation survival. Further investigation is required to determine the mechanism of organ damage in airplane transported livers. However in the meantime it would seem prudent to minimise donor liver trauma and atmospheric pressure change effects by transporting isolated organs in a pressure sealed cooler that has an appropriate organ harness and that is isolated from floor vibrations. Clearly these observations have similar and important implications for other donor organs that are transported by airplane.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

In the current environment of donor scarcity donor sharing between large geographical areas is increasing. Airplane transportation is commonly used for long distance donor transportation. However, no studies have evaluated the potential effect of airplane transportation on post liver transplantation survivals.

### Research frontiers

The geographic isolation of Perth allows a unique opportunity to evaluate the effect of long distance airplane transport of donor livers on post liver transplantation outcomes.

### Innovations and breakthroughs

This study demonstrates for the first time a significantly decreased graft and patient survival for patients who received an airplane transported donor liver compared to a local donor liver not requiring airplane transport. This effect was independent of the cold ischaemic time.

### Applications

This study raised an interesting clinical question and leads to further investigations to determine the mechanism of organ damage in airplane transported livers. In the meantime, transporting isolated organs in a pressure sealed cooler that has an appropriate organ harness and that is isolated from floor vibrations should be considered to minimise the potential damage caused by airplane transportation.

### Peer-review

This manuscript compared survival of liver transplant recipients that received a local organ donor versus an airplane transported donor liver in Australia. This is an interesting exploratory and novel study that has not been reported previously. The study finding is very topical in an era of increasing transport of donor livers that aims to redistribute organs in a fair way.

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ORIGINAL ARTICLE

Retrospective Study

# First-line endoscopic treatment with over-the-scope clips significantly improves the primary failure and rebleeding rates in high-risk gastrointestinal bleeding: A single-center experience with 100 cases

Hans-Jürgen Richter-Schrag, Torben Glatz, Christine Walker, Andreas Fischer, Robert Thimme

Hans-Jürgen Richter-Schrag, Christine Walker, Andreas Fischer, Robert Thimme, Center of Interdisciplinary Gastrointestinal Endoscopy, Department of Medicine II, Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg, D-79106 Freiburg, Germany

Torben Glatz, Department of General and Visceral Surgery, Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg, D-79106 Freiburg, Germany

Author contributions: Richter-Schrag HJ designed the research and wrote the paper; Glatz T performed and analyzed the data; Walker C and Fischer A contributed to the evaluation; Thimme R critically revised the manuscript.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board and the Ethics Committee of Albert-Ludwigs-University-Freiburg, Germany.

Informed consent statement: All study participants, or their legal guardians, provided informed written consent prior to study enrollment and treatment. The identity of all subjects, including all details, was anonymized.

Conflict-of-interest statement: The authors declare that there is no conflict of interest with the paper presented.

Data sharing statement: No additional data are available. Informed consent for data sharing was not obtained, but the present data are anonymized, and the risk of identification is low. Questions regarding the technical aspects and data set are available from the corresponding author at hans-juergen.schrag@uniklinik-freiburg.de.



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Correspondence to: Hans-Jürgen Richter-Schrag, MD, Professor of Medicine, Center of Interdisciplinary Gastrointestinal Endoscopy, Department of Medicine II, Medical Center - University of Freiburg, Faculty of Medicine, University of Freiburg, Hugstetter Street 55, D-79106 Freiburg, Germany. hans-juergen.schrag@uniklinik-freiburg.de
Telephone: +49-761-27032020
Fax: +49-761-27027750

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## Abstract

### *AIM*

To evaluate rebleeding, primary failure (PF) and mortality of patients in whom over-the-scope clips (OTSCs) were used as first-line and second-line endoscopic treatment (FLET, SLET) of upper and lower gastrointestinal bleeding (UGIB, LGIB).

### *METHODS*

A retrospective analysis of a prospectively collected database identified all patients with UGIB and LGIB in a tertiary endoscopic referral center of the University of Freiburg, Germany, from 04-2012 to 05-2016 (*n*

= 93) who underwent FLET and SLET with OTSCs. The complete Rockall risk scores were calculated from patients with UGIB. The scores were categorized as < or ⩾ 7 and were compared with the original Rockall data. Differences between FLET and SLET were calculated. Univariate and multivariate analysis were performed to evaluate the factors that influenced rebleeding after OTSC placement.

***RESULTS***

Primary hemostasis and clinical success of bleeding lesions (without rebleeding) was achieved in 88/100 (88%) and 78/100 (78%), respectively. PF was significantly lower when OTSCs were applied as FLET compared to SLET (4.9% *vs* 23%, $P$ = 0.008). In multivariate analysis, patients who had OTSC placement as SLET had a significantly higher rebleeding risk compared to those who had FLET (OR 5.3; $P$ = 0.008). Patients with Rockall risk scores ⩾ 7 had a significantly higher in-hospital mortality compared to those with scores < 7 (35% *vs* 10%, $P$ = 0.034). No significant differences were observed in patients with scores < or ⩾ 7 in rebleeding and rebleeding-associated mortality.

***CONCLUSION***

Our data show for the first time that FLET with OTSC might be the best predictor to successfully prevent rebleeding of gastrointestinal bleeding compared to SLET. The type of treatment determines the success of primary hemostasis or primary failure.

**Key words:** Gastrointestinal bleeding; Rockall risk score; Over-the-scope clip; First-line endoscopic treatment; Second-line endoscopic treatment



**Core tip:** In the present study, the over-the-scope clip (OTSC) was evaluated for first-line and second-line endoscopic treatment (FLET, SLET) of high-risk upper and lower gastrointestinal bleeding. One hundred OTSCs were applied in 93 patients. Primary hemostasis and clinical success was achieved in 88% and 78%, respectively. Statistical analysis shows no significant influence of anticoagulants on the rebleeding rate. The total mortality was 21.5%. Primary failure was significantly reduced by the use of OTSC as FLET (4.9% *vs* 23.1%, $P$ = 0.008). This largest single-center data of OTSC-placement in high-risk GI bleeding published to date shows, for the first time, that FLET is a significant predictor of reduced rebleeding (OR = 5.2; $P$ = 0.009).

Richter-Schrag HJ, Glatz T, Walker C, Fischer A, Thimme R. First-line endoscopic treatment with over-the-scope clips significantly improves the primary failure and rebleeding rates in high-risk gastrointestinal bleeding: A single-center experience with 100 cases. *World J Gastroenterol* 2016; 22(41): 9162-9171 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i41/9162.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i41.9162

## INTRODUCTION

Since the introduction of the Over-the-Scope Clip (OTSC®, Ovesco, Tübingen, Germany) in 2006 and the first clinical experience reported by Kirschniak *et al*[1] in 2007, the spectrum of indications has extended successively. Currently, the system, which is characterized by a circular tissue compression, is used for emergency endoscopy in acute gastrointestinal bleeding, acute perforation and fistula-closure in the upper and lower gastrointestinal (GI) tracts. The reported success rates range between 42% and 100% depending on the indication[2,3].

Particularly with regard to reducing the high risk of undesirable outcomes, especially rebleeding and mortality, of upper gastrointestinal bleeding (UGIB) secondary to peptic ulcer disease and other lesions, new endoscopic techniques such as the OTSC are increasingly the focus of interest.

Table 1 gives an overview of the last 9 years of experience with OTSC treatment of UGIB and lower non-variceal gastrointestinal bleeding (LGIB)[3-16]. The data were processed to distinguish between first- and second-line endoscopic treatments (FLET/SLET).

Actually, the published data of OTSC in FLET and SLET are difficult to compare because of non-uniform and small sample sizes and the often insufficiently defined risk profiles of patients.

From recent results, four most important questions arise: (1) Are FLET and SLET with OTSC actually comparable in terms of outcomes? (2) Can any independent risk factors be defined with respect to the rate of rebleeding? (3) Do patients with UGIB and high Rockall risk scores (⩾ 7) benefit from OTSC treatment with respect to morality compared to those with scores < 7? and (4) Is the outcome of OTSC in patients with UGIB comparable to the standard of care (conventional treatment modalities) regarding rebleeding events and rebleeding-associated mortality?

Consequently, the present single-center study retrospectively analyzed the outcome of FLET and SLET with OTSC in patients with UGIB and LGIB, to elicit differences in the quality and location of the bleeding lesions and the application of dual antiplatelet or anticoagulation drugs. In addition, patients with UGIB were categorized by complete Rockall risk score[17], and the data were used to calculate predictors of OTSC success and mortality.

## MATERIALS AND METHODS

### *The OTSC device*

The OTSC device consists of a transparent applicator cap carrying the circular nitinol clip, a release thread, a hand wheel and a thread retriever. The applicator cap can be fixed on the distal end of the endoscope. The application mechanism is similar to common endoscopic variceal band-ligation systems. The clip application can be carried out after the tissue has been

**Table 1 Data overview of first- and second-line treatment with over-the-scope clips in patients with upper and lower non-variceal gastrointestinal bleeding (2009-2016)**

| Ref. | *n* | Primary success (%) | Patients/clinical success (*n*/%) | | Follow up, mean (mo) | Rebleeding, *n* | UGIB/LGIB, *n* | Design |
|---|---|---|---|---|---|---|---|---|
| | | | FLET | SLET | | | | |
| Wedi *et al*[4], 2016 | 44 | 85.4 | 31/? | 13/? | - | 6 | 41/3 | Single center Retro |
| Manno *et al*[3], 2015 | 40 | 100 | 40/100 | - | 1 | - | 40/0 | Single center Retro |
| Manta *et al*[5], 2013 | 30 | 97 | - | 93.3 | 1 | 2 | 23/7 | Multicenter Retro |
| Kirschniak *et al*[6], 2011 | 27 | 100 | 27/92.6 | - | 0.13 | 2 | 12/15 | Single center Retro |
| Skinner *et al*[7], 2014 | 12 | 100 | 12/83.4 | - | 1 | 2 | 12 | Single center Retro |
| Chan *et al*[8], 2013 | 9 | 100 | 3/100 | 6/77.7 | 2 | 2 | 9 | Single center Pro |
| Nishiyma *et al*[9], 2013 | 9 | 77.8 | 9/77.7 | - | 2.2 | 2 | 8/1 | Single center Retro |
| Baron *et al*[10], 2012 | 7 | 100 | - | 7/100 | 1 | 0 | 6/1 | Multicenter Retro |
| Albert *et al*[11], 2011 | 7 | 100 | - | 7/57 | 1 | 3 | 6/1 | Single center Pro |
| Repici *et al*[12], 2009 | 7 | 100 | 3/100 | 4/100 | 3 | 0 | 3/4 | Single center Retro |
| Mönkemüller *et al*[13], 2014 | 6 | 100 | - | 6/100 | 10 | 0 | 6 | Multicenter Retro |
| Alcaide *et al*[14], 2014 | 2 | 100 | - | 2/100 | - | - | 1/1 | Single center Retro |
| Jayaraman *et al*[15], 2013 | 2 | 100 | 2/100 | - | 2.9 | 0 | 0/2 | Single center Retro |
| Sulz *et al*[16], 2014 | 1 | 100 | 1/100 | - | - | 0 | 1 | Single center Pro |

Retro: Retrospective; Pro: Prospective.

mobilized in the cap through grasping or suctioning (Figure 1). The compression force is 7-8 Newtons.

In our hospital, only the traumatic OTSC clip with sharp teeth is used in sizes of 11 and 14 mm diameter. The suction method was applied in all cases.

### Study design

In this observational, single-center case series, the data of all patients with UGIB and LGIB who were treated with OTSC® (Ovesco Endoscopy GmbH, Tuebingen, Germany) between April 2012 and May 2016 were obtained from of our prospectively maintained database and retrospectively analyzed. The data were evaluated by patient demographics, indications, previous therapy and secondary treatments after failure of OTSC placement. The Rockall risk score was used to categorize patients with UGIB by risk of recurrent bleeding and mortality. Furthermore, we calculated total mortality, rebleeding events and rebleeding-associated mortality in patients with OTSC-treated UGIB compared to patients who received best standard-of-care (using the original Rockall data) based on a complete Rockall risk score < or ⩾ 7. The same parameters were calculated from the original data published by Rockall and colleagues[17].

Written informed consent was obtained to the extent possible in emergency situations.

All procedures were performed with propofol sedation. OTSC was used primarily in cases of acute UGIB and recurrent UGIB after failure of conventional endoscopic therapies. The conventional treatment consisted of single-use or combinations such as epinephrine (1:10.000 dilution in saline)/fibrin glue (FG), epinephrine solution/through-the-scope clipping (TTSC) and thermal contact modalities such as argon plasma coagulation (APC). If recurrent bleeding was suspected during the subsequent hospital stay (hematemesis, melena, lack of increasing hemoglobin after transfusion, or a drop in hemoglobin of more than 2 g/dL within 24-h after transfusion or unclear or unstable cardiovascular parameters and shock), emergency endoscopy was performed. The procedures were performed by highly skilled endoscopists of our interdisciplinary team, using standard Olympus endoscopes (gastroscope GIF- H180J/XTQ160 and colonoscope CF-H180, Tokyo, Japan).

Independent of previous conventional treatments, the indication for OTSC placement was determined by the endoscopist in charge depending on the individual situation.

### Definitions

The following study endpoints were defined: (1) Primary hemostasis: No rebleeding immediately after OTSC

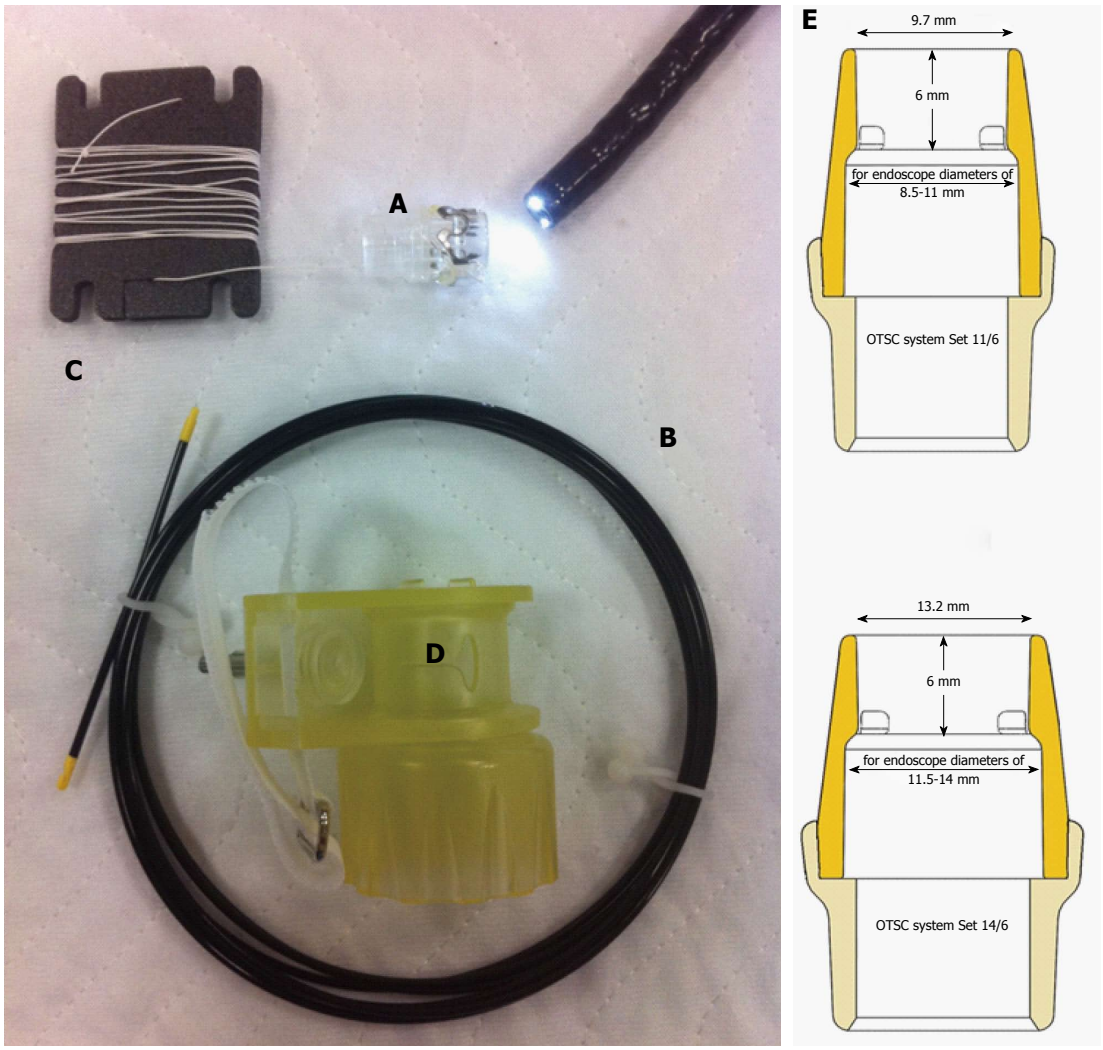


**Figure 1 Details of the over-the-scope clip-system.** A: Applicator cap with clip; B: Thread retriever; C: Thread; D: Hand wheel; E: Dimensions and cross-section of the applicator cap (without clip), sizes used in the present study.

placement; (2) Primary failure: Continued bleeding after OTSC placement; (3) Rebeeding: In-hospital rebleeding after primary hemostasis with OTSC; (4) Clinical success: Having no primary failure and no in hospital rebleeding; (5) Technical success: Successful placement of the OTSC on the target lesion; (6) Mortality (no rebleed): Mortality of patients without rebleeding after OTSC placement; (7) Mortality (rebleeding): Mortality of patients with continued bleeding or rebleeding after OTSC placement and rebleeding; and (8) Total Mortality: Total in-hospital mortality.

The number of OTSC clips used per patient was evaluated as well as adverse events due to the OTSC application. Secondary complications that had no effect on the primary result were not considered treatment failures.

### *Statistical analysis*

The results of our study were obtained by retrospective analysis of our prospectively maintained database. IBM SPSS Statistics for Windows, (Version 23.0 Armonk, IBM Corp. NY, United States) was used for the statistical analysis. Categorical variables are given as absolute and relative frequencies; differences were evaluated by Fisher's exact test. The Fisher exact test tends to be employed instead of Pearson's chi-square test when sample sizes are small (calculation of the Freiburg *vs* the Rockall patient population, respectively). Univariate analysis was performed by using the $\chi^2$ test. Quantitative values are expressed as medians with interquartile range (IQR), and differences were measured using the Mann-Whitney-*U*-test or Kruskal-Wallis-test as appropriate. Multivariate logistic regression analysis with a forward stepwise selection strategy using a likelihood ratio, including the report of relative risks and their 95%CIs, was used to identify independent risk factors for rebleeding. The inclusion *P* for multivariate analysis was 0.10. A *P*-value < 0.05 was considered statistically significant. The null hypothesis asserts the independence of the variables under consideration.

## RESULTS

A total of 93 patients [58 males, median age (IQR),

**Table 2 Type of bleeding lesions in the upper and lower gastrointestinal tracts, *n***

| Lesions UGIB | FIa | FIb | FIIa | FIIb | Spurting | Oozing | Total (*n* = 69) | FLET (*n* = 39) | SLET (*n* = 33) |
|---|---|---|---|---|---|---|---|---|---|
| Ulcer | | | | | | | | | |
| Cardiac | 4 | 1 | 3 | | | | 8 | 5 | 3 |
| Gastric | 2 | 8 | | 1 | | | 11 | 7 | 4 |
| Duodenal | 8 | 11 | 7 | | | | 26 | 17 | 9 |
| Jejunal | | 3 | | | | | 3 | 2 | 1 |
| Polypectomy | | | | | | | | | |
| Gastric | | | | | | 3 | 3 | 3 | |
| Duodenal | | | | 2 | | | 2 | | 2 |
| Anastomoses | | | | | 2 | 3 | 5 | 1 | 4 |
| Gastrojejunal | | | | | | | | | |
| Mallory-Weiss | | | | | | 2 | 2 | 2 | |
| Vascular Malformation | | | | | | 1 | 1 | | 1 |
| Heart device | | | | | | | | | |
| Dieulafoy | | | | | 4 | | 4 | 2 | 2 |
| Metastasis | | | | | | | | | |
| Gastric | | | | | | 4 | 4 | | 4 |
| **Lesions LGIB** | **FIa** | **FIb** | **FIIa** | **FIIb** | **Spurting** | **Oozing** | **Total (*n* = 31)** | **FLET (*n* = 22)** | **SLET (*n* = 9)** |
| Ulcer | | | | | | | | | |
| Rectal | 6 | 3 | 2 | 1 | | | 12 | 9 | 3 |
| Cecal | 1 | 1 | | | | | 2 | 2 | |
| Polypectomy | | | | | | | | | |
| Rectal | | | | | 2 | | 2 | | 2 |
| Colonal | | | | | 1 | 3 | 4 | 4 | |
| Anastomoses | | | | | | | | | |
| Ileocolonic | | | | 1 | 2 | 1 | 4 | 3 | 1 |
| Hemorrhoidal | | | | | 2 | 1 | 3 | 2 | 1 |
| Diverticular | | | | | | 3 | 3 | 1 | 2 |
| Tumor | | | | | | | | | |
| Colonic | | | 1 | | | | 1 | 1 | |

UGIB: Upper gastrointestinal bleeding; LGIB: Lower gastrointestinal bleeding; FLET: First-line endoscopic treatment; SLET: Second-line endoscopic treatment.

**Table 3 Demographics and characteristics *n* (%)**

| | Total (*n* = 93) | UGI (*n* = 63) | LGI (*n* = 30) | *P* value |
|---|---|---|---|---|
| Sex, male | 58 (62) | 38 (60) | 20 (67) | 0.361 |
| Age (yr), median (IQR) | 72 (19-98) | 68 (27-92) | 74 (19-93) | 0.580 |
| Complete Rockall score, median (IQR) | - | 7 (3-10) | - | - |
| Anticoagulation | 46 (50) | 29 (46) | 17 (56) | 0.231 |
| In-Hospital-Mortality | 20 (22) | 17 (27) | 3 (10) | 0.051 |
| Lesions and clips | (*n* = 100) | (*n* = 69) | (*n* = 31) | |
| Bleeding source | | | | |
| Ulcers | 66 | 50 (72) | 16 (52) | 0.018 |
| Other | 34 | 19 (27) | 15 (48) | |
| Active bleeding[1] | 82 | 56 (81) | 26 (84) | 0.492 |
| First-line-therapy | 61 | 39 (57) | 22 (71) | 0.125 |
| Primary failure (including technical failure *n* = 2) | 12 | 8 (12) | 4 (13) | 0.545 |
| Rebleeding | | | | |
| complete | 16 (16) | 11 (16) | 5 (16) | 0.597 |

[1]Forrest classification of ulcers Ia/Ib and spurting/oozing bleeding lesions. IQR: Interquartile range; UGI: Upper gastrointestinal tract; LGI: Lower gastrointestinal tract.

72 (19-98)] with 100 different severe acute UGIB and LGIB lesions were treated with OTSC as FLET or SLET. The types of bleeding lesions are shown in Table 2. One patient had 3 OTSC applications, and five other patients had 2 OTSCs on different lesions. Twenty-four patients were hospitalized primary due to an acute hemorrhage of the GI tract. The mean hospital stay was 19.8 d (range 1-79). Primary hemostasis and clinical success of UGIB and LGIB lesions was achieved in 88/100 (88%) and 78/100 (78%), respectively. An overview of the demographics, characteristics and OTSC results is given in Table 3.

In patients with SLET the median previous FG injection volume was 2.25 mL (1-8) (Tesseel 1 ml Duo

**Table 4 Complete Rockall risk score of patients with upper gastrointestinal bleeding**

| Rockall risk score | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8+ | Total |
|---|---|---|---|---|---|---|---|---|---|
| *n* | 0 | 0 | 1 | 0 | 6 | 13 | 13 | 30 | 63 |

**Table 5 Comparison of total mortality, rebleeding-associated mortality and rebleeding events between upper gastrointestinal bleeding patients who received best standard of care (original Rockall group) and those who underwent first-line or second-line endoscopic treatment over-the-scope clips (Freiburg group)**

| Total mortality Rockall < 7 | Total mortality Rockall ⩾ 7 |
|---|---|
| Rockall[1] 5.8% *vs* Freiburg[2] 10%; *P* = 0.327 | Rockall 32.8% *vs* Freiburg 34.8%; *P* = 0.865 |
| 145 of 2499 *vs* 2 of 20 | 150 of 457 *vs* 15 of 43 |
| Rebleeding-associated mortality | Rebleeding-associated mortality |
| **Rockall < 7** | **Rockall ⩾ 7** |
| Rockall 2.8% *vs* Freiburg 5%; *P* = 0.436 | Rockall 22.3% *vs* Freiburg 13.9%; *P* = 0.247 |
| 70 of 2499 *vs* 1 of 20 | 102 of 355 *vs* 6 of 43 |
| **Rebleeding events Rockall < 7** | **Rebleeding events Rockall ⩾ 7** |
| Rockall 13.8% *vs* Freiburg 15%; *P* = 0.750 | Rockall 46.8% *vs* Freiburg 18.6%; *P* = 0.0003 |
| 345 of 2499 *vs* 3 of 20 | 214 of 457 *vs* 8 of 43 |

[1]Best standard of care; [2]OTSC FLET and SLET. OTSC: Over-the-scope clips; FLET: First-line endoscopic treatment; SLET: Second-line endoscopic treatment.

S, Baxter, Unterschleißheim, Germany).

### *Rockall score, rebleeding and predictors of clinical success*

The median Rockall risk score of patients with UGIB was 7 (3-10; Table 4). No significant difference was observed between patients with a complete Rockall risk score < and ⩾ 7 with respect to rebleeding (15% *vs* 19%, respectively, *P* = 0.51) and rebleeding-associated mortality (5% *vs* 14%, respectively, *P* = 0.27).

Table 5 compares total mortality, rebleeding-associated mortality and rebleeding events of patients who received standard-of-care (the Rockall group) with patients who underwent OTSC treatment (the Freiburg group).Total mortality and rebleeding associated mortality were comparable in both groups, but patients with a high risk profile (⩾ 7) had significantly fewer rebleeding events when treated with OTSC (original Rockall data group 46.8% *vs* Freiburg group 18.6%, *P* = 0.0003).

In univariate analysis, type of lesion, location (UGIB, LGIB), bleeding activity (active, non-active) and anticoagulants were not statistically significant predictors for rebleeding (Table 6). However, our data shows that the clinical success of patients with both UGIB and LGIB was significantly higher when OTSCs were applied as FLET compared to SLET (8.2% *vs* 28.2%, *P* = 0.009, Figure 2). In multivariate analysis,

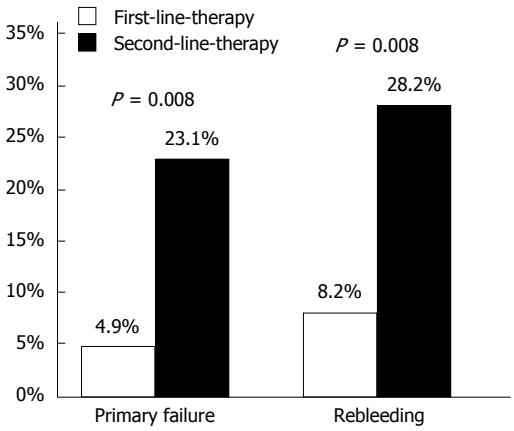


**Figure 2 Outcome of over-the-scope clip in the upper and lower gastrointestinal tract.** Primary failure: Persistent bleeding after over-the-scope clip.

compared to SLET, only FLET continued to be a significant factor related to reduced rebleeding (OR = 5.2; *P* = 0.009).

### *Primary failure, technical failure and adverse events*

Primary failure was significantly reduced by the use of OTSC placement as FLET (4.9% *vs* 23.1%, *P* = 0.008, Figure 2). In one patient with a posterior duodenal wall ulcer, over 50% of the circumference the tissue was so strongly indurated that the clip could not grasp the tissue (technical failure). The same phenomenon was observed with an ileo-transversostomy anastomosis, during which the clip bounced off, and which was treated with multiple adrenalin injections. No malfunction of the device was registered. One adverse event was observed 1.8 mo after a duodenal OTSC application. The resulting lumen obstruction was released with 3 balloon-dilatations.

### *Anticoagulation and antiplatelet drugs*

In total, 47 patients (50%) had no anticoagulants. Nine (10%) patients were treated with one platelet aggregation inhibitor, and 40 patients had anticoagulants (43%) with or without one or more antiplatelet drugs.

Statistical analysis showed no significant influence of anticoagulants on the rebleeding rate after OTSC placement (Table 6).

### *Mortality*

The total mortality was 21.5%. Causes of death included respiratory insufficiency (*n* = 3), cardiogenic shock (*n* = 2), stroke (*n* = 3), hemorrhagic shock (*n* = 4), multi-organ failure and septic shock (*n* = 8).

Two patients died during the hemostasis. One of them died due to a cardiogenic shock during endoscopic clip application and the other due to an aeroembolism after radiological embolization of the gastroduodenal artery.

The in-hospital mortality of patients with UGIB and a complete Rockall risk score of ⩾ 7 was significant

**Table 6 Univariate and multivariate analyses of predictors of rebleeding after over-the-scope clips placement**

| Predictor | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|
| | OR (CI) | *P* value | OR (CI) | *P* value |
| Bleeding (active/non active) | 0.94 (0.24-3.72) | 0.586 | 1.43 (0.33-6.21) | 0.636 |
| Localization (UGIB/LGIB) | 0.99 (0.31-3.13) | 0.597 | 1.67 (0.45-6.15) | 0.451 |
| Anticoagulation (Y/N) | 1.41 (0.48-4.15) | 0.359 | 1.34 (0.43-4.20) | 0.611 |
| Lesion (ulcers/others) | 1.65 (0.53-5.17) | 0.282 | 2.03 (0.56-7.27) | 0.275 |
| Treatment (SLET/FLET) | 4.40 (1.39-13.90) | 0.009 | 5.29 (1.53-18.24) | 0.008 |

UGIB: Upper gastrointestinal bleeding; LGIB: Lower gastrointestinal bleeding; FLET: First-line endoscopic treatment; SLET: Second-line endoscopic treatment.

higher compared to that of those with scores < 7 (35% *vs* 10%, *P* = 0.034). The mortality rate of patients with UGIB without rebleeding and rebleeding-associated mortality was 15.9% and 11%, respectively.

## DISCUSSION

Acute gastrointestinal bleeding is a common and potentially life-threatening emergency with an wide-ranging annual incidence of hospitalization of 42-172 and 20-87 per 100000 for upper and lower GI tract, respectively, and it has a mortality rate as high as 10%[17-26].

In the last 10 years, the experience with OTSC to treat high-risk non-variceal bleeding (NVGIB) of the upper and lower GI tract remains limited, and the published data include small-case series[3-16].

Especially for new technologies, the question arises whether their effectiveness is comparable to established endoscopic techniques, in particular to avoid rebleeding and perhaps to reduce rebleeding-associated mortality in multimorbid patients.

To the best of our knowledge, we present the largest single-center study of OTSCs to date to differentiate FLET and SLET techniques in the management of acute GI bleeding. The data of this series are the first to provide the following answers to important questions with respect to the application and performance of the new device:

First, FLET and SLET are not comparable with respect to the prevention of rebleeding. Importantly, primary failure was attained significantly more in the SLET group of patients compared with FLET patients and in particular if the GI bleeding was pretreated with FG. In these patients, a mean volume-injection of 2.25 portions of FG was administered. Moreover, some underwent APC, and previously applied TTSC had to be removed before OTSC placement. The resulting alterations of the tissue (hardening and fibrosis) can disguise the bleeding source of the target lesion and therefore make the identification difficult. Additionally, the tissue cannot be (or at least not sufficiently) suctioned into the applicator cap, which can cause primary and technical failure. Other authors have reported similar experiences.

A decreased clinical success was observed by OTSC rescue therapy due to the fibrotic nature of leaks and fistulae[2,27,28], and primary therapy was found to be a significant predictor for clinical success of defect closures. In this context, Baron *et al*[28] postulated the use of an anchoring device (Ovesco Endoscopy AG, Tübingen, Germany) could be helpful.

Second, our data show that type of treatment (FLET *vs* SLET) is an independent predictor to prevent rebleeding.

Previous studies have identified a first and second bleeding endoscopic failure rate of 16% and 33.3%, respectively. Furthermore, unsuccessful endoscopic hemostasis was found to be an independent risk factor for rebleeding and was associated with increased 30-d mortality in patients with NVGIB[17,29,30]. These findings support the role of a primary effective endoscopic hemostasis.

In multivariate analysis, OTSCs performed significantly better across all GI bleeding types (UGIB, LGIB) when applied as FLET as opposed to rescue therapy (SLET).

Thus, according to our experience, the use of the OTSC device is preferable in active bleeding from lesions of visible vessels and ulcers, which have never been treated previously with conventional endoscopic treatments, and which are equal to or less than 3 cm in diameter. Less preferable is its use in diffuse bleeding from polypoid metastases, vascular malformations and hardened and fibrotic lesions.

Third, our data suggest that patients with UGIB and a complete Rockall risk score $\geqslant$ 7 might not benefit from OTSC treatment compared to patients with score values of < 7. The total mortality rates of patients with Rockall scores < *vs* $\geqslant$ 7 in the current study are in accordance with those from a prior large, multicenter series published by Rockall *et al*[17]. Furthermore, no significant differences were observed in relation to the rebleeding rate and rebleeding-associated mortality in both groups (< 7 *vs* $\geqslant$ 7) treated with OTSC.

Fourth, based on the original Rockall data, patients with UGIB and complete Rockall risk scores $\geqslant$ 7 could benefit from OTSC placement with respect to the reduction of rebleeding events, compared to endoscopic established treatment modalities. The mortality remains unaffected.

Currently, there are no published prospective trials

regarding the use of OTSC for endoscopic hemostasis compared to conventional techniques.

Indeed, established interventions, including FG, TTSC, band ligators, thermal modalities alone or in combination with epinephrine solution or recently commercially introduced hemostatic granules or powders, show a wide range of permanent hemostasis varying from 10%to 85%[31-35]. However, combined modalities such as hemoclip and injection therapy or thermal coagulation and injection therapy appear to be superior to the use of injection or thermal techniques alone[36].

Therefore, it is generally difficult to compare the outcome of OTSC with previously published data of established endoscopic techniques regarding the reduction of undesirable outcomes of UGIB and LGIB, including rebleeding and mortality and independent of GI localization and bleeding activity.

For this reason, we calculated total mortality, re-bleeding events and rebleeding-associated mortality in relation to patients with Rockall scores < or ⩾ 7 of both the OTSC and original Rockall sample sizes. However, no differences in patients with Rockall scores < 7 were observed with respect to above-mentioned parameters. Only in patients with Rockall scores ⩾ 7 were the rebleeding events significant reduced compared to the calculated original Rockall data, which might indicates a selection bias because our collective mainly consisted of predominantly spurting and oozing bleeding lesions. Prospective randomized trials are needed to confirm this hypothesis.

Finally, durability and exact placement of hemostatic clips on the bleeding source are important factors for successful hemostasis and to reduce rebleeding, other adverse events and emergency surgery.

Modern TTS clips for example can be rotated and reopened, and they open at a wide angle. However, TTSCs have several disadvantages:

It is commonly known that more than one TTSC is necessary for the treatment of large bleeding lesions or blood vessels with large diameters because a single clip can only compress small tissue areas. In addition, adequate space is required properly to release the TTSC. That is why TTSC release in angulated positions such as the duodenum can be tricky.

Due to this and our results, and considering the costs of modern single-use TTS clip systems, we strive for a paradigm shift. Thus, in patients with circumscribed lesions of high-risk UGIB and LGIB, we employ OTSC as FLET.

On the other hand, it is important to understand the mechanism of the OTSC device. The degree of mobilization of the tissue into the applicator cap is crucial for therapeutic success. For patients who require an endoscopic full-thickness resection (FTRD), for example, a similar problem exists. In these cases, we identify the target tissue to resect and mobilize this tissue using a specially designed cap (FTRD prOVE Cap, Ovesco Endoscopy AG, Tübingen, Germany)[37].

Certainly, our study has some limitations, *e.g.*, its retrospective nature. Moreover, only experienced endoscopists with a high level of expertise performed the procedures with the OTSC device. Nevertheless, the present study also has many strengths. It is a large, single-center study with a broad spectrum of bleeding lesions in the upper and lower GI tracts. The large number of lesions, most of which were characterized by a spurting and oozing quality, were treated only with the suction method, and the traumatic type of OTSC represents a homogeneous study cohort that allowed, for the first time, statistically substantiated hypotheses on the effectiveness of the OTSC-device.

In conclusion, the reduction of primary failure was best achieved in patients undergoing treatment of UGIB and LGIB when OTSC was used for FLET. In our series, FLET seems to be a predictor of successful reduction of rebleeding rates.

## ACKNOWLEDGMENTS

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## COMMENTS

### *Background*

Over-the-scope clips (OTSC) are used for emergency endoscopy in acute perforation and fistula-closure in the upper and lower GI tracts. Furthermore, the OTSCs have become an important tool in hemostasis of upper and lower gastrointestinal bleeding (UGIB, LGIB) as first-line and second-line endoscopic treatment (FLET, SLET). However, direct comparisons of OTSCs in FLET and SLET are limited.

### *Research frontiers*

In this retrospective analysis of 93 patients, the authors compared the results of first-line and second-line endoscopic treatment of 100 OTS clips in UGIB and LGIB. To the best of our knowledge, this is the largest single-center series on the use of OTSCs in GI bleeding to date.

### *Innovations and breakthroughs*

Primary failure and rebleeding occurred significantly more often in patients undergoing second-line endoscopic treatment with OTSC than in first-line treated patients. The better outcome of first-line treated patients is comparable with the known data in patients with fistula, who were treated with OTSC.

### *Applications*

By understanding the mechanism, advantages and limits of the OTSCs device, the present study might provide a new prognostic factor for clinical success and hemostatic treatment of acute GI bleeds treated with OTSCs as first-line therapy.

### *Peer-review*

The manuscript presents the outcome of first- and second-line endoscopic treatment with OTSC in high risk gastrointestinal bleeding. It is a topic of interest to the researchers in the related areas but the paper needs some improvement before acceptance for publication.

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ORIGINAL ARTICLE

Retrospective Study

# Presepsin teardown - pitfalls of biomarkers in the diagnosis and prognosis of bacterial infection in cirrhosis

Maria Papp, Tamas Tornai, Zsuzsanna Vitalis, Istvan Tornai, David Tornai, Tamas Dinya, Andrea Sumegi, Peter Antal-Szalmas

Maria Papp, Tamas Tornai, Zsuzsanna Vitalis, Istvan Tornai, Department of Internal Medicine, Division of Gastroenterology, University of Debrecen, Faculty of Medicine, H-4032 Debrecen, Hungary

David Tornai, Peter Antal-Szalmas, Department of Laboratory Medicine, University of Debrecen, Faculty of Medicine Debrecen, H-4032 Debrecen, Hungary

Tamas Dinya, Institute of Surgery, University of Debrecen, Faculty of Medicine, H-4032 Debrecen, Hungary

Andrea Sumegi, Vascular Biology, Thrombosis and Haemostasis Research Group, Hungarian Academy of Sciences, H-4032 Debrecen, Hungary

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Correspondence to: Maria Papp, MD, PhD, Department of Internal Medicine, Division of Gastroenterology, University of Debrecen, Faculty of Medicine, Nagyerdei krt. 98, H-4032 Debrecen, Hungary. papp.maria@med.unideb.hu
Telephone: +36-52-255152
Fax: +36-52-255152

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## Abstract

***AIM***

To evaluate the diagnostic and prognostic value of presepsin in cirrhosis-associated bacterial infections.

***METHODS***

Two hundred and sixteen patients with cirrhosis were enrolled. At admission, the presence of bacterial infections and level of plasma presepsin, serum C-reactive protein (CRP) and procalcitonin (PCT) were evaluated. Patients were followed for three months to assess the possible association between presepsin level and short-term mortality.

***RESULTS***

Present 34.7 of patients had bacterial infection. Presepsin levels were significantly higher in patients with infection than without (median, 1002 pg/mL *vs* 477 pg/mL, $P < 0.001$), increasing with the severity of infection [organ failure (OF): Yes *vs* No, 2358 pg/mL *vs* 710 pg/mL, $P < 0.001$]. Diagnostic accuracy of presepsin for severe infections was similar to PCT and superior to CRP (AUC-ROC: 0.85, 0.85 and 0.66, respectively, $P$ = NS for presepsin *vs* PCT and $P < 0.01$ for presepsin *vs* CRP). At the optimal cut-off value of presepsin > 1206 pg/mL sensitivity, specificity, positive predictive values and negative predictive values were as follows: 87.5%, 74.5%, 61.8% and 92.7%. The accuracy of presepsin, however, decreased in advanced stage of the disease or in the presence of renal failure, most probably because of the significantly elevated presepsin levels in non-infected patients. 28-d mortality rate was higher among patients with > 1277 pg/mL compared to those with ⩽ 1277 pg/mL (46.9% *vs* 11.6%, $P < 0.001$). In a binary logistic regression analysis, however, only PCT (OR = 1.81, 95%CI: 1.09-3.01, $P = 0.022$) but neither presepsin nor CRP were independent risk factor for 28-d mortality after adjusting with MELD score and leukocyte count.

***CONCLUSION***

Presepsin is a valuable new biomarker for defining severe infections in cirrhosis, proving same efficacy as PCT. However, it is not a useful marker of short-term mortality.

**Key words:** Presepsin; Cirrhosis; Bacterial infection; Organ failure; Mortality



**Core tip:** C-reactive protein (CRP) and procalcitonin (PCT) are broadly used in clinical practice to aid early diagnosis of bacterial infections, but they have limitations in cirrhosis. Additional biomarkers with enhanced accuracy are highly needed. Presepsin is a novel biomarker of infection and sepsis, but has not been assessed in cirrhosis so far. In the present study we evaluated the diagnostic and prognostic performance of presepsin in cirrhosis-associated infections in comparison with classic acute phase proteins. Presepsin measurement enhanced diagnostic utility of CRP and reflected the severity of infections more accurately, with a similar efficacy as PCT. Advanced disease stage and renal failure limited the diagnostic accuracy. The increase in PCT level but not in presepsin concentration was an independent predictor of short-term mortality during infectious episodes.

Papp M, Tornai T, Vitalis Z, Tornai I, Tornai D, Dinya T, Sumegi A, Antal-Szalmas P. Presepsin teardown - pitfalls of biomarkers in the diagnosis and prognosis of bacterial infection in cirrhosis. *World J Gastroenterol* 2016; 22(41): 9172-9185 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i41/9172.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i41.9172

## INTRODUCTION

Infectious episodes represent particularly important causes of progression of liver failure and the development of liver-related complications[1]. Due to altered sensitivity, the end-organ damaging effect of bacterial infection is greater in cirrhosis and often culminates in newly developed liver and/or extrahepatic organ failures, which is associated with a very high short-term mortality rate[2,3].

Early recognition of bacterial infections is essential, however, in the clinical practice their accurate identification is challenging from both the clinical[4] and the laboratory point of view[5]. In cirrhosis, usual clinical presentations lack up to 50% of the bacterial infections and are replaced by non-specific complaints or just revealed by organ dysfunctions. Due to some disease specific characteristics, there is an evident lack of sensitivity and specificity of the conventional laboratory and clinical parameters for the definition of systemic inflammatory response (SIRS)[6,7], which often makes it difficult to diagnose sepsis.

Currently C-reactive protein (CRP) and procalcitonin (PCT) are broadly used in the clinical practice to aid the early diagnosis of bacterial infection[8]. In cirrhosis, these conventional markers, however, perform somewhat differently in comparison with the non-cirrhotic patient populations for various reasons. For the first, if the main source of the molecule is the liver, as in the case of CRP, synthesis of the molecule is affected by liver failure and its severity. As a result the diagnostic accuracy of liver synthesised acute phase proteins (APPs) decreases in advanced stage of cirrhosis[9]. Moreover, peak levels can be misleading and do not indicate the severity of the infection adequately, since the more severe the underlying liver dysfunction, the lower the CRP response to bacteraemia is[10]. Secondly, elimination of certain molecules can be affected by renal failure and also renal replacement therapy. Acute kidney injury (AKI) is frequent in patients with cirrhosis, especially in bacterial infections[11]. While CRP has a high molecular weight (MW) (115-kDa) and its renal clearance is negligible[12,13], PCT is small with a MW of 13 kDa, and renal elimination is thought to be one of the major pathways for its elimination[14]. Accordingly, false or inappropriate increase of the PCT level has been reported in end-stage renal disease patients due to the prolonged elimination rate[15,16]. Similarly, artificial reduction of the PCT level was also found after renal replacement therapy (HCO-CVVHDF). Proteins with MW < 60 kDa are filtered out by the dialysis membrane[17]. Thirdly, inflammatory state sustained by

bacterial translocation (BT) and without overt infection is alone sufficient to elevate inflammatory markers to a significant level[5,9]. Bacterial translocation is an increasing problem with disease severity[18].

Accordingly, data are not homogeneous about the optimal cut-off for either of CRP and PCT to differentiate patients with infection from those without[19-23]. Probably using a single threshold is not appropriate. Additional biomarkers are highly needed to optimize the rule in and rule out processes necessary for the diagnosis and also for the severity assessment of the infectious episodes in cirrhosis.

Presepsin (soluble CD14 subtype, sCD14-ST) is a 13-kDa-cleavage product of CD14 receptor that recognizes different cell surface structure of both Gram-negative and positive bacteria. Presepsin in the circulation can be perceived as a witness of activated monocyte-macrophage in response to pathogens[24]. Several recent clinical studies have shown that presepsin is a specific and sensitive novel marker for the diagnosis of sepsis[25], for evaluating the severity of sepsis and for predicting the outcome[26,27]. Beyond sepsis, presepsin is worthy of studying in those clinical settings, where systemic infections are frequently associated with severe diseases course such in cirrhosis [acute decompensation (AD), organ failure]. Contributive role of presepsin for the diagnosis and prognosis of cirrhosis associated bacterial infection has not been assessed extensively so far.

In the present study, we aimed to assess (1) performance of presepsin in the diagnosis of cirrhosis associated bacterial infections in comparison with routinely used APPs such as CRP and PCT; (2) whether presepsin is devoid of the limitations of classic APPs related to cirrhosis; and (3) whether presepsin is able to provide prognostic information during infectious episodes in cirrhosis.

## MATERIALS AND METHODS

### Patient population

Two hundred and sixteen well-characterized patients with cirrhosis (male/female: 118/99, age: 57.5 ± 10.3 years, disease duration: 3.9 ± 4.2 years) were included consecutively from our in- and outpatient Gastroenterology Department between May 2010 and April 2011. The diagnosis of cirrhosis was considered either histologically proven or considered obvious by clinical, biochemical and morphological criteria[28].

### Data collection

The clinical and laboratory characteristics of the patients are presented in Table 1. Clinical data were recorded at enrolment. These included demographics, co-morbidities (including cardio- and cerebrovascular, respiratory, renal disorders, diabetes and extrahepatic cancers), etiology of cirrhosis, history and severity of liver disease, presence of hepatocellular carcinoma (HCC), reason for AD, clinical status of patients, and also presence, type and location of bacterial infection. Severity of the cirrhosis was graded according to liver-oriented scores [Child-Pugh score (CPs) and the model for end-stage liver disease (MELD)][29]. Episodes of AD were defined by acute development of large ascites, hepatic encephalopathy, gastrointestinal hemorrhage, bacterial infection or any combination of these warranting hospital admission[3]. The enrolled patients were followed for 90 d or until death. Presence and grade of organ system failure(s) [OF] were determined retrospectively based on the available clinical and laboratory data after accessibility of CLIF-C Organ Failure Score[30].

The diagnosis of bacterial infection was established by assessment of clinical symptoms and laboratory reports, including microbiological culture results (if available), compatible findings of imaging techniques, and the effect of antibiotic treatment by two independent gastroenterologists (M.P. and Zs.V.). Following bacterial infections were considered and diagnosed on the basis of conventional criteria: (1) skin and soft tissue infections[31]; (2) lower respiratory tract infections (acute bronchitis, pneumonia)[32]; (3) urinary tract infections (UTI) (cystitis, pyelonephritis)[33]; (4) some rare causes of infections, such as biliary tract infections (cholecystitis, cholangitis, liver abscess), osteomyelitis, and endocarditis; (5) spontaneous bacterial peritonitis (SBP), diagnosis of which was based on ascitic fluid polymorphonuclear cell (PMN) count exceeding 250/mm$^3$ and$^3$/or positive culture if secondary causes of peritonitis were excluded (EASL guidelines[34]); and (6) bacterial infection of unknown origin was defined when clinical symptoms and signs of infection were present and confirmed by microbiological demonstration of the causative organism from blood culture in the absence of site-specific infection. Bacterial infection was considered severe when the infectious episode was complicated by OF.

### Measurements of presepsin and other laboratory parameters

Venous blood samples were captured at enrolment. Routine laboratory data, such as liver biochemistry, renal function, blood count and serum CRP and PCT levels were determined directly at the Department of Laboratory Medicine. Methods for qualitative assessment of serum CRP and PCT levels were reported previously[9].

For presepsin measurements, blood samples were immediately centrifuged at 3000 *g* for 10 min, and plasma was stored at -70 ℃ until use. Presepsin levels were measured by means of a PATHFAST® presepsin analyzer (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) which is based on chemiluminescent enzyme immunoassay, with a detection limit of 20 pg/mL.

### Outcome

For outcome assessment, a follow-up examination was set up at the 28$^{th}$ day after enrolment in the study.

**Table 1 Demographic, clinical and laboratory characteristics of patients with or without bacterial infections**

| | | | Non-infected | Infected | *P* value |
|---|---|---|---|---|---|
| *n* | | | 141 | 75 | |
| Gender (male/female) | | | 77/64 | 41/34 | NS |
| Age (yr) | | | 57.3 ± 10.7 | 58.1 ± 9.7 | NS |
| Child-Pugh score | | | 6.9 ± 1.7 | 9.3 ± 2.2 | < 0.001 |
| Child-Pugh stage, *n* (%) | | | | | |
| A | | | 58 (41.1) | 6 (8.0) | < 0.001 |
| B | | | 65 (46.1) | 28 (37.3) | |
| C | | | 18 (12.8) | 41 (54.7) | |
| MELD score | | | 12.3 ± 4.1 | 19.1 ± 9.1 | < 0.001 |
| Serum bilirubin (μmol/L) | | | 41.4 ± 38.3 | 124.1 ± 147.4 | < 0.001 |
| Serum albumin (g/L) | | | 35.4 ± 7.1 | 28.1 ± 6.5 | < 0.001 |
| INR | | | 1.3 ± 0.2 | 1.6 ± 0.5 | < 0.001 |
| Serum creatinine (μmol/L) | | | 83.8 ± 74.3 | 131.2 ± 129.9 | < 0.001 |
| Ascites present, *n* (%) | | | 58 (41.1) | 59 (78.7) | < 0.001 |
| HCC, *n* (%) | | | 4 (2.8) | 11 (14.7) | 0.003 |
| Comorbidities present, *n* (%) | | | 72 (51.1) | 45 (60.0) | NS |
| Type of bacterial infections, *n* (%) | | | | | |
| UTI | | | | 25 (29.4) | |
| SBP | | | | 20 (23.5) | |
| Pneumonia | | | | 18 (21.2) | |
| SSTI | | | | 4 (4.7) | |
| Miscellaneous | | | | 18 (21.2) | |
| Multiple | | | | 9 (10.6) | |
| Acute phase proteins, median (IQR) | | | | | |
| Presepsin (pg/mL) | Overall | | 477 (332-680) | 1002 (575-2149) | < 0.001 |
| | OF absent | OF present | | 710 (533-1277) 2357 (1398-3666) | < 0.001 |
| CRP (mg/L) | Overall | | 4.6 (1.8-8.8) | 30.1 (11.3-57.4) | < 0.001 |
| | OF absent | OF present | | 25 (9.6-40.5) 52.2 (23.4-84) | 0.027 |
| PCT (μmol/L) | Overall | | 0.1 (0.1-0.2) | 0.4 (0.1-1.2) | < 0.001 |
| | OF absent | OF present | | 0.2 (0.1-0.5) 1.7 (0.6-5.3) | < 0.001 |

Data are presented as mean ± SD or *n* (%) if not otherwise indicated. CRP: C-reactive protein; HCC: Hepatocellular carcinoma; IQR: Interquartile range; INR: International normalized ratio; MELD: Model for end-stage liver disease score; NS: Non-significant; PCT: Procalcitonin; SD: Standard deviation; UTI: Urinary tract infection; SBP: Spontaneous bacterial peritonitis; SSTI: Skin and soft tissue infection.

Patients who survived until follow-up were counted as survivors, whereas patients who died within the follow-up period were counted as non-survivors.

### *Statistical analysis*

Variables were tested for normality using Shapiro Wilk's W test. Continuous variables were summarized as mean ± SD or as medians [interquartile range (IQR)] according to their homogeneity. Categorical variables were compared with the $\chi^2$ test or $\chi^2$ test with Yates correction as appropriate. Continuous variables were compared with the Mann-Whitney *U* test or Student's *t* test. Relationship between continuous variables was assessed with the non-parametric Spearman correlation. Diagnostic accuracy of presepsin and other APPs for defining various study-endpoints: (1) presence of bacterial infection; (2) presence of severe infection; and (3) short-term mortality was estimated using receiver operating curve (ROC) analysis by plotting sensitivity *vs* 1-specificity. Area under the curve (AUC-ROC) and corresponding 95%CI were calculated. ROC curves were compared with the method of DeLong *et al*[35] in Medcalc. Youden index was chosen, calculated as the maximum (sensitivity + specificity - 1) value, to estimate the best discriminate thresholds. Sensitivities, specificities, positive predictive values (PPV) and negative predictive values (NPV) were calculated to determine the predictive power of individual APPs or their combinations in all the three clinical settings. Binary logistic regression was used to assess the relationship between APPs and short-term mortality adjusted for the MELD score and WBC count. For the analysis APPs were $\log_e$-transformed to ensure normal distribution. Associations are given as odds ratios (OR) or likelihood ratios (LR) with a 95%CI. A 2-sided probability value < 0.05 was considered to be significant. For statistical analysis and graphical presentation SPSS 22.0 (SPSS Inc., Chicago, IL, United States) and GraphPad Prism 7 (San Diego, CA, United States) were used.

The statistical methods of this study were reviewed by Professor Elek Dinya, PhD, DSc, Semmelweis University, Institute of Health Informatics, Budapest, Hungary.

### *Review of the literature*

We performed a systematic review of studies reporting on CRP and PCT in prognosis of cirrhosis. Papers were eligible if they presented original research in adult patients with cirrhosis and reported association of CRP and/ or PCT with the disease outcome either in patients with or without bacterial infection. Studies had

**Table 2 Association of classic acute phase proteins with mortality in patients with cirrhosis**

| Ref. | Year | Journal | Population | *n* | Outcome | Measure | Value |
|---|---|---|---|---|---|---|---|
| C-reactive protein | | | | | | | |
| Reuken *et al*[72] | 2013 | Liver Int | Ascites | 108 | 90-d mortality | AUC | 0.69 (0.59-0.79), $P = 0.001$ |
| Moreno *et al*[69] | 2013 | Liver Int | Non-septic | 95 | 1-yr mortality | AUC | 0.71 (0.6-0.8), $P < 0.001$ |
| Mortensen *et al*[70] | 2012 | Eur J Gastroenterol Hepatol | Stable alcohol cirrhosis | 45 | Long term mortality (about 6- yr) | HR[1] | 1.074 (1.001-1.153), $P = 0.046$ |
| Wiese *et al*[74] | 2014 | Liver Int | Stable cirrhotic | 193 | 1- yr survival | HR | 1.18, $P = 0.048$[3] |
| Lim *et al*[68] | 2014 | Plos One | SBP | 75 | 30-d mortality | AUC<br>HR | 0.64 (0.49-0.79), $P = 0.076$<br>ND, $P = 0.064$ |
| Schwabl *et al*[73] | 2015 | Liver Int | SBP | 168 | 30-d mortality | HR[1] | 1.067 (1.004-1.134), $P = 0.037$ |
| Ha *et al*[65] | 2011 | Korean J Intern Med | Bacteraemia | 202 | 30-d mortality | Difference | Survivor *vs* non-survivor[2]<br>37.8 *vs* 34.3 mg/L, $P = 0.721$ |
| Cervoni *et al*[20] | 2012 | J Hepatol | CPs > 7 | 148 | 180-d mortality | AUC<br>HR[1,4] | 0.63 (0.51-0.73), $P =$ ND<br>2.73 (1.41-5.26), $P = 0.003$ |
| Di Martino *et al*[64] | 2015 | Liver Transplant | CPs > 7 | 109 | 90-d mortality | HR[1,4] | 2.21 (1.03-4.76), $P = 0.042$ |
| Cervoni *et al*[63] | 2016 | Eur J Gastroenterol Hepatol | CPs > 7 | 583 | 90-d mortality | HR[1,4] | 1.69 (1.01-2.81), $P = 0.046$ |
| Park *et al*[71] | 2015 | J Korean Med Sci | Alcoholic cirrhosis various reasons for admission | 409 | 30-d mortality | OR | "CRP > 20 not independent predictor" |
| Ximenes *et al*[75] | 2016 | Am J Emerg Med | Hepatic decompensation | 149 | Inhospital mortality | OR | OR: ND, $P > 0.100$ |
| Kwon *et al*[67] | 2015 | BMC Gastroenterol | Acute decompensation | 184 | 30 d survival | OR | OR: ND, $P = 0.122$ |
| Lazzarotto *et al*[19] | 2013 | Ann Hepatol | Acute decompensation | 64 | 90 d survival | Difference | Survivor *vs* non-survivor[2]<br>7 *vs* 41 mg/L, $P = 0.026$ |
| Kronenberger *et al*[66] | 2012 | BMC Med | Acute decompensation + outpatients | 120 | Overall survival (196 ± 165 d) | HR | Univariate: 0.314 (0.141-0.702) , $P = 0.005$<br>Multivariate: ND, $P = 0.077$ |
| Procalcitonin | | | | | | | |
| Lin *et al*[80] | 2015 | J Crit Care | Acute decompensation | 96 | Sepsis in-hospital mortality | AUC | 0.692 |
| Kotecha *et al*[79] | 2013 | Eur J Gastroenterol Hepatol | Cirrhosis + SIRS | 100 | In-hospital survival | OR[1] | 0.949 (0.868-1.037), $P = 0.249$ |
| Connert *et al*[78] | 2003 | Z Gastroenterol | Compensated + decompensated | 100 | 60-d mortality | Percent died | < 0.58 *vs* ⩾ 0.58:<br>9.1% *vs* 46.7%, $P < 0.001$ |
| Al-Dorzi *et al*[76] | 2014 | Clin Lab | Septic shock | 45 | 28-d mortality | Difference | Low PCT *vs* high PCT:<br>80% *vs* 77%, $P = 0.61$ |
| Berres *et al*[77] | 2009 | Liver Int | Critically ill | 38 | ICU mortality | Difference | NA, $P =$ NS |

[1]Adjusted for MELD score; [2]Median values; [3]Not significant in the log-rank test; [4]CRP variation over 15 d. SBP: Spontaneous bacterial peritonitis; HR: Hazard ratio; AUC: Area under the curve; OR: Odds ratio; CPs: Child-Pugh score; ICU: Intensive care unit; SIRS: Systemic inflammatory response syndrome.

to have been published in peer-reviewed journals. We started searching PubMed using the following search terms: ["C-reactive protein" OR "procalcitonin"] AND "liver cirrhosis". Limits were human and time ranging from 1991 until 2016 (1st June). Only articles reporting short or long-term outcome in cirrhosis were included. This search revealed 20 articles. In Table 2 we summarize the clinical significance of CRP and PCT in the prediction of disease course in cirrhosis based on findings reported in relevant literature.

### *Ethical permission*

All patients were informed of the nature of the study and signed an informed consent form. The regional and national committee (DEOEC RKEB/IKEB 5306-9/2011, 3885/2012/EKU [60/PI/2012]) for research ethics approved the study protocol.

## RESULTS

### *Study population*

Two hundred and sixteen patients with cirrhosis were enrolled in this cohort. The main characteristics of patients with or without infection are summarized in Table 1. There were 118 men with a mean age of 57.6 ± 10.3 years. The median Child-Pugh and MELD score were 7 (95%CI: 6-9) and 13 (95%CI: 10-17), respectively. The main baseline characteristics were as follows: alcoholic liver disease in 159 patients (73.6%), HCC in 15 patients (6.9%) and renal impairment in 33 (15.3%) based on creatinine cut-off values ⩾ 133 μmol/L. One-hundred and seventeen patients (54.2%) had extrahepatic co-morbidities. Acute decompensation of the disease warranting hospital admission occurred in a total of 101 patients (46.8%)

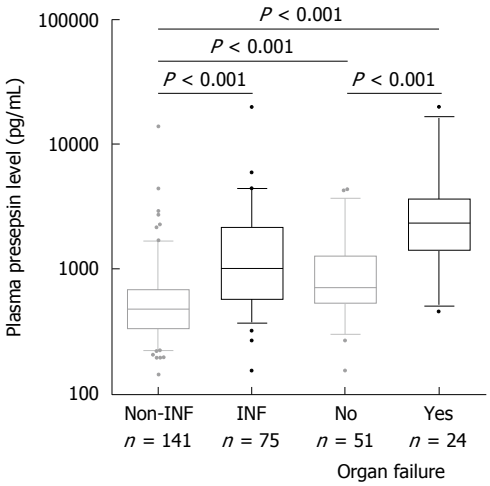


**Figure 1 Presepsin levels in patients with cirrhosis according to presence or absence and severity of bacterial infections (*n* = 216).** Median presepsin levels are significantly higher in patients with infection as compared to those without and associated to the severity of the infection. Lines denote median values, boxes represent 25th-75th percentiles and whiskers indicate the 5th-95th range. *P* values were calculated by Mann-Whitney *U*-test or Kruskal-Wallis *H*-test as appropriate. INF: Bacterial infection.

of whom 27 (26.7%) had at least one OF.

Documented bacterial infection was present in 75 (34.7%) patients of whom 9 (12.0%) suffered from multifocal episode. Bacteria were Gram-negative in 52.6% and Gram-positive in 47.4% of culture-positive cases. No cases of invasive fungal infections were detected. The distribution of infections is shown in Table 1. The infected and the non-infected patient groups did not differ in gender, age, and presence of comorbidities. However, patients with infections had more advanced disease stage, as indicated by median values of Child-Pugh and MELD score and presence of ascites. Renal impairment (29.3% *vs* 7.8%, $P < 0.001$) and occurrence of HCC (14.7% *vs* 2.8%, $P = 0.003$) were more frequent in patients with infection as compared to those without as well. The occurrence of AD episodes and the development of OF were more common in the presence of infections (AD: INF *vs* non-INF, 85.3% *vs* 26.2%, $P < 0.001$ and OF: INF *vs* non-INF, 37.5% *vs* 8.1%, $P = 0.001$).

### *Association between presepsin levels and bacterial infections*

Presepsin values ranged from 142 to 5950 pg/mL [median (IQR), 576 pg/mL (376-972)] and were significantly higher in patients with infection as compared to those without [1002 (575-2149) pg/mL *vs* 477 (332-680) pg/mL, $P < 0.001$] (Figure 1). This association was also confirmed in the different disease severity subgroups according to Child-Pugh stage (Figure 2A) or the presence of ascites (Figure 2B). In the subgroup of patients with renal failure, presepsin levels were also different numerically between infected and non-infected patient groups however it did not reach statistically significance ($P = 0.08$) (Figure 2C).

**Table 3 Correlations between presepsin and different laboratory parameters or liver-orientated scores**

| Variable | Spearman's rho | *P* value |
|---|---|---|
| CRP | 0.63 | < 0.001 |
| PCT | 0.53 | < 0.001 |
| Leucocyte count | 0.27 | < 0.001 |
| Serum creatinine | 0.36 | < 0.001 |
| Serum bilirubin | 0.28 | < 0.001 |
| Serum albumin | -0.40 | < 0.001 |
| INR | 0.15 | 0.032 |
| CPs | 0.42 | < 0.001 |
| MELD score | 0.45 | < 0.001 |

CRP: C-reactive protein; CPs: Child-Pugh score; INR: International normalized ratio; MELD score: Model for end-stage liver disease; PCT: Procalcitonin.

Further evaluating non-infected patients, a significant increase was observed in presepsin levels in case of more advanced disease stage and also in the presence of renal failure ($P < 0.001$ for both).

Presepsin level was positively correlated with classic markers of bacterial infections, such as CRP, PCT, and different WBC parameters, but also with renal and liver function tests (Table 3) and accordingly with liver liver-oriented scores (CPs and MELD).

Considering the type of infectious episodes, presepsin level was not different according to the location or Gram specificity of the infection (data not shown). Patients with multifocal infections (10.6%) showed numerically higher presepsin levels than those with unifocal ones without reaching statistically significance [2470 pg/mL (729-2671) *vs* 983 pg/mL (560-1774), $P = 0.065$]. Nonetheless, presepsin level was associated with the severity of the infection. Twenty-four infections (32%) were complicated with at least one OF. Presepsin level was significantly higher in patients with OF as compared to those without [2358 pg/mL (1398-3666) *vs* 710 pg/mL (533-1277), $P < 0.001$] (Figure 1).

### *Accuracy of presepsin level in the diagnosis of bacterial infections compared to classic acute phase proteins*

The diagnostic accuracy of presepsin for identifying patients with infection was established by ROC analysis and compared to CRP and PCT. Presepsin was a similar predictor of bacterial infection in overall [AUC-ROC, 95%CI: 0.79 (0.73-0.84)] *vs* PCT [0.77 (0.71-0.83), $P = 0.668$] but somewhat lower than CRP [0.86 (0.80-0.90), $P = 0.057$] (Figure 3A). Combination of CRP with presepsin, however, increased the sensitivity and NPV, compared with CRP on its own, by 9 % and 4 % respectively. A similar trend was found with the combination of CRP and PCT (Table 4). On the contrary, the diagnostic accuracy of presepsin [AUC-ROC 95%CI: 0.85 (0.74-0.92)] for identifying patients with infection complicated by OF was similar to PCT [0.85 (0.74-0.92)] and clearly superior to CRP [0.66 (0.54-0.77), $P = 0.994$ for presepsin *vs* PCT, and *P*

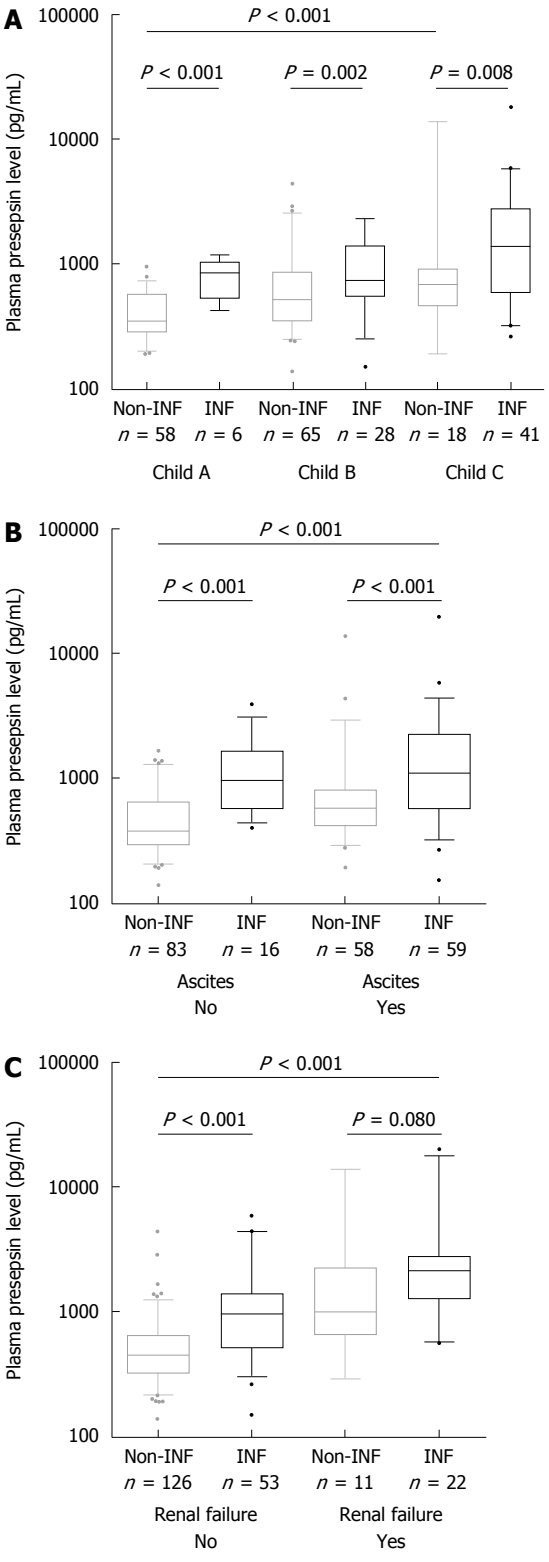


**Figure 2 Presepsin levels in subgroups of patients with different diseases severity according to presence or absence of bacterial infections (*n* = 216).** Significant differences in median presepsin levels between non-infected and infected patients are observed in all disease severity subgroups according to Child-Pugh stage (A) or the presence of ascites (B); However, no significant difference is observed in renal failure subgroup (C). Among non-infected patients, a significant increase in median presepsin levels is observed according to disease severity or in the presence of renal failure. Lines denote median values, boxes represent 25$^{th}$-75$^{th}$ percentiles and whiskers indicate the 5$^{th}$-95$^{th}$ range. *P* values were calculated by the Mann-Whitney *U* or the Kruskal-Wallis *H*-test as appropriate. Creatinine values of 4 patients were missing in the non-infected group.

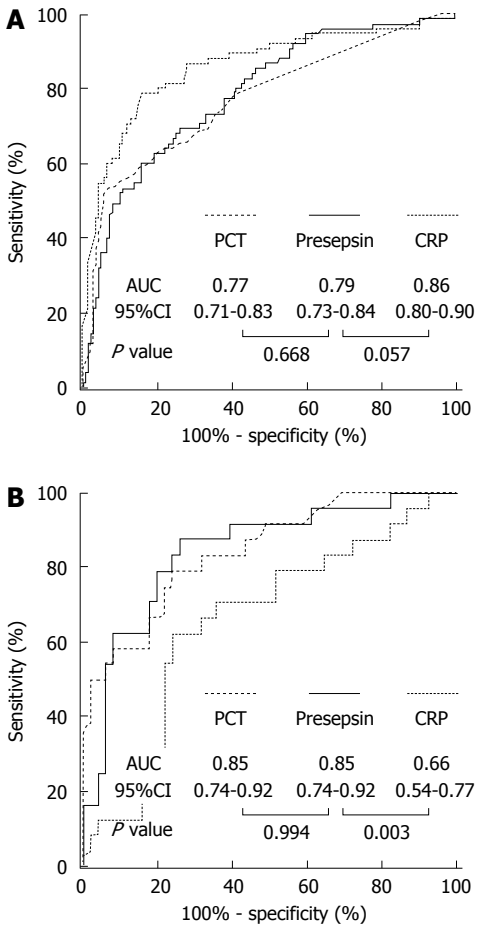


**Figure 3 Receiver-operating characteristic curves of presepsin, procalcitonin and C-reactive protein for the identification of bacterial infection overall (A) or bacterial infection complicated by organ failure (B).** ROC analysis were performed (A) in the whole cohort (n = 216) or (B) in patients with bacterial infection (*n* = 75). The control group comprised (A) patients without bacterial infection (*n* = 141), or (B) patients with bacterial infection without organ failure (*n* = 51). AUC: Area under curve; CI: Confidence interval; CRP: C-reactive protein; PCT: Procalcitonin.

< 0.01 for both presepsin *vs* CRP and PCT *vs* CRP] (Figure 3B). The optimum diagnostic thresholds for each individual biomarker based on ROC analysis and their performances belonging to the cut-off points are shown in Table 4.

### *Diagnostic accuracy of presepsin level according to the disease severity*

Diagnostic accuracy of presepsin for identifying patients with infection decreased in advanced stage of the disease and also in the presence of renal failure. Specificity and LR values of presepsin > 844 pg/mL were obviously lower in patients with Child B or C stage cirrhosis (74%; LR+: 2.28, LR-: 0.56) compared with those with Child A (96%; LR+: 15.6, LR-: 0.31), whereas the difference in sensitivity was somewhat less (70% *vs* 58%). A similar trend was found when the performance of presepsin > 844 pg/mL was evaluated in patients with or without renal failure (specificity: 46% *vs* 87%; sensitivity: 86% *vs* 49%;

**Table 4 Performance characteristics of presepsin and other acute phase proteins during bacterial infections in patients with cirrhosis in various clinical settings**

| | Variable | Cut-off values | Sensitivity | Specificity | PPV | NPV | LR+ | LR- |
|---|---|---|---|---|---|---|---|---|
| INF overall | Presepsin (pg/mL) | 844 | 60.0% | 84.45 | 67.2% | 79.9% | 3.85 | 0.47 |
| | PCT (μmol/L) | 0.39 | 53.3% | 93.65 | 81.6% | 79.0% | 8.36 | 0.50 |
| | CRP (mg/L) | 10.8 | 78.7% | 84.4% | 78.2% | 88.1% | 5.04 | 0.25 |
| | At least one marker positive (Presepsin/CRP) | | 88.0% | 74.5% | 64.7% | 92.1% | 3.45 | 0.16 |
| | At least one marker positive (PCT/CRP) | | 81.3% | 84.2% | 69.3% | 91.1% | 5.15 | 0.22 |
| INF + OF | Presepsin (pg/mL) | 1206 | 87.5% | 74.5% | 61.8% | 92.75 | 3.43 | 0.17 |
| | PCT (μmol/L) | 0.5 | 79.2% | 76.5% | 61.3% | 88.6% | 3.36 | 0.27 |
| | CRP (mg/L) | 40.5 | 62.5% | 76.5% | 55.6% | 81.2% | 2.66 | 0.49 |

PPV: Positive predictive value; NPV: Negative predictive value; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; PCT: Procalcitonin; CRP: C-reactive protein; MELD: Model for end-stage liver disease; INF: Infection; OF: Organ failure.

**Table 5 Performance characteristics of presepsin, procalcitonin and C-reactive protein for prediction of short-term (28-d) mortality in patients with bacterial infection ($n$ = 75)**

| Variable | Cut-off values | AUC-ROC (95%CI) | Sensitivity | Specificity | PPV | NPV | LR+ | LR- |
|---|---|---|---|---|---|---|---|---|
| Presepsin (pg/mL) | 1277 | 0.76 (0.64-0.85) | 75.0% | 69.1% | 46.9% | 88.4% | 2.43 | 0.36 |
| PCT (μmol/L) | 0.48 | 0.87 (0.77-0.93) | 90.0% | 74.6% | 56.2% | 95.3% | 3.54 | 0.13 |
| CRP (mg/L) | 39.6 | 0.74 (0.63-0.84) | 75.0% | 74.6% | 51.7% | 89.1% | 2.95 | 0.34 |

PPV: Positive predictive value; NPV: Negative predictive value; LR+: Positive likelihood ratio; LR-: Negative likelihood ratio; PCT: Procalcitonin; CRP: C-reactive protein; MELD: Model for end-stage liver disease.

**Table 6 Association of presepsin, procalcitonin and C-reactive protein levels with short-term (28-d) mortality in patients with cirrhosis and bacterial infection**

| | Binary logistic regression analysis | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Univariate | | Multivariate | | | | | |
| | Unadjusted | *P* value | Adjusted for MELD | *P* value | Adjusted for leucocyte count | *P* value | Adjusted for MELD and leucocyte count | *P* value |
| ln(Presepsin) | 3.59 (1.65-7.84) | 0.001 | 1.9 (0.81-4.43) | 0.138 | 2.91 (1.28-6.64) | 0.011 | 1.61 (0.65-3.97) | 0.303 |
| ln(PCT) | 2.54 (1.55-4.16) | < 0.001 | 1.89 (1.14-3.14) | 0.014 | 2.33 (1.42-3.83) | 0.001 | 1.81 (1.09-3.01) | 0.022 |
| ln(CRP) | 2.17 (1.23-3.81) | 0.007 | 1.73 (0.93-3.21) | 0.081 | 1.84 (1.03-3.31) | 0.040 | 1.56 (0.81-2.99) | 0.180 |

Associations are expressed as odds ratios and 95%CI per 1 loge-unit increase. MELD: Model for end-stage liver disease; PCT: Procalcitonin; CRP: C-reactive protein.

LR+: 1.58 *vs* 3.86, LR-: 0.30 *vs* 0.58, respectively).

### *Association of presepsin level with short-term mortality during infectious episodes*

Seventy-five patients with bacterial infection were eligible for evaluation of short-term mortality. Twenty-three patients (31.5%) died within 3 mo of follow-up. Of these, 20 patients (27.4%) died within the first 28 d. Plasma presepsin levels at admission were significantly higher in non-survivors than in survivors at the 28-d follow-up [2323 (1172-3688) pg/mL *vs* 852 (549-1451) pg/mL, $P < 0.001$]. Discriminative ability (AUC-ROC) of presepsin was 0.76 with the best cut-off value of 1277 pg/mL. 28-d mortality rate was significantly higher among patients with presepsin level above this threshold (46.9% *vs* 11.6%, $P < 0.001$). The optimum cut-off values and the belonging sensitivities, specificities, PPVs and NPVs of all three APPs for identifying non-survivors are summarized in Table 5.

In the univariate logistic regression analysis, increased presepsin level was found to be a risk factor of short-term mortality during bacterial infection [OR = 3.59 (95%CI: 1.65-7.84), $P = 0.001$] similarly to CRP and PCT. Presepsin level however lost it significance after adjusting for MELD score and leukocyte count [OR = 1.61, (95%CI: 0.65-3.97), $P = 0.303$], with multivariate binary logistic regression analysis. PCT was the only APP that was independently associated with the risk of short-term mortality [OR = 1.81, (95%CI: 1.09-3.01), $P = 0.022$] in this model (Table 6).

## DISCUSSION

Infected patients with cirrhosis can be asymptomatic at initial stages, but highly susceptible to dissemination of infections due to their immunocompromised state that often leads to development of severe disease specific complications with significant mortality rate[36,37]. Accurate laboratory markers are of importance to maximize the efficacy of diagnostic procedure of bacterial infections and thus making early intervention possible. C-reactive protein is the most widely used APP in the everyday clinical practice; however, it has some limitations in patients with cirrhosis[5]. Thus identification of novel biomarkers is required to reach this unmet need in this patient group.

Primary aim of our study was to assess the performance of presepsin - a recently reported novel sepsis marker - in the diagnosis of cirrhosis-associated bacterial infections in comparison with routinely used APPs (CRP, PCT) in such a patient cohort that represents the everyday clinical practice. To the best of our knowledge, this is the first study in cirrhosis, reporting the feasibility and the usefulness of presepsin in these clinical settings. We evaluated a large cohort of patients, in which not only severe but also mild forms of infections were represented. One-third of patients had mild infections and mainly localized to the urinary tract, while another subgroup of patients (32%) suffered from severe infectious episodes. In our study severe infectious episodes were defined by the presence of hepatic and/or extrahepatic OF(s), since currently accepted clinical definition of SIRS and hence sepsis[38] is not entirely applicable to cirrhotic patients for various reasons[5]. New definition of OFs has directly been elaborated for cirrhotic patient population recently[3,30] that uses simple measures and is easy to apply in everyday clinical practice. Moreover presence of OF is predictive of worse outcome[2].

For the diagnosis of bacterial infections, the best cut-off level of presepsin was 844 pg/mL in our cirrhotic patient cohort. Diagnostic cut-off levels were different in previous studies in non-cirrhotic populations, but most reports suggest an approximate level of 400-600 pg/mL[39,40].

Presepsin alone was not suitable as a screening tool to search for infection, however adding it to CRP, we found that presepsin was clinically useful. For the first, this combination amended efficacy of identification of the infectious episode. Sensitivity and NPV were increased by 9% and 4% compared to CRP alone. Secondly, presepsin was able to distinguish severe infectious episodes from non-severe ones more properly compared to CRP; AUC-ROC values were 0.85 and 0.66, respectively. Performance of presepsin corresponds to those reported in non-cirrhotic septic patient populations. In a recent meta-analysis of Zheng *et al*[41], comprising a total of 8 studies and 1757 patients, the AUC of the summary ROC (SROC) was 0.82. In contrast, weak predictive power of CRP with an AUC-ROC of 0.64 was reported for the infections in critically ill patients with cirrhosis in intensive care unit (ICU)[22], which is also in agreement with our results. Regarding CRP, patients with cirrhosis may present reduced CRP in response to infection[5,10].

It is acknowledged that level of certain APPs are different according to the pathogens causing infections, while others are not. In a landmark study of Angeletti *et al*[42] level of PCT and mid-regional pro-adrenomedullin (MR-proADM) were found to be significantly higher in patients with sepsis caused by Gram-negative than Gram-positive strains. These data are also confirmed by other studies[43-45]. Some reports also highlighted differences in circulating cytokine levels in bloodstream infections according to Gram specificity, *i.e.,* Gram-negative infections leaded to higher increase in the level of interleukin (IL)-6, TNF-alpha or IL-10[46]. On the contrary, levels of other APPs, such as C-reactive protein, soluble (s)CD14, sCD163 or soluble urokinase plasminogen activator receptor (SuPAR) are not in relation with the Gram specificity of the infection[47-50]. In the present study, presepsin level was not different according to Gram specificity of the infection, which is in agreement with previous literature findings[51-53].

Overall, presepsin was indisputably a valuable complementary tool in our cirrhotic patient cohort from a clinical point-of-view, but cost issues might compromise their joint use in the laboratory screening procedure of infections. Adding presepsin to CRP significantly increased the cost, from 0.9 to 12.5 $. Medico-economic evaluation, however is lacking at this time and should be performed before proposing introduction of their combined use into routine clinical practice. Presepsin had very similar discriminative ability as PCT in both above-mentioned clinical settings. Furthermore, prices of presepsin and PCT are also comparable (12.5 and 10.7 $) suggesting their interchangeability in this patient population.

Secondary aim of our study was to evaluate whether presepsin is devoid of the limitations of classic APPs in cirrhosis. Previously in a small case-control study of Park *et al*[10] showed that the more severe the underlying liver dysfunction, the lower the CRP response to bacteremia was. Equally in a former study[9], we reported that the diagnostic accuracy of both CRP and PCT for identifying patients with infection obviously decreased in advanced stage of the disease or in presence of ascites. Correspondingly, presepsin behaved similarly in the present study. Presepsin is not primarily synthesized in the liver, thus not the decreasing synthetic capacity is the major limitation of their diagnostic performance in advanced cirrhosis. Ongoing chronic inflammatory state is a characteristic feature of cirrhosis that is potentially able to induce the synthesis of APPs in the absence of infection[54,55] and inevitably limits their clinical utility in the diagnostic procedure of bacterial infections. Out of various explanations, BT has major importance.

Bacterial translocation is frequently reported in patients with cirrhosis-associated severe liver dysfunction or ascites[56,57]. It is likely that this process resulted in higher presepsin level in our non-infected cirrhotic patients compared to healthy controls in previous studies[39,58]. Furthermore, presepsin levels were associated with diseases severity (Child A: 361, Child B: 530 and Child C: 703 pg/mL, $P < 0.001$) or presence of ascites (Yes *vs* No: 382 pg/mL *vs* 575 pg/mL, $P < 0.001$) in the non-infected patient group as well.

Another important, but rarely considered issue is the effect of renal function on the levels of APPs. Acute kidney injury (AKI) is a frequent complication of cirrhosis, occurring in up to 50% of hospitalized patients with cirrhosis[59]. Exact clearance mechanism of presepsin is unknown but considering its low MW it is presumably filtered by the glomeruli, reabsorbed, and catabolized within the proximal tubular cells[60]. From a clinical point of view, little information is available on the accurate association between presepsin level and kidney function. Nagata *et al*[61] reported that presepsin levels tend to increase with decreasing glomerular filtration rate - assessed by inulin renal clearance measurements - and are markedly high in patients with chronic renal failure or receiving hemodialysis. Nakamura *et al*[62] retrospectively analyzed presepsin levels in patients with or without sepsis presenting in the ICU, and found that presepsin levels were markedly high in patients with renal failure and end-stage kidney disease. Accordingly, we evaluated the impact of kidney function on presepsin levels. Significant correlation was found between presepsin and serum creatinine level (Spearman's rho: +0.36, $P < 0.001$). Furthermore, in a small subgroup of patients with renal failure, presepsin values were markedly high even in the absence of infection, at comparable levels to those of bacterial infection but without renal failure (1011 *vs* 774 pg/mL). These results suggest that the evaluation of presepsin levels in cirrhosis warrants special consideration during AD episodes complicated by AKI, and probably a different cut-off is needed for diagnosing infection in such patients.

Third aim of our study was to assess whether presepsin is able to provide prognostic information in cirrhosis associated bacterial infections. Studies in this clinical setting only exist regarding CRP[19,20,63-75] and PCT[76-80] and their findings are not without controversies. In Table 2 we summarized available data on clinical significance of CRP and PCT in short or long-term mortality of patients with cirrhosis. Most of the studies included both stable outpatients and patients with ongoing AD episodes with or without bacterial infections. Furthermore, evaluations often were done as a whole of these non-homogenous patient groups rendering direct comparison and a single conclusion rather difficult. Recently, an important concept has been derived from the CANONIC study[3]. Acute-on-chronic liver failure (ACLF) is associated with systemic inflammation and robust inflammatory response as judged by presence of elevated CRP or elevated leukocyte count results in worse outcome. Higher leukocyte count was found to be an independent predictor of 28-d transplant-free mortality. Based on these results it was reasonable to assume that excessive increase in the APP levels, as a representative of the exaggerated inflammatory process could be associated with higher risk of short-term mortality in cirrhosis during bacterial infections. In patients with increased level of PCT, CRP and presepsin, short-term mortality was significantly higher. Indeed, higher level of PCT, CRP and presepsin were associated with short-term mortality in our study. However, after adjusting for diseases severity and leukocyte count, this association was only preserved for PCT and not for CRP or presepsin. From biological point of view this finding might be explained by the fact that presepsin has a different profile. It belongs to a distinctive class of molecules, so-called "hormonkines" [81]. Procalcitonin has a cytokine-like behaviour during inflammation and infection. It is produced primarily in neuroendocrine cells of various organs and represents involvement of several instead of one organ into the pro-inflammatory response[82]. Lastly, it has been demonstrated that PCT has various toxic effects and pose harm to the host. Administration of PCT to septic animals greatly increases mortality. Antibodies directed against PCT are able to ameliorate harmful effects of PCT with a marked decrease symptomatology and mortality of sepsis[83]. Presepsin represents activation of the monocyte-macrophage system during inflammatory process. Macrophages have a dual effect: production of excessive amount of inflammatory cytokines can cause tissue damage but involvement in the resolution of the inflammation promote tissue repair. This latter process is driven by M2-type macrophages in the presence of local microenvironmental anti-inflammatory signals such as IL-10[84].

Plasma presepsin was only assessed at enrolment, and thus dynamic changes of the concentration were unknown, which is inevitably one of the limitations of the present study. For this reason, additional clinical study will be needed to further investigate serial changes in presepsin levels and their possible association with worse outcome during infection.

To conclude, the present study suggests that presepsin is a promising biomarker during diagnostic procedure of bacterial infections in cirrhosis by enhancing the diagnostic capacity of CRP and reflecting more accurately the severity of infections. Performance of presepsin is equal to PCT in these clinical settings. Diagnostic accuracy of presepsin, however, decreases in advanced stage of the disease or in the presence of renal failure. Level of presepsin is not associated with the pathogens causing infections. Procalcitonin, but not presepsin, is a biomarker for predicting infection-related short-term mortality in patients with cirrhosis.

## COMMENTS

### Background

Bacterial infections are frequent complications in cirrhosis and often culminate in newly developed liver and/or extrahepatic organ failures, which is associated with significant mortality. Early laboratory diagnosis of these episodes is essential but challenging. There is an evident lack of sensitivity and specificity of the conventional laboratory markers due to disease specific characteristics. Advanced stage of cirrhosis affects diagnostic accuracy of liver synthesised acute phase proteins (*e.g.,* C-reactive protein) whereas acute kidney injury affects renal clearance of small molecules (*e.g.,* procalcitonin). Enhanced bacterial translocation induces significant elevation of inflammatory markers as well. Additional biomarkers are highly needed to optimize the diagnostic procedure and severity assessment of the infectious episodes in cirrhosis. Presepsin is a novel biomarker of activated monocyte-macrophage in response to pathogens and specific and sensitive marker of the sepsis.

### Research frontiers

Presepsin is worthy of studying in cirrhosis, where systemic infections are frequently associated with severe disease course, such as acute development of liver and/or extrahepatic organ failures. Contributive role of presepsin for the diagnosis and prognosis of cirrhosis associated bacterial infection, however, has not been assessed extensively so far.

### Innovations and breakthroughs

To the knowledge of the authors, this is the first study in cirrhosis to investigate the performance of presepsin in the diagnosis and prognosis of cirrhosis associated bacterial infections. Presepsin is a promising biomarker of infection in terms of diagnostic, but not the prognostic procedure. Presepsin enhances diagnostic capacity of C-reactive protein and reflects more accurately severity of infections. In these clinical settings its performance is equal to procalcitonin. Diagnostic accuracy of presepsin, however, decreases in advanced stage of the disease or in the presence of renal failure. Level of presepsin is not associated with the pathogens causing infections. A clear strength of our study is the large study population that represents the everyday clinical practice and assessment of presepsin in comparison with routinely used acute phase proteins. The authors also provide a profound overview about the significance of routinely used acute phase proteins in the prognosis of cirrhosis.

### Applications

In every day clinical practice, presepsin is a useful complementary adjunct to C-reactive protein and promising alternate of procalcitonin during the diagnostic procedure of cirrhosis associated bacterial infection. However, it is not devoid of the limitations of these classic acute phase proteins in the presence of advanced stage or certain acute complications of the disease. Larger prospective studies including serial changes in presepsin levels are needed to further investigate any possible association of presepsin level with worse outcome during infection or any suggestion for more aggressive or pre-emptive antibiotic therapy according to presepsin level.

### Terminology

Presepsin or soluble CD14 subtype (sCD14-ST) is a 13-kDa-cleavage product of CD14 receptor of monocyte-macrophage that recognizes different cell surface structure of both Gram-negative and positive bacteria. Bacterial translocation is defined as an enhanced passage of bacteria and/or bacterial products from the intestinal tract to systemic circulation.

### Peer-review

This study suggests for the first time that presepsin is a promising biomarker during diagnostic procedure of bacterial infections in cirrhosis for enhancing diagnostic capacity of C-reactive protein and reflecting more accurately the severity of infections. Performance of presepsin is equal to procalcitonin in these clinical settings. In contrast to procalcitonin and certain cytokines, presepsin level is not associated with the pathogens causing infections. Moreover, procalcitonin but not presepsin is a biomarker for predicting infection-related short-term mortality in patients with cirrhosis. Acute phase proteins are not simply surrogate markers of on-going inflammatory processes of the host organism but might also be active participants, hence exerting harmful or beneficial effects.

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ORIGINAL ARTICLE

Retrospective Study

# Impact of IL28B and *OAS* gene family polymorphisms on interferon treatment response in Caucasian children chronically infected with hepatitis B virus

Krzysztof Domagalski, Małgorzata Pawłowska, Agnieszka Zaleśna, Małgorzata Pilarczyk, Paweł Rajewski, Waldemar Halota, Andrzej Tretyn

Krzysztof Domagalski, Centre for Modern Interdisciplinary Technologies, Nicolaus Copernicus University, 87-100 Toruń, Poland

Małgorzata Pawłowska, Małgorzata Pilarczyk, Waldemar Halota, Department of Paediatric Infectious Diseases and Hepatology, Faculty of Medicine, Collegium Medicum Bydgoszcz, Nicolaus Copernicus University Torun, 85-030 Bydgoszcz, Poland

Agnieszka Zaleśna, Paweł Rajewski, Provincial Infectious Diseases Hospital in Bydgoszcz, 85-030 Bydgoszcz, Poland

Andrzej Tretyn, Department of Plant Physiology and Biotechnology, Nicolaus Copernicus University, 87-100 Toruń, Poland

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Correspondence to: Małgorzata Pawłowska, MD, PhD, Department of Paediatric Infectious Diseases and Hepatology, Faculty of Medicine, Collegium Medicum Bydgoszcz, Nicolaus Copernicus University Torun, Floriana 12, 85-030 Bydgoszcz, Poland. mpawlowska@cm.umk.pl
Telephone: +48-523-255605
Fax: +48-523-255650

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## Abstract

***AIM***

To investigate the impact of IL28B and *OAS* gene polymorphisms on interferon treatment responses in children with chronic hepatitis B.

***METHODS***

We enrolled 52 children (between the ages of 4 and 18) with hepatitis B e antigen-negative chronic hepatitis B (CHB), who were treated with pegylated interferon alfa for 48 wk. Single nucleotide polymorphisms in the OAS1 (rs1131476), OAS2 (rs1293747),

OAS3 (rs2072136), OASL (rs10849829) and IL28B (rs12979860, rs12980275 and rs8099917) genes were studied to examine their associations with responses to IFN treatment in paediatric patients. We adopted two criteria for the therapeutic response, achieving an hepatitis B virus (HBV) DNA level < 2000 IU/mL and normalization of ALT activity (< 40 IU/L). To perform the analyses, we compared the patients in terms of achieving a partial response (PR) and a complete response (CR) upon measurement at the 24-wk post-treatment follow-up.

***RESULTS***

The PR and CR rates were 80.8% and 42.3%, respectively. Factors such as age, gender and liver histology had no impact on the type of response (partial or complete). A statistically significant relationship between higher baseline HBV DNA and ALT activity levels and lower rates of PR and CR was shown ($P$ < 0.05). The allele association analysis revealed that only the IL-28B rs12979860 (C *vs* T) and IL28B rs12980275 (A *vs* G) markers significantly affected the achievement of PR ($P$ = 0.021, OR = 3.3, 95%CI: 1.2-9.2 and $P$ = 0.014, OR = 3.7, 95%CI: 1.3-10.1, respectively). However, in the genotype analysis, only IL-28B rs12980275 was significantly associated with PR (AA *vs* AG-GG, $P$ = 0.014, OR = 10.9, 95%CI: 1.3-93.9). The association analysis for CR showed that the TT genotype of IL28B rs12979860 was present only in the no-CR group ($P$ = 0.033) and the AA genotype of OASL rs10849829 was significantly more frequent in the no-CR group ($P$ = 0.044, OR = 0.26, 95%CI: 0.07-0.88). The haplotype analysis revealed significant associations between PR and CR and OAS haplotype ($P$ = 0.0002 and $P$ = 0.001, respectively), but no association with IL28B haplotype was observed.

***CONCLUSION***

IL28B and OAS polymorphisms are associated with different clinical outcomes in CHB children treated with interferon.

**Key words:** Chronic hepatitis B; IL28B; OAS; Single-nucleotide polymorphisms; IFN therapy; Children



**Core tip:** The limited efficacy and side effects associated with IFN treatment limit its clinical use in paediatric patients with chronic hepatitis B (CHB). Therefore, pretreatment predictors are required to identify those patients at highest risk for treatment response failure. OAS and IL28B are well-known IFN-induced antiviral pathway players; however, the impact of host-related genetic variability in the IL28B and *OAS* genes on response rates to IFN therapy in CHB paediatric patients has not been studied. The results of our study show an association between IL28B rs12979860, OASL rs10849829 and OAS haplotypes and final IFN-treatment response in Caucasian CHB children.

Domagalski K, Pawłowska M, Zaleśna A, Pilarczyk M, Rajewski P, Halota W, Tretyn A. Impact of IL28B and *OAS* gene family polymorphisms on interferon treatment response in Caucasian children chronically infected with hepatitis B virus. *World J Gastroenterol* 2016; 22(41): 9186-9195 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i41/9186.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i41.9186

## INTRODUCTION

It is estimated that at least 2 billion people worldwide have serologic evidence of contact with HBV, including approximately 350 million people who develop chronic hepatitis B (CHB)[1,2]. The largest proportion of chronic cases occurs in children infected in the first years of life; this figure reaches 90% in the case of infection in the perinatal period[1,3,4]. Despite the significant decrease in the number of new cases of CHB in children because of the introduction of compulsory vaccination in many countries, we are still struggling with the treatment of adolescent patients, especially in developing countries[5-7].

A 48-wk course of interferon therapy is recommended as a first-line treatment option for select HBeAg-negative patients, especially young patients with increased aminotransferase activity, which indicates the activation of the immune system to eliminate infected hepatocytes, and providing a therapy with antiviral and immunomodulatory activity in the form of interferon may enhance this effect[8,9]. However, PEG-IFN-based therapy is modestly effective in suppressing viral replication in comparison to nucleos(t)ide analogues, which are highly effective in suppressing HBV replication. In contrast to nucleos(t)ide analogues, PEG-IFN-based therapy has a higher HBsAg seroconversion rate[10-12]. Therefore, pretreating patients to identify those with the highest probability of success is of great clinical importance to IFN therapy.

The antiviral activity of interferons is associated with their ability to induce virus targeting proteins such as 2′-5′-oligoadenylate synthetase (2′,5′-OAS)[13]. Analytic studies in human hepatocytes have confirmed that both interferon alfa and interferon lambda, which includes interleukin 28B, have activating effects[14]. The OAS proteins are well-known IFN-induced antiviral pathway players involved in the cleavage of viral RNA molecules, resulting in the inhibition of viral replication. The human OAS family contains the OAS1, OAS2, OAS3, and OASL genes, which are located on chromosome 12 (in the 12q24.1 region)[15,16]. Single-nucleotide polymorphisms (SNPs) in the OAS family genes have been identified as a factor associated with

susceptibility to viral infection and antiviral effects during IFN-based therapy in patients infected with HCV[17-20].

The influence of host-related genetic variability on differences in response rates to IFN therapy in CHB patients is not well understood. Unlike many studies confirming that SNPs in the interleukin 28B (*IL28B*) gene play a primary role in IFN-based treatment outcomes in patients with chronic hepatitis C, the association between IL28B SNPs and the result of IFN monotherapy in CHB has been a subject of very few studies[21-23]. Additionally, there is limited information about the role of SNPs in 2',5'-oligoadenylate synthetase (OAS) family genes in the IFN response in hepatitis B patients[24,25]. Currently, there are no available results concerning the impact of IL28B or OAS SNPs on the results of IFN therapy in a group of paediatric patients with CHB.

The aim of this study was to determine the relationship between IL28B and OAS gene family SNPs and the biochemical and virological response rates to PEG-IFN alfa-2a monotherapy in a cohort of Caucasian children chronically infected with HBV.

## MATERIALS AND METHODS

### Patients

We retrospectively enrolled a cohort of CHB children of Caucasian ethnicity who were treated with PEG-IFN alfa-2a (Pegasys) at a dose of 180 μg per week for 48 wk at the Department of Paediatric Infectious Diseases and Hepatology.

The inclusion criteria were as follows: HBsAg positive for more than 6 mo, confirmed detection of HBV DNA > 2000 IU/mL or abnormal liver biochemistry (alanine aminotransferase (ALT) > 40 IU/L) for a period of one year prior to the start of therapy. All the included patients were treatment naïve, HBeAg-negative, and had completed a 48-wk course of PEG-IFN α-2a monotherapy with a minimum of 24 wk post-therapy follow-up.

The exclusion criteria included hepatitis C virus (HCV) coinfection, human immunodeficiency virus (HIV) coinfection, a coexisting autoimmune disease, cirrhosis, hepatocellular carcinoma (HCC) or chronic liver disease other than CHB. There were no pregnant women in the study group. Blood samples from these patients were used for IL28B and OAS genotyping. Detailed demographic characteristics and other standard clinical data, including the ALT level, HBsAg, HBeAg, and anti-HBe status, HBV DNA level, and liver histology, were obtained from the patients' clinical documentation. Pre-treatment liver histological analysis was carried out by only one pathologist using the modified Scheuer scoring system (F0–F4; A0–A4).

The study was reviewed and approved by the NCU Bioethics Committee at Collegium Medicum NCU. All procedures conformed to the ethical guidelines of the 1975 Declaration of Helsinki. The patients' legal guardians and all patients older than 16 signed a written informed consent.

### IL28B and OAS genotyping

Host genomic DNA was prepared using the QIAamp DNA Mini Kit (Qiagen) from peripheral whole-blood samples collected in 0.5 M EDTA tubes. The detection of IL28B (rs12979860 CT, rs12980275 AG and rs8099917 TG), OASL (rs10849829), OAS1 (rs1131476), OAS2 (rs1293747), and OAS3 (rs2072136) SNPs was carried out with a real-time polymerase chain reaction (real-time PCR) using the TaqMan SNP Genotyping Assays (Applied Biosystems by Life Technologies). For all SNPs, genotyping was performed on a LightCycler® 480 Instrument (Roche Diagnostics) with the following standard reaction conditions: 95 ℃ for 10 min, 92 ℃ for 15 s, followed by 35 cycles of 60 ℃ for 1 min. A population analysis for each genetic marker was performed for all patients.

### Treatment responses

The main focus of this study was to determine the predictive value of IL28B and OAS SNPs in treatment outcomes. The impact of other prognostic factors on the results of therapy was also analysed. To evaluate the therapeutic effects, we assessed virological, serological and biochemical responses by analysing HBV DNA levels, HBsAg status and ALT activity before, during and 24 wk after the end of therapy. A limit of 2000 IU HBV DNA/mL, which has been used in the literature, was adopted in the virological response analysis[26]. In our study, we determined the impact of genetic markers on the efficacy of therapy by comparing patients in terms of achieving a defined therapeutic response. We adopted two criteria for the therapeutic response, achieving an HBV DNA level < 2000 IU/mL and normalization of ALT activity (< 40 IU/L). To perform the analyses, we compared the patients in terms of achieving a partial response (PR) and a complete response (CR). CR was defined as the suppression of viral replication to an HBV DNA level < 2000 IU/mL and normalization of ALT activity (< 40 IU/L) 24 wk after completing the treatment. To be classified as PR, patients were required to achieve a positive result for at least one of the two parameters. Based on the PR criterion, patients were divided into those who achieved at least one of the two considered parameters and those who did not achieve any positive response 24 wk after completing the treatment. HBV DNA level was assessed with a quantitative polymerase chain reaction assay (cobas AmpliPrep/cobas TaqMan HBV Test; Roche Diagnostics).

### Statistical analysis

The summary statistics for the continuous variables are presented as the median and range. The categorical variables are presented as frequencies.

**Table 1 Characteristics of hepatitis B e antigen-negative chronic hepatitis B children**

| Characteristic | |
|---|---|
| *n* | 52 |
| Age (yr) | |
| Median (range) | 16 (4-18) |
| Gender | |
| Female/male | 16 (30.8)/36 (69.2) |
| Staging (F) | |
| F0/F1/F2/F3 | 2 (3.9)/31 (59.6)/16 (30.7)/3 (5.8) |
| Grading (A) | |
| A0/A1/A2 | 2 (3.9)/ 22 (42.3)/28 (53.8) |
| IL28B SNPs | |
| rs12979860 CC/CT/TT | 20 (38.5)/26 (50.0)/6 (11.5) |
| rs12980275 AA/AG/GG | 24 (46.2)/24 (46.2)/4 (7.6) |
| rs8099917 TT/TG/GG | 30 (57.7)/19 (36.5)/3 (5.8) |
| OAS SNPs | |
| rs10849829 AA/AG/GG | 19 (36.5)/27 (52.0)/6 (11.5) |
| rs1131476 AA/AG/GG | 26 (50.0)/17 (32.7)/9 (17.3) |
| rs1293747 GG/GA/AA | 32 (61.5)/17 (32.7)/3 (5.8) |
| rs2072136 GG/GA/AA | 30 (57.7)/20 (38.5)/2 (3.8) |
| Baseline HBV DNA (IU/mL) | |
| median (range), log | 4.6 (3.4-8.0) |
| < 20000 | 31 (59.6) |
| Baseline ALT (U/L) | |
| median (range) | 42 (12-210) |
| < 40 | 14 (26.9) |
| At week 24 of treatment | |
| HBV DNA < 100 (IU/mL) | 32 (61.5) |
| ALT < 40 (U/L) | 13 (25.0) |
| At the end of treatment | |
| HBV DNA < 2000 (IU/mL) | 39 (75.0) |
| ALT < 40 (U/L) | 28 (53.9) |
| 24 wk post-treatment | |
| HBV DNA < 2000 (IU/mL) | 27 (51.92) |
| ALT < 40 (U/L) | 37 (71.15) |
| Partial response | |
| < 2000 IU/mL HBV DNA or < 40 IU/L ALT | 42 (80.8) |
| Complete response | |
| < 2000 IU/mL HBV DNA with < 40 IU/L ALT | 22 (42.3) |

Data are presented as the number of patients (%) unless otherwise indicated.

To identify factors predicting treatment response, we evaluated statistical significance using univariate analysis. Differences between continuous variables, such as HBV viral load and ALT activity, were analysed with the Mann-Whitney *U* test (the distributions were not parametric). The Pearson $\chi^2$ test or Fisher's exact test, where appropriate, was used for categorical variables. IL28B and OAS SNP comparisons were made using dominant and recessive models based on the minor allele frequency in the presented study group. For IL28B and OAS in the dominant model, patients carrying one or two copies of the minor allele were compared with the others, and for IL28B and OAS in the recessive model, patients carrying two copies of the minor allele were compared with the others. Multivariate analysis was performed using logistic regression models that included the variables determined to be significant in univariate analysis. The analysis of linkage disequilibrium (LD) and associations of haplotype with treatment response were performed using SHEsis online software[27]. The results were considered statistically significant when the *P* value was less than 0.05. Odds ratios (OR) and 95%CI were also calculated for the statistically significant results of compared binary clinical variables. The statistical analysis and graphing were completed with the use of IBM SPSS 20 and GraphPad Prism 6 software. The statistical methods of this study were reviewed by a biomedical statistician.

## RESULTS

### *Patient characteristics and treatment responses*

This study recruited 52 Caucasian children chronically infected with HBV, including girls (30.8%) and boys (69.2%), with a median age of 16 years. The clinical characteristics of all the patients treated for CHB are presented in Table 1. All the patients in the present study had a baseline HBV DNA level above 2000 IU/mL; 31 of them had values under 20000 IU/mL (59.6%) at baseline. The median baseline HBV DNA of the entire group was 4.6 $\log_{10}$ IU/mL. Fourteen (26.9%) patients had normal ALT activity (< 40 U/L) at baseline. The median ALT activity was 42 U/L. The pretreatment liver biopsy sample data, which was assessed in all patients, showed that most of the patients had stage F1 (59.6%) and F2 (30.7%) fibrosis and grade A1 (42.3%) and A2 (53.8%) inflammation, according to the modified Scheuer score. None of these patients had cirrhosis. The genotype distributions for the IL28B and OAS family genes are presented in Table 1.

The HBV DNA level < 2000 IU/mL and ALT normalization response rates were 75.0% and 53.9%, respectively, at the end of therapy and 51.9% and 71.2%, respectively, 24 wk post-treatment. The partial and complete response rates were 80.8% and 42.3%, respectively. Two (3.8%) of the analysed patients group achieved HBsAg seroclearance (data not shown).

### *Factors associated with PR*

At first, we evaluated the association between clinical baseline characteristics, including the analysed polymorphisms, and PR (Table 2). In general, the univariate analysis showed no relationship between age at start of therapy, gender, liver histology and partial response. In contrast, the baseline HBV DNA level and ALT activity significantly affect the PR. Higher baseline HBV DNA levels and ALT activity were associated with decreased rates of PR.

Analysis of the IL28B and OAS gene family SNPs showed that only the IL28B SNPs had an impact on partial response. However, only the IL28B rs12980275 marker was significantly different in the PR group (AA *vs* AG-GG, *P* = 0.014, OR = 10.9, 95%CI: 1.3-93.9). The other two markers had borderline significance (0.068 and 0.075 for CC *vs* CT-TT of rs12979860 and

**Table 2 Impact of clinical and genetic factors on partial response in HBeAg-negative chronic hepatitis B children**

| Characteristic | PR, *n* = 42 | No-PR, *n* = 10 | *P* value | OR (95%CI) |
|---|---|---|---|---|
| Age (yr) | | | | |
| Median (range) | 16 (4-18) | 16 (5-18) | 0.803 | |
| Gender | | | | |
| Female | 14 (33.3) | 2 (20.0) | 0.412 | |
| Staging (F) | | | | |
| F0-F1 | 26 (61.9) | 7 (70.0) | 0.729 | |
| Grading (A) | | | | |
| A0-A1 | 20 (47.6) | 5 (50.0) | 0.892 | |
| Baseline HBV DNA (IU/mL) | | | | |
| Median (range), log | 4.1 (3.4-8.0) | 5.9 (3.6-8.0) | 0.017 | |
| < 20000 | 28 (66.7) | 3 (30.0) | 0.069 | |
| Baseline ALT (U/L) | | | | |
| Median (range) | 44 (10-120) | 63 (22-118) | 0.003 | |
| < 40 | 13 (31.0) | 1 (10.0) | 0.207 | |
| IL28B rs12979860 | | | | |
| CC | 19 (45.2) | 1 (10.0) | 0.068 | |
| CT | 20 (47.6) | 6 (60.0) | | |
| TT | 3 (7.2) | 3 (30.0) | 0.077 | |
| IL28B rs12980275 | | | | |
| AA | 23 (54.8) | 1 (10.0) | 0.014 | 10.9 (1.3-93.9) |
| AG | 17 (40.6) | 7 (70.0) | | |
| GG | 2 (4.8) | 2 (20.0) | 0.163 | |
| IL28B rs8099917 | | | | |
| TT | 27 (64.2) | 3 (30.0) | 0.075 | |
| TG | 13 (31.0) | 6 (60.0) | | |
| GG | 2 (4.8) | 1 (10.0) | 0.481 | |
| OASL rs10849829 | | | | |
| AA | 16 (38.1) | 5 (50.0) | 0.500 | |
| AG | 20 (47.6) | 5 (50.0) | | |
| GG | 6 (14.3) | 0 (0.0) | 0.582 | |
| OAS1 rs1131476 | | | | |
| AA | 19 (45.2) | 7 (70.0) | 0.291 | |
| AG | 15 (35.7) | 2 (20.0) | | |
| GG | 8 (19.1) | 1 (10.0) | 0.670 | |
| OAS2 rs1293747 | | | | |
| GG | 25 (59.5) | 7 (70.0) | 0.481 | |
| GA | 15 (35.7) | 2 (20.0) | | |
| AA | 2 (4.8) | 1 (10.0) | 0.722 | |
| OAS3 rs2072136 | | | | |
| GG | 23 (54.8) | 7 (70.0) | 1.000 | |
| GA | 17 (40.6) | 3 (30.0) | | |
| AA | 2 (4.8) | 0 (0.0) | 0.488 | |

Data are presented as the number of patients (%) unless otherwise indicated.

**Table 3 Impact of clinical and genetic factors on partial response in HBeAg-negative chronic hepatitis B children**

| Characteristic | CR, *n* = 22 | No-CR, *n* = 30 | *P* value | OR (95%CI) |
|---|---|---|---|---|
| Age (yr) | | | | |
| Median (range) | 16 (12-18) | 17 (4-18) | 0.141 | |
| Gender | | | | |
| Female | 8 (36.4) | 8 (26.7) | 0.454 | |
| Staging (F) | | | | |
| F0-F1 | 14 (63.6) | 19 (63.3) | 0.942 | |
| Grading (A) | | | | |
| A0-A1 | 10 (45.5) | 15 (50.0) | 0.746 | |
| Baseline HBV DNA (IU/mL) | | | | |
| Median (range), log | 4.6 (3.4-8.0) | 4.7 (3.5-8.0) | 0.671 | |
| < 20000 | 14 (63.6) | 17 (56.7) | 0.613 | |
| Baseline ALT (U/L) | | | | |
| Median (range) | 43 (10-110) | 48 (17-126) | 0.116 | |
| < 40 | 8 (36.3) | 6 (20.0) | 0.189 | |
| IL28B rs12979860 | | | | |
| CC | 8 (36.4) | 12 (40.0) | 0.790 | |
| CT | 14 (63.6) | 12 (40.0) | | |
| TT | 0 (0.0) | 6 (20.0) | 0.033 | NA |
| IL28B rs12980275 | | | | |
| AA | 11 (50.0) | 13 (43.3) | 0.634 | |
| AG | 11 (50.0) | 13 (43.3) | | |
| GG | 0 (0.0) | 4 (13.4) | 0.128 | |
| IL28B rs8099917 | | | | |
| TT | 15 (68.2) | 15 (50.0) | 0.190 | |
| TG | 7 (31.8) | 12 (40.0) | | |
| GG | 0 (0.0) | 3 (10.0) | 0.253 | |
| OASL rs10849829 | | | | |
| AA | 5 (22.7) | 16 (53.3) | 0.026 | 0.26 (0.07-0.88) |
| AG | 14 (63.6) | 11 (36.7) | | |
| GG | 3 (13.6) | 3 (10.0) | 0.685 | |
| OAS1 rs1131476 | | | | |
| AA | 10 (45.5) | 16 (53.3) | 0.575 | |
| AG | 8 (36.4) | 9 (30.0) | | |
| GG | 4 (18.1) | 5 (16.7) | 0.887 | |
| OAS2 rs1293747 | | | | |
| GG | 14 (63.6) | 18 (60.0) | 0.253 | |
| GA | 8 (36.4) | 9 (30.0) | | |
| AA | 0 (0.0) | 3 (10.0) | 0.790 | |
| OAS3 rs2072136 | | | | |
| GG | 15 (68.2) | 15 (50.0) | 1.000 | |
| GA | 6 (27.3) | 14 (46.7) | | |
| AA | 1 (4.5) | 1 (3.3) | 0.190 | |

Data are presented as the number of patients (%) unless otherwise indicated. NA: Not applicable.

TT *vs* TG-GG of rs8099917, respectively). However, the distributions of allele frequencies for IL28B rs12979860 (C *vs* T) were significantly different between the PR and no-PR groups ($P$ = 0.021, OR = 3.3, 95%CI: 1.2-9.2), in contrast to rs8099917 (T *vs* G, $P$ = 0.082) (data not shown). The factors that were significantly associated with PR in the univariate analysis were analysed by logistic regression analysis. We found significant associations for baseline HBV DNA level ($P$ = 0.037, OR = 2.2, 95%CI: 1.1-4.7) and IL28B rs12980275 AA ($P$ = 0.020, OR = 18.8, 95%CI: 1.6-123.2), but not for ALT activity ($P$ = 0.70).

### *Factors associated with complete response*

In the next step, we analysed the significance of the association between baseline factors and complete response (Table 3). The presented data revealed that overall there were no statistically significant differences in the baseline characteristics of the complete response (CR) and no-CR groups in the analysed group of children. However, ALT activity was higher in patients in the no-CR group (48 U/L) compared with patients with the CR group (43 U/L). Additionally, the percentage patients with ALT < 40 U/L was higher in the patients with a CR (36.3% *vs* 20.0%).

Analyses of the examined polymorphisms showed that only the IL28B rs12979860 and OASL rs10849829

**Table 4 Association of OAS haplotypes with treatment response**

| Haplotype | CR | No-CR | *P* value | OR (95%CI) | PR | No-PR | *P* value | OR (95%CI) |
|---|---|---|---|---|---|---|---|---|
| A A A A | 0.11 | 0.16 | 0.402 | 0.60 (0.18-1.98) | 0.16 | 0.00 | 0.041 | NA |
| A A G A | 0.03 | 0.05 | 0.473 | 0.47 (0.06-3.78) | 0.05 | 0.00 | 0.283 | NA |
| A A G G | 0.17 | 0.35 | 0.031 | 0.35 (0.13-0.92) | 0.21 | 0.57 | 0.002 | 0.21 (0.07-0.61) |
| A G G G | 0.24 | 0.12 | 0.156 | 2.11 (0.74-6.00) | 0.17 | 0.13 | 0.543 | 1.55 (0.37-6.48) |
| G A G G | 0.28 | 0.03 | 0.0003 | 11.96 (2.38-59.97) | 0.16 | 0.05 | 0.173 | 3.91 (0.48-31.8) |
| G G G G | 0.05 | 0.17 | 0.046 | 0.24 (0.05-1.06) | 0.15 | 0.05 | 0.194 | 3.70 (0.45-30.25) |

Data are presented as the haplotype frequency; NA: Not applicable.

markers had an impact on CR results. The recessive genotype distributions for the IL28B rs12979860 polymorphism (CC-CT *vs* TT) were significantly different between the CR and no-CR groups ($P$ = 0.033). These results were observed because none of the 6 children with the rs12979860 TT genotype achieved CR and the proportion of patients with CC-CT genotypes was comparable between the CR and no-CR groups. Despite the lack of statistical significance such as that observed in rs12979860 for the other two IL28B polymorphisms, none of the patients carrying homozygous genotypes for the minor alleles (GG for rs8099917 and GG for rs12980275) achieved CR. In contrast to the PR results, there were no statistically marginal or significant differences in CR rates for the dominant genotype and allele frequency distributions of the IL28B markers. The genotype distributions for the OASL rs10849829 polymorphisms (AA *vs* AG-GG) were significantly different between the CR and no-CR groups. In our series, for rs10849829, the OR of being a responder for the AA genotype compared to the AG and GG genotypes was 0.26 (95%CI: 0.07-0.88). CR was achieved in 5/21 (23.8%) of patients with the AA genotype at rs10849829, 14/25 (56.0%) patients with the genotype AG, and 3/6 (50.0%) patients with the genotype GG. Multivariate analysis for factors significantly associated with CR in univariate analysis showed only borderline significance for OASL rs10849829 AA ($P$ = 0.061) in predicting a complete response.

### *Impact of OAS and IL28B haplotypes on treatment responses*

Because all four OAS SNPs were located in a cluster on chromosome 12, we analysed the association of 4 SNPs with treatment response. First, we determined whether the four analysed SNPs were in strong linkage disequilibrium (LD). Overall, LD analysis demonstrated that rs1131476 OAS1, rs1293747 OAS2, rs2072136 OAS3 and rs10849829 OASL were in slight linkage disequilibrium (ranges, D′ = 0.358 - 0.999, $r^2$ = 0.023 - 0.382). The strongest LD was noted for the OAS1 and OAS3 markers (D′ = 0.635, $r^2$ = 0.152) and for the OAS2 and OAS3 markers (D′ = 0.999, $r^2$ = 0.382). In the next step, we conducted a haplotype analysis of the 4 OAS SNPs. There were 6 common haplotypes with a frequency exceeding 5% in our study cohort (Table 4). The global tests revealed significant associations between OAS haplotype and PR and CR ($P$ = 0.0002 and $P$ = 0.001, respectively). Significant differences in haplotype frequencies between the CR and no-CR groups were noted for the G-A-G-G haplotype (28% *vs* 3%, $P$ = 0.0003), the A-A-G-G haplotype (17% *vs* 34%, $P$ = 0.031) and the G-G-G-G haplotype (5% *vs* 17%, $P$ = 0.046). In addition, the haplotypes A-A-A-A and A-A-G-G were associated with the PR group rather than the no-PR group (16% *vs* 0%, $P$ = 0.041 and 21% *vs* 57%, $P$ = 0.002, respectively).

In our study cohort, the IL28B SNPs were in strong LD (D′ = 0.999 for all pairs; $r^2$ ranges from 0.550 to 0.712). The global IL28B haplotype analyses indicated that the response groups (PR *vs* no-PR and CR *vs* no-CR) did not contain significantly different haplotype frequencies. However, the C-A-T haplotype (major alleles of all markers) was significantly associated with achieving PR (69% in the PR group *vs* 40% in the no-PR group, $P$ = 0.015). In addition, for the T-G-G haplotype (minor alleles of all markers) there was a borderline significant difference between the CR and no-CR groups (16% *vs* 30%, $P$ = 0.09) and the PR and no-PR groups (21% *vs* 40%, $P$ = 0.06).

## DISCUSSION

The limited efficacy, high cost and side effects associated with PEG-IFN treatment limit its clinical use in patients with CHB; however, this therapy is currently the only option for the permanent elimination of the virus[28]. Although viral clearance is rarely achieved with IFN treatment, it is more common with treatment than the natural rate of HBsAg seroclearance, which remains at a level of 2%-3%[29-31]. In this study of a group of paediatric patients, two (3.8%) patients permanently eliminated the virus.

Since there is no method of eradicating HBV, the current realistic goal of antiviral therapy is to achieve the permanent suppression of HBV replication to a level enabling the inhibition or retardation of inflammation and fibrosis of the liver and to protect against the development of hepatocellular carcinoma[32,33]. In the case of HBeAg-negative patients treated with IFN, reducing the viral load of HBV DNA after treatment to less than 2000 IU/mL is usually considered a

therapeutic success. The other endpoint of IFN therapy is a reduction in ALT to levels considered normal and the improvement of liver histology[28]. In our study, 42.3% of the patients achieved a complete response (HBV < 2000 IU/mL with ALT normalization), which is a result similar to the results obtained in other tests carried out among adult patients negative for HBe antigen[11,34,35].

Identification of the molecular mechanisms affecting the efficacy of interferon therapies and the severity of side effects of the treatment remain one of the main objectives of the study. In the current study, we analysed the relationship between polymorphisms in the interleukin 28B gene and OAS gene family and the response to interferon therapy in children with CHB. In our previous study, which included adult patients, we demonstrated that IL28B markers are associated with response to interferon therapy in patients with HBeAg negative CHB[36]. Other previous studies tried to determine whether having favourable IL28B genotype markers is associated with a better response to interferon therapy in HBeAg-negative adult patients, but the results were inconclusive. Some reports have suggested that the presence of favourable IL28B genotypes is associated with a better response to treatment[31,37], while others have indicated that there is no association between IL28B SNPs and the response to treatment with interferon[26,38]. In the present study, we demonstrated a statistically significant relationship between the rs12979860 marker and the response to treatment with interferon in patients with CHB. However, the results indicate the importance of the unfavourable TT genotype in estimating the chances of treatment failure.

Oligoadenylate synthetase plays a critical role in innate immunity, controlling the outcome of virus production. Consistent with the important role of OAS proteins in viral infection, it was shown that polymorphisms in the OAS genes are associated with increased susceptibility to HCV infection[18] and IFN treatment outcomes[17,19,20]. However, the molecular basis for this association is not well understood. In several studies, it was shown that polymorphisms in the OAS genes result in variations in enzyme activity by influencing basal enzyme activity[39], gene expression[17] or cellular localization[40].

In our study, we tried determine whether SNPs within the OAS genes may also affect the efficacy of interferon therapy in paediatric patients with CHB. In this study, a total of four SNPs located in the OAS1 (rs1131476), OAS2 (rs1293747), OAS3 (rs2072136) and OASL (rs10849829) genes were selected. The results of our study indicate that only the OASL rs10849829 marker has an impact on final therapeutic success, which was defined as the suppression of viral replication to an HBV DNA level < 2000 IU/mL and normalization of ALT activity 24 wk after completing treatment. The connection between the OAS gene polymorphisms and the efficacy of interferon therapy has not been extensively studied in patients with CHB. A small number of available studies have been carried out only in Han Chinese treatment-naïve CHB patients, who are representative of the Asian population[24,25]. The study of Wu *et al*[24] demonstrated an association between interferon treatment and the haplotype G-T-G-A within the rs3177979G, rs1293747T, rs4767043G, and rs10849829A alleles, which occur in the OAS1, OAS2, OAS3, and OASL genes, respectively, instead of with OASL rs10849829. In this study, the authors suggested that patients with a G-T-G-A OAS haplotype were less responsive to IFN treatment. In agreement with the results of Wu *et al*[24] we demonstrated that the AA genotype of rs10849829 was associated with failure of IFN therapy; however, there were no significant differences in allele frequencies between the different response groups. Additionally, the authors showed that the allele frequencies and genotype distributions of all the examined OAS SNPs were not correlated with treatment outcomes in patients who underwent IFN therapy. In another study conducted by Ren *et al*[25], the influence of 4 SNPs - rs2285934 OAS1, rs2072138 OAS2, rs2072136 OAS3 and rs10849829 OASL - on the outcome of a 48-week IFN treatment of Han Chinese treatment-naïve CHB patients (265 HBeAg-positive, 55 HBeAg-negative, and 43 inactive HBsAg carriers) was evaluated. In this study, treatment response was defined as HBsAg seroconversion or HBeAg seroconversion for the patients who were HBeAg-positive, without HBsAg seroconversion. In contrast to our results, the allele frequencies and genotype distribution analysis of these SNPs showed that only the OAS3 SNP (rs2072136 T/C) was independently associated with IFN treatment response. However, OAS haplotype analysis demonstrated significant associations between haplotypes and response to IFN treatment. The most common haplotype C-C-T-A (rs2285934C, rs2072138C, rs2072136T and rs10849829A) was associated with non-response. Based on the results of these two studies, in our study we conducted a haplotype analysis of the 4 SNPs. Although the sample size was relatively small, we identified an association between the OAS haplotypes and PR and CR to IFN treatment.

In addition to the different endpoints of therapy, the reason for the discrepancies in the results of our work may be, as for other OAS gene polymorphisms, differences in the distribution of alleles between Caucasian and Asian populations. While the most common genotype for the rs10849829 marker is genotype AA (approx. 60%[25]) in the Asian population, in our study, the percentage of patients with the AA genotype did not exceed 40%. The differences in the distribution of alleles between populations and their role in predicting response to interferon therapy in patients infected with HCV have been documented

for IL28B polymorphisms[41]. Additionally, Lampertico *et al*[42] suggest that studies of IL28B genotype and response to peginterferon in chronic hepatitis B should be stratified by HBV genotype. Although comparing the results for the Caucasian population with the results of the Asian population is difficult due to differences in the human alleles and HBV genotype distributions, it indicates the direction required for further studies, including those in Caucasian patients.

In summary, the presented study demonstrated a relationship between the IL28B rs12979860 and rs10849829 OASL genotypes, OAS haplotypes and the final IFN treatment outcome in paediatric patients infected with CHB. We noted that the unfavourable IL28B SNP genotype was present only in the no-CR group. Unlike adult studies, paediatric studies are definitely less frequent and are carried out in relatively small populations. Even the group we studied was not large enough to produce results that can be considered final. Nevertheless, this study indicates the potential markers we should focus on during further studies involving larger groups of patients.

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## COMMENTS

### Background

The 48-wk course of interferon therapy is recommended as a first-line treatment option for selected HBeAg-negative patients, especially in young patients with increased aminotransferase activity. The limited efficacy and side effects associated with IFN treatment limit its clinical use in paediatric patients with chronic hepatitis B (CHB). Therefore, a pretreatment identifying children with the highest probability of success is of great clinical importance to IFN therapy.

### Research frontiers

The influence of host-related genetic variability on differences in response rates to IFN therapy in CHB patients is not well understood. The association between interleukin 28B (IL28B) and 2',5'-oligoadenylate synthetase (OAS) polymorphisms and the result of IFN monotherapy in adults with CHB has been the subject of very few studies. Currently, there are no available results concerning the impact of the IL28B or OAS SNPs on the results of IFN therapy in a group of paediatric patients with CHB.

### Innovations and breakthroughs

The association between OASL rs10849829 and OAS haplotypes with IFN-treatment response in Caucasian CHB children is an important finding of this study. The results of the study confirm a most significant role of IL28B rs12979860 in predicting treatment response in Caucasian patients; however, the results indicate the importance of the unfavourable TT genotype in estimating the chances of treatment failure.

### Applications

The results of the study indicate potential markers that may be useful to confirm the eligibility of children with CHB for IFN treatment.

### Terminology

2,5 -*oligoadenylate synthetases* are the family of interferon-induced enzymes that play a critical role in controlling the production of viral proteins. The human OAS family contains the OAS1, OAS2, OAS3, and OASL genes located on chromosome 12. Interleukin 28B is a cytokine belonging to the interferon lambda family that plays a role in immune defence against virus infection, including inducing the transcription of antiviral genes such as the *OAS* genes.

### Peer-review

This study examined the association between SNPs in OAS and IL28B and the biochemical and virological response rates to PEG-IFN alfa-2a monotherapy in a cohort of Caucasian children chronically infected with HBV. The results of this study revealed that patients with the AA allele at OASL rs10849829 and the TT allele at IL28B rs12979860 have lower chances of a complete response. In addition, this study found that the OAS1 (rs1131476), OAS2 (rs1293747), OAS3 (rs2072136), and OASL (rs10849829) haplotype had an impact on estimating the treatment response.

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ORIGINAL ARTICLE

Retrospective Study

# Simplified criteria for diagnosing superficial esophageal squamous neoplasms using Narrow Band Imaging magnifying endoscopy

Akira Dobashi, Kenichi Goda, Noboru Yoshimura, Tomohiko R Ohya, Masayuki Kato, Kazuki Sumiyama, Masato Matsushima, Shinichi Hirooka, Masahiro Ikegami, Hisao Tajiri

Akira Dobashi, Kenichi Goda, Noboru Yoshimura, Tomohiko R Ohya, Kazuki Sumiyama, Department of Endoscopy, The Jikei University School of Medicine, Tokyo 105-8461, Japan

Masayuki Kato, Department of Endoscopy, The Jikei University Katsushika Medical Center, Toky 125-0062, Japan

Masato Matsushima, Division of Clinical Epidemiology, The Jikei University School of Medicine, Tokyo 105-8461, Japan

Shinichi Hirooka, Masahiro Ikegami, Department of Pathology, The Jikei University School of Medicine, Tokyo 105-8461, Japan

Hisao Tajiri, Department of Innovative Interventional Endoscopy Research, The Jikei University School of Medicine, Tokyo 105-8461, Japan

Author contributions: Dobashi A designed and performed the research and wrote the paper; Goda K designed and performed the research and wrote the paper; Yoshimura N, Sumiyama K, Hirooka S, Ikegami M, Tajiri H supervised the report; Kato M, Matsushima M designed the research and contributed to statistical analysis; Ohya TR was an English narrator and supervised the report.

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Informed consent statement: This study was carried out as a *post-hoc* analysis by using the data from our previous study of randomized controlled trial. All study participants provided informed written consent prior to study enrollment.

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Correspondence to: Kenichi Goda, MD, PhD, Associate Professor, Department of Endoscopy, The Jikei University School of Medicine, 3-25-8 Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan. kengoendoscopy@hotmail.co.jp
Telephone: +81-3-34331111-3181
Fax: +81-3-34594524

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## Abstract

### *AIM*

To simplify the diagnostic criteria for superficial esophageal squamous cell carcinoma (SESCC) on Narrow Band Imaging combined with magnifying endoscopy (NBI-ME).

### *METHODS*

This study was based on the post-hoc analysis of a randomized controlled trial. We performed NBI-ME for 147 patients with present or a history of squamous cell carcinoma in the head and neck, or esophagus between January 2009 and June 2011. Two expert endoscopists

detected 89 lesions that were suspicious for SESCC lesions, which had been prospectively evaluated for the following 6 NBI-ME findings in real time: "intervascular background coloration"; "proliferation of intrapapillary capillary loops (IPCL)"; and "dilation", "tortuosity", "change in caliber", and "various shapes (VS)" of IPCLs (*i.e.*, Inoue's tetrad criteria). The histologic examination of specimens was defined as the gold standard for diagnosis. A stepwise logistic regression analysis was used to identify candidates for the simplified criteria from among the 6 NBI-ME findings for diagnosing SESCCs. We evaluated diagnostic performance of the simplified criteria compared with that of Inoue's criteria.

***RESULTS***

Fifty-four lesions (65%) were histologically diagnosed as SESCCs and the others as low-grade intraepithelial neoplasia or inflammation. In the univariate analysis, proliferation, tortuosity, change in caliber, and VS were significantly associated with SESCC ($P < 0.01$). The combination of VS and proliferation was statistically extracted from the 6 NBI-ME findings by using the stepwise logistic regression model. We defined the combination of VS and proliferation as simplified dyad criteria for SESCC. The areas under the curve of the simplified dyad criteria and Inoue's tetrad criteria were 0.70 and 0.73, respectively. No significant difference was shown between them. The sensitivity, specificity, and accuracy of diagnosis for SESCC were 77.8%, 57.1%, 69.7% and 51.9%, 80.0%, 62.9% for the simplified dyad criteria and Inoue's tetrad criteria, respectively.

***CONCLUSION***

The combination of proliferation and VS may serve as simplified criteria for the diagnosis of SESCC using NBI-ME.

**Key words:** Simplified criteria; Narrow Band Imaging; Magnifying endoscopy; Esophageal cancer; Squamous cell carcinoma; Endoscopic diagnosis; Classification; Superficial squamous cell carcinoma; Stepwise logistic regression analysis



**Core tip:** Narrow Band Imaging combined with magnifying endoscopy (NBI-ME) significantly improves the diagnostic accuracy for superficial esophageal squamous cell carcinoma (SESCC). However, currently used NBI-ME diagnostic criteria may confuse endoscopists and inhibit the widespread use of NBI-ME. The findings of this study suggest that simplified dyad criteria composed of the presence of "proliferation of intrapapillary capillary loops (IPCL)" and "various shapes (VS) of IPCLs" had comparable diagnostic performance to Inoue's tetrad criteria, which are the most popular diagnostic criteria for SESCC. Proliferation and VS may serve as simplified NBI-ME criteria for diagnosing SESCC.

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## INTRODUCTION

Esophageal cancer is one of the most common cancers, and the number of deaths related to esophageal cancer ranks sixth worldwide[1]. The histology of esophageal cancer mainly consists of squamous cell carcinoma (SCC) and adenocarcinoma. Especially in developing countries and Asian countries, SCC accounts for about 90% of esophageal cancer[2]. Esophageal cancer at an advanced stage brings a dismal prognosis; thus, the detection of esophageal cancer at an early stage is needed to obtain a higher quality of life and better prognosis[3,4].

However, it is difficult to detect esophageal SCC at an early stage using white light imaging endoscopy (WLI), because most of the superficial esophageal SCCs (SESCC) appear flat and/or isochromatic[4]. Lugol staining has high sensitivity for the detection of SESCCs but low specificity, as well as the potential for adverse effects including severe discomfort and allergic reaction[5].

Narrow Band Imaging (NBI) is a revolutionary technology utilizing optical imaged-enhanced endoscopy for diagnosing SESCCs[6,7]. The NBI can visualize a SESCC clearly as a well-demarcated brownish area without the use of Lugol staining. However, the brownish appearance occasionally represents a false-positive SESCC, because esophageal mucosa with inflammation also appears brown under NBI[8,9].

In the normal esophageal mucosa, loop-like vessels arise from the subepithelial capillary network beneath the epithelium. These microvessels are inside the epithelial papillae and named "intrapapillary capillary loops (IPCLs)"[6]. The IPCLs or abnormal microvessels in the superficial layer of the lesion are clearly visualized on magnifying endoscopy in combination with NBI.

SESCC lesions have abnormal microvessels with severe irregularity. The severe irregularity is defined as having morphological changes as follows: "dilation", "tortuosity", "change in caliber", and "various shapes (VS)"[6,10]. When SCC invades up to the lamina propria mucosae, the abnormal microvessels with severe irregularity show a loop-like formation. When the tumor invasion reaches the muscularis mucosae or shallowly invades the submucosae, abnormal microvessels show no loop-like formation that has a stretched and markedly elongated transformation. The tumor deeply invading the submucosae has abnormal vessels with

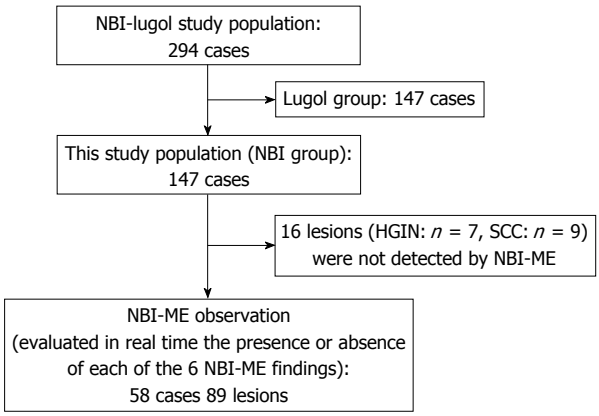


**Figure 1 Overview of the study design.** HGIN: High-grade intraepithelial neoplasia; SCC: Squamous cell carcinoma; NBI-ME: Narrow Band Imaging magnifying endoscopy.

severe dilation and green color. Thus, the identification of IPCLs and abnormal microvessels can contribute to accurate diagnosis and reduced false-positive results in the diagnosis of SESCC[6,8]. Previous studies have shown a significantly higher diagnostic accuracy for SESCC with NBI combined with magnifying endoscopy (NBI-ME) when compared with WLI and Lugol chromoendoscopy[4,8].

NBI-ME is not widely used despite its high diagnostic performance[11]. Previous studies proposed a total of 6 NBI-ME findings that are significantly suggestive of SESCC. The 6 NBI-ME findings comprise complicated diagnostic criteria that may prove confusing for general endoscopists and prevent the widespread application of NBI-ME[4,6,12,13]. We considered that simplifying the NBI-ME diagnostic criteria. It may overcome the issues on NBI-ME if the NBI-ME diagnostic criteria can be simplified. The aim of this study was to identify NBI-ME findings for construction of simplified criteria for diagnosing SESCC.

## MATERIALS AND METHODS

### Study design and patients

This study was carried out as a post-hoc analysis by using the data from our previous study of RCT (NBI-Lugol study)[8]. The NBI-Lugol study was approved by the institutional review board at the Jikei University School of Medicine and was compliant with the Declaration of Helsinki. The patients who had a histologically confirmed present or past history of SCC in the head and neck, or esophagus from January 2009 to June 2011 were randomly assigned to the NBI group ($n$ = 147) or Lugol group ($n$ = 147). Only patients in the NBI group underwent the NBI-ME inspection. Therefore, the data from NBI group patients was analyzed in this study (Figure 1). The exclusion criteria in the NBI-Lugol study were as follows: (1) prior esophagectomy; (2) history of chemotherapy or radiotherapy for SESCC; (3) recent history of chemotherapy for any malignancy; (4) history of intolerance to Lugol chromoendoscopy or allergic reaction to iodine; (5) concurrent presentation of an esophageal varix; (6) current pregnancy in women; and (7) contraindication to stopping antiplatelet or anticoagulant medication.

### Endoscopic examination and biopsy protocol

NBI-ME was performed using a high-definition magnifying endoscope (GIF-H260Z; Olympus, Tokyo, Japan) and a 19-inch high-resolution liquid-crystal monitor (OEV19H, Olympus). The combination of the endoscope and the monitor enabled endoscopic examination at a maximum magnification of 90-fold. A black rubber attachment (MB-46, Olympus) was mounted on the tip of the endoscope to maintain an adequate distance between the tip of the endoscope and the targeted lesion. The NBI-ME inspections were all performed by two experts (Goda K or Yoshimura N) who both had experience in NBI-ME observation of more than 200 SESCC cases.

Under NBI without magnification, we detected all well-demarcated brownish areas > 5 mm in diameter and non-brownish areas with elevation or depression that were suspicious for SESCC. Once the suspicious lesion was detected, we performed further inspection subsequently by adding magnification with NBI (*i.e.,* NBI-ME), and the suspicious lesions was evaluated in real time the presence or absence of each of the 6 NBI-ME findings (Figure 2). Finally, we applied Lugol solution, and biopsies were taken from all target lesions that were detected under NBI and irregularly shaped lesions > 5 mm diameter identified with Lugol staining. The biopsied lesions were removed by endoscopic or surgical resection if histology demonstrated high-grade intraepithelial neoplasia (HGIN) or SCC. We analyzed lesions that were detected by NBI inspection and excluded lesions that were detected only by Lugol chromoendoscopy in this study.

### Diagnostic criteria of SESCC using NBI-ME

NBI enhances the contrast between microvessels and background mucosa in the esophagus, so that microvessels can be observed clearly using NBI-ME[6]. Inoue *et al*[6,12-14] observed the last branch of the superficial vascular structures in the esophagus and named it the IPCL. Moreover, they proposed a close relationship between morphological changes of IPCLs and histological atypia. The lesions were highly suspicious for SESCC in the lesions had the four following morphological changes: "dilation," "tortuosity," "change in caliber," and "VS" (*i.e.,* Inoue's tetrad criteria)[6,12,15]. Inoue's tetrad criteria are the most popular NBI-ME diagnostic criteria for SESCC[10].

Proliferation[8,16,17] and intervascular background coloration (IBC) (*i.e.,* background coloration[18,19], brownish epithelium[13,20,21], and inter-vascular brownish epithelium[8,16]) are also NBI-ME findings strongly

| Magnified NBI endoscopy finding | Definition | Schema |
|---|---|---|
| Normal | Superficial microvessels with a single loop but no changes in caliber or no various shapes in the normal whitish mucosa [*i.e.*, intrapapillary capillary loops (IPCL)] |  |
| Intervascular background coloration | Brownish coloration between microvessels that differed from whitish epithelium of surrounding normal mucosa |  |
| Proliferation | The presence of a group of higher dense microvessels compared with a density of IPCL on surrounding normal mucosa |  |
| Morphological change of IPCLs<br>Dilation | Diameters of a group of microvessels were at least twice compared with those of IPCL on surrounding normal mucosa |  |
| Tortuosity | The presence of a group of microvessels that are more greatly or sharply twisted or bent compared with IPCL on surrounding normal mucosa |  |
| Changes in caliber | The presence of abrupt changes in vessel diameter (*i.e.*, thickening or narrowing) in a group of microvessels |  |
| Various shapes | The presence of highly diverse morphologies in a group of microvessels |  |

**Figure 2 Definitions of Narrow Band Imaging magnifying endoscopy findings.** NBI: Narrow Band Imaging; IPCL: Intrapapillary capillary loop.

**Table 1 Demographics of patients and lesions, *n***

| | |
|---|---|
| Number of patients | *n* = 147 |
| Age (yr), median (range) | 67 (39-86) |
| Male, *n* (%) | 130 (88%) |
| History of cancers (head and neck/esophagus) | 85/74 |
| Drinking history | |
| Number of drinkers | 134 |
| Drinking duration (yr), median (range) | 40 (10-68) |
| Number of flushers | 102 |
| Smoking history | |
| Number of smokers | 129 |
| Smoking (yr), median (range) | 35 (3-70) |
| Number of lesions | 89 |
| Diameter of lesions (mm), median (range) | 15 (4-100) |
| Histology (SCC/HGIN/LGIN/inflammation) | 48/6/27/8 |
| Superficial esophageal squamous cell carcinoma (including HGIN) | 54 |
| Diameter (mm), median (range) | 19 (4-100) |
| Macroscopic type; *n*, (0-Ⅰ/0-Ⅱa/0-Ⅱb/0-Ⅱc/0-Ⅲ) | 5/2/25/21/1 |
| Depth of invasion; *n*, (HGIN/EP, LP/MM-SM1/SM2/unknown[1]) | 3/29/7/3/12 |
| Treatment; *n*, (ER/SR/CRT/others) | 32/11/5/6 |

[1]Only biopsy was obtained. SCC: Squamous cell carcinoma; HGIN: High-grade intraepithelial neoplasia; LGIN: Low-grade intraepithelial neoplasia; EP: Epithelium; LPM: Lamina propria mucosae; MM: Muscularis mucosae; SM: Submucosae; ER: Endoscopic resection; SR: Surgical resection; CRT: Chemoradiotherapy.

suggestive of SECCC. These 6 NBI-ME findings were prospectively evaluated in the NBI-Lugol study[8]. The definitions of the NBI-ME findings are listed in Figure 2.

### *Outcome and measurement*

The primary goal was to identify a minimal number of NBI-ME criteria for SESCC. The secondary goal was to evaluate diagnostic performance of the simplified criteria compared to Inoue's tetrad criteria.

### *Histological evaluation*

The histology from biopsied or endoscopically/surgically resected specimens was evaluated by a pathologist (Ikegami M) who was experienced in diagnosing early esophageal cancers and blinded to any endoscopic findings. The histological diagnosis was established according to the Japanese classification of esophageal cancer[22]. SESCC was histologically defined as HGIN or SCC invading up to the submucosa. If the histology results were different between the biopsied and resected specimens in the same lesion, the worse histology was considered the final histology of the lesion.

### *Statistical analysis*

To assess the relationship between each NBI-ME finding and the diagnosis of SESCC, the Pearson $\chi^2$ test was used for comparisons of variables in a univariate analysis. We did not perform a multivariate analysis because a stepwise logistic regression analysis was used to estimate the association between histologically determined SESCC and the 6 NBI-ME findings, as well as to identify the best combination of NBI-ME findings for diagnosing SESCC.

The associations were evaluated in terms of odds ratio with 95%CI. The diagnostic performances of the simplified and Inoue's tetrad criteria were evaluated by means of the area under the curve (AUC) from the receiver operating characteristic (ROC) curve. The diagnostic values (sensitivity, specificity, and overall accuracy) were calculated based on the per lesion analysis. Categorical variables were compared using the $\chi^2$ test or Fisher's exact test. Statistical significance was defined as a two-tailed *P* value of $< 0.01$. Stata Version 11 software (Stata Corp, College Station, Texas, United States) was used for all statistical analyses.

## RESULTS

The demographics of patients and lesions are listed

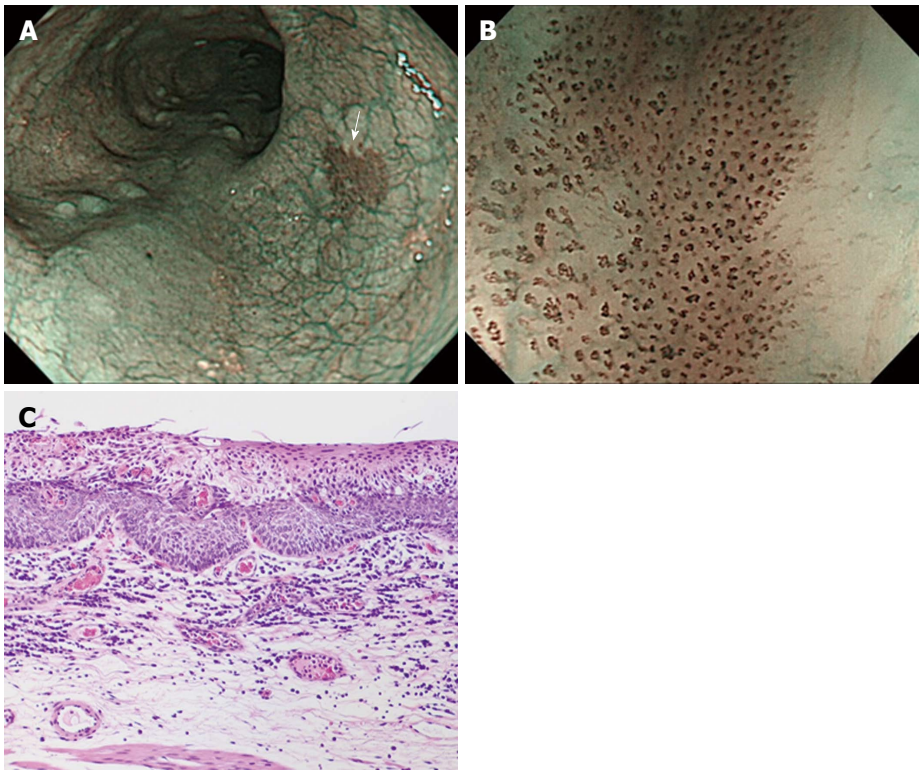


**Figure 3 Representative case of superficial squamous cell carcinoma.** A: On non-magnifying NBI endoscopy, the lesion demonstrated a well-demarcated brownish area; B: The lesion has all six of the diagnostic findings obtained by using NBI-ME; C: Histology from endoscopic submucosal dissection showing squamous cell carcinoma invading up to the lamina propria mucosae. NBI: Narrow Band Imaging; NBI-ME: Narrow Band Imaging combined with magnifying endoscopy.

**Table 2 The results of the univariate analysis of the Narrow Band Imaging combined with magnifying endoscopy findings for the diagnosis of superficial esophageal squamous cell carcinoma, *n* (%)**

| NBI-ME findings | Esophagitis (*n* = 8) | LGIN (*n* = 27) | SESCC (*n* = 54) | Odds ratio (95%CI) | *P* value |
|---|---|---|---|---|---|
| IBC | 1 (13) | 19 (70) | 45 (83) | 3.3 (1.2-8.9) | NS |
| Proliferation | 3 (38) | 21 (78) | 50 (93) | 5.0 (1.4-17.5) | < 0.01 |
| Dilatation | 3 (38) | 20 (74) | 48 (89) | 3.6 (1.2-11.1) | NS |
| Tortuosity | 2 (25) | 14 (52) | 44 (81) | 5.2 (2.0-13.6) | < 0.01 |
| Change in caliber | 2 (25) | 6 (22) | 31 (57) | 4.5 (1.7-11.8) | < 0.01 |
| Various shapes | 3 (38) | 14 (52) | 46 (85) | 5.4 (2.0-14.8) | < 0.01 |

NBI-ME: Narrow Band Imaging combined with magnifying endoscopy; LGIN: Low-grade intraepithelial neoplasia; SESCC: Superficial esophageal squamous cell carcinoma; IBC: Intervascular background coloration; NS: Not significant.

Table 1. The median age of patients was 67 year-old, and 88% of patients were male. Eighty-five patients had a history of head and neck SCCs, while seventy-four had a history of SESCCs. The median lesion size was 15 mm. The histology of 54 lesions (65%) was SESCC, 27 were low-grade intraepithelial neoplasia (LGIN), and 8 were inflammation. In the 54 SESCC lesions, the median diameter was 19 mm, the majority (87%) had a flat appearance (0-IIb) on endoscopy, and more than half of the SESCCs (59%) were confined to the lamina propria mucosae and treated with endoscopic resection (Figures 3 and 4).

In the univariate analysis (Table 2), proliferation, tortuosity, change in caliber, and VS were significantly associated with SESCC ($P < 0.01$). The combination of VS and proliferation was statistically extracted from the 6 NBI-ME findings by using a stepwise logistic regression model. We defined the combination of VS and proliferation as a simplified "dyad criteria" for the NBI-ME diagnosis of SESCC.

The diagnostic performance of the simplified dyad criteria was compared with that of Inoue's tetrad criteria using the ROC curve. The AUC of the simplified dyad criteria and Inoue's tetrad criteria were 0.70 (95%CI: 0.60-0.80) and 0.73 (95%CI: 0.63-0.84), respectively. No significant difference was shown between them (Figure 5).

The sensitivity of a brownish area alone and simplified dyad criteria for diagnosing SESCC were 92.6% and 77.8%. There was no difference between them.

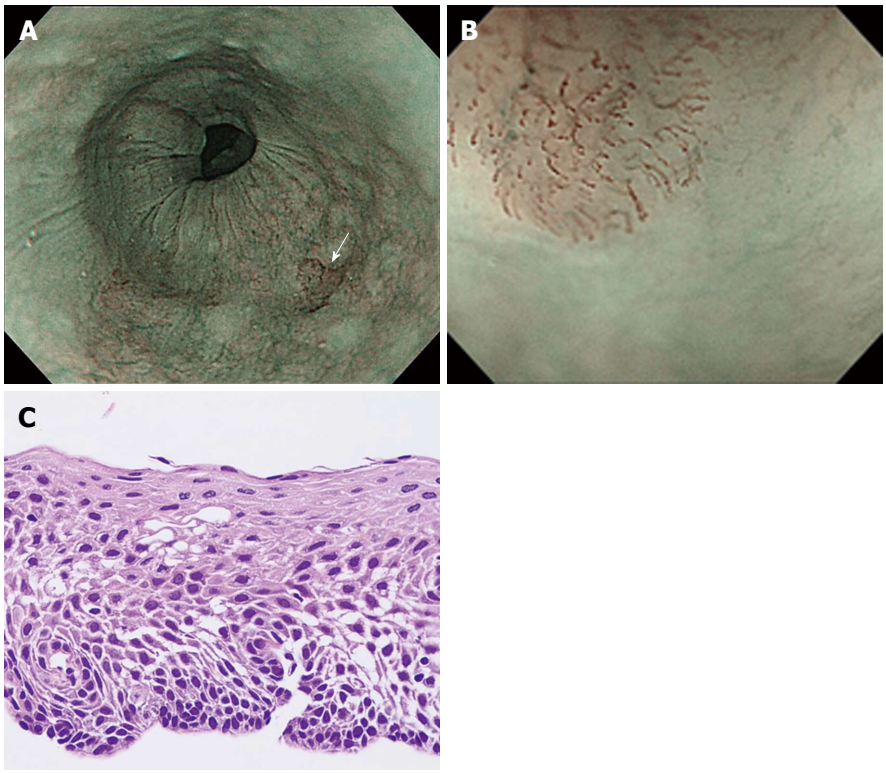


**Figure 4 Representative case of low-grade intraepithelial neoplasia.** A: On non-magnifying NBI endoscopy, the lesion demonstrated a well-demarcated brownish area; B: Under magnifying NBI observation, the lesion had only two of the six findings, "intervascular background coloration" and "dilation" of IPCLs; C: The histology from biopsy showed low-grade intraepithelial neoplasia. NBI: Narrow Band Imaging; IPCL: Intrapapillary capillary loop.

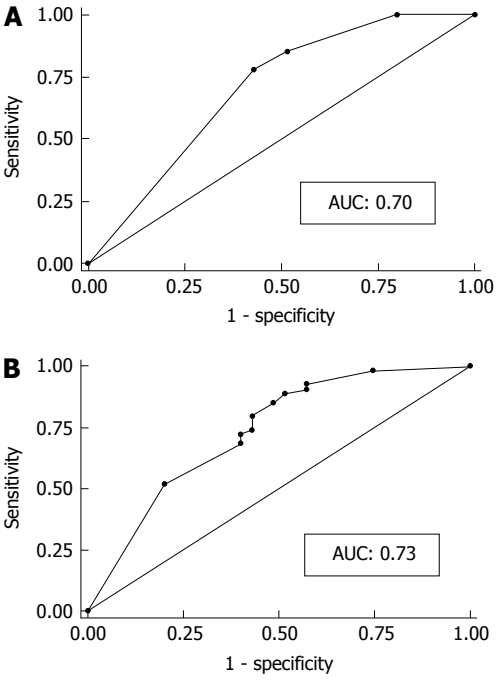


**Figure 5 Diagnostic performance based on the receiver operating characteristic analysis.** The area under the curve of the simplified dyad criteria (A) was 0.70 and that of Inoue's tetrad criteria was 0.73 (B). No significant difference was found between them. AUC: Area under the curve.

The specificity of the simplified dyad criteria was significantly greater than that of a brownish area alone ($P < 0.01$, Table 3). There was no difference between the specificity of simplified dyad criteria and that of Inoue's criteria (57.1% *vs* 80.0%).

**Table 3 Diagnostic performance of each criteria**

| | Brownish area alone (95%CI) | Simplified dyad criteria (95%CI) | *P* value |
|---|---|---|---|
| Sensitivity | 92.6% (82.1-97.9) | 77.8% (64.4-88.0) | NS[1] |
| Specificity | 8.57% (1.8-23.1) | 57.1% (39.4-73.7) | $P < 0.01$[2] |
| Overall accuracy | 59.6% (48.6-69.8) | 69.7% (59.0-79.0) | NS[1] |

[1]$\chi^2$ test; [2]Fisher's exact test; NS: Not significant.

## DISCUSSION

Using the data from the RCT (NBI-Lugol study) and a stepwise logistic regression model, VS and proliferation were extracted from the 6 NBI-ME findings that had been reported to be strongly suggestive of SESCC[6,8,13]. VS and proliferation might be candidates for simplified criteria with diagnostic performance comparable to Inoue's tetrad criteria.

We evaluated 6 NBI-ME findings that have been previously reported as useful for diagnosing SESCC[6,8,13]. The 6 NBI-ME findings can be divided into the following three categories: 4 morphological changes (*i.e.,* dilation, tortuosity, change in caliber, VS), an increasing number of IPCLs (proliferation), and color change of background mucosa (*i.e.,* IBC). VS was statistically extracted as

one of the simplified dyad criteria for the prediction of SESCC. VS may be indicative of the presence of the other 3 morphological changes of IPCLs, because 95% of the lesions with VS also had dilation, tortuosity, or change in caliber. Thus, VS may more efficiently represent 3 morphological changes of IPCLs.

In contrast, proliferation is not related to IPCL morphology but to newly developed microvasculature. A previous study showed that vasculature increases during the early phases of cancer development[23]. Kumagai *et al*[24] already indicated that the microvessel density in SESCC gradually increased in proportion to the depth by using immunohistochemical staining with CD34 and CD105. Their study will support our result that proliferation of IPCLs is one of two highly suggestive NBI-ME findings to diagnose SESCC. This may be the reason why proliferation was extracted as the other factor of the simplified dyad criteria. Proliferation may complement the diagnostic value of IPCL morphological changes, including VS. Thus, it seemed reasonable that these two findings based on IPCL morphology and newly developed microvasculature were the best combination for diagnosing SESCC.

Several studies have shown a close relationship between IBC and SESCC[18-21]; however, IBC was not extracted from the 6 NBI-ME findings and not included in the simplified dyad criteria. IBC might be caused by an extravascular component of hemoglobin that is produced within tumor cells[25], as well as thinning of the keratinous layer or epithelium caused by neoplastic cell proliferation[19]. LGIN cells may be the source of the hemoglobin, while intraepithelial infiltration of inflammatory cells may cause the thinning the keratinous layer or epithelium. Thus, LGIN and esophageal mucosa with inflammation may demonstrate IBC, and this may be the reason why IBC was not extracted in this study.

A brownish area is a reliable landmark in the detection of SESCC, as the presence of a brownish area alone showed a very high sensitivity (92.6%). However, since SESCC detection based on a brownish area alone involved a high false-positive rate as seen in a previous study[8,9], the specificity of a brownish area was considerably low (8.6%) in this study. Simplified and Inoue's criteria showed a much higher specificity but lower sensitivity than the presence of a brownish area alone. As with Inoue's tetrad criteria, the simplified dyad criteria of NBI-ME will reduce the high false-positive rate of a brownish area alone.

Concerning the esophagus, we propose an ideal endoscopic examination as follows. Initially, we should pay attention to elevated or depressed lesions and brownish areas using an NBI non-magnifying endoscopy. We then evaluate for the presence of VS and proliferation under NBI-ME for all suspicious lesions. This strategy may allow endoscopists to detect and discriminate SESCC from inflammation and LGIN easily, quickly, and accurately. The dyad criteria may enable a simplified diagnosis of SESCC and lead to widespread use of the NBI-ME.

This study has several limitations. First, this is a retrospective study based on statistical analysis. A prospective study with a clinical setting is needed to validate these study results. Second, NBI-ME findings were evaluated only by expert endoscopists. The diagnostic utility of the simplified dyad criteria should be validated by non-experts as well as experts. Finally, the lesions detected only by Lugol chromoendoscopy were not included in this study. However, it may not affect the results, as the number of SESCCs only detected by Lugol chromoendoscopy was small.

In conclusion, the combination of VS and proliferation may be considered simplified criteria for diagnosis of SESCC based on NBI-ME findings.

## ACKNOWLEDGMENTS

A part of this paper was presented as an oral presentation at the United European Gastroenterology Week, October 12-16th, 2013 in Berlin, Germany.

## COMMENTS

### Background

The prognosis of patients with esophageal squamous cell carcinoma (SCC) is improved if the tumor is detected at an early stage. The usefulness of Narrow Band Imaging combined with magnifying endoscopy (NBI-ME) for early detection and characterization of superficial esophageal SCC (SESCC) has been previously reported. However, NBI-ME is somewhat limited in clinical application because the current criteria for NBI-ME findings are complicated. Thus, simplified criteria are needed.

### Research frontiers

The following 6 NBI-ME findings have been reported as significant diagnostic markers for SESCCs: "dilation", "tortuosity", "change in caliber", "various shapes (VS)", "proliferation" of intrapapillary capillary loops (IPCLs), and "intervascular background coloration". No reports have provided simplified criteria for diagnosing SESCC by using NBI-ME. The goal of this study was to simplify the diagnostic criteria.

### Innovations and breakthroughs

If simplified criteria are newly created, the process of diagnosing SESCCs using NBI-ME would be facilitated, which would result in widespread use of magnifying endoscopy.

### Applications

The simplified dyad criteria were compatible in diagnostic performance to Inoue's tetrad criteria. If a brownish area or other suspicious lesion is found during NBI endoscopy without magnification, the simplified criteria may be useful to differentiate a neoplasia from a non-neoplastic lesion.

### Terminology

NBI is a revolutionary optical image-enhanced technology. NBI enhances the contrast between the microvessels and background mucosa in the esophagus. Thus, visibility of the microvessels is improved when NBI is combined with magnifying endoscopy. Inoue's tetrad criteria are the most popular NBI-ME diagnostic criteria for SESCC and consist of the four following morphological

changes: "dilation", "tortuosity", "change in caliber", and "VS" of IPCLs. On the other hand, the simplified criteria are composed of proliferation and VS of IPCLs.

### Peer-review

The authors pointed out the difficulty of Inoue's tetrad criteria and proposed simplified diagnostic criteria for the changes of the IPCL. From the statistical analysis, the authors extracted "various shapes" from the tetrad criteria as the most significant parameter for the prediction of SESCC. In addition, the authors also focused on the "proliferation of the IPCLs" and proposed simplified criteria by combining the "VS of the IPCLs" and "proliferation of the IPCLs".

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ORIGINAL ARTICLE

Retrospective Study

# Assessment of scoring systems for acute-on-chronic liver failure at predicting short-term mortality in patients with alcoholic hepatitis

Hee Yeon Kim, Chang Wook Kim, Tae Yeob Kim, Do Seon Song, Dong Hyun Sinn, Eileen L Yoon, Young Kul Jung, Ki Tae Suk, Sang Soo Lee, Chang Hyeong Lee, Tae Hun Kim, Jeong Han Kim, Hyung Joon Yim, Sung Eun Kim, Soon Koo Baik, Byung Seok Lee, Jae Young Jang, Young Seok Kim, Sang Gyune Kim, Jin Mo Yang, Joo Hyun Sohn, Heon Ju Lee, Seung Ha Park, Eun Hee Choi, Dong Joon Kim; Korean Acute-on-Chronic Liver Failure Study Group

Hee Yeon Kim, Chang Wook Kim, Do Seon Song, Jin Mo Yang, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul 06591, South Korea

Tae Yeob Kim, Institute of Medical Science, Hanyang University, Seoul, 04763, South Korea

Dong Hyun Sinn, Department of Internal Medicine, Samsung Medical Center, Seoul 06351, South Korea

Eileen L Yoon, Department of Internal Medicine, Inje University Sanggye Paik Hospital, Seoul 01757, South Korea

Young Kul Jung, Hyung Joon Yim, Department of Internal Medicine, Korea University Ansan Hospital, Ansan 15355, South Korea

Ki Tae Suk, Dong Joon Kim, Department of Internal Medicine, Hallym University Chuncheon Sacred Heart Hospital, Chuncheon 24253, South Korea

Sang Soo Lee, Department of Internal Medicine, Gyeongsang National University Hospital, Jinju 52727, South Korea

Chang Hyeong Lee, Department of Internal Medicine, Catholic University of Daegu School of Medicine, Daegu 42472, South Korea

Tae Hun Kim, Department of Internal Medicine, Ewha Womans University School of Medicine, Seoul 07985, South Korea

Jeong Han Kim, Department of Internal Medicine, Konkuk University School of Medicine, Seoul 05030, South Korea

Sung Eun Kim, Department of Internal Medicine, Hallym University Sacred Heart Hospital, Anyang 14068, South Korea

Soon Koo Baik, Department of Internal Medicine, Yonsei University Wonju College of Medicine, Wonju 26426, South Korea

Byung Seok Lee, Department of Internal Medicine, Chungnam National University, School of Medicine, Daejeon 35015, South Korea

Jae Young Jang, Department of Internal Medicine, Soonchunhyang University College of Medicine, Seoul 04401, South Korea

Young Seok Kim, Sang Gyune Kim, Department of Internal Medicine, Soonchunhyang University Bucheon Hospital, Bucheon 14584, South Korea

Joo Hyun Sohn, Department of Internal Medicine, Hanyang University Guri Hospital, Guri 11923, South Korea

Heon Ju Lee, Department of Internal Medicine, Yeungnam University College of Medicine, Daegu 42415, South Korea

Seung Ha Park, Department of Internal Medicine, Inje University Haeundae Paik-Hospital, Inje University College of Medicine, Busan 48108, South Korea

Eun Hee Choi, Institute of Lifestyle Medicine, Yonsei University Wonju College of Medicine, Wonju 26426, South Korea

Author contributions: Kim HY, Kim CW and Kim TY proposed the idea and designed the research; Kim DJ designed and supervised the study; Kim HY, Song DS, Sinn DH, Yoon EL, Jung YK, Suk KT, Lee SS, Lee CH, Kim TH, Kim JH, Yim HJ, Kim SE, Baik SK, Lee BS, Jang JY, Kim YS, Kim SG, Yang JM, Sohn JH, Lee HJ and Park SH enrolled patients and collected the data; Kim HY, Kim CW, Kim TY and Choi EH performed the statistical analysis and interpreted the data; Kim HY and Kim DJ wrote the draft; all authors have read and approved the final manuscript.

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Correspondence to: Dong Joon Kim, MD, PhD, Department of Internal Medicine, Hallym University Chuncheon Sacred Heart Hospital, Sakju-ro 77, 200-704, Chuncheon 24253, South Korea. djkim@hallym.ac.kr
Telephone: +82-33-2405646
Fax: +82-33-2418064

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## Abstract

***AIM***

To assess the performance of proposed scores specific for acute-on-chronic liver failure in predicting short-term mortality among patients with alcoholic hepatitis.

***METHODS***

We retrospectively collected data from 264 patients with clinically diagnosed alcoholic hepatitis from January to December 2013 at 21 academic hospitals in Korea. The performance for predicting short-term mortality was calculated for Chronic Liver Failure-Sequential Organ Failure Assessment (CLIF-SOFA), CLIF Consortium Organ Failure score (CLIF-C OFs), Maddrey's discriminant function (DF), age, bilirubin, international normalized ratio and creatinine score (ABIC), Glasgow Alcoholic Hepatitis Score (GAHS), model for end-stage liver disease (MELD), and MELD-Na.

***RESULTS***

Of 264 patients, 32 (12%) patients died within 28 d. The area under receiver operating characteristic curve of CLIF-SOFA, CLIF-C OFs, DF, ABIC, GAHS, MELD, and MELD-Na was 0.86 (0.81-0.90), 0.89 (0.84-0.92), 0.79 (0.74-0.84), 0.78 (0.72-0.83), 0.81 (0.76-0.86), 0.83 (0.78-0.88), and 0.83 (0.78-0.88), respectively, for 28-d mortality. The performance of CLIF-SOFA had no statistically significant differences for 28-d mortality. The performance of CLIF-C OFs was superior to that of DF, ABIC, and GAHS, while comparable to that of MELD and MELD-Na in predicting 28-d mortality. A CLIF-SOFA score of 8 had 78.1% sensitivity and 79.7% specificity, and CLIF-C OFs of 10 had 68.8% sensitivity and 91.4% specificity for predicting 28-d mortality.

***CONCLUSION***

CLIF-SOFA and CLIF-C OF scores performed well, with comparable predictive ability for short-term mortality compared to the commonly used scoring systems in patients with alcoholic hepatitis.

**Key words:** Acute-on-chronic liver failure; Alcoholic hepatitis; Mortality; Prognosis; Scoring system



**Core tip:** Alcoholic hepatitis (AH) often leads to acute-on-chronic liver failure (ACLF), which is characterized by acute hepatic decompensation of chronic liver disease, organ failure, and high short-term mortality. We investigated the prognostic utilities of proposed scores specific for ACLF in predicting short-term mortality among patients with AH. Chronic Liver Failure (CLIF)-Sequential Organ Failure Assessment and CLIF Consortium Organ Failure score performed well, and showed comparable predictive ability for short-term mortality compared to commonly used scoring systems proposed for AH. The present study suggests that scores proposed for ACLF could be useful in predicting short-term morality in patients with AH.

Kim HY, Kim CW, Kim TY, Song DS, Sinn DH, Yoon EL, Jung YK, Suk KT, Lee SS, Lee CH, Kim TH, Kim JH, Yim HJ, Kim SE, Baik SK, Lee BS, Jang JY, Kim YS, Kim SG, Yang JM, Sohn JH, Lee HJ, Park SH, Choi EH, Kim DJ; Korean Acute-on-Chronic Liver Failure Study Group. Assessment of scoring systems for acute-on-chronic liver failure at predicting short-term mortality in patients with alcoholic hepatitis. *World J Gastroenterol* 2016; 22(41): 9205-9213 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i41/9205.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i41.9205

## INTRODUCTION

Alcoholic hepatitis (AH) is an acute inflammatory syndrome that occurs in patients after long-term alcohol misuse. The clinical spectrum of AH is diverse, ranging from mild to severe[1]. AH may deteriorate rapidly in its severe form, which has a high 30-d mortality of up to 50%[2,3]. AH often presents with acute deterioration superimposed on chronic liver disease[4], comprising acute-on-chronic liver failure (ACLF). ACLF is a distinct entity that was recently defined on the basis of acute decompensation, organ failure, and high short-term mortality. One of the leading causes of ACLF is active alcoholism, presenting in about 25% of patients with ACLF[5].

Given that short-term mortality is high in severe AH, it is crucial to assess disease severity and identify patients at greater risk of death in the management of patients with AH. Various scoring systems have been proposed to assess the severity of AH and to predict prognosis in these patients. Maddrey's discriminant function (DF) has proven helpful in scoring disease severity and guiding specific treatment in AH[6]. DF $\geqslant$ 32 is associated with a high short-term mortality, therefore it is used as the threshold for corticosteroid or pentoxifylline therapy[7]. The age, bilirubin, international normalized ratio (INR), and creatinine score (ABIC) was proposed to risk stratify patients with AH into low, intermediate, and high risk of death using cut-off values of 6.71 and 9.0[8]. The Glasgow Alcoholic Hepatitis Score (GAHS) is based on age, serum bilirubin, blood urea, prothrombin time (PT), and peripheral blood white blood cell (WBC) count[9]. A GAHS $\geqslant$ 9 identifies patients with a high risk of death[10]. In addition to these disease-specific models, the model for end-stage liver disease (MELD) and modified MELD including sodium (MELD-Na) have been found to predict prognosis in AH with good accuracy[11-14].

Meanwhile, several models have been proposed to predict mortality in patients with ACLF. The European Association for Study of Liver/Chronic Liver Failure Consortium (EASL-CLIF Consortium) has modified the sequential organ failure assessment (SOFA) score to include factors specific to liver disease. This modified SOFA score adapted for patients with cirrhosis (CLIF-SOFA score) was shown to predict mortality in acute deterioration of chronic liver disease[5]. A simplified CLIF-SOFA score (CLIF Consortium Organ Failure score, CLIF-C OFs) is easy to calculate, and has similar prognostic accuracy compared to the CLIF-SOFA score[15].

The ability of scores proposed for ACLF to predict survival in patients with AH is largely unknown. The aim of this study was to validate the utility of ACLF scoring systems and compare the predictive ability of these scores with that of other commonly used prognostic models in predicting outcomes for patients with AH.

## MATERIALS AND METHODS

### Study population

Consecutive patients with acutely decompensated alcoholic liver disease and active alcoholism were retrospectively enrolled from 21 Korean academic hospitals from January to December 2013. The inclusion criteria were history of recent excess alcohol consumption within the last 2 mo (> 50 g/d for males and > 40 g/d for females) and a clinical diagnosis of alcoholic hepatitis. Alcoholic hepatitis was clinically diagnosed as the combination of serum bilirubin more than 3 mg/dL, elevated aspartate aminotransferase (AST) but < 400 U/L, and an AST to alanine aminotransferase (ALT) ratio of > 1.5[16]. Key exclusion criteria were the presence of other causes of liver disease, infection, gastrointestinal bleeding, drug-induced hepatitis, and hepatocellular carcinoma. Medical treatment for severe AH was left to the physician's discretion at each institute, although it usually included corticosteroids and/or pentoxifylline. Baseline clinical characteristics, laboratory findings, and survival 28 and 90 d following hospitalization were retrospectively identified by chart review. This study was approved by the Institutional Ethics Committee of all participating institutions.

### Scoring systems

For each patient, DF[6], ABIC[8], GAHS[9], MELD[14], MELD-Na[17], CLIF-SOFA[5], and CLIF-C OF[15] were calculated using laboratory data from the day of hospitalization. The formulas used to calculate prognostic models are listed in Table 1.

### Statistical analysis

Data are expressed as mean ± SD for continuous variables. Categorical variables are expressed as frequencies (percentage). Differences between two groups were compared using a *t*-test for continuous variables and $\chi^2$-test for categorical variables. Cumulative survival curves were estimated by the Kaplan-Meier method. The log-rank statistic was used to test for significant differences between the curves. The prognostic utility of various scoring systems for predicting mortality at 28 or 90 d was assessed using the area under receiver operating characteristics curves (AUROCs). Comparison between AUROCs was performed by DeLong's test. For each model, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using originally proposed cut-off values: 32 for DF, 9.0 for ABIC, 9 for GAHS, 21 for MELD, and 28 for MELD-Na[6,8,9,13,18]. For CLIF-SOFA and CLIF-C OFs, predictive performance was calculated using an optimal cut-off point with the best sensitivity and specificity from the cohort[19]. Analyses were performed using SPSS version 18 (SPSS, IBM, Chicago, IL, USA). The comparisons between AUROCs were performed using MedCalc version 16.4.3 (Medisoftware, Mariakerke,

**Table 1 Formulas for scores used in alcoholic hepatitis**

| Scores | Formulas | | | | |
|---|---|---|---|---|---|
| DF[6] | 4.6 × [patient's prothrombin time-control prothrombin time (s)] + total bilirubin (mg/dL) | | | | |
| ABIC score[8] | (age × 0.1) + [serum bilirubin (mg/dL) × 0.08] + [serum creatinine (mg/dL) × 0.3] + (INR × 0.8) | | | | |
| GAHS[9] | Age, blood urea nitrogen, white blood cell count, serum bilirubin, and INR; each scored 1-3 | | | | |
| MELD score[14] | MELD = 3.78 ln[bilirubin (mg/dL)] + 11.20ln(INR)+ 9.57ln[creatinine (mg/dL)] + 6.43 | | | | |
| MELD-Na score[17] | MELD + 1.59 (135-Na), with maximum and minimum Na of 135 and 120 mEq/L, respectively | | | | |
| CLIF-SOFA score[5] | 0 | 1 | 2 | 3 | 4 |
| Liver (bilirubin, mg/dL) | < 1.2 | ⩾ 1.2 to < 2.0 | ⩾ 2.0 to < 6.0 | ⩾ 6.0 to < 12.0 | ⩾ 12.0 |
| Renal (creatinine, mg/dL) | < 1.2 | ⩾ 1.2 to < 2.0 | ⩾ 2.0 to < 3.5 | ⩾ 3.5 to < 5.0 or use of RRT | ⩾ 5.0 |
| CNS (HE grade) | No HE | I | II | III | IV |
| Coagulation (INR) | < 1.1 | ⩾ 1.1 to < 1.25 | ⩾ 1.25 to < 1.5 | ⩾ 1.5 to < 2.5 | ⩾ 2.5 or platelet ⩽ 20 × $10^9$/L |
| Cardiovascular (hypotension) | MAP ⩾ 70 | MAP < 70 | Dopamine ⩽ 5 or dobutamine (any dose)[1] or terlipressin | Dopamine > 5 or epi ⩽ 0.1 or norepi ⩽ 0.1[1] | Dopamine > 15 or epi > 0.1 or norepi > 0.1[1] |
| Respiration | | | | | |
| $PaO_2/FiO_2$ | > 400 | > 300 to ⩽ 400 | > 200 to ⩽ 300 | > 100 to ⩽ 200 | ⩽ 100 |
| Or $SpO_2/FiO_2$ | > 512 | > 357 to ⩽ 512 | > 214 to ⩽ 357 | > 89 to ⩽ 214 | ⩽ 89 |
| CLIF-C OF score[15] | 1 | 2 | 3 | | |
| Liver (bilirubin, mg/dL) | < 6 | ⩾ 6.0 to < 12.0 | ⩾ 12 | | |
| Renal (creatinine, mg/dL) | < 2 | ⩾ 2.0 to < 3.5 | ⩾ 3.5 or RRT | | |
| CNS (HE grade) | 0 | 1-2 | 3-4 | | |
| Coagulation (INR) | < 2.0 | ⩾ 2.0 to < 2.5 | ⩾ 2.5 | | |
| Cardiovascular (hypotension) | MAP ⩾ 70 | MAP < 70 | Vasopressors | | |
| Respiration | | | | | |
| $PaO_2/FiO_2$ | > 300 | ⩽ 300 and > 200 | ⩽ 200 | | |
| Or $SpO_2/FiO_2$ | > 357 | > 214 and ⩽ 357 | ⩽ 214 | | |

[1]Adrenergic agents administered for at least 1 h (disease are given in μg/kg/min). DF: Discriminant function; ABIC: Age, bilirubin, INR creatinine; GAHS: Glasgow Alcoholic Hepatitis Score; MELD: Model for end-stage liver disease; MELD-Na: Modified MELD including sodium; CLIF-SOFA: Chronic Liver Failure-Sequential Organ Failure Assessment; CLIF-C OF: Chronic Liver Failure Consortium Organ Failure score; INR: International normalized ratio; RRT: Renal replacement therapy; HE: Hepatic encephalopathy; MAP: Mean arterial pressure; $PaO_2$: Partial pressure of arterial oxygen; $FiO_2$: Fraction of inspired oxygen; $SpO_2$: Pulse oximetric saturation.

Belgium).

## RESULTS

### *Baseline characteristics*

The study population comprised 264 consecutive patients with a clinical diagnosis of AH who met the inclusion criteria. Table 2 describes baseline characteristics and prognostic scores of enrolled patients. The mean age was 48.8 ± 9.1 years, and males were predominant (77.3%). Overall, 28-d mortality was 12.0% and 90-d mortality was 19.0%. The differences between 28-d survivors and non-survivors are presented in Table 2. Patients who died within 28 d had higher baseline WBC count, bilirubin, INR, creatinine, and lower albumin and gammaglutamyl transferase (GGT) compared to patients who survived. Prognostic scores including DF, ABIC, GAHS, MELD, MELD-Na, CLIF-SOFA, and CLIF-C OFs, were significantly higher in non-survivors compared to 28-d survivors.

### *Performance of different scores in predicting short-term mortality*

The ROC curves for various scores are shown in Figure 1. Table 3 shows the predictive accuracy of DF, ABIC, GAHS, MELD, MELD-Na, CLIF-SOFA, and CLIF-C OFs. The AUROCs of CLIF-SOFA and CLIF-C OF for 28-d mortality were 0.86 (0.81-0.90) and 0.89 (0.84-0.92) respectively. The AUROC of CLIF-SOFA was comparable to those of other scoring systems for alcoholic hepatitis in predicting 28-d mortality, such as DF, ABIC, GAHS, MELD, and MELD-Na score [AUROC (95%CI): 0.79 (0.74-0.84) for DF, 0.78 (0.72-0.83) for ABIC, 0.81 (0.76-0.86) for GAHS, 0.83 (0.78-0.88) for MELD, and 0.83 (0.78-0.88) for MELD-Na]. The AUROC of CLIF-C OFs was superior to those of DF, ABIC, and GAHS ($P$ = 0.005 for DF, $P$ = 0.006 for ABIC, $P$ = 0.046 for GAHS), but was comparable to those of MELD and MELD-Na scores in predicting 28-d mortality. There were no significant differences between predictive abilities of the DF, ABIC, GAHS, MELD, MELD-Na, CLIF-SOFA, and CLIF-C OFs for 90-d mortality (except between CLIF-C OFs and DF; $P$ = 0.02).

Using a DF cut-off score of ⩾ 32, the sensitivity and specificity were 81.3% and 50.9%, respectively, at predicting 28-d mortality. The ABIC score of 9 had 43.7% sensitivity and 93.5% specificity at predicting 28-d mortality. The sensitivity and specificity of MELD ⩾ 21 for predicting 28-d mortality were 87.5% and 68.8%, respectively. A MELD-Na of 28 had 68.8% sensitivity and 80.2% specificity. The optimal cut-off

**Table 2 Baseline characteristics of patients**

| Variable | Total cohort (*n* = 264) | 28-d survivors (*n* = 232) | 28-d nonsurvivors (*n* = 32) | *P* value |
|---|---|---|---|---|
| Age (yr) | 48.8 ± 9.1 | 48.6 ± 9.2 | 48.3 ± 8.1 | 0.865 |
| Men, *n* (%) | 204 (77.3%) | 175 (75.4%) | 29 (90.6%) | 0.070 |
| Presence of cirrhosis, *n* (%) | 240 (90.9%) | 208 (89.7%) | 32 (100%) | 0.092 |
| SIRS, *n* (%) | 76 (28.8%) | 63 (27.2%) | 13 (40.6%) | 0.144 |
| Mean blood pressure (mmHg) | 89.3 ± 15.9 | 90.1 ± 15.1 | 83.5 ± 20.0 | 0.082 |
| WBC count (× $10^9$/L) | 9.6 ± 6.0 | 9.2 ± 5.9 | 12.0 ± 6.7 | 0.013 |
| Platelet count (× $10^9$/L) | 109.2 ± 71.5 | 110.6 ± 72.0 | 98.7 ± 68.0 | 0.377 |
| Albumin (g/dL) | 2.7 ± 0.6 | 2.7 ± 0.5 | 2.3 ± 0.6 | < 0.0001 |
| Bilirubin (mg/dL) | 11.3 ± 8.6 | 10.3 ± 8.0 | 18.7 ± 9.7 | < 0.0001 |
| AST (U/L) | 157.7 ± 86.6 | 160.5 ± 88.4 | 137.3 ± 70.7 | 0.098 |
| ALT (U/L) | 49.9 ± 32.4 | 50.4 ± 33.3 | 46.4 ± 24.6 | 0.514 |
| GGT (U/L) | 428.4 ± 437.5 | 455.4 ± 448.4 | 236.9 ± 290.5 | 0.001 |
| Prothrombin time (s) | 18.0 ± 5.2 | 17.3 ± 3.9 | 22.8 ± 9.6 | 0.004 |
| INR | 1.7 ± 0.6 | 1.6 ± 0.4 | 2.3 ± 0.9 | 0.001 |
| Creatinine (mg/dL) | 1.2 ± 1.5 | 0.9 ± 0.7 | 2.9 ± 3.6 | 0.005 |
| Sodium (mEq/L) | 133.5 ± 6.6 | 133.8 ± 6.3 | 131.1 ± 8.2 | 0.078 |
| DF score | 38.8 ± 28.2 | 34.9 ± 21.6 | 68.4 ± 48.0 | 0.001 |
| ABIC score | 7.5 ± 1.4 | 7.3 ± 1.1 | 9.0 ± 1.9 | < 0.0001 |
| GAHS | 7.4 ± 1.5 | 7.2 ± 1.3 | 9.1 ± 1.6 | < 0.0001 |
| MELD score | 21.6 ± 6.9 | 20.3 ± 5.5 | 30.6 ± 9.1 | < 0.0001 |
| MELD-Na score | 24.3 ± 6.8 | 23.1 ± 5.9 | 32.3 ± 7.2 | < 0.0001 |
| CLIF-SOFA score | 6.8 ± 3.1 | 6.1 ± 2.1 | 11.5 ± 4.6 | < 0.0001 |
| CLIF-C OF score | 7.9 ± 2.0 | 7.4 ± 1.4 | 11.2 ± 2.9 | < 0.0001 |

Data are presented as mean ± SD or *n* (%). SIRS: Systemic inflammatory response syndrome; WBC: White blood cell; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyltransferase; INR: International normalized ratio; DF: Discriminant function; ABIC: Age, bilirubin, INR, creatinine; GAHS: Glasgow Alcoholic Hepatitis Score; MELD: Model for end-stage liver disease; MELD-Na: Modified MELD including sodium; CLIF-SOFA: Chronic Liver Failure-Sequential Organ Failure Assessment; CLIF-C OF: Chronic Liver Failure Consortium Organ Failure.

**Table 3 Performance characteristics of various scoring systems in predicting 28- and 90-d mortality**

| Scores | AUROC (95%CI) | Cutoff | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|---|---|
| 28-d mortality | | | | | | |
| CLIF-SOFA | 0.86 (0.81-0.90) | 8 | 78.1% | 79.7% | 34.7% | 96.4% |
| CLIF-C OF | 0.89 (0.84-0.92) | 10 | 68.8% | 91.4% | 52.4% | 95.5% |
| DF | 0.79 (0.74-0.84) | 32 | 81.3% | 50.9% | 18.6% | 95.2% |
| ABIC | 0.78 (0.72-0.83) | 9 | 43.7% | 93.5% | 48.3% | 92.3% |
| GAHS | 0.81 (0.76-0.86) | 9 | 65.6% | 83.2% | 35.0% | 94.6% |
| MELD | 0.83 (0.78-0.88) | 21 | 87.5% | 57.8% | 22.2% | 97.1% |
| MELD-Na | 0.83 (0.78-0.88) | 28 | 68.8% | 80.2% | 32.4% | 94.9% |
| 90-d mortality | | | | | | |
| CLIF-SOFA | 0.81 (0.76-0.86) | 8 | 66.7% | 84.3% | 54.8% | 89.8% |
| CLIF-C OF | 0.83 (0.78-0.88) | 10 | 52.9% | 94.4% | 73.0% | 87.5% |
| DF | 0.77 (0.71-0.82) | 32 | 78.4% | 53.4% | 32.5% | 89.6% |
| ABIC | 0.78 (0.72-0.83) | 9 | 39.2% | 96.1% | 74.1% | 84.7% |
| GAHS | 0.82 (0.77-0.87) | 9 | 68.6% | 88.8% | 63.6% | 90.8% |
| MELD | 0.81 (0.77-0.87) | 21 | 82.4% | 61.8% | 38.2% | 92.4% |
| MELD-Na | 0.87 (0.82-0.91) | 28 | 66.7% | 87.6% | 60.7% | 90.2% |

AUROC: Area under receiver operating characteristic curve; PPV: Positive predictive value; NPV: Negative predictive value; CLIF-SOFA: Chronic Liver Failure-Sequential Organ Failure Assessment; CLIF-C OF: Chronic Liver Failure Consortium Organ Failure; DF: Discriminant function; ABIC: Age, bilirubin, INR, creatinine; GAHS: Glasgow Alcoholic Hepatitis Score; MELD: Model for end-stage liver disease; MELD-Na: Modified MELD including sodium.

points were chosen for CLIF-SOFA and CLIF-C OFs based on receiver operating characteristics curves. Using a CLIF-SOFA cut-off of 8, the sensitivity and specificity of CLIF-SOFA for predicting 28-d mortality were 78.1% and 79.7%, respectively. The sensitivity and specificity of CLIF-C OFs ⩾ 10 were 68.8% and 91.4%, respectively, for predicting 28-d mortality.

### *Survival analysis*

Figure 2 illustrates a survival curve comparing mortality based on CLIF-SOFA score ⩾ 8 and < 8 ($P < 0.05$) with CLIF-C OFs ⩾ 10 and < 10 ($P < 0.05$). Cumulative survival rates differed significantly for patients with a CLIF-SOFA score ⩾ 8 and < 8. In addition, patients with CLIF-C OFs < 10 had a

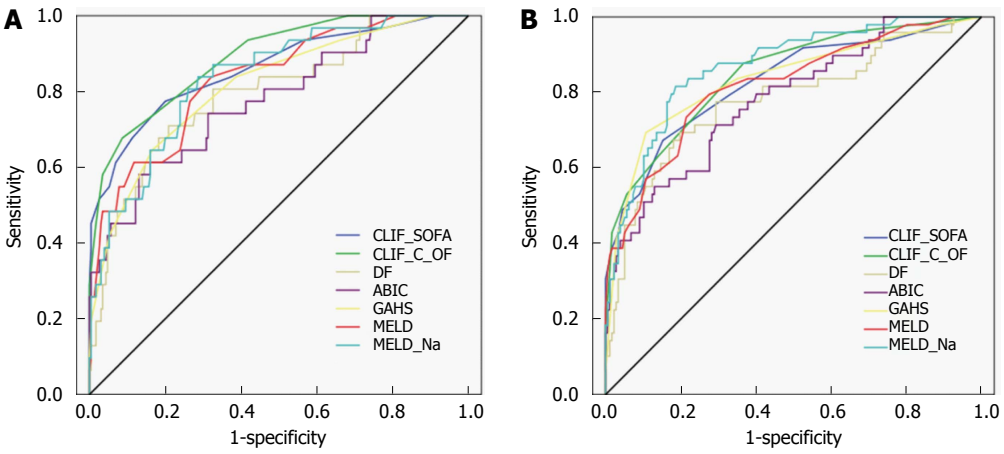


**Figure 1 Receiver operating characteristics curve comparison between scoring systems in predicting short-term mortality.** A: 28-d mortality; B: 90-d mortality. Chronic Liver Failure-Sequential Organ Failure Assessment (CLIF-SOFA) area under receiver operating characteristics curve (AUROC), 0.86; 95%CI: 0.81-0.90; CLIF Consortium Organ Failure score (CLIF-C OFs) AUROC, 0.89; 95%CI: 0.84-0.92; DF AUROC, 0.79; 95%CI: 0.74-0.84; ABIC AUROC, 0.78; 95%CI: 0.72-0.83; GAHS AUROC, 0.81; 95%CI: 0.76-0.86; MELD AUROC, 0.83; 95%CI: 0.78-0.88; MELD-Na AUROC, 0.83; 95%CI: 0.78-0.88 for 28-d mortality.

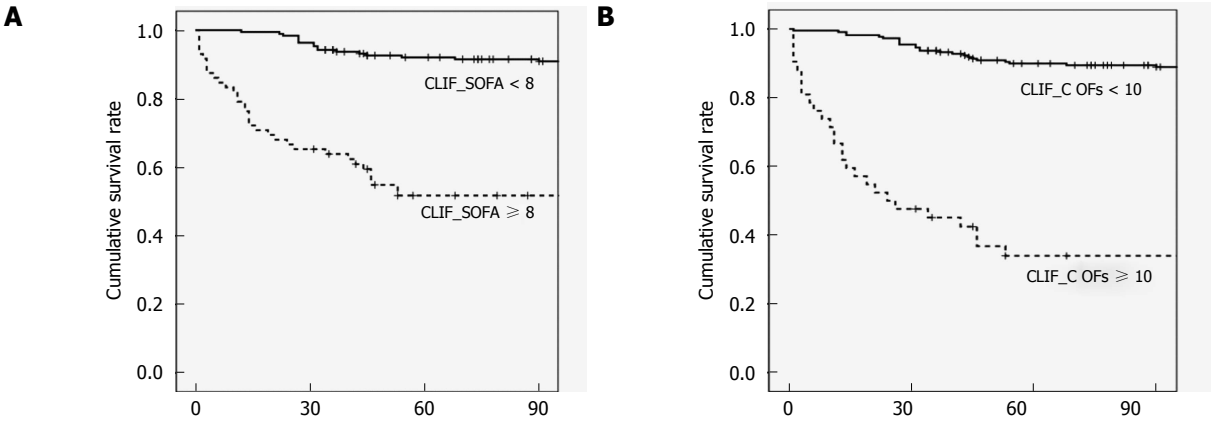


**Figure 2 Cumulative survival of patients in relation to Chronic Liver Failure-Sequential Organ Failure Assessment (A) and Chronic Liver Failure Consortium Organ Failure score (B).** CLIF-SOFA: Chronic Liver Failure-Sequential Organ Failure Assessment; CLIF-C OFs: CLIF Consortium Organ Failure score.

significantly better survival rate than those with CLIF-C OFs ⩾ 10.

## DISCUSSION

ACLF is a recently established syndrome characterized by acute deterioration of chronic liver disease resulting in organ failure and high short-term mortality. ACLF usually develops following a precipitating insult on cirrhosis, and AH is a common triggering event[5]. The CLIF-SOFA score and CLIF-C OFs are newly proposed scoring systems for cirrhotic patients with acute decompensation[5,15]. However, the value of these scores in predicting outcome for patients with AH remains unclear.

We assessed the prognostic utility of CLIF-SOFA score and CLIF-C OFs for predicting short-term mortality in patients with AH. We also compared the predictive ability of CLIF-SOFA score and CLIF-C OFs with that of DF, ABIC, GAHS, MELD, and MELD-Na in predicting short-term mortality in a multicenter cohort of patients with AH. Our study showed that the CLIF-SOFA score and CLIF-C OFs were excellent for predicting 28- or 90-d mortality with comparable discriminatory power as DF, ABIC, GAHS, MELD, and MELD-Na scores. In particular, the predictive ability of CLIF-C OFs was superior to that of DF, ABIC, and GAHS in predicting 28-d mortality.

In a considerable proportion of cases, the mortality rate of patients with AH is high due to hepatic inflammation and progression to organ failure[2,3]. Early identification of patients with poor outcomes could be used to risk stratify hospitalized patients with AH, ultimately guiding intensive treatment for such cases[20]. Several scoring systems have been introduced to assess severity and predict prognosis in patients with AH.

DF, initially described in 1978, has undergone

modifications to replace the absolute value of PT with the prolongation of PT over control[6,7]. DF is easy to calculate and has been validated in many clinical studies. However, the poor standardization of PT and inter-laboratory variation are limitations. Other prognostic models have been proposed for patients with AH, such as the ABIC, GAHS, MELD, and MELD-Na score. Initial research demonstrated that these scores have excellent diagnostic accuracy in predicting short-term outcomes[8,11-14].

CLIF-SOFA score is a newly proposed modified version of the SOFA score applicable to patients with acute decompensation of underlying chronic liver disease[15]. Like the original SOFA score, the CLIF-SOFA score is based on assessment of six organ systems. However, the CLIF-SOFA score also accounts for some special situations in end-stage liver disease by substituting the INR of PT for platelet count and substituting hepatic encephalopathy for Glasgow coma scale. In addition, the use of terlipressin and renal replacement therapy is included. CLIF-C OFs is a simplified version of the CLIF-SOFA score[15]. Respiratory, cardiac, and central nervous (hepatic encephalopathy) systems are components of the CLIF-SOFA score and CLIF-C OFs, but not the DF, ABIC, GAHS, MELD, or MELD-Na scores. Indeed, organ failure is highly associated with increased mortality in ACLF including AH. Therefore, prognostic models incorporating organ failure are promising for use in patients with AH. Many studies have analyzed available prognostic scores that assess AH severity[13,21-26]. Our results are in line with previous observations on the utility of DF, ABIC, GAHS, MELD, and MELD-Na in predicting short-term mortality[21,24-26]. To the best of our knowledge, we are the first to assess scoring systems proposed for ACLF in predicting short-term mortality in AH patients. In our cohort, CLIF-SOFA and CLIF-C OFs perform well for predicting short-term mortality in AH. The AUROCs of CLIF-SOFA in predicting 28- or 90-d mortality were comparable to those of commonly used prognostic scores such as DF, ABIC, GAHS, MELD, and MELD-Na. In addition, the performance characteristic of CLIF-C OFs in predicting 28-d mortality was comparable to those of MELD and MELD-Na scores, and superior to those of DF, ABIC, and GAHS.

In our cohort, the optimal CLIF-SOFA cut-off was 8, which predicted 28-d mortality with 78.1% sensitivity and 79.7% specificity. Moreover, the CLIF-C OFs cutoff at 10 had sensitivity of 68.8% and specificity of 91.4%. The originally proposed cut-offs for DF and MELD lacked specificity (50.9% for DF $\geqslant$ 32, 57.8% for MELD $\geqslant$ 21). The unoptimized threshold for DF and MELD partially account for the low specificity of DF and MELD score. The NPV of all prognostic models were excellent, in most instances > 90%. In most cases, PPV was lower than 50% (Table 3). This implies that these prognostic models are useful for excluding low-risk patients, rather than identifying those at high risk of death.

Our study has limitations. First, AH was diagnosed based on clinical presentation. Clinical diagnosis poses a 10%-50% risk of erroneous classification as AH[27,28]. Second, medical treatment was determined by physician judgment. Corticosteroid effects may interfere with the predictive ability of prognostic scores, and there is some controversy in the survival benefit provided by corticosteroid treatment[29,30]. Third, we did not evaluate the alcohol relapse rate which might influence mortality and the predictive value of scoring systems. Finally, sequential values of scoring systems were not obtained. Therefore, the dynamic phase of clinical disease may not be reflected. Nevertheless, the strength of this study lies in its multi-center retrospective analysis of consecutive cases. This structure reduces selection bias and improves generalizability. Moreover, the present study is the first investigation of scoring systems proposed for ACLF at predicting the mortality of patients with AH.

In conclusion, the prognostic scores proposed for ACLF, such as CLIF-SOFA or CLIF-C OFs, proved excellent for predicting 28- and 90-d mortality. CLIF-SOFA and CLIF-C OFs had comparable discriminatory power for predicting short-term mortality compared to DF, ABIC, GAHS, MELD, and MELD-Na scores in patients with AH. CLIF-SOFA scores $\geqslant$ 8 or CLIF-C OFs $\geqslant$ 10 on the day of hospitalization should be regarded as negative short-term prognostic factors. Prospective studies validating the prognostic utility of CLIF-SOFA and CLIF-C OFs in AH are warranted.

## COMMENTS

### Background

Alcoholic hepatitis (AH) occurs in patients after long-term alcohol misuse. AH often presents with acute deterioration superimposed on chronic liver disease, comprising acute-on-chronic liver failure (ACLF), which is characterized by acute decompensation, organ failure, and high short-term mortality. Given that short-term mortality is high in severe AH, various scoring systems have been proposed to assess the severity of AH and to predict prognosis in these patients. Meanwhile, several models have been proposed to predict mortality in patients with ACLF. The ability of scores proposed for ACLF to predict mortality in patients with AH is largely unknown. The aim of this study was to evaluate the utility of ACLF scoring systems and compare the predictive ability of these scores with that of other commonly used prognostic models in predicting outcomes for patients with AH.

### Research frontiers

ACLF is a recently recognized syndrome, frequently related to active alcoholism. Few studies have analyzed scoring systems proposed for ACLF in patients with AH. The results of this study contribute to evaluating the potential of ACLF scoring systems for AH prognostication.

### Innovations and breakthroughs

In this study, Chronic Liver Failure-Sequential Organ Failure Assessment (CLIF-SOFA) and CLIF Consortium Organ Failure score (CLIF-C OFs) performed well in predicting short-term mortality in AH patients. In addition CLIF-SOFA and CLIF-C OFs showed comparable predictive ability for short-term mortality

compared to commonly used scoring systems proposed for AH.

### Applications

This study suggests that scores proposed for ACLF could be useful in predicting short-term morality in patients with AH. In patients with AH, CLIF-SOFA scores ⩾ 8 or CLIF-C OFs ⩾ 10 could be used as one of the negative short-term prognostic factors.

### Terminology

ACLF: a distinct syndrome with high short-term mortality which is used to characterize patients hospitalized for acute decompensation of cirrhosis who have organ failure. CLIF-SOFA: a modified SOFA score adapted for patients with cirrhosis which is based on the number and type of organ failure to define ACLF.

### Peer-review

The author of this paper explored the ability of new scores proposed for patients with cirrhosis and acute decompensation in predicting mortality in alcoholic hepatitis. A promising performance of scores proposed for ACLF in predicting short-term mortality in patients with AH was found, and further prospective studies are needed.

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ORIGINAL ARTICLE

Prospective Study

# Molecular detection of *Helicobacter pylori* antibiotic resistance in stool *vs* biopsy samples

Denise E Brennan, Joseph Omorogbe, Mary Hussey, Donal Tighe, Grainne Holleran, Colm O'Morain, Sinéad M Smith, Deirdre McNamara

Denise E Brennan, Joseph Omorogbe, Mary Hussey, Donal Tighe, Grainne Holleran, Colm O'Morain, Sinéad M Smith, Deirdre McNamara, Department of Clinical Medicine, School of Medicine, Trinity College Dublin, 2 Dublin, Ireland

Sinéad M Smith, School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, 2 Dublin, Ireland

Author contributions: Smith SM and McNamara D contributed equally this work; Brennan DE, Omorogbe J, Hussey M, Tighe D, Holleran G, O'Morain C and McNamara D recruited patients and collected samples for the study; Brennan DE and Smith SM conducted experiments, analysed data and wrote the manuscript; all authors critically reviewed and approved the manuscript prior to publication.

Institutional review board statement: The study was reviewed and approved by the Adelaide and Meath Hospital Research Ethics Committee.

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Correspondence to: Dr. Sinéad M Smith, PhD, Assistant Professor, Department of Clinical Medicine, School of Medicine, Trinity College Dublin, Tallaght, 2 Dublin, Ireland. smithsi@tcd.ie
Telephone: +35-318-961385
Fax: +35-318-962988

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## Abstract

### *AIM*

To compare (1) demographics in urea breath test (UBT) *vs* endoscopy patients; and (2) the molecular detection of antibiotic resistance in stool *vs* biopsy samples.

### *METHODS*

Six hundred and sixteen adult patients undergoing endoscopy or a UBT were prospectively recruited to the study. The GenoType HelicoDR assay was used to detect *Helicobacter pylori* (*H. pylori*) and antibiotic resistance using biopsy and/or stool samples from CLO-positive endoscopy patients and stool samples from UBT-positive patients.

### *RESULTS*

Infection rates were significantly higher in patients referred for a UBT than endoscopy (overall rates: 33% *vs* 19%; treatment-naïve patients: 33% *vs* 14.7%, respectively). *H. pylori*-infected UBT patients were younger than *H. pylori*-infected endoscopy patients (41.4 *vs* 48.4 years, respectively, $P < 0.005$), with a higher percentage of *H. pylori*-infected males in the endoscopy-compared to the UBT-cohort (52.6% *vs* 33.3%, $P = 0.03$). The GenoType HelicoDR assay was more accurate at detecting *H. pylori* infection using biopsy samples than stool samples [98.2% ($n$ = 54/55) *vs* 80.3% ($n$ =53/66), $P < 0.005$]. Subset analysis using stool and biopsy samples from CLO-positive endoscopy patients revealed a higher detection rate of

resistance-associated mutations using stool samples compared to biopsies. The concordance rates between stool and biopsy samples for the detection of *H. pylori* DNA, clarithromycin and fluoroquinolone resistance were just 85%, 53% and 35%, respectively.

***CONCLUSION***

Differences between endoscopy and UBT patients provide a rationale for non-invasive detection of *H. pylori* antibiotic resistance. However, the GenoType HelicoDR assay is an unsuitable approach.

**Key words:** *Helicobacter pylori*; Antibiotic resistance; Clarithromycin; Fluoroquinolone; Molecular methods



**Core tip:** The successful detection of clarithromycin and/or fluoroquinolone resistant *Helicobacter pylori* (*H. pylori*) infections by non-invasive methods would enable a widespread assessment of resistance rates. Here we evaluate the GenoType HelicoDR assay for the detection of clarithromycin and fluoroquinolone resistance using DNA isolated from stool samples compared to biopsy samples. Although results using this assay on biopsy tissue have previously been shown to correspond well with culture and antimicrobial susceptibility testing, there was weak correlation between results obtained using biopsy *vs* stool samples in our study. Further studies are required to optimise the non-invasive detection of clarithromycin and fluoroquinolone resistant *H. pylori* infection.

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a gram-negative bacterium that specifically colonizes the epithelium of the human stomach, in particular the gastric antrum. It infects approximately 50% of the world's population. The prevalence of *H. pylori* varies globally, increasing with older age and lower socio-economic status. Most infected individuals will not develop any clinically significant complications; however the most common symptoms of infection are gastritis and gastric or duodenal ulcers. The diagnosis and treatment of *H. pylori* infection are critical factors in the prevention and management of these conditions[1-3]. *H. pylori* infection can be detected by invasive and non-invasive means, using a variety of diagnostic tests. The Maastricht IV/Florence Consensus Report recommends the "Test and Treat" strategy for patients presenting with uncomplicated dyspepsia with no alarm symptoms associated with an increased risk of gastric cancer[2]. In the Irish healthcare setting, the urea breath test (UBT) is the current gold standard non-invasive test for *H. pylori* infection in patients managed by the "Test and Treat" strategy. The UBT is highly accurate with a sensitivity of 88%-95% and specificity of 95%-100%[4]. For patients presenting with new onset dyspepsia (above 45 years; European guidelines) or dyspepsia along with accompanying alarm symptoms such as weight loss, gastrointestinal bleeding, abdominal mass or iron deficient anaemia, endoscopy is recommended[2]. When an endoscopy is performed, *H. pylori* infection can be diagnosed using gastric biopsy specimens. The most common test employed is the rapid urease-test for *Campylobacter*-like organisms (CLO), which has a sensitivity of > 90% and specificity of > 95%[5].

Treatment for *H. pylori* infection is recommended in all symptomatic individuals. However, eradication rates have fallen in many countries in recent years[6-8] mainly due to poor patient compliance and the emergence of antibiotic resistant strains of *H. pylori*, particularly to clarithromycin and levofloxacin[9-11]. The European *Helicobacter* and Microbiota Study Group (EHMSG) and the most recent Maastricht IV/Florence Consensus recommend local surveillance of existing and emerging antibiotic resistance and that the combination of antibiotics for *H. pylori* eradication should be chosen according to the local resistance patterns[2,10]. Clarithromycin-based first-line triple therapy is no longer recommended in regions where antibiotic resistance surveillance indicates that clarithromycin resistance is above 15%-20%[2]. Since *H. pylori* is a fastidious bacterium, culture and antimicrobial sensitivity testing is time-consuming. The sensitivity of culture of *H. pylori* from gastric biopsy samples has been reported to be as low as 55%[11]. Molecular testing represents an attractive alternative to culture-based methods and has been recommended by the Maastricht Consensus guidelines to detect *H. pylori* and both clarithromycin and fluoroquinolone resistance when standard culture and sensitivity testing are unavailable[2]. Single point mutations (most commonly A2146C, A2146G and A2147G) within the *H. pylori rrl* gene that encodes the 23S ribosomal subunit confer clarithromycin resistance[11,12]. The most significant mutations conferring fluoroquinolone resistance are located at positions 87 (N87K) and 91 (D91N, D91G, D91Y) of the *H. pylori gyrA* gene, which encodes the A subunit of the DNA gyrase enzyme[11,12]. The GenoType HelicoDR assay allows for the molecular genetic identification of *H. pylori* and its resistance to clarithromycin and fluoroquinolones, such as levofloxacin. The assay has been reported to be efficient at detecting mutations predictive of antibiotic resistance when applied to *H. pylori* cultures or gastric biopsy

specimens[13-16], with a sensitivity and specificity of 94%-100% and 86%-99% for detecting clarithromycin resistance and 83%-87% and 95%-98.5% for detecting fluoroquinolone resistance, respectively[16,17].

Currently, *H. pylori* antibiotic resistance surveillance is based primarily on patients undergoing invasive testing by means of endoscopy. However, most patients are diagnosed by non-invasive methods such as the UBT. As such, antibiotic resistance data obtained solely from endoscopy patients may not reflect the true prevalence of *H. pylori* infection and the rates of antibiotic resistance in symptomatic patients. The aims of this study were to (1) compare demographics and prevalence of *H. pylori* infection in patients referred for endoscopy with those of patients referred for a UBT; and (2) evaluate the potential use of the GenoType HelicoDR assay for the non-invasive detection of *H. pylori* and antibiotic resistant infection using stool samples.

## MATERIALS AND METHODS

### Study design and ethics

A prospective study was carried out in a tertiary referral teaching hospital (Adelaide and Meath Hospital, Dublin, Ireland) affiliated with Trinity College Dublin. Patients who had been referred to the endoscopy clinic were included from August 2014 until March 2016. The study received ethical approval from the Adelaide and Meath Hospital Research Ethics Committee. Informed consent was obtained from all patients before enrolment.

### Study population

Inclusion criteria were (1) ability and willingness to participate in the study and to provide informed consent; and (2) confirmed *H. pylori* infection by UBT or a positive rapid urease test (TRI-MED Distributors, PTY LTD, Washington, United States) at 60 min performed and/or histology.

Exclusion criteria were (1) age less than 18 years; (2) pregnancy or lactation; (3) severe inter-current illness; (4) current PPI use or recent antibiotic use (within 4 wk); and (5) bleeding problems or use of blood thinning drugs (for endoscopy patients).

### Sample collection and antimicrobial susceptibility genotyping

A single corpus and antrum biopsy from each patient was placed into DENT transport medium (brain heart infusion broth containing 2.5% (w/v) yeast extract, 5% sterile horse serum and Dent *Helicobacter* Selective Supplement; Oxoid, Basingstoke, United Kingdom) for transport to the research laboratory. Biopsies were placed into fresh collection tubes and stored at -20 ℃ until processed for genomic DNA isolation using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. Patients attending for endoscopy or the UBT were invited to provide a stool sample collected within 24 h of their appointment. Stool samples were stored at 4 ℃ until processed for genomic DNA isolation using the PSP Spin Stool DNA Plus Kit (STRATEC Molecular GmbH, Berlin, Germany) according to the manufacturers' instructions. All isolated DNA was stored -20 ℃ until genotyping for clarithromycin and fluoroquinolone-mediating mutations was performed using the Genotype HelicoDR assay (Hain Lifescience GmbH, Nehren, Germany). Multiplex amplification of DNA regions of interest was performed using the biotinylated primers supplied in the GenoType HelicoDR kit and the Hotstart Taq DNA polymerase kit (Qiagen). PCR products were reverse hybridised to DNA strips containing probes for gene regions of interest, developed and interpreted according to the manufacturers' instructions. Briefly, all strips were analysed for the presence of a conjugate control band (to indicate successful conjugate binding and substrate reaction), an amplification control band (to indicate a successful amplification reaction), a *H. pylori* control band (to document the presence of a *H. pylori* strain) and gene locus control bands for *gyrA* and *23S* (to indicate successful detection of the gene regions of interest). In addition, the strips were analysed for the presence of wild type and/or mutation bands. An infection was considered clarithromycin sensitive when the 23S wild-type probe stained positive and clarithromycin resistant if one of the 23S mutation probes stained positive. As per manufacturers' instructions, results of both positions of the *gyrA* gene were combined to draw conclusions about fluoroquinolone resistance. Thus, an infection was only considered fluoroquinolone sensitive when one of the wild-type probes for codon 87 of the *gyrA* gene stained positive together with a positive wild-type probe for codon 91. Fluoroquinolone resistance was indicated if either the wild-type probes for codon 87 or the wild-type probe for codon 91 stained negative, or if one of the mutant codon 87 or 91 probes stained positive. For all mutations probes, only bands whose intensities were equal to or stronger than the amplification control were considered positive.

### Statistical analysis

Statistical analysis was carried out using GraphPad Prism (GraphPad Software Inc., CA, United States). Continuous variables are presented as arithmetic mean and SD. *P* values for continuous variables were calculated and compared using the two-tailed independent *t*-test. Categorical variables are presented as percentages and 95% confidence intervals (95%CI). *P* values for categorical variables were calculated using the Fisher's exact test/Pearson $\chi^2$ test. In all cases, a *P* value less than 0.05 was considered significant.

## RESULTS

### Prevalence of H. pylori infection and demographics of endoscopy and UBT patients

A schematic of patient inclusion and analysis is presented in Figure 1. In all, 616 patients were

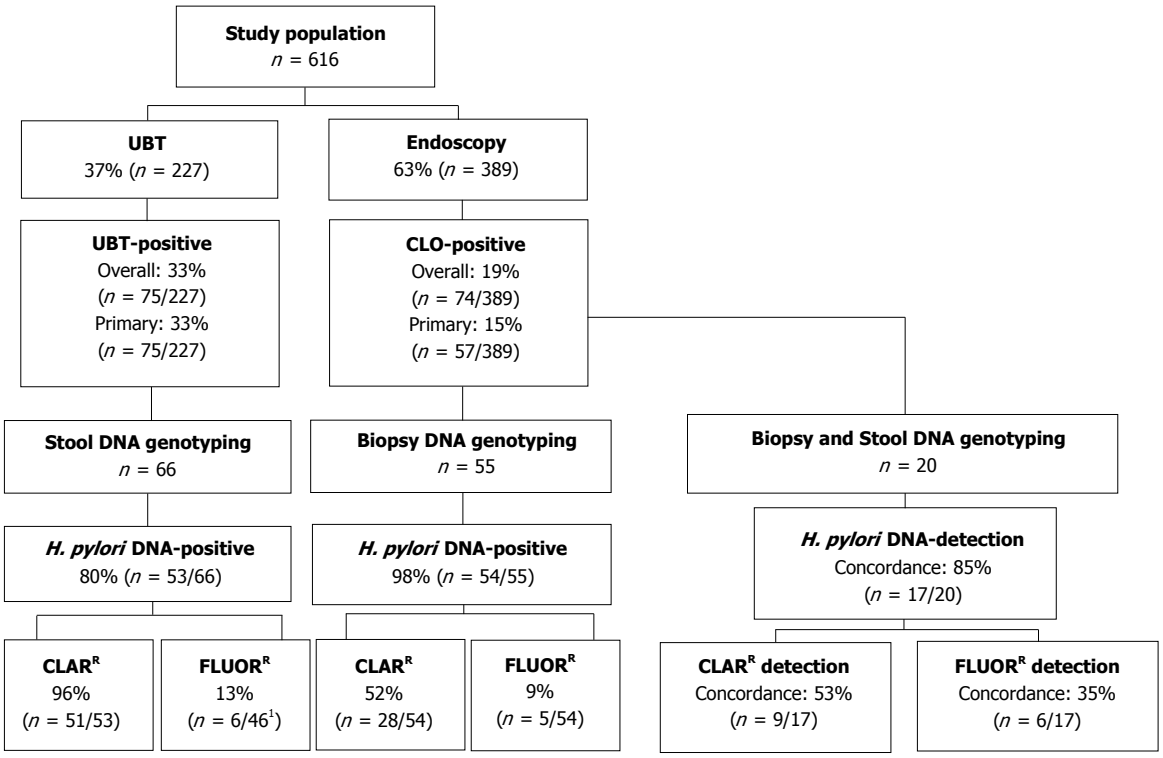


**Figure 1 Flow chart of patient inclusion and analysis.** [1]Only samples that were positive for the control gene locus for the fluoroquinolone resistance gene were included. CLAR$^R$: Clarithromycin resistant; FLUOR$^R$: Fluoroquinolone resistant.

**Table 1 Demographics of endoscopy and urea breath test patients**

| | Endoscopy | UBT | *P* value | 95%CI |
|---|---|---|---|---|
| Total | *n* = 389 | *n* = 227 | | |
| Mean age (SD) | 52.3 (16.4) | 39.6 (12.6) | < 0.001 | 10.22-15.18 |
| Male | 42.2% (*n* = 164) | 30.4% (*n* = 69) | < 0.005 | 3.68-19.60 |
| Primary *H. pylori* infection | 14.7% (*n* = 57) | 33.0% (*n* = 75) | < 0.001 | 11.07-25.65 |
| Mean age (SD) | 48.4 (14.9) | 41.4 (13.0) | < 0.005 | 2.19-11.81 |
| Male | 52.6% (*n* = 30) | 33.3% (*n* = 25) | 0.03 | 1.23-36.29 |

included in the study between August 2014 and March 2016; 389 patients (mean age 52.3 years, 42.2% male) underwent endoscopy and 227 patients (mean age 39.6 years, 30.4% male) a UBT (Table 1). The overall prevalence of *H. pylori* infection was significantly higher in the UBT cohort than the endoscopy cohort [33.0% (*n* = 75) *vs* 19% (*n* = 74), *P* < 0.001; 95%CI: 6.58-21.54] (Figure 1). Of the *H. pylori*-positive endoscopy patients (CLO-positive), 17 had been previously treated for *H. pylori* infection, therefore the prevalence of primary *H. pylori* infection was 14.7% (*n* = 57). All of the *H. pylori*-positive UBT patients were treatment naïve, thus the prevalence of primary *H. pylori* infection was also significantly higher in patients referred for UBT than for endoscopy (33.0% *vs* 14.7%, *P* < 0.001, 95%CI: 11.07-25.65; Figure 1 and Table 1). In keeping with the guidelines recommending endoscopy for symptomatic patients over 45 years, *H. pylori*-positive patients in the endoscopy cohort were significantly older than those in the UBT cohort (48.4 years *vs* 41.4 years; *P* < 0.005, 95%CI: 2.19-11.81). There were a greater number of *H. pylori*-positive men in the endoscopy cohort than the UBT cohort (52.6% *vs* 33.3%, *P* = 0.03, 95%CI: 1.23-36.29; Table 1). Taken together, these findings indicate significant differences in demographics and the prevalence of both overall and primary *H. pylori* infection rates in patients referred for endoscopy and those referred for the UBT.

### *Comparison of H. pylori detection and the prevalence of antibiotic resistance-mediating mutations using the GenoType HelicoDR assay in endoscopy vs UBT patients using biopsies and stool samples, respectively*

The GenoType HelicoDR assay is based on DNA strip technology that enables the molecular genetic identification of *H. pylori* and resistance to fluoroquinolones and/or clarithromycin by detecting the most frequent mutations in the *gyrA* gene (codons 87 and 91) and the *23S* gene (positions 2146 and 2147), respectively. Previous studies have demonstrated strong correlations between results obtained using the GenoType

**Table 2 Molecular detection of *Helicobacter pylori* infection and resistance-mediating mutations using biopsies from CLO-positive endoscopy patients and stool samples of Urea Breath Test-positive patients**

| | Biopsy Specimens | Stool specimens | *P* value | 95%CI |
|---|---|---|---|---|
| Total analysed | *n* = 55 | *n* = 66 | | |
| *H. pylori* DNA positive | 98.2% (*n* = 54/55) | 80.3 % (*n* = 53/66) | < 0.005 | 6.10-29.66 |
| Clarithromycin resistant | 51.9% (*n* = 28/54) | 96.2 % (*n* = 51/53) | < 0.001 | 27.70-58.71 |
| Fluoroquinolone resistant | 9.3 % (*n* = 5/54)[1] | 13 % (*n* = 6/46)[1] | 0.55 | -9.99-18.28 |

[1]Fluoroquinolone and clarithromycin mutations present. *H. pylori*: *Helicobacter pylori*.

**Table 3 Concordance in the detection of *Helicobacter pylori* and antibiotic resistance-mediating mutations between results obtained using biopsies *vs* stool samples from individual *Campylobacter*-like organisms-positive endoscopy patients**

| | Biopsy Specimens | Stool specimens | Concordance |
|---|---|---|---|
| Total analysed | *n* = 20 | *n* = 20 | |
| *H. pylori* DNA positive | 95% (*n* =19/20) | 90% (*n* = 18/20) | 85% (*n* = 17/20) |
| Clarithromycin resistant[1] | 52.9% (*n* = 9/17) | 100.0% (*n* = 17/17) | 52.9% (*n* = 9/17) |
| Fluoroquinolone resistant[1] | 23.5% (*n* = 4/17) | 52.9% (*n* = 9/17) | 35.3% (*n* = 6/17) |

[1]Only samples where *H. pylori* DNA was detected were analysed. *H. pylori*: *Helicobacter pylori*.

HelicoDR assay on biopsy specimens compared to culture and antimicrobial testing[14,16,17]. In order to evaluate the GenoType HelicoDR assay for the non-invasive detection of *H. pylori* using stool samples, we first set out to compare the detection rate of *H. pylori* infection using stool samples from *H. pylori*-positive UBT patients with that obtained using biopsy samples from CLO-positive endoscopy patients. Initial control experiments showed that the assay did not detect *H. pylori* DNA in stool samples from 2 uninfected UBT-negative patients (not shown). In *H. pylori*-infected patients, the GenoType HelicoDR assay was significantly more accurate at detecting *H. pylori* infection using biopsy samples than stool samples [98.2% (*n* = 54/55) *vs* 80.3% (*n* = 53/66), *P* < 0.005, 95%CI: 6.10-29.66] (Figure 1 and Table 2).

Next, the prevalence of antibiotic resistance-mediating mutations was compared using stool samples from *H. pylori*-positive UBT patients and biopsy samples from CLO-positive endoscopy patients. Using the GenoType HelicoDR assay, the *23S* gene locus control was positive in all of *H. pylori*-positive DNA samples isolated from either biopsy tissue (100%, *n* = 54/54) or stool specimens (100%, *n* = 53/53). A significantly higher level of clarithromycin resistance-mediating mutations was detected using stool samples from UBT-positive patients than biopsy samples from CLO-positive patients [96.2% (*n* = 51/53) *vs* 51.9% (*n* = 28/54), *P* < 0.001, 95%CI: 27.70-58.71] (Figure 1 and Table 2).

In terms of *gyrA* genotyping, the *gyrA* locus control probe was positive in all DNA samples isolated from biopsy tissue (100%, *n* = 54/54), but only 86.8% (*n* = 46/53) of *H. pylori*-positive DNA samples isolated from stool. Fluoroquinolone resistance-mediating mutations were detected in 9.3% (*n* = 5/54) of biopsy samples from CLO-positive patients compared to 13% (*n* = 6/46) of stool samples from UBT-positive patients (*P* = 0.56, 95%CI: -9.99-18.28; Figure 1 and Table 2). For both endoscopy and UBT patients, all samples that were positive for fluoroquinolone resistance mutations were positive for clarithromycin resistance mutations (Table 2). Taken together, these findings indicate that the GenoType HelicoDR assay is more accurate at detecting *H. pylori* DNA using biopsies from CLO-positive endoscopy patients than stool DNA isolated from UBT-positive patients. In addition, the assay detected a significantly higher rate of clarithromycin resistance using stool samples from patients diagnosed by the UBT than that obtained when biopsy samples from CLO-positive endoscopy patients were analysed.

### *Evaluation of the GenoType HelicoDR assay for the detection of resistance-mediating mutations by comparing stool and biopsy analyses from individual patients*

Given the high rate of clarithromycin resistance detected using stool specimens from UBT-positive patients (96.2%; Table 2) and the lack of published data on the use of the GenoType HelicoDR assay for stool sample analysis, we next set out to directly compare a stool DNA sample with that of a biopsy DNA sample isolated from a subset of the CLO-positive endoscopy patients. In all, stool and biopsy samples from 20 CLO-positive patients were analysed (mean age 46.8 ± 15.8 years, 50% male). *H. pylori* DNA was detected in 95% (*n* = 19/20) of biopsy samples and 90% (*n* = 18/20) of stool samples from the CLO-positive patients. Concordance between results from biopsy and stool samples of individual patients for the detection of *H. pylori* DNA was 85% (*n* = 17/20; Figure 1 and Table 3). In terms of antibiotic resistance, results were compared in the 17 patients with concordant results for the presence of *H. pylori* DNA in

both their stool and biopsy samples. Concordance for the analysis of stool and biopsy samples of individual patients was just 52.9% ($n$ = 9/17) for clarithromycin resistance and 35.3% ($n$ = 6/17) for fluoroquinolone resistance (Figure 1, Table 3). Higher rates of both clarithromycin and fluoroquinolone resistance were detected in stool samples compared to biopsy samples obtained from the same patient (Table 3), suggesting a lack of specificity of the assay for the detection of antibiotic resistance-mediating mutations using DNA isolated from stool samples.

## DISCUSSION

As the recommended first-line therapy for *H. pylori* infection should be guided by the local prevalence of primary clarithromycin resistance and third-line and subsequent treatment regimens should be guided by antimicrobial susceptibility testing[2], methods for detecting antibiotic resistance are of great interest. Antimicrobial susceptibility testing for *H. pylori* is mainly performed using biopsy specimens obtained by invasive means at endoscopy. As a result, findings on the prevalence of *H. pylori* infection and antibiotic resistance based solely on this patient cohort may not represent the true rates of resistance in a given population. In order to determine whether *H. pylori*-infected endoscopy patients are representative of the wider *H. pylori*-infected population, we compared the prevalence of infection and patient demographics between endoscopy patients with those referred for non-invasive *H. pylori* diagnoses by the UBT. Indeed we found significant differences between the two patient cohorts. Both the overall infection rate and the prevalence of primary infection in *H. pylori* treatment-naïve patients were significantly higher in patients referred for a UBT than endoscopy (overall infection rates of 33% *vs* 19% respectively, and primary infection rates of 33% *vs* 14.7%, respectively). *H. pylori*-infected UBT patients were also significantly younger than *H. pylori*-infected endoscopy patients (41.4 *vs* 48.4 years, respectively), with a higher percentage of *H. pylori* infected males in the endoscopy compared to UBT cohort (52.6% *vs* 33.3%). Both age and sex have been reported as risk factors for *H. pylori* antibiotic resistance, for example age > 50 years has been reported as a risk factor for levofloxacin resistance and being female has been associated with metronidazole resistance in the most recent pan-European study on antimicrobial resistance[10]. Thus the statistically significant differences in age and sex between endoscopy and UBT patients in our study suggests that *H. pylori*-infected endoscopy patients are likely not representative of the wider *H. pylori*-positive cohort, providing a strong rationale for performing more widespread antimicrobial susceptibility testing.

Successfully extending molecular-based methods to diagnose *H. pylori* non-invasively would greatly enhance our ability to more accurately assess the prevalence of resistance to a range of antibiotics, and enable clinicians to offer personalised antimicrobial susceptibility-based therapy to a wider number of patients. *H. pylori* DNA has been detected in a number of clinical specimens including blood, stool samples and oral cavity specimens [18-22]. Analysis of stool samples has shown the most promise for the molecular detection of clarithromycin resistance-mediating mutations to date[18,23-28]. Studies have demonstrated sensitivity and specificity values of 83%-98% and 98%-100%, respectively, for the detection clarithromycin resistance using the *H. pylori* ClariRes Assay (Ingenetix) to analyse stool samples[23-26]. However, data on the detection of *H. pylori* fluoroquinolone resistance using stool samples is lacking. Although the GenoType HelicoDR assay has proven useful for detecting clarithromycin and fluoroquinolone resistance in biopsy or culture specimens[13-17], evaluation of the assay for the analysis of stool specimens presented herein proved suboptimal. Firstly, the assay detected *H. pylori* infection in a significantly lower percentage of *H. pylori*-infected patients when stool rather than biopsy specimens were analysed (80%-90% *vs* 95%-98.2%; respectively Tables 2 and 3). As *H. pylori* specifically colonizes the stomach and is not an intestinal bacterium, it is present only in low numbers in the stool, a factor which may have impacted the sensitivity of *H. pylori* detection using stool samples in our study. Additionally, *H. pylori* DNA may be exposed to enzymatic or mechanical degradation during transit from the stomach through the intestines[22]. When results using biopsy samples from individual *H. pylori*-infected patients were directly compared with those obtained from their stool samples, concordance scores for clarithromycin and fluoroquinolone resistance were just 52.9% and 35.6%, respectively. In addition, a higher rate of clarithromycin and fluoroquinolone resistance was detected in DNA isolated from the stool samples compared to DNA isolated from biopsy samples from the same patient (Table 3). Given that previous studies have demonstrated strong correlations between results obtained using the GenoType HelicoDR assay on biopsy specimens compared to culture and antimicrobial testing[14,16,17], this would suggest that stool sample analysis using the GenoType HeliocDR assay is less sensitive than biopsy sample analysis, providing an explanation for the high rates of antibiotic resistance obtained using stool samples from the UBT patients in Table 2. The presence of large amounts of diverse commensal bacteria in the stool may hamper the specificity of the Genotype HelicoDR assay in detection of *H. pylori* antibiotic resistance-mediating mutations. Our findings suggest it is currently unsuitable for the accurate detection of clarithromycin and fluoroquinolone resistance-mediating mutations in stool specimens. Further studies are required to extend approaches for the non-invasive detection of *H. pylori* resistance to include multiple antibiotics. Recent advances in next generation DNA sequencing technologies may provide

more robust opportunities for the accurate analysis of specific resistance-associated DNA regions. The successful optimisation of molecular-based antimicrobial susceptibility testing methods will enable resistance data obtained from patients managed by the "Test and Treat" strategy to be utilised in choosing effective antibiotics for the treatment of *H. pylori*. In this way, eradication rates for *H. pylori* may be improved.

## COMMENTS

### Background

Currently antimicrobial susceptibility testing for *Helicobacter pylori* (*H. pylori*) is mainly performed using cultures isolated from tissue biopsy samples obtained at endoscopy by invasive means. However, many patients are diagnosed with *H. pylori* infection by non-invasive means, such as the urea breath test. As such, antibiotic resistance data based solely on endoscopy patients may not truly reflect the prevalence of antibiotic resistance in the wider *H. pylori* infected population.

### Research frontiers

Molecular methods for the detection of *H. pylori* antibiotic resistance-mediating mutations offer a more rapid alternative to standard culture-based methods. Studies have shown that data generated using molecular methods on tissue biopsy samples correlates well with culture and antimicrobial susceptibility testing. Data on the use of molecular methods, in particular the GenoType HelicoDR assay, for the analysis of stool samples is limited.

### Innovations and breakthroughs

The present findings suggest that the GenoType HelicoDR assay is not suitable for the accurate detection of antibiotic resistance-mediating mutations using stool samples from *H. pylori* infected patients. Alternative PCR or DNA sequencing-based methods may show more potential.

### Applications

While the GenoType HelicoDR assay has been shown to be accurate for the analysis of clarithromycin- and fluoroquinolone-mediating mutations using biopsy tissue samples, the present findings indicate that this assay is not suitable for the analysis of stool samples.

### Peer-review

The authors described an examination of antibiotic resistance in both gastric biopsy and stool samples obtained from patients who underwent testing for a urea breath test or had a gastroscopy performed. The main conclusion is that the Genotype HelicoDR assay is not appropriate for use on stool samples. This seriously limits its use and thus the paper is of importance and deserves to be published. It would have been useful to include formal sensitivity testing to the bacteria isolated on gastric biopsy.

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CASE REPORT

# Two cases of pancreatic ductal adenocarcinoma with intrapancreatic metastasis

Yusuke Fujita, Minoru Kitago, Yohei Masugi, Osamu Itano, Masahiro Shinoda, Yuta Abe, Taizo Hibi, Hiroshi Yagi, Yoko Fujii-Nishimura, Michiie Sakamoto, Yuko Kitagawa

Yusuke Fujita, Minoru Kitago, Osamu Itano, Masahiro Shinoda, Yuta Abe, Taizo Hibi, Hiroshi Yagi, Yuko Kitagawa, Department of Surgery, Keio University School of Medicine, Shinjuku-ku, Tokyo 160-8582, Japan

Yohei Masugi, Yoko Fujii-Nishimura, Michiie Sakamoto, Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Author contributions: Fujita Y, Kitago M and Masugi Y wrote the paper; Masugi Y, Fujii-Nishimura Y and Sakamoto M evaluated the pathological findings; all other members equally contributed medical treatment.

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Correspondence to: Minoru Kitago, MD, PhD, Department of Surgery, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. dragonpegasus@a3.keio.jp
Telephone: +81-3-53633802
Fax: +81-3-33554707

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## Abstract

There are no standardized diagnostic criteria for intrapancreatic metastasis of pancreatic ductal adenocarcinoma (PDAC). Here, we report two cases of patients with PDAC who were pathologically diagnosed as harboring intrapancreatic metastasis. In both cases, the main lesions were located in the pancreatic body, and no other lesion was detected preoperatively. The patients were diagnosed with pancreatic body cancers and distal pancreatectomy was performed. Pathological findings revealed microscopic cancer nests, which had connections to neither the main lesion nor the premalignant lesion in the pancreatic tail parenchyma. In both cases, the histological type of the daughter lesion was quite similar to that of the main lesion. Hence, we diagnosed the daughter lesions as metastatic foci in the pancreas. Although intrapancreatic metastasis of PDAC has been regarded as a poor prognostic factor, few reports of intrapancreatic metastasis are available. This article reports two such cases and provides a review of the literature.

**Key words:** Carcinoma; Pancreatic ductal; Neoplasm micrometastasis; Recurrence; Carcinogenesis



**Core tip:** Although intrapancreatic metastasis (IPM) of pancreatic ductal adenocarcinoma has been regarded as a poor prognostic factor, few reports of IPM are available. Furthermore, the diagnostic criteria and the

clinicopathological significance of IPM still need to be clarified. It should be remembered that IPM is present at a constant rate, and may be located in the remnant pancreas or in resected specimens other than the main lesion. IPM could be a cause of early recurrence. Here, we have presented two cases of IPM and provided suggestions regarding the foundation of the diagnosis of IPM.

Fujita Y, Kitago M, Masugi Y, Itano O, Shinoda M, Abe Y, Hibi T, Yagi H, Fujii-Nishimura Y, Sakamoto M, Kitagawa Y. Two cases of pancreatic ductal adenocarcinoma with intrapancreatic metastasis. *World J Gastroenterol* 2016; 22(41): 9222-9228 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i41/9222.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i41.9222

## INTRODUCTION

Despite progress in diagnosis and treatment, the prognosis of pancreatic ductal adenocarcinoma (PDAC) has remained dismal. The disease prevalence and age-adjusted death rate of pancreatic cancer are increasing yearly, and pancreatic cancer is the fourth-leading cause of cancer death in Japan[1].

Based on studies of hepatocellular carcinoma, intrahepatic cholangiocarcinoma, and lung cancer, tumor metastasis in the primary organ is a poor prognostic factor. Furthermore, it contributes to the T factor in the Union for International Cancer Control TNM classification. In clinical practice, we occasionally encounter patients with two or more PDACs in the pancreas (preoperatively or postoperatively), a situation that bears some resemblance to tumor metastasis in the primary organ. However, there is no consensus or handling convention for multiple lesions in cases of PDAC.

There are two forms of multiple lesions in PDAC: multicentric carcinogenesis and intrapancreatic metastasis (IPM). It is difficult to discriminate between multicentric carcinogenesis and IPM because there are no diagnostic criteria for their pathological findings. Furthermore, few reports have specifically described IPM of PDAC, and its clinicopathological significance has remained unclear. On a search of the literature, we found only two prior reports about IPM of PDAC[2,3].

In this article, we have reported two further cases of IPM of PDAC that were diagnosed pathologically. We have also reviewed the prior literature and discussed the importance of IPM.

## CASE REPORT

### *Case 1*

A 30-year-old Japanese man visited his doctor with the complaint of epigastric pain. Abdominal enhanced computed tomography (CT) revealed a hypovascular tumor in his pancreatic body. He was referred to our hospital for further examination and treatment. He had no significant past medical history and no surgical history, but had a family history of pancreatic cancer (his uncle had had this disease). Although he had no history of smoking, he regularly consumed alcohol.

On examination, his abdomen was soft and flat, without any evident mass or tenderness. His laboratory data were unremarkable, except for the carbohydrate antigen 19-9 (CA-19-9) level, which was elevated to 139 U/mL. Abdominal enhanced CT revealed an 18-mm hypovascular tumor in the pancreatic body and a dilated main pancreatic duct in the tail side of this tumor (Figure 1A). The tumor was compressing the splenic vein. Endoscopic ultrasound showed a 15-mm low echoic tumor that had ill-defined borders and was located next to the splenic vein. Endoscopic retrograde pancreatography demonstrated disruption of the main pancreatic duct in the pancreatic body (Figure 1B). We diagnosed PDAC with invasion of the splenic vein and performed distal pancreatectomy, lymph node dissection, and splenectomy.

Macroscopic findings of the resected specimen showed a 35 mm × 18 mm tumor in the pancreatic body and no other lesion (Figure 2). Pathological findings revealed a moderately differentiated tubular adenocarcinoma with invasion of neutrophil in the main lesion (Figure 3A). The tumor had infiltrated the tunica externa of the splenic vein. At a 20-mm distance to the tail side from the main lesion, there was a 0.6-mm cancer nest, which was a moderately differentiated adenocarcinoma with invasion of neutrophil (in resemblance with the main lesion) (Figure 3B). There was no connection to the main lesion, and we diagnosed this small lesion as intrapancreatic micrometastasis of PDAC. The patient was administered 5-fluorouracil and heparin-based infusion chemotherapy combined with cisplatin and mitomycin C (PI4W) as perioperative chemotherapy[4], and was discharged without any complications. He was administered S-1 (tegafur, gimeracil, and oteracil potassium combination) for 6 mo as adjuvant chemotherapy. However, he developed a recurrence in the liver 6 mo after surgery and underwent FOLFIRINOX therapy following GEM and nab-PTX therapy. Nonetheless, he died 25 mo after surgery.

### *Case 2*

The physician of a 70-year-old Japanese woman noted the carbohydrate antigen 19-9 (CA-19-9) level, which was elevated to 112 U/mL, and CT revealed a tumor in the pancreatic body. She was referred to our hospital for further examination and treatment. She had diabetes mellitus and no family history of cancer. She had no smoking history or alcohol consumption.

On examination, her abdomen was soft and flat without any apparent mass or tenderness. Blood tests

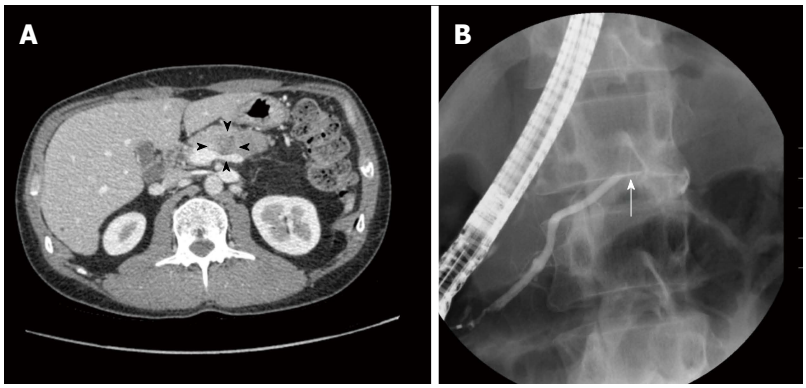


**Figure 1 Enhanced abdominal computed tomography scan (A) and endoscopic retrograde cholangiopancreatography image (B) of case 1.** A: The hypovascular tumor in the pancreatic body (arrowheads); B: Disruption of the main pancreatic duct (arrow).

**Figure 2 Image of the resected pancreas from case 1.** The resected specimen showed a 35 mm × 18 mm tumor in the pancreatic body (arrows). Pathological findings revealed a small lesion at a 20-mm distant to the tail side from the main lesion (arrowhead).

demonstrated elevated tumor markers (CA-19-9, 112 U/mL; Span-1, 41 U/mL). Abdominal enhanced CT revealed an 18-mm hypovascular tumor in the pancreatic body and a dilated main pancreatic duct in the tail side of this tumor (Figure 4A). The tumor was located next to the splenic vein. There was a 7-mm cystic lesion without a nodule in the pancreatic head. Endoscopic ultrasound showed an 18.5-mm low echoic heterogeneous tumor in the pancreatic body and a 10-mm branch duct intraductal papillary mucinous neoplasm (IPMN) in the uncinate process of the pancreas. The main pancreatic duct was narrowed at the pancreatic body and dilated in the tail side in endoscopic retrograde pancreatography (Figure 4B). We diagnosed PDAC in the pancreatic duct with a branch duct type IPMN in the pancreatic uncus and performed distal pancreatectomy, splenectomy, and lymph node dissection.

Macroscopic findings of the resected specimen showed a 32 mm × 20 mm tumor in the pancreatic body and a small lesion in the pancreatic tail, 15 mm away from the main tumor (Figure 5). Pathological findings revealed a poorly differentiated tubular adenocarcinoma with invasion of the splenic vein at the main tumor (Figure 6A). Carcinoma *in situ* continued in the main pancreatic duct, in the range of 15 mm from the invasive cancer. A 1-mm poorly differentiated tubular adenocarcinoma was present in the pancreatic tail parenchyma, 20 mm away from the main invasive cancer (Figure 6B). There was no continuity between this small lesion and the main tumor or carcinoma *in situ*, and we diagnosed the small lesion as an intrapancreatic micrometastasis of PDAC. We administered PI4W as perioperative chemotherapy[4] and discharged the patient without any complications. She was administered gemcitabine for 6 mo as adjuvant chemotherapy. However, she developed a recurrence in the liver at 16 mo after surgery, and underwent gemcitabine and TS-1 therapy. Nonetheless, she died 35 mo after surgery.

## DISCUSSION

In each of the cases described in this report, we found a small lesion separated from the main tumor in the resected specimens, and diagnosed IPM of PDAC pathologically. The current cases and a previously reported case are compared in Table 1. Although there are no guidelines or diagnostic criteria for IPM, we reached the diagnosis of IPM in our cases for several specific reasons. First, although the small lesions had no connection to the main tumors, the small lesions and main tumors showed similar histological findings. The small lesions were separated from the main tumors by 10 mm or more, and a few sections of the intermediate regions included no invasive cancer. Second, there were no premalignant lesions in or around the small lesions. Third, the histological type of the small lesions was monotonous. PDAC essentially consists of various differentiated carcinomas, reflecting its multistep carcinogenesis[5-7]. Although there is a possibility of *de novo* carcinogenesis for such small and poorly differentiated carcinomas, it seems to be a rare occurrence.

In a report of 21 cases of IPM, Oguro *et al*[3] sug-

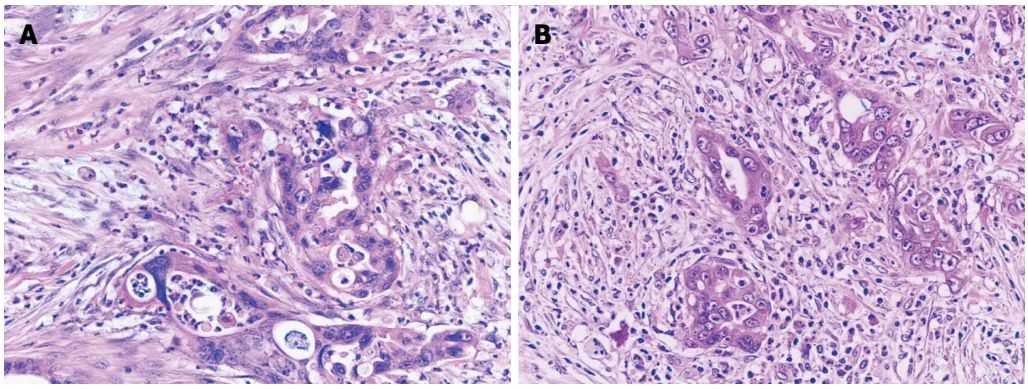


**Figure 3 Microscopic findings of the main lesion (A) and the daughter lesion (B) of case 1.** A: Tumor cells form irregular glands with marked infiltration of neutrophils. Hematoxylin and eosin staining. Objective magnification, × 40; B: The microscopic lesion of the pancreatic tail demonstrated similar morphology to the main lesion. Hematoxylin and eosin staining. Objective magnification, × 40.

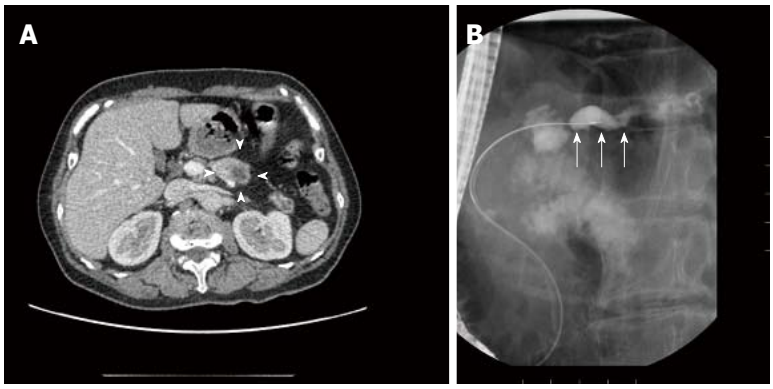


**Figure 4 Enhanced abdominal computed tomography scan (A) and endoscopic retrograde cholangiopancreatography image (B) of case 2.** A: The hypovascular tumor in the pancreatic body (arrowheads); B: Dilation and disruption of the main pancreatic duct (arrows).

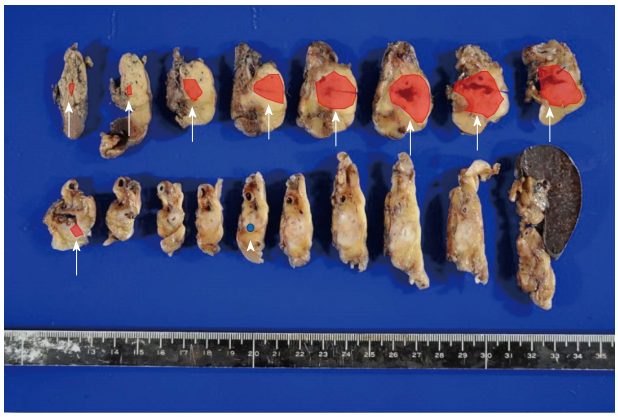

**Figure 5 Image of the resected pancreas from case 2.** The resected specimen showed a 32 mm × 20 mm tumor in the pancreatic body (arrows) and a small lesion at a 15 mm distance at the tail side from the main lesion (arrowhead).

gested the following diagnostic criteria: (1) located within the pancreatic parenchyma and separated from the dominant, primary tumor by a distance of 5 mm or more; (2) showing a histologic appearance identical to that of the dominant, primary tumor; (3) differentiated to the same degree as or less than the dominant, primary tumor; and (4) unaccompanied by premalignant lesions of PDAC, such as pancreatic intraepithelial neoplasia (PanIN) and IPMN. Oguro *et al*[3] also commented that IPM is an invasive lesion that is separated by a distance of 5 mm or more from PanIN-3 or noninvasive IPMN with high-grade dysplasia. Each of our two cases was consistent with first three criteria of them. With regard to premalignant lesions, there was an IPMN in the pancreatic head and an intraepithelial lesion connected to main tumor in case 2, but no connections existed between the IPM and these lesions.

There are some commonalities between our two cases. Both patients had lymph node metastasis and splenic vein invasion, both patients developed early recurrence in the liver, and both IPMs were located in the tail of pancreas. In addition, the lymphatic and venous invasions in the area of the IPM were ly1, v1 in case 1 and ly0, v1 in case 2. Oguro *et al*[3] observed no significant relationship between IPMs and tumor location, lymph node status, portal vein invasion, or other pathological factors. However, IPM was an independent poor prognostic factor, and lymphatic and venous invasion in the area of the IPM were observed for 35% and 62% of IPMs.

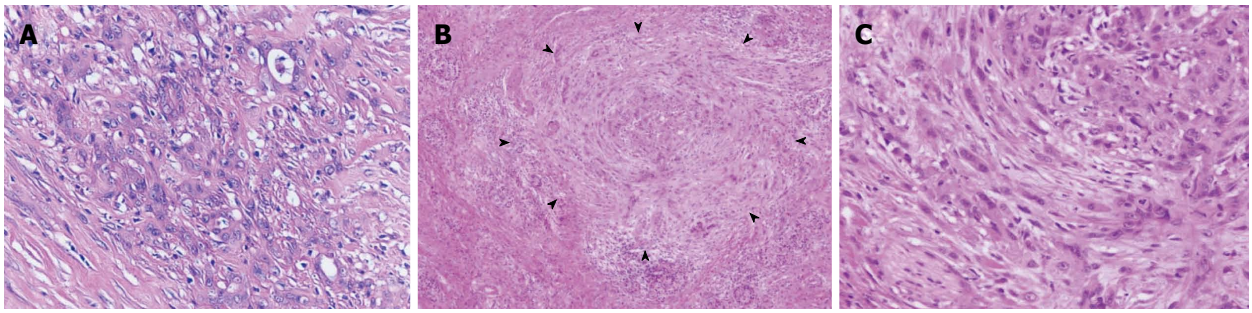


**Figure 6 Microscopic findings of the main lesion (A) and the daughter lesion (B, C) of case 2.** A: Carcinoma cells form trabecular or ill-defined structure with fibrosis. Hematoxylin and eosin staining. Objective magnification, × 40; B, C: A tiny cancer nest was observed in the pancreatic tail without distinct spatial connection with the main tumor. Hematoxylin and eosin staining. Objective magnification, × 10 (B) and × 40 (C).

**Table 1 Intrapancreatic metastasis of pancreatic ductal adenocarcinoma**

| | Main tumor | | | | Stage (UICC) | Metastatic tumor | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Location | Size (mm) | Tumor differentiation | Major vascular invasion | | Location | Distance from main tumor | Size (mm) | Tumor differentiation | Postoperative course |
| Ogawa (2011) | Body | 15 × 12 | Moderate | Unknown | Unknown | Body | 2 mm | 3 × 2 | Unknown | 12 mo NED |
| Case 1 | Body | 35 × 18 | Moderate | PVsp | IIB | Tail | 25 mm | 0.6 | Moderate | 6 mo REC (liver) |
| Case 2 | Body | 32 × 20 | Poor | PVsp | IIB | Tail | 20 mm | 1 | Poor | 16 mo REC (liver) |

NED: No evidence of disease; PVsp: Portal vein (splenic vein); REC: Recurrence; UICC: Union for International Cancer Control.

Between January 2012 and March 2014, 48 patients with PDAC underwent initial surgical resection at our institution. All specimens were cut into serial slices of 5-mm thickness and were examined by a pathologist. Among the 48 patients, 2 (4.2%) had IPM, whereas Oguro *et al*[3] reported an IPM incidence of about 5%. However, our results may underestimate the rate of incidence for two reasons. First, most IPMs are too small to detect preoperatively using imaging tests such as CT and magnetic resonance imaging. Furthermore, our cases were less than 2 mm in size, like micrometastasis in breast cancer or melanoma. We were able to find the small lesions only after the specimens had been cut into serial slices of 5-mm thickness and all sections had been examined in detail by the pathologist. If a micrometastasis happens to occur between the slices, then we cannot identify it. Second, because we generally perform pancreatoduodenectomy or distal pancreatectomy, we cannot examine the remnant pancreas pathologically. Therefore, we think that some cases of early recurrence in the remnant pancreas of PDAC may include IPM that is overlooked at the time of first surgery. For example, Kleeff *et al*[8] reported the cases of 22 patients who underwent pancreatic surgery for recurrence of PDAC in the remnant pancreas. Among the 22 cases, there were 13 cases of recurrence within 12 mo and 2 cases of recurrence within 6 mo. It was too early to diagnose these cases as recurrence of PDAC, and it is possible that IPM was present at the time of first surgery. It should be remembered that IPM is present in a certain proportion of cases, and that the pancreas on the opposite side of the main tumor should be checked to the extent that is possible.

To try to detect small lesion of IPM, we suggest two methods of imaging: endoscopic ultrasound (EUS) and intraoperative ultrasound (IOUS). Ogawa *et al*[2] reported that they could diagnose IPM (2 mm) preoperatively by EUS. EUS has higher sensitivity than other imaging modalities for the detection of pancreatic small lesions, in particular solid lesions[9,10]. IOUS in which the transducer is in direct contact with the pancreas can provide higher resolution images than extracorporeal ultrasound because the pancreas is located deep in the body cavity. Marcal *et al*[11] reported that IOUS provides high spatial and contrast resolutions alongside its real-time imaging capabilities, and this imaging method has enabled us to detect additional lesions, which were not identified on preoperative imaging. Furthermore, the procedures of IOUS are easier than those of EUS. If IPM is recognized preoperatively or intraoperatively, the physician should begin to consider management methods for this lesion, for example including diagnosis by ultrasound-guided needle biopsy; a change to the operative method, such as additional resection or total pancreatectomy; or close observation after the operation. Although it may be difficult to detect micrometastasis, it is worth making the attempt.

Regarding the pathogenesis of PDAC, it is known that

multicentric carcinogenesis is derived from premalignant lesions, PanIN, or IPMN[6,12]. We discriminate IPM from multicentric carcinogenesis pathologically on the basis of discontinuity with the premalignant lesion. Premalignant lesions like IPMN have heterogeneity in the base sequence of the same gene mutation, and multicentric cancers from the same IPMN may have different base sequences in the gene mutation[13-15]. On the other hand, a metastatic lesion and its primary lesion may have the same base sequence in the gene mutation. This means that IPM and multicentric carcinogenesis can be discriminated by analyzing and comparing the base sequences of gene mutations in the two lesions. It is important that future investigations clarify the mechanisms behind the occurrence of IPM and multicentric carcinogenesis.

In conclusion, we have documented two cases of IPM of PDAC. It should be remembered that IPM is present at a constant rate, and may be located in the remnant pancreas or in resected specimens other than main lesion. Although the diagnostic criteria for IPM and the clinicopathological significance of IPM still need to be clarified, we have only encountered a small number of subjects. Therefore, further accumulation of cases is necessary.

## COMMENTS

### Case characteristics

The patient in case 1 was a 30-year-old man with the complaint of epigastric pain, and the patient in case 2 was a 70-year-old woman with an elevated carbohydrate antigen 19-9 level.

### Clinical diagnosis

The pancreatic ductal adenocarcinoma (PDAC) lesions were diagnosed as intrapancreatic metastasis.

### Differential diagnosis

The differential diagnosis of the multiple PDAC lesions was multicentric carcinogenesis.

### Laboratory diagnosis

No abnormal laboratory test results were observed, except for an elevated carbohydrate antigen 19-9 level.

### Pathological diagnosis

Pathology revealed small monotonous lesions that were separated from the main tumors by ⩾ 10 mm, with no premalignant lesions in or around it.

### Treatment

Both patients underwent distal pancreatectomy and adjuvant chemotherapy.

### Related reports

Oguro *et al* reported 21 cases of PDAC with intrapancreatic metastasis in 2013.

### Term explanation

Intrapancreatic metastasis of PDAC is one type of lesion associated with PDAC.

### Experiences and lessons

Intrapancreatic metastasis of PDAC is present at a constant rate, and may be located in the remnant pancreas or in resected specimens other than the main lesion; although it is difficult to identify multicentric carcinogenesis, pathological diagnostic criteria of intrapancreatic metastasis of PDAC have been suggested in this report.

### Peer-review

There have been only a few reports of intrapancreatic metastasis of PDAC. This study introduces the clinicopathological characteristics and follow-up information of intrapancreatic metastasis of PDAC.

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CASE REPORT

# Collision tumor of hepatocellular carcinoma and neuroendocrine carcinoma involving the liver: Case report and review of the literature

Gyu Ho Choi, Sun Young Ann, Soon Il Lee, Suk Bae Kim, Il Han Song

Gyu Ho Choi, Sun Young Ann, Soon Il Lee, Suk Bae Kim, Il Han Song, Dankook University College of Medicine, Cheonan 31116, South Korea

Author contributions: Choi GH and Ann SY collected the patients' clinical data; Choi GH, Lee SI, Kim SB and Song IH designed the report and wrote the paper.

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Correspondence to: Suk Bae Kim, MD, Dankook University College of Medicine, 119 Dandae-ro, Dongnam-gu, Cheonan 31116, South Korea. dryakson@hanmail.net
Telephone: +82-41-5503910
Fax: +82-41-5563256

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## Abstract

Primary hepatic neuroendocrine carcinoma (NEC) with concurrent occurrence of hepatocellular carcinoma (HCC) of the liver is very rare. Only 8 cases have been reported in the literature. Concurrent occurrence of HCC and NEC in the liver is classified as combined type or collision type by histological distributional patterns; only 2 cases have been reported. Herein, we report a case of collision type concurrent occurrence of HCC and NEC, in which primary hepatic NEC was in only a small portion of the nodule, which is different from the 2 previously reported cases. A 72-year-old male with chronic hepatitis C was admitted to our hospital for a hepatic mass detected by liver computed tomography (CT) at another clinic. Because the nodule was in hepatic segment 3 and had proper radiologic findings for diagnosis of HCC, including enhancement in the arterial phase and wash-out in the portal and delay phases, the patient was treated with laparoscopic left lateral sectionectomy. The pathology demonstrated that the nodule was 2.5 cm and was moderately differentiated HCC. However, a 3 mm-sized focal neuroendocrine carcinoma was also detected on the capsule of the nodule. The tumor was concluded to be a collision type with HCC and primary hepatic NEC. After the surgery, for follow-up, the patient underwent a liver CT every 3 mo. Five multiple nodules were found in the right hepatic lobe on the follow-up liver CT 6 mo post-operatively. As the features of the nodules in the liver CT and MRI were different from that of HCC, a liver biopsy was performed. Intrahepatic recurrent NEC was proven after the liver biopsy, which showed the same pathologic features with the specimen obtained 6 mo ago. Palliative chemotherapy with a combination

of etoposide and cisplatin has been administered for 4 months, showing partial response.

**Key words:** Collision tumor; Hepatocellular carcinoma; Neuroendocrine carcinoma; Chronic hepatitis C



**Core tip:** Only 2 cases of collision tumor of hepatocellular carcinoma and primary hepatic neuroendocrine carcinoma involving the liver have been reported in the literature. This case shows different clinical characteristics from the previous cases. And we analyzed total 8 previous cases reported as concurrent occurrence of hepatocellular carcinoma and neuroendocrine carcinoma. This report will be helpful to elucidate the features of collision tumors.

Choi GH, Ann SY, Lee SI, Kim SB, Song IH. Collision tumor of hepatocellular carcinoma and neuroendocrine carcinoma involving the liver: Case report and review of the literature. *World J Gastroenterol* 2016; 22(41): 9229-9234 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i41/9229.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i41.9229

## INTRODUCTION

Although the liver is the most common metastatic lesion of NEC, primary NEC is very rare[1]. The concurrent occurrence of NEC and HCC in the liver is extremely rare with only 8 cases reported[2-9]. They are classified as combined type or collision type based on the presentation of histological distribution. Only 2 cases of the collision type have been reported. Here, we report a rare case of a NEC and HCC collision tumor in the liver of a chronic hepatitis C patient, and we discuss the previous 8 cases.

## CASE REPORT

A 72-year-old man presented with a hepatic mass that had been detected by liver computed tomography (CT) at another clinic. The patient had a 3-year history of chronic hepatitis C without treatment but with regular check-ups. His initial vital signs were stable. He had no specific symptoms, such as abdominal discomfort, body weight loss, or jaundice. A complete blood count revealed a white blood cell count of 3310/μL, hemoglobin of 16.0 g/dL, and a platelet count of 191000 /μL. Serum chemistry test results showed normal ranges of total protein 7.4 g/dL, albumin 4.2 g/dL, and total bilirubin 0.65 mg/dL. However, the levels of serum aspartate transaminase (AST) 141 IU/L and alanine transaminase (ALT) 140 IU/L were elevated. A coagulation test was within normal limits, including international normalized ratio (INR) 0.92. Alpha-fetoprotein (AFP) was 3.8 ng/mL, but protein induced by vitamin K antagonist-II (PIVKA-II) was very high at 1059 mAU/mL. Viral markers showed negative serum hepatitis B surface antigen/antibody (HBsAg/anti-HBs) and positive serum hepatitis C antibody (anti-HCV). His serum HCV RNA titer was 77464503 IU/mL with 1b genotype. The prior liver CT revealed a 2.2 cm × 2.0 cm sized nodule in hepatic segment 3, which featured mild external protrusion. It also showed slight enhancement compared to the surrounding liver parenchyma with a subtle border in the arterial phase, and low density with clear border in the portal and delayed phases after wash-out of the contrast medium. Additional dynamic liver magnetic resonance imaging (MRI) revealed greater enhancement than the CT scan in the arterial phase, and low density after wash-out of the contrast medium, similar to the CT scan in the portal and delay phases (Figure 1). Therefore, the patient proceeded to undergo laparoscopic left lateral sectionectomy under a diagnosis of HCC. The actual size of the nodule was 2.5 cm × 2.0 cm. Pathology demonstrated that the nodule was mostly moderately differentiated HCC with clear cytoplasm and positive immunohistochemical staining for hepatocyte paraffin-1, CD31, and CD34. A 3 mm-sized focal neuroendocrine differentiation was also detected and was separated by a fibrous band within one nodule. Cells obtained from this portion featured little cytoplasm, a high nucleus/cytoplasm (N/C) ratio, and salt and pepper chromatin in their nuclei (Figure 2A). Immunohistochemistry and special staining were negative for hepatocyte paraffin-1, CD31, and CD34 but positive for CD56 (Figure 2B and C). The mitotic count was 20 mitoses per 10 high power fields (20/10 HPF), so high grade NEC was diagnosed. After the surgery, the patient was followed-up with a liver CT every 3 mo. Five multiple nodules were found in the right hepatic lobe in a follow-up liver CT 6 months post-operatively. The nodules were presumed not to be HCC but another different tumor, as the largest one was 3.3 cm in size with rim enhancement and no other enhancement on liver CT and on liver MRI (Figure 3). Fine-needle aspiration guided by ultrasound revealed the lesion to be consistent with high grade NEC, and it showed the same pathologic features with the specimen obtained 6 mo ago (Figure 4). To exclude metastatic NEC from other organs, gastrofibroscopy, colonoscopy and chest CT were performed; there was no evidence of another origin site in the examinations. Eventually, recurrent NEC was proven after collision tumor of HCC and primary hepatic NEC surgery. Palliative chemotherapy with a combination of etoposide and cisplatin has been administered for 4 mo, showing partial response.

## DISCUSSION

Concurrent occurrence of two different tumors in the

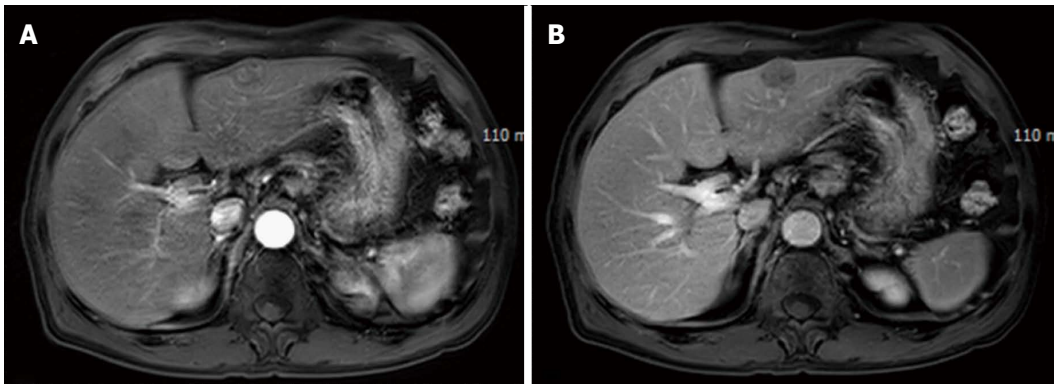


**Figure 1 Magnetic resonance imaging of the liver.** A 2.2 cm × 2.2 cm sized lobular contoured mass was found on segment 3. It showed mild enhancement in the arterial phase (A) and a washed-out pattern in the portal phase (B).

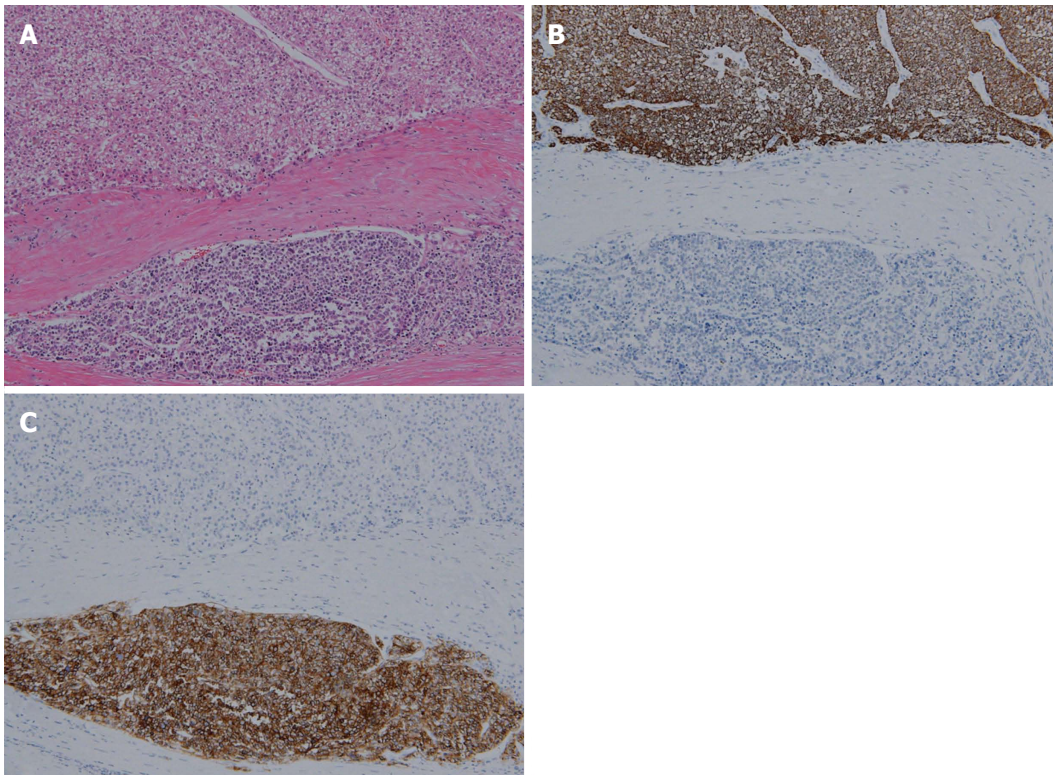


**Figure 2 Microscopic findings.** A: Moderately differentiated hepatocellular carcinoma (HCC) is found in the upper portion. The malignant cells show a clear and rich cytoplasm. It is separated from neuroendocrine carcinoma (NEC) by a fibrous band. Poorly differentiated NEC is found in the lower portion. The cytoplasm is barely seen, and the N/C (nucleus/cytoplasm) is very high (hematoxylin eosin staining, magnification × 100); B: Immunohistochemical staining of hepatocyte paraffin-1 is positive in the upper HCC portion (magnification × 100); C: Immunohistochemical staining of CD56 is positive in the lower NEC portion (magnification × 100).

liver is classified as combined type or collision type by histological distribution. It can present as a combined tumor in which components of both tumors intermingle and cannot be clearly separated in the transitional area within a single tumor nodule. A collision tumor shows two histologically distinct tumors involving the same organ with no histologic admixture. They co-exist with distance or adherence, in which the tumors are separated by a fibrous band. The combined type of HCC plus cholangiocarcinoma is most common, representing 2.0% to 3.6% of all primary hepatic malignancies[10].

In contrast to the HCC plus cholangiocarcinoma type in the liver, the concurrent occurrence of HCC and NEC is rarer because the incidence of primary hepatic NEC is very rare in contrast to occasional intrahepatic metastasis of NEC. Only 8 cases have been reported in the literature (Table 1)[2-9]. The 8 cases include 6 cases of the combined type and 2 cases of the collision type. In the present case, it was difficult to classify the tumor as a combined or collision type. The collision type is definitively distinguished by a fibrous band without a transition zone. In comparison with other collision types in which each tumor has some volume,

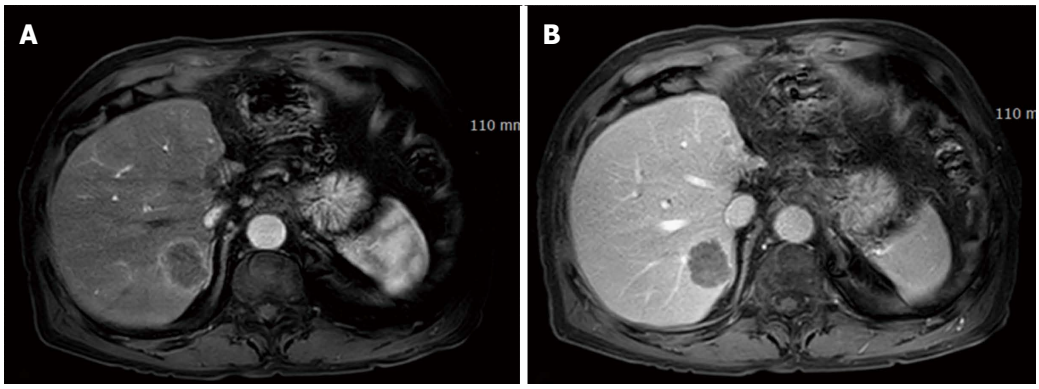


**Figure 3 Magnetic resonance imaging of the liver after 6 mo.** Five nodules were detected in the right lobe. The biggest was 3.3 cm. The nodules showed rim enhancement on the arterial phase (A) and low density on the portal phase (B).

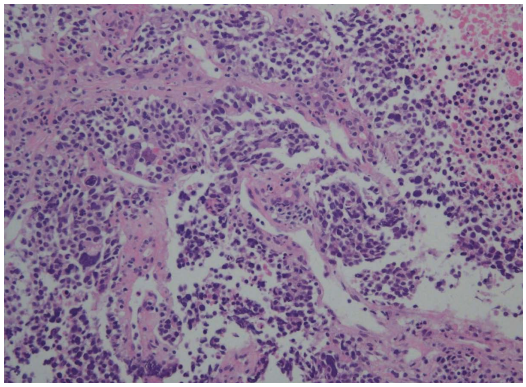

**Figure 4 Microscopic findings of fine-needle biopsy.** The size of malignant cells is variable, and the shape is very bizarre. They are clustered, forming nests, and the mitosis rate is above 20 per 10 HPF (hematoxylin eosin, magnification × 100).

the volume of the NEC portion in the present case was small (only 3 mm). However, it is still reasonable to consider collision type rather than combined type because of the presence of a fibrous band between the two tumors without a transition zone.

The present case involved a patient with chronic hepatitis C, as did the other 2 reported cases of the collision type. Garcia *et al*[2] reported a case of collision type tumor with HCC and primary hepatic NEC in a 50-year-old male patient. The tumor was 5 cm in size. The tumor was 70% NEC and 30% HCC, which differed from the present case. A similarity was the division of the tumors by a fibrous band in microscopic examination[2]. On the other hand, the case reported by Ishida *et al*[3] showed different features from the other cases in that the collision type tumor occurred in other hepatic segments: the 3 cm NEC and the 1.5 cm HCC were in segment 8 and segment 5, respectively.

With both the combined and collision type, it is important to make a clear distinction between primary intrahepatic NEC and metastatic NEC from extrahepatic organs because the incidence of primary intrahepatic NEC is rare. Surveys and evaluations to rule out metastatic NEC were essential in the case reported by Garcia *et al*[2] because the tumors developed in different hepatic segments. On the other hand, in the present case, it was difficult to consider the NEC as a metastatic lesion because the HCC and NEC regions existed within the same capsule separated only by a fibrous band.

The previous 8 cases of concurrent HCC and primary hepatic NEC all featured male patients with underlying liver disease, involving chronic hepatitis C in 4 cases, chronic hepatitis B in 3 cases, and cirrhosis of unknown cause in one case. The age distribution included the 40 s (one case), 50 s (2 cases), 60 s (3 cases), and 70 s (3 cases). More frequent diagnosis with age is evident. Surgery was performed after the diagnosis in 7 cases (Table 1).

The histogenesis of primary hepatic neuroendocrine tumors (NETs) is unclear. There are two theories. One is that such tumors originate from neuroendocrine cells in the intrahepatic bile duct epithelium. The other is that stem cell precursors of malignant cells from another hepatic malignant tumor differentiate into a neuroendocrine tumor[11,12]. The first theory can explain the histogenesis when there is no other malignant tumor in the liver but only for primary hepatic neuroendocrine tumors. The second theory is more convincing in the case of concurrent occurrence of HCC and primary hepatic NET, whether combined or collision type. The combined type is more amenable to the second theory because the two types of malignant cells mingle in the transition zone.

There has been no established treatment for the combined and collision types because few cases have been reported. Although it is difficult to find out which tumor (HCC or NEC) determines the poor prognosis of the disease, previous cases and our case implicate NEC in the poor prognosis due to its aggressive course, which can include metastasis. Another reason for the association of NEC with poor prognosis is the relatively short survival time of patients, even with regular liver ultrasonography or liver CT for surveillance of their underlying liver disease, such as chronic viral hepatitis, and early surgical management. Consequently, when the pathologic grade of NEC is high, adjuvant

**Table 1 Summary of previously reported cases of hepatocellular carcinoma plus neuroendocrine carcinoma involving the liver**

| Ref. | Age/sex | Clinical manifestations | Underlying liver disease | Tumor size | Type | Treatment | Survival |
|---|---|---|---|---|---|---|---|
| Barsky/1984[4] | 43/M | RUQ swelling | Hepatitis B | Huge[1] | Combined | Chemotherapy | 26 mo |
| Artopoulos/1994[5] | 69/M | RUQ pain | Hepatitis B | 10 cm | Combined | Operation | NM |
| Vora/1999[6] | 63/M | Abdominal pain and jaundice | Liver cirrhosis | 10 cm | Combined | Operation | Died during admission |
| Ishida/2003[3] | 72/M | No symptom | Hepatitis C | 3 cm, 1.5 cm | Collision | Operation | NM |
| Yamaguchi/2004[7] | 71/M | No symptom | Hepatitis C | 4.1 $cm^2$, 4.5 $cm^3$ | Combined | Operation | NM |
| Garcia/2006[2] | 50/M | No symptom | Hepatitis C | 5 cm | Collision | Operation and chemotherapy | NM |
| Yang/2009[8] | 65/M | Epigastric pain | Hepatitis B | 7.5 cm | Combined | Operation | Died after 1 yr |
| Aboelenen/2014[9] | 51/M | Abdominal pain | Hepatitis C | 7.5 cm | Combined | Operation | No recurrence up to 6 mo |

[1]Large mass involving the right lobe; [2]Combined tumor of hepatocellular carcinoma (HCC) and neuroendocrine carcinoma; [3]Pure HCC. RUQ: Right upper quadrant; NM: Not mentioned.

chemotherapy is needed to increase life expectancy whereas NEC was small as in this case.

In summary, we experienced a case of concurrent occurrence of HCC and NEC collision type in a chronic hepatitis C patient with multiple intrahepatic metastases 6 mo after the surgical procedure in spite of the very small size of the NEC, which had been completely removed. In consideration of the present case and previous cases, aggressive chemotherapy is necessary in concurrent occurrence of HCC and NEC. More cases need to be documented to better define treatment.

## COMMENTS

### Case characteristics

Neuroendocrine carcinoma recurred at 6 mo after operation for collision tumor of hepatocellular carcinoma and neuroendocrine tumor in 72-year-old man with chronic hepatitis C.

### Clinical diagnosis

A few hepatic masses that was detected by liver computed tomography during follow-up after operation.

### Differential diagnosis

Hepatocellular carcinoma, neuroendocrine carcinoma, hepatic metastasis of other malignancy.

### Laboratory diagnosis

There was no specific laboratory findings for recurred neuroendocrine carcinoma.

### Imaging diagnosis

The largest mass was 3.3 cm in size with rim enhancement and no other enhancement on the liver CT and the liver MRI.

### Pathological diagnosis

The pathology showed little cytoplasm, high nucleus/cytoplasm (N/C) ratio, and salt and pepper chromatins in their nuclei with positive for CD56.

### Treatment

Chemotherapy with a combination of etoposide and cisplatin.

### Related reports

The concurrent occurrence of NEC and hepatocellular carcinoma in the liver are classified as combined type and collision type. Only 2 cases of the collision type and 6 cased of combined type have been reported.

### Term explanation

Combined type -components of both tumors intermingle and cannot be clearly separated in the transitional area within a single tumor nodule.

### Experiences and lessons

Please summarize experiences and lessons learnt from the case in one sentence. Adjuvant chemotherapy should be done for collision or combined tumor of hepatocellular carcinoma and neuroendocrine carcinoma although the portion of neuroendocrine carcinoma is very small.

### Peer-review

Good job. Very rare case actually.

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CASE REPORT

# Reoperation in an adult female with "right-sided" Hirschsprung's disease complicated by refractory hypertension and cough

Zhi-Jian Wei, Lei Huang, A-Man Xu

Zhi-Jian Wei, Lei Huang, A-Man Xu, Department of Gastrointestinal Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui Province, China

Lei Huang, German Cancer Research Center, 69120 Heidelberg, Germany

A-Man Xu, Department of General Surgery, The Fourth Affiliated Hospital of Anhui Medical University, Hefei 230000, Anhui Province, China

Author contributions: All authors diagnosed and treated the patients, acquired the data, and wrote and revised the paper; Wei ZJ and Huang L contributed equally to this work.

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Institutional review board statement: This case report was approved by the Institutional Review Board in The First Affiliated Hospital of Anhui Medical University in Hefei.

Informed consent statement: The patient involved in this study gave her written informed consent authorizing use and disclosure of her health information.

Conflict-of-interest statement: All the authors have no conflict-of-interest to disclose.



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Correspondence to: A-Man Xu, MD, Professor, Department Head, Department of Gastrointestinal Surgery, The First Affiliated Hospital of Anhui Medical University, 218 Jixi Road, Hefei 230022, Anhui Province, China. amanxu@163.com
Telephone: +86-551-65334247
Fax: +86-551-63633742

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## Abstract

Hirschsprung's disease (HD) is an intestinal malformation caused by the innate absence of ganglion cells in the neural plexus of the colorectal wall, and is most common in male infants. It is rare in adult, and is usually left-sided. Herein we reported based on the CARE guidelines a case of a 47-year-old adult female suffering from "right-sided" HD complicated by refractory hypertension and cough. The patient with a history of cesarean section and with digestive unfitness (abdominal pain, distention, and constipation) only since 20 years old had recurrence of HD after initial surgery due to the incomplete removal of the HD-affected bowel based on a diagnosis of "chronic ileus", leading to the relapse of the digestive symptoms and the emergence of some intractable circulatory and respiratory complications which could be hardly controlled by conservative treatment. During the long interval before coming to our department for help, she had been re-hospitalized for several times with various misdiagnoses and supplied merely with symptomatic treatment which could only achieve temporary symptomatic relief. At her admission to our department, the imaging examinations strongly indicated recurrent HD which was further supported

by pathological examinations, and right hemi-colectomy was performed to remove the remnant aganglionic intestinal segment. Intraoperative and postoperative pathology supported the completeness of the definitive resection. Post-operation, the patient's bowel motility significantly improved, and interestingly, the complications disappeared. For adult patients with long-term constipation combined with cough and hypertension, rare diseases like HD which requires definite surgery and which could be "right-sided" should not be overlooked. It is vital to diagnose and cure HD patients in childhood. Through the comparison of the two surgeries, it is noteworthy that for diagnosed HD, sufficient removal of the non-functional intestine confirmed by intraoperative pathology is essential.

**Key words:** Adult Hirschsprung's disease; Reoperation; Ileus; Chronic constipation; Hypertension; Cough; CARE



**Core tip:** Hirschsprung's disease (HD) is most common in male infant, and is usually left-sided. Herein we reported a case of a 47-year-old adult female suffering from "right-sided" recurrent HD complicated by refractory hypertension and cough and receiving reoperation. The patient with digestive unfitnesses only since 20 years old had recurrence of HD after the initial surgery due to the incomplete removal of the HD-affected bowel. At her admission to our department, right hemi-colectomy was performed to completely remove the remnant aganglionic intestinal segment, which was confirmed by intraoperative pathology. Post-operation, the patient's bowel motility significantly improved, and interestingly, the complications disappeared.

Wei ZJ, Huang L, Xu AM. Reoperation in an adult female with "right-sided" Hirschsprung's disease complicated by refractory hypertension and cough. *World J Gastroenterol* 2016; 22(41): 9235-9241 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i41/9235.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i41.9235

## INTRODUCTION

Congenital megacolon (Hirschsprung's disease, HD) is an intestinal malformation caused by the innate absence or decrease of ganglion cells in the submucosal (Meissner) and myenteric (Auerbach) neural plexuses of the colorectal wall, and dates back to a defect in the craniocaudal migration of the neuroblast originating from the neural crest that occurs during the first 3 mo of gestation[1]. It is most common in male neonates and infants, and usually affects the left colon and part of the rectum. Rarely the whole colon could be aganglionic, which is known as the Zuelzer-Wilson syndrome[2]. HD patients mostly suffer from long-term constipation, distention, and bellyache which are not relevant to diet. Adult HD, which is rare, often develops from misdiagnosed infant HD with mild symptoms, and is easily considered intractable constipation[3]. Conservative treatment does not work satisfactorily for adult HD, which usually requires surgery[4].

Herein we report a case of a female adult patient with 'right-sided' HD complicated by refractory hypertension and cough who had undergone several misdiagnoses and who received re-surgery in our department 17 years after the initial operation due to continuous digestive, circulatory and respiratory unfitness, which, to the best of our knowledge, has not been previously reported.

Written informed consent was obtained from the patient, and this report was approved by the Institutional Review Board of the First Affiliated Hospital of Anhui Medical University and was in accordance with the Declaration of Helsinki[5], the Good Clinical Practice[6], and the CARE Statement[7] for clinical case reports.

## CASE REPORT

On April 23rd 2015, a 47-year-old female patient with a surgical history of cesarean section in 1992 was admitted to our department complaining about postprandial pain and distention of the right lower abdomen for 17 years after the resection of most part of the transverse colon. She suffered from constipation since 1988, with the average defecation frequency once 3-4 d, and the lowest once a week, and received no special treatment then. However, she did not experience any abdominal symptoms during the first 20 years of her life.

On 10th February 1998, she was hospitalized in Department of Emergency Surgery in our hospital because of bellyache and constipation, and was discharged after conservative treatment. However, she was re-hospitalized due to intestinal obstruction and was suspected as "adhesive ileus" based on her operation history only 6 d later. The transverse colon resection was planned. The pre-surgical blood and urine tests were normal. The endoscopic pathological examination and the routine fecal examination were not conducted. The surgery was conducted on 12th March. During surgery, it was observed that the middle of the transverse colon was significantly distended to approximately 10 cm in diameter. Around 25 cm of the dilated colon was removed, and the splenic flexure was rectified. The post-operational pathological examination showed infiltration of acute and chronic inflammation cells and focal hemorrhage (the immunohistochemistry and specific staining tests were not performed, possibly due to the fact that HD was not considered, or to the unavailability of such a test then). However, after the first surgery, she still experienced constipation, and postprandial bloating

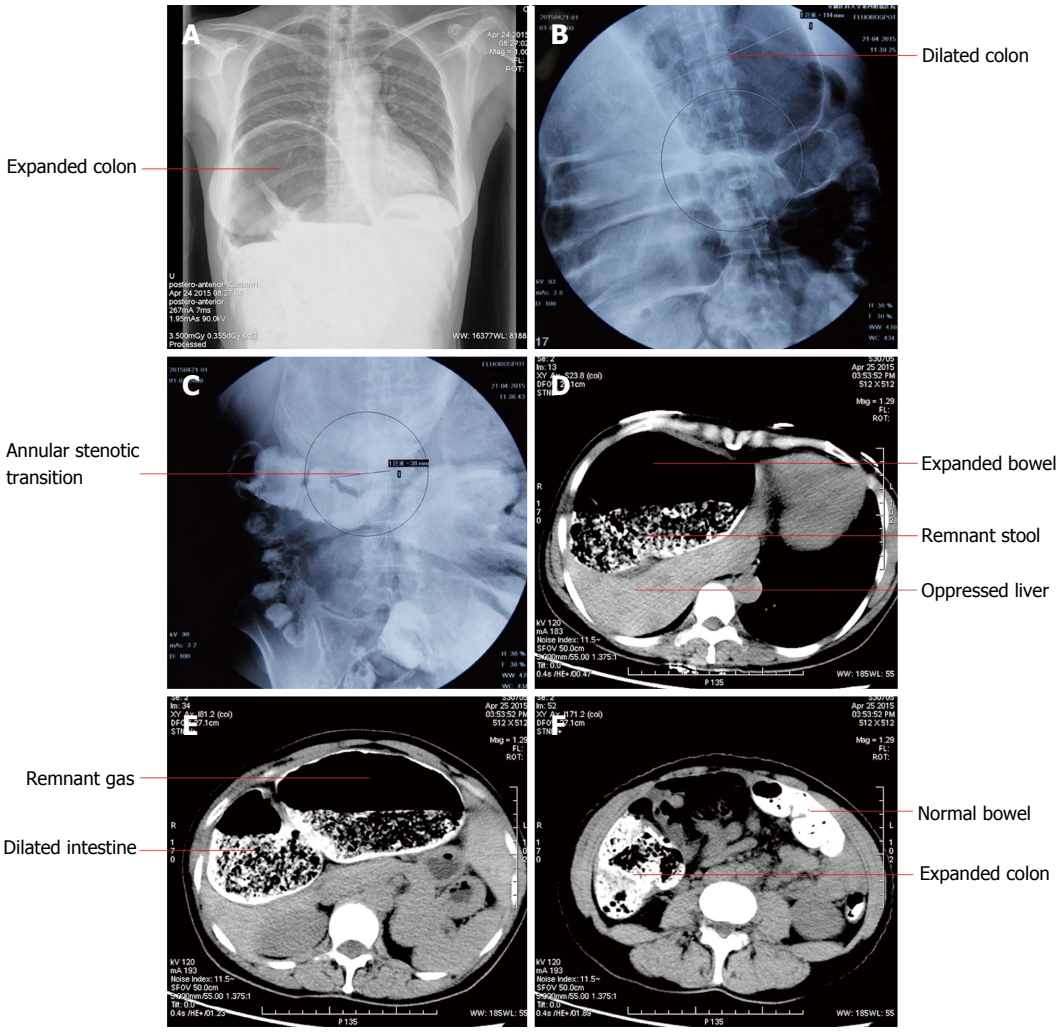


**Figure 1 Imaging examinations.** The radiography (A) showed right pleural effusion and thickening, and potential interposition of the dilated colon. The colon double contract pneumobarium radiography (B and C) and the abdominopelvic computed tomography (D-F) revealed significant expansion of the cecum, the ascending colon, the hepatic flexure of colon, and the remnant transverse colon, with the most dilated area 13.6 cm wide. Abundant residual stool, gas, and liquid existed. An annular stenotic transitional segment (only 3.8 cm wide) could be perceived. The other intestines were normal. The neighboring organs and tissues underwent marked displacement and deformation under pressure.

and bellyache, and soon felt obvious aggravation of the symptoms. She was then prescribed with laxatives. She was hospitalized to our hospital again in 2000 due to ileus, and refused the suggested reoperation. She was discharged when feeling better after conservative management, and depended on laxatives thereafter. From 2000 to 2015, the patient was admitted to hospital for several times due to intestinal obstruction, and the symptoms were relieved after symptomatic treatment like gastrointestinal decompression, enema, and anti-inflammation. Unfortunately, during this period, she had not been proposed the diagnosis of HD.

The patient had hypertension since 2002, and the blood pressure (BP) could be initially well-controlled by oral nitrendipine treatment. However, later on the BP gradually elevated, and had to be antagonized by the addition of captopril. In recent 2 years, the patient experienced aggravated anorexia and post-meal symptoms, obvious weight loss (about 20 kg), progressive continuous cough, and significantly elevated BP (146/100 mmHg at admission, and 180/110 mmHg as the highest level during drug administration), which could be poorly controlled by oral symptomatic treatment drugs. On April 21$^{st}$ 2015, the colon contrast pneumobarium conducted in the Fourth Affiliated Hospital of Anhui Medical University indicated giant right hemi-colon, and she came to our department seeking for medical management. The patient weighed 44 kg at admission. The physical examination showed a bulging abdomen with an about 15 cm-long old surgical scar on the right upper abdomen. The drum sound was obvious during percussion, and the frequency of the bowel sound was 5 times per min. All the other physical examinations were normal.

For the hospitalization in our department, the routine blood and fecal tests were normal, and the urine test showed the presence of bacteria (429/μL). Imaging examinations offered informative clues for diagnosis (Figure 1). The chest radiography showed

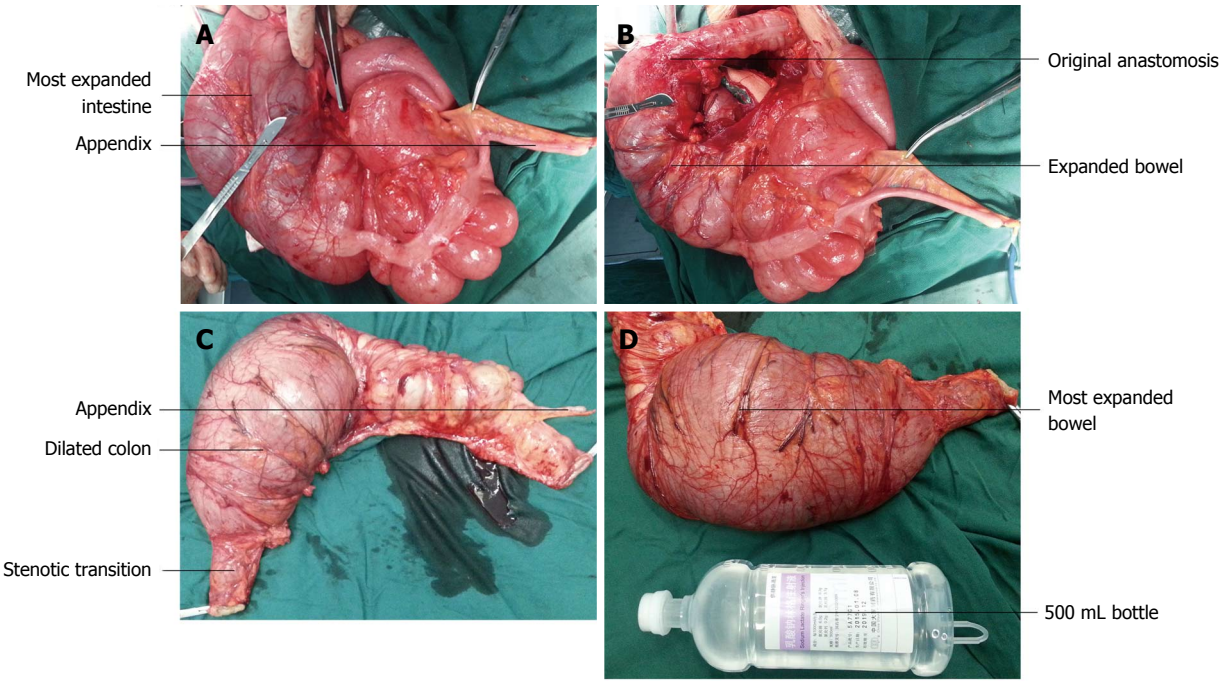


**Figure 2 Surgical pictures.** In the second surgery, right colectomy was conducted to remove the aganglionic and the dilated fragments (A and B), and the resected intestine included the non-dilated bowel 6 cm beyond the anastomosis of the initial surgery (C), ensuring the completeness and definitiveness of the removal. The affected colon was obviously outstretched (D).

right pleural effusion and thickening, and potential colon interposition. The abdominopelvic computed tomography (CT) and the colon double contract pneumobarium radiography revealed significant expansion of the cecum, the ascending colon, the hepatic flexure of colon, and the remnant transverse colon, with the most dilated area 13.6 cm wide. Abundant residual stool, gas, and liquid existed. An annular stenotic transitional segment (only 3.8 cm wide) could be perceived, and the original transverse-descending colon anastomosis was slightly narrow with limited distension capability. All the other intestines were normal. The neighboring organs and tissues underwent marked displacement and deformation under pressure.

Right colectomy was conducted for the patient to remove the aganglionic and the dilated fragments based on the initial consideration of HD followed by ileum-colon anastomosis, and the resected intestine included the non-dilated bowel 6 cm beyond the anastomosis of the initial surgery. The intraoperative frozen section pathological examination convinced us of the completeness and definitiveness of the eradication of all the remnant aganglionic bowel segment. An intestine segment with normal ganglion cells could be seen in the resected specimen pathologically. During surgery, we observed mild adhesion of the abdominal cavity, the obviously outstretched colon, and the deformed liver due to extrusion (Figure 2). The right lobe of the liver markedly shifted upwards, and the left lobe underwent compensatory enlargement. The left colon and rectum were all fine in morphology and motility with satisfactory tension perceived and without abnormal dilation or constriction, and the intraoperative pathology further supported that they were normal in structure with sufficient ganglion cells and should not be removed, which seems different from the common understanding that they are usually affected in HD[1]. The postsurgical pathological report supported the HD diagnosis, showed the remnant aganglionic section left behind by the first surgery, and importantly, confirmed the definitiveness of the reoperation based on the presence of normal bowel segment with normal ganglion cells in the resected specimen (Figure 3).

Interestingly, after reoperation, the BP dropped to 138/69 mmHg at discharge without the assistance of any drug, and cough was also cured. Symptomatic treatment has thus been ceased since then. Up till now, the patient has been followed up once every month. No adverse event was observed. She reported satisfactory diet, without distention and ache of the abdomen, cough, or hypertension. The defecation frequency is 3-4 times per day without the assistance of laxative, the bowel movement is active and smooth, and she gains weight of about 6 kg with satisfactory fecal control. She feels much better concerning quality of life (QoL), especially in the digestive aspect. Contrary to the initial surgery, the patient did not feel recurrence/worsening of any HD-related symptoms. The abdominal CT scan and X-ray examination conducted in May 2016 did not reveal abnormality. Her condition was still closely and carefully watched on, and she expressed great willingness to share her case.

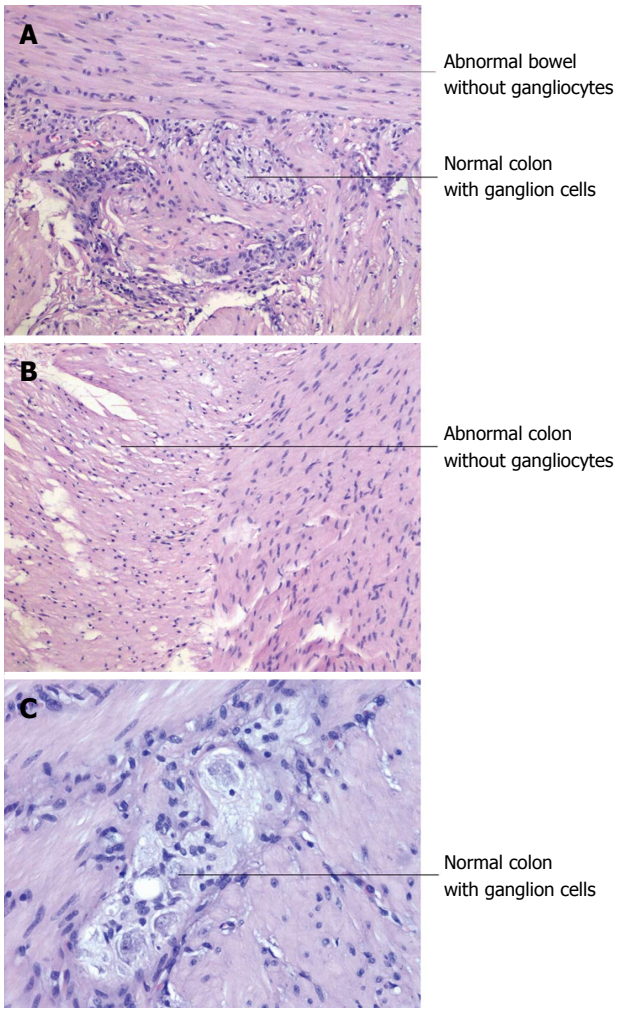


**Figure 3 Postsurgical pathological examination.** A: Shows the junction of the normal and the abnormal bowel contained in the resected specimen (magnification × 100); B: Indicates the resected abnormal bowel segment without ganglion cells (magnification × 100). The cutting edge contained abundant normal ganglion cells (C, magnification × 200), ensuring the total and complete removal of the abnormal and aganglioniccolon segment.

## DISCUSSION

Adult HD was first described by Rosin *et al*[8] in 1950 concerning a 54-year-old man with absence of colorectal gangliocytes. Most of the HD patients take laxatives or receive enemas to facilitate defecation, with the hope to reduce the digestive unfitness, which they suffer since childhood, and which could provide key clues for diagnosis. HD could lead to serious and even lethal complications like enterocolitis, bowel volvulus and necrosis, which require urgent surgical intervention[9]. The major diagnostic method of HD is barium enema with the mark of a narrowed transition zone, which might however worsen ileus at the initial stage of the disease[10]. Nowadays, CT scan has become a commonly used diagnostic approach, and could reveal the thickening extent of the colon and the uniformity during enhancement scanning[11]. The golden diagnostic standard is endoscopic biopsy. A multimodal investigation approach is usually required for diagnosis[12].

Rectum and distal sigmoid colon are usually affected by HD which is mostly a left-sided disease[4,13], while in this case only the right hemi-colon was the influenced bowel as confirmed by pathology and intraoperative observation, which is scarce compared to the previous literature reports, and the initial suspicious diagnosis was even "colon interposition" by experts based on the imaging. Hypoganglionosis and intestinal neuronal dysplasia are the common allied disorders of HD, and the whole digestive tract might be affected to be disordered in motility, causing refractory anorexia and constipation[14,15], which however slightly affects the QoL in the long term[16]. The tolerance of HD is relatively good due to a usually short aganglionic bowel segment[17]. Unfortunately, the initial surgery in this case only removed most part of the dilated colon, and ignored the silent aganglionic part just adjacent to the structurally-abnormal bowel. Older female HD patients with a longer aganglionic segment might have a poorer bowel function and QoL[4,18,19]. The patient in this case experienced apparent unfitness after her adolescence presenting like the pseudo-HD[20], which could be explained by the gradual proceeding of the disease, or by the fact that adult HD might be acquired rather than congenital[21]. Interestingly, she did not experience obvious discomfort during her first 20 years of life. The defecation frequency of this HD adult was more frequently than reported by literature (1/7-10 d)[22].

For HD surgery, it is important to completely remove the bowel without functional ganglion cells[23]. The major surgical methods to treat distal HD include the one- or two-stage Duhamel, Swenson, Soave, Rehbein, and some modified approaches, and the choice was influenced by surgeons' experience[4]. The laparoscopic approach could also be applied[24]. The modified Duhamel operation is considered satisfactory with a few manageable adverse events and nice functional outcomes when treating adult HD patients[16,25], and the subtotal colectomy combined with the modified Duhamel procedure is also effective and safe when dealing with adult distal HD[26]. However, few literatures have reported the surgical management of proximal/"right-sided" HD. In this case, the left colon and the rectum were normal, and it is obviously inappropriate to remove them as the reported surgical method for distal HD, since it is principle to preserve as many as healthy tissues for our patients, otherwise the postsurgical QoL would be very low with very poor fecal control. We herein reported our case with the hope to add some novel knowledge to the literature. For the second surgery of this patient, right colectomy was performed to resect the remnant aganglionic segment, which had not been carefully removed by the initial operation. It turns out to be successful based on the improvement of the patient's syndromes and imaging findings. The biofeedback therapy could be additionally supplied post-surgery to further recover the intestinal function[27].

In this case, the patient's colon was significantly aggrandized due to the long-term constipation. Her symptoms were not significantly relieved after the initial surgery, and later experienced refractory complications (hypertension and cough), which has been rarely reported before. The potential reasons for the recurrence are that the intraoperative frozen pathological examination, which should be highly demanded for such patients, and which could avoid insufficient extent of resection, was not conducted during the first surgery potentially due to the limited medical condition then, thus leading to the reoperation. The cough was possibly caused by the stimulation of the diaphragm elevated by the ectatic colon, and the hypertension might be attributed to the disturbed circulation due to the imbalanced pleuroperitoneal pressure and the oppressed liver. For the second surgery, the patient received right colectomy, which effectively eliminated the initial symptoms, as well as the hypertension and cough, and effectively increased the postoperative defecation frequency and QoL without causing any complications.

The lessons from this case are key to prevent patients with similar situations from unnecessary pain and suffering. Constipation is a common digestive symptom[28]. For adult patients with long-term constipation complicated by cough and hypertension, rare diseases like HD which requires curative and definitive surgery to remove all the aganglionic segment should not be overlooked. The potential reasons for specific symptoms should be actively explored, and notably, symptomatic treatment could not solve the fundamental problems. It is vital to diagnose and cure HD patients in childhood, which could significantly improve the satisfactory functional outcomes[13]. For diagnosed HD, sufficient removal of the non-functional intestineconfirmed by intraoperative frozen section pathology with regular follow-up is essential, and remaining aganglionic segment should be considered if symptoms persist or recur.

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## COMMENTS

### *Case characteristics*

A 47-year-old adult female with a history of cesarean section and with digestive unfitness (abdominal pain, distention, and constipation) only since 20 years old had recurrence of digestive symptoms after initial surgery due to the incomplete removal of the affected bowel based on a diagnosis of "chronic ileus", leading to the relapse of the digestive symptoms and the emergence of some intractable circulatory and respiratory complications which could be hardly controlled by conservative treatment.

### *Clinical diagnosis*

"Right-sided" Hirschsprung's disease (HD) complicated by refractory hypertension and cough with incomplete removal of the affected bowel.

### *Differential diagnosis*

Chronic ileus, chronic constipation, intestinal tuberculosis, inflammatory bowel disease, and toxic megacolon could be differentiated from this case mainly based on pathological examinations.

### *Laboratory diagnosis*

All laboratory tests were within normal limits except the presence of bacteria in urine (429/μL).

### *Imaging diagnosis*

The abdominopelvic computed tomography and the colon double contract pneumobarium radiography revealed significant expansion of the cecum, the ascending colon, the hepatic flexure of colon, and the remnant transverse colon, with the most dilated area 13.6 cm wide.

### *Pathological diagnosis*

The pathological report supported the HD diagnosis, showing the remnant aganglionic bowel section left behind by the first surgery.

### *Treatment*

Right colectomy was conducted to remove the aganglionic and the dilated fragments followed by ileum-colon anastomosis, and the resected intestine included the non-dilated bowel 6 cm beyond the anastomosis of the initial surgery.

### *Related reports*

HD is an intestinal malformation caused by the innate absence of ganglion cells in the neural plexus of the colorectal wall, and is most common in male infants. It is rare in adult, and is usually left-sided. HD patients mostly suffer from long-term constipation, distention, and bellyache which are not relevant to diet. Conservative treatment does not work satisfactorily for adult HD, which usually requires surgery.

### *Term explanation*

Congenital megacolon (Hirschsprung's disease) is an intestinal malformation caused by the innate absence or decrease of ganglion cells in the submucosal (Meissner) and myenteric (Auerbach) neural plexuses of the colorectal wall, and dates back to a defect in the craniocaudal migration of the neuroblast originating from the neural crest that occurs during the first 3 mo of gestation. Rarely the whole colon could be aganglionic, which is known as the Zuelzer-Wilson syndrome.

### *Experiences and lessons*

For adult patients with long-term constipation combined with cough and hypertension, rare diseases like HD which requires definite surgery and which could be "right-sided" should not be overlooked, and it is noteworthy that for diagnosed HD, sufficient removal of the non-functional intestine confirmed by intraoperative pathology is essential.

### *Peer-review*

In this case report, the authors reported a case of a female adult patient with "right-sided" congenital megacolon HD complicated by refractory hypertension and cough. It could be helpful to clinical study.

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CASE REPORT

# Metabolic imaging for guidance of curative treatment of isolated pelvic implantation metastasis after resection of spontaneously ruptured hepatocellular carcinoma: A case report

Bing Hao, Wei Guo, Na-Na Luo, Hao Fu, Hao-Jun Chen, Long Zhao, Hua Wu, Long Sun

Bing Hao, Wei Guo, Na-Na Luo, Hao Fu, Hao-Jun Chen, Long Zhao, Hua Wu, Long Sun, Department of Nuclear Medicine and Minnan PET Center, Xiamen Cancer Hospital, the First Hospital of Xiamen University, Xiamen 361003, Fujian Province, China

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Correspondence to: Long Sun, MD, Department of Nuclear Medicine and Minnan PET Center, Xiamen Cancer Hospital, the First Hospital of Xiamen University, Xiamen 361003, Fujian Province, China. 13178352662@163.com
Telephone: +86-0592-2137077

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## Abstract

Spontaneous rupture of hepatocellular carcinoma (HCC) is a life-threatening complication and its prognosis is significantly poor because of the high recurrence rate after initial hepatectomy. Resection of isolated extrahepatic metastasis of HCC has been advocated to obtain a possibility of long-term survival. However, it is a challenge for clinicians to detect implantation metastasis of spontaneously ruptured HCC. Accurate re-staging plays the most important role in making a decision on isolated metastasis resection. $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) positron emission tomography/computed tomography (PET/CT) is useful in detecting intra-abdominal implantation metastasis from a variety of malignancies and shows superior accuracy to conventional imaging modalities in determining the location of metastasis. We present one patient with a new isolated pelvic implantation metastasis detected by $^{18}$F-FDG PET/CT and pathologically confirmed by PET/CT-guided percutaneous biopsy, who had a history of resection of spontaneously ruptured HCC two years ago. The patient's condition was stable at the 6-mo follow-up after resection of the isolated pelvic metastasis.

**Key words:** Fluorodeoxyglucose; Positron emission tomography/computed tomography; Spontaneously

ruptured hepatocellular carcinoma; Isolated pelvic implant metastasis; Re-staging; Surgical resection



**Core tip:** Spontaneous rupture of hepatocellular carcinoma is a life-threatening complication and its prognosis is significantly poor. It is a challenge for clinicians to detect implantation metastasis. Accurate re-staging plays the most important role in making a decision on isolated metastasis resection. $^{18}$F-fluorodeoxyglucose positron emission tomography/computed tomography is useful in detecting intra-abdominal implantation metastasis from a variety of malignancies and shows superior accuracy to conventional imaging modalities in determining the location of metastasis.

Hao B, Guo W, Luo NN, Fu H, Chen HJ, Zhao L, Wu H, Sun L. Metabolic imaging for guidance of curative treatment of isolated pelvic implantation metastasis after resection of spontaneously ruptured hepatocellular carcinoma: A case report. *World J Gastroenterol* 2016; 22(41): 9242-9246 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i41/9242.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i41.9242

## INTRODUCTION

Spontaneous rupture is a fatal complication seen in 3%-15% of all patients with hepatocellular carcinoma (HCC) and has a high mortality rate[1-3]. Currently, there is no consensus about the best treatment for spontaneously ruptured HCC. Low-risk curative hepatic resection may be the best treatment option for Child-Pugh A-B patients with spontaneously ruptured HCC. Small tumor length and number, and early Barcelona Clinic Liver Cancer (BCLC) stage are the most crucial predictors associated with satisfactory overall survival. However, resection of isolated extrahepatic metastasis of HCC has been advocated to obtain a possibility of long-term survival. Intra-abdominal implantation metastasis can be seen anywhere in the abdominal/pelvic cavity. Therefore, it is logical that whole body $^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) positron emission tomography/computed tomography (PET/CT) should be a tool for monitoring recurrence of HCC rupture. We present here a patient with spontaneously ruptured HCC resected two years ago and a new pelvic metastasis recently identified by $^{18}$F-FDG PET/CT. Pelvic implantation metastasis of spontaneously ruptured HCC was confirmed by PET/CT guided percutaneous biopsy. A relatively good prognosis was achieved in this patient after surgical resection of the isolated pelvic implantation metastasis.

## CASE REPORT

Two years ago, a 38-year-old man with a family history of HCC was referred to our hospital after an episode of sudden upper abdominal pain. Laboratory examination revealed that he was a hepatitis B virus (HBV) carrier and his alpha-fetoprotein (AFP) level was more than 1000 ng/mL. Contrast-enhanced CT showed multiple nodules in the right lobe of the liver (Figure 1) and segments V-VI-VII hepatectomy was performed in October 2013. Histology showed an HCC (Edmondson-Steiner grade III) with a diameter of 12.0 cm and early cirrhosis. No microscopic vascular invasion was found. After partial liver resection, his AFP level returned to normal. During 25 mo of follow-up, contrast-enhanced magnetic resonance imaging (MRI) every 3 mo did not reveal any evidence of intrahepatic recurrence or extrahepatic metastasis; however, his AFP level gradually increased to 418.18 ng/mL. To find out the reason for the AFP increase, the patient was referred to our center for a whole body $^{18}$F-FDGPET/CT examination. $^{18}$F-FDG PET/CT detected an isolated hypermetabolic lesion with a diameter of 2.4 cm between the right side of the seminal vesicle and the rectum, which suggested a pelvic implantation metastasis. A PET/CT-guided percutaneous biopsy of the hypermetabolic lesion (Figure 2) was performed and confirmed a real pelvic implantation metastasis of the spontaneously ruptured HCC. After the isolated hypermetabolic metastasis was resected, his AFP level decreased to the accepted level. There was no recurrence or metastasis in the 6-mo follow-up period after resection of the isolated pelvic implantation metastasis.

## DISCUSSION

Spontaneous rupture is one of the most serious complications of HCC, and its mortality rates range from 25% to 75%[4]. Early-stage elective hepatectomy is the first-choice treatment for patients with spontaneously ruptured HCC, with 1-, 3- and 5-year overall survival rates being 85.4%, 63.2% and 46.3%, respectively, compared with 66.3%, 23.4% and 10.1% in non-surgical patients[5]. The prognosis of spontaneously ruptured HCC has been reported to be poor, and most (30%-70%) of the patients died within 30 d after resection of the spontaneous ruptured HCC[6]. The patients with younger age, better liver function and earlier tumor stage after resection have a good prognosis[7]. In our case, the 38-year-old patient with good liver function underwent a curative hepatectomy and achieved a chance of long-term survival.

Intra-abdominal implantation metastasis is a significantly unfavorable factor for long-term survival in patients with spontaneously ruptured HCC. HCC rupture

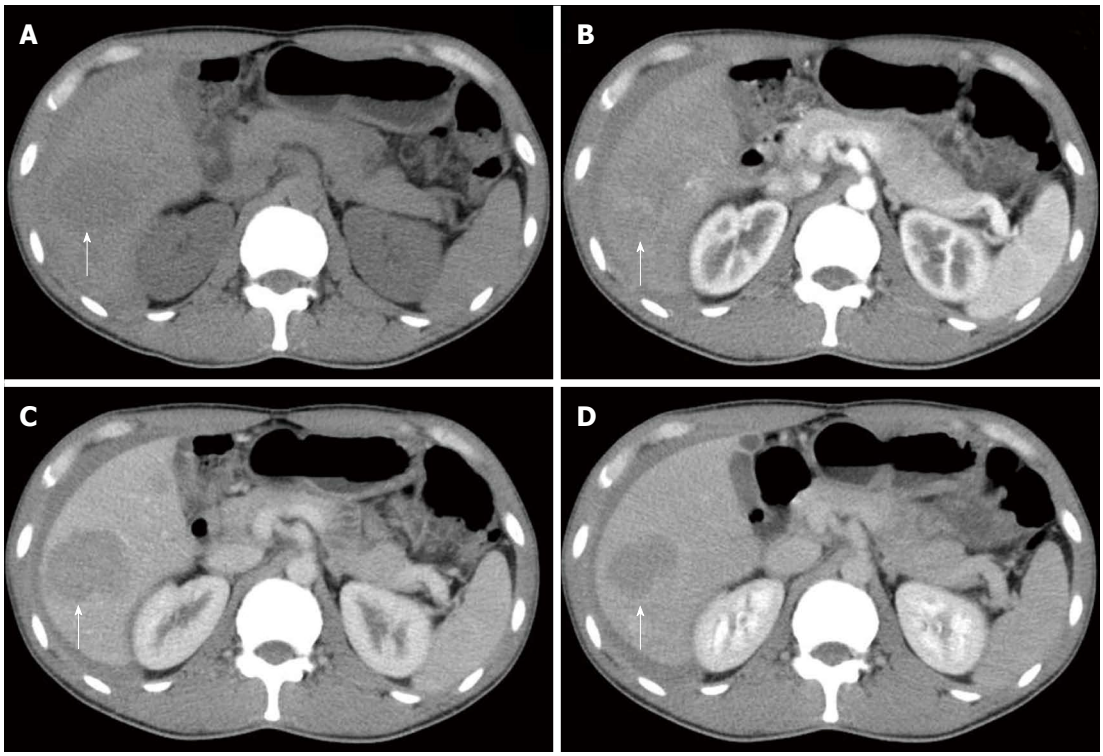


**Figure 1 Contrast-enhanced computed tomography performed two years ago demonstrated multiple nodules at the right lobe of the liver.** A: Non-contrast-enhanced computed tomography scan; B: Arterial phase; C: Portal phase; and D: Venous phase.

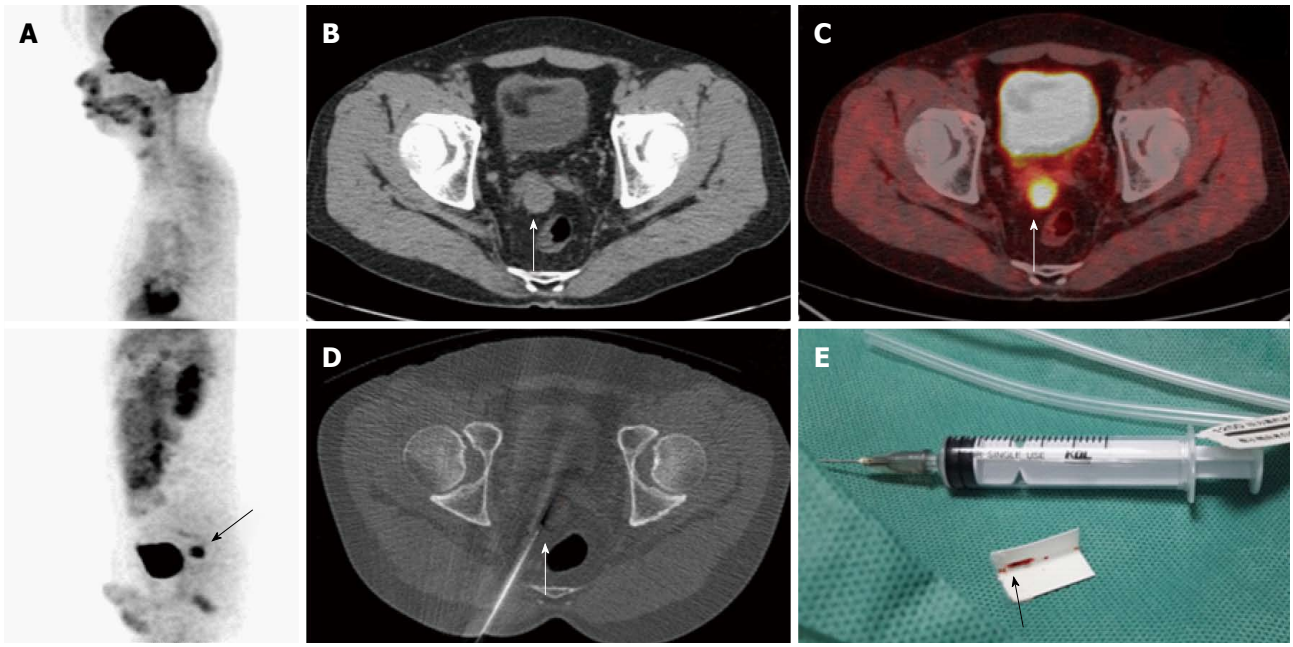


**Figure 2 Whole body positron emission tomography.** A: An isolated hypermetabolic focus is located behind the bladder (black arrow); B: Non-enhanced CT detecting a median density lesion in the pelvic cavity (white arrow); C: Fused imaging of PET/CT revealing a hypermetabolic lesion at the same position; D and E: PET/CT-guided biopsy confirmed HCC metastasis (black arrow).

with intra-abdominal hemorrhage is accompanied with neoplastic cell implantation metastasis. Intra-abdominal implantation metastases are divided into two types: isolated and diffuse. The isolated implantation metastasis of spontaneously ruptured HCC is suitable for curative surgical treatment while diffuse metastasis does not. Most reported cases of peritoneal metastases were documented months after HCC rupture[8]. Hung *et al*[9] and Yunoki *et al*[10] reported peritoneal metastasis in the omentum occurring one year after HCC rupture. Shirabe *et al*[11] reported a patient who had previously undergone hepatic resection for ruptured HCC but

developed a solitary peritoneal recurrence at the incision site 105 mo later. Lin *et al*[12] reported a patient who had diffuse intraperitoneal metastasis after spontaneous rupture of HCC. In contrast, pelvic implantation metastasis is rarely reported. Nakashima *et al*[13] noted that metastases to the pouch of Douglas occurred in 6.2% of 232 consecutive autopsy cases of HCC. We present here one patient with isolated implantation metastasis occurring two years after resection of the initial spontaneously ruptured HCC.

The early diagnosis of recurrence and metastasis of spontaneously ruptured HCC is very important for further treatment to improve the prognosis. At present, the main methods used for detecting recurrence and metastasis in clinical practice include monitoring serum AFP value and conventional imaging. An unexplained gradual rise of AFP after treatment is a sensitive early indicator of tumor recurrence or extrahepatic metastasis; however, AFP cannot localize the lesion. Although conventional imaging has been generally acknowledged in detecting intrahepatic recurrence, it has many limitations in detecting extrahepatic recurrence and metastasis. Nowdays, $^{18}$F-FDG PET/CT can not only detect the host residual liver recurrence, but also is the most effective imaging method for detecting distant metastasis of tumor[14].

$^{18}$F-FDG PET/CT is a useful metabolic imaging tool for monitoring recurrence and re-staging when AFP is increasing again after the resection of spontaneously ruptured HCC, especially for the isolated extrahepatic implantation metastasis[15]. It is not only an effective whole-body imaging technique but also detects metabolic changes that differentiate malignant from benign tumors through the precise localization of suspected $^{18}$F-FDG uptake foci and their characterization compared to conventional imaging structural findings[16]. In our previous study[17], we have found that merged PET/CT images provide a complementary role in the assessment of whether the detected disease is resectable. It is crucial for our patient that the detected isolated pelvic implantation metastasis by $^{18}$F-FDG PET/CT scan was curatively re-resected two years after the resection of spontaneously ruptured HCC.

The isolated implantation metastasis, if possible, should be curatively resected after initial resection of spontaneously ruptured HCC, which might offer long-term survival benefit[18,19]. $^{18}$F-FDG PET/CT provide important information for the detection of extrahepatic implantation metastasis and precise location to complete preoperative re-staging. This case is sensationally practical for patient follow-up and re-staging after the resection of spontaneously ruptured HCC, especially when a gradual rise of AFP that conventional imaging cannot explain is present.

## COMMENTS

### Case characteristics

A 38-year-old man was in good condition and asymptomatic two years after the resection of spontaneously ruptured hepatocellular carcinoma (HCC).

### Clinical diagnosis

It is highly suspected as recurrence after the resection of spontaneously ruptured HCC due to an AFP increase.

### Differential diagnosis

Pelvic primary benign tumor, pelvic malignant tumor, and germ cell tumor.

### Laboratory diagnosis

The AFP gradually increased from the normal level to 418.18 ng/mL.

### Imaging diagnosis

$^{18}$F-fluorodeoxyglucose ($^{18}$F-FDG) positron emission tomography/computed tomography (PET/CT) detected an isolated hypermetabolic lesion with a diameter of 2.4 cm between the right side of the seminal vesicle and the rectum.

### Pathological diagnosis

PET/CT-guided percutaneous biopsy of the hypermetabolic lesion was performed and confirmed pelvic implantation metastasis of the spontaneously ruptured HCC.

### Treatment

The isolated hypermetabolic metastasis in the pelvic cavity was excised for curative surgical treatment.

### Related reports

Pelvic implantation metastasis has not been reported, and it only was noted that metastases to the pouch of Douglas occurred in autopsy cases of HCC.

### Term explanation

Spontaneously ruptured hepatocellular carcinoma has a poor prognosis, and resection of isolated extrahepatic metastasis of HCC has been advocated to obtain a possibility of long-term survival.

### Experiences and lessons

$^{18}$F-FDG PET/CT is a useful metabolic imaging tool for monitoring recurrence of spontaneous ruptured HCC and its re-staging when AFP is increasing again after the resection of spontaneously ruptured HCC, especially for the isolated extrahepatic implantation metastasis.

### Peer-review

Imaging supports the findings. It is well-documented, written and presented.

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# Hepatic epithelioid hemangioendothelioma: Dilemma and challenges in the preoperative diagnosis

Hai-Jie Hu, Yan-Wen Jin, Qiu-Yang Jing, Anuj Shrestha, Nan-Sheng Cheng, Fu-Yu Li

Hai-Jie Hu, Yan-Wen Jin, Anuj Shrestha, Nan-Sheng Cheng, Fu-Yu Li, Department of Biliary Surgery, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

Qiu-Yang Jing, Department of Pathology, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China

Anuj Shrestha, Department of General Surgery, Gandaki Medical College, Pokhara 33700, Nepal

Author contributions: Hu HJ and Jin YW contributed equally to this paper; Hu HJ and Jin YW contributed to data acquisition and drafted the manuscript; Jing QY contributed to data acquisition; Shrestha A and Cheng NS were involved in the revision of the manuscript; Li FY conceived and designed the study and revised the manuscript.

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Correspondence to: Fu-Yu Li, MD, PhD, Department of Biliary Surgery, West China Hospital of Sichuan University, 37 Guoxue Xiang, Wuhou District, Chengdu 610041, Sichuan Province, China. lfy_74@hotmail.com
Telephone: + 86-28-85422465
Fax: +86-28-85422465

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## Abstract

Hepatic epithelioid hemangioendothelioma (HEHE) is a rare category of vascular tumor with uncertain malignant potential. It commonly presents nonspecific and variable clinical manifestations, ranging from asymptomatic to hepatic failure. In addition, laboratory measurements and imaging features also lack specificity in the diagnosis of HEHE. The aim of the present study is to highlight the dilemma and challenges in the preoperative diagnosis of HEHE, and to enhance awareness of the range of hepatobiliary surgery available in patients with multiple hepatic nodular lesions on imaging. In these patients, HEHE should at least be considered in the differential diagnosis.

**Key words:** Hepatic epithelioid hemangioendothelioma; Vascular tumors; Diagnosis; Dilemma; Challenges



**Core tip:** Hepatic epithelioid hemangioendothelioma (HEHE) is a rare category of vascular tumor with uncertain malignant potential. In the present study, by illustrating a case, we aimed to highlight the dilemma and challenges in the preoperative diagnosis of HEHE, and to enhance awareness of the range of hepatobiliary surgery available in patients with multiple hepatic nodular lesions on imaging. In these patients, HEHE should at least be considered in the differential diagnosis.

Hu HJ, Jin YW, Jing QY, Shrestha A, Cheng NS, Li FY. Hepatic epithelioid hemangioendothelioma: Dilemma and challenges in the preoperative diagnosis. *World J Gastroenterol* 2016; 22(41): 9247-9250 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i41/9247.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i41.9247

## TO THE EDITOR

Originating from endothelial cells, hepatic epithelioid hemangioendothelioma (HEHE) is a rare category of vascular tumor with uncertain malignant potential, and some present as slow-growing lesions while others are rapidly progressive tumors[1,2]. HEHE was first identified by Ishak *et al*[3] in 1984 and usually presents as a multi-nodular lesion imitating metastases with low-to-intermediate grade malignancy[1,3-5]. Based on radiological imaging, HEHE can be classified into a solitary nodular or diffuse nodular phenotype. The clinical biological features of HEHE are similar to those of a benign hemangioma and malignant angiosarcoma[6]. HEHE is resistant to chemotherapy and radiotherapy, thus, complete surgical resection is performed in patients with early stage monolobar disease, and liver transplantation is the only curative treatment in specific patients with diffuse liver involvement[7-9]. However, HEHE commonly presents nonspecific and variable clinical manifestations, ranging from asymptomatic to portal hypertension, Budd-Chiari syndrome or hepatic failure[10,11]. In addition, laboratory measurements also lack specificity in the diagnosis of HEHE, which typically manifests as a "halo" sign and "capsular retraction" on imaging[3,12]. However, most lesions have nonspecific features. Thus, the preoperative diagnosis of HEHE is difficult and most previously published cases were misdiagnosed as metastatic carcinoma, hepatocellular carcinoma, cholangiocarcinoma, or other types of vascular lesions preoperatively[13,14].

We report a patient whose primary diagnosis was metastatic carcinoma while the final pathological diagnosis was HEHE. The 40-year-old female patient was investigated due to persistent right epigastric pain for more than two months. She had no previous history of gastrointestinal or immunological diseases or previous surgical history. Physical examination was unremarkable. Laboratory tests, including liver biochemical tests, routine blood examination and serum tumor markers, were all within the normal range. Serological testing for hepatitis B and C were also negative. Abdominal contrast-enhanced computerized tomography revealed multiple low density nodular lesions scattered in the liver parenchyma, involving the right lobe and left medial segment, with inhomogeneous enhancement (Figure 1). Contrast-enhanced ultrasonography indicated multiple hypoechoic nodules with peripheral hyper-enhancement during the arterial phase and hypo-enhancement during the portal phase (Figure 2). Thus, an initial diagnosis of metastatic carcinoma was made and $^{18}$F-fluorodeoxyglucose positron emission tomography/computed tomography ($^{18}$F-FDG PET/CT) was then performed. The hepatic masses showed low glycometabolism (Figure 3) and no specific primary lesion was found, precluding metastases. We then obtained intraoperative frozen sections and the patient underwent extended right hemihepatectomy with complete resection of all lesions under the guidance of intraoperative ultrasonography. The lesions were grey-white in color and ranged in size from 0.7 to 5 cm. The results of hematoxylin/eosin staining were suspicious for a tumor of vascular origin (Figure 4A). Immunohistochemically, the lesions were positive for CD34 (Figure 4B), and CD31 (Figure 4C), supporting the diagnosis of HEHE.

For patients with liver masses, correct preoperative diagnosis is necessary to guarantee an appropriate therapeutic approach. Given the rarity and unpredictable natural of HEHE, it is not possible to make an accurate

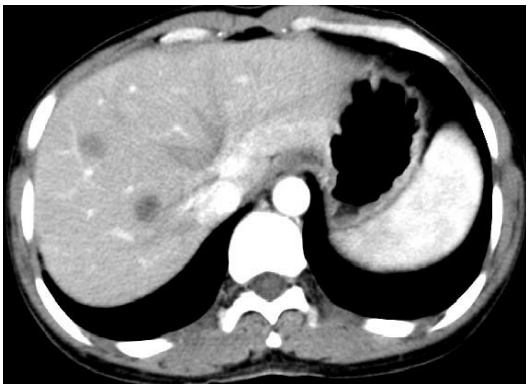

**Figure 1 Abdominal contrast-enhanced computerized tomography findings.** Abdominal contrast-enhanced computerized tomography (CT) revealed multiple low density nodular lesions scattered in the liver parenchyma, involving the right lobe and left medial segment, with inhomogeneous enhancement.

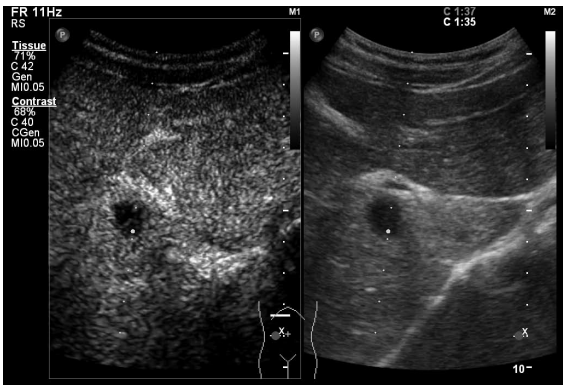


**Figure 2 Contrast-enhanced ultrasonography findings.** Contrast-enhanced ultrasonography indicated multiple hypoechoic nodules with peripheral hyper-enhancement during the arterial phase and hypo-enhancement during the portal phase.

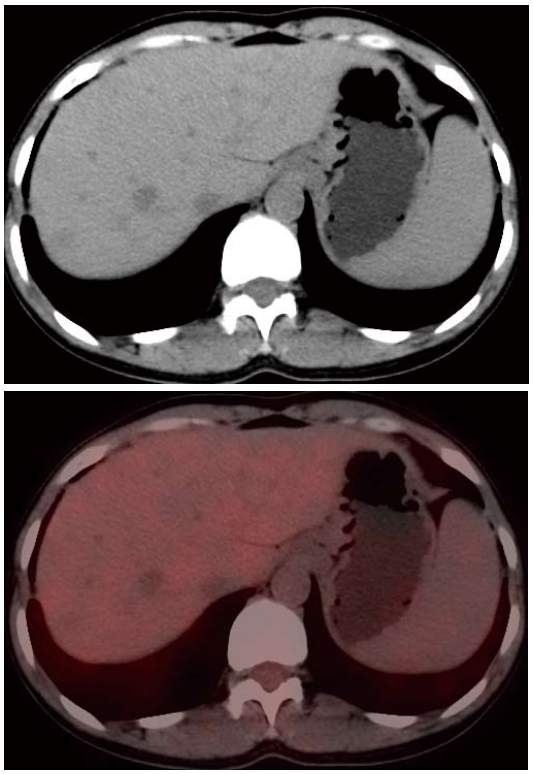

**Figure 3 PET-CT findings.** PET-CT showed that the hepatic masses had low glycometabolism.

diagnosis from heterogeneous clinical manifestations and nonspecific laboratory measurements. Most of these lesions also lack typical manifestations such as a "halo" sign and "capsular retraction" on imaging and present with nonspecific features. Thus, only histopathological results can guarantee an accurate diagnosis. In this study, the patient who had multiple hepatic nodules was examined using preoperative contrast-enhanced computerized tomography and contrast-enhanced ultrasonography, which indicated a diagnosis of metastatic carcinoma. PET-CT was then performed to detect the primary lesions. Surprisingly, no primary lesion was identified, and the hepatic lesions with low glycometabolism did not support the diagnosis of metastatic carcinoma. Thus, a tumor of vascular origin was then suspected and after clinical discussion, an extended right hemihepatectomy was carried out and the final histological results confirmed the diagnosis of HEHE.

HEHE has heterogeneous clinical features, nonspecific radiological characteristics and a variable natural history with a highly unpredictable clinical course. PET-CT may provide more information to help with the preoperative differential diagnosis; however, PET-CT is associated with high costs and sometimes only provides us with a reference and cannot guarantee an accurate diagnosis. One of the aims of the current study was to enhance awareness of the range of hepatobiliary surgery available in patients with multiple nodular lesions on imaging. In these patients,

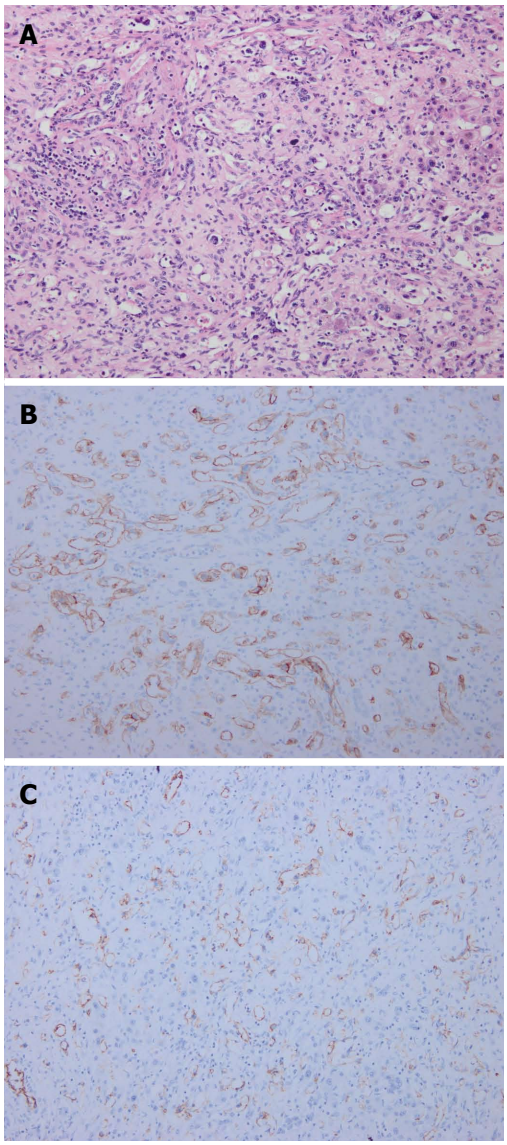


**Figure 4 Pathological findings.** Pathological investigations identified hepatic epithelioid hemangioendothelioma: A: Hematoxylin-eosin staining revealed abnormal hyperplasia of fibrous tissue combined with vessel-like formations, with scattered neoplastic epithelioid cells; B: Immunohistochemistry showed that the tumor was positive for CD34; C: Immunohistochemistry showed that the tumor was positive for CD31.

HEHE should at least be considered in the differential diagnosis. Multicenter studies based on the analysis of more practical and economic diagnostic tools are required to establish better regimens and subsequently guide the preoperative diagnosis of HEHE. Further studies focusing on the etiology of HEHE to improve preoperative diagnosis are also required.

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